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The skin microbiome in allergen-induced canine atopic dermatitis

Felipe Pierezan*,[1], Thierry Olivry†, Judith S. Paps†, Sara D. Lawhon*, Jing Wu*, Jörg M. Steiner*, Jan S. Suchodolski* and Aline Rodrigues Hoffmann*

*Texas A&M University, College of Veterinary Medicine and Biomedical Sciences, College Station, TX 77843, USA
†Department of Clinical Sciences, College of Veterinary Medicine and Comparative Medicine Institute, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, USA

Correspondence: Aline Rodrigues Hoffmann, Dermatopathology Specialty Service, Department of Veterinary Pathobiology, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX 77843, USA. E-mail: arodrigues@cvm.tamu.edu

Background – Studies focusing on next-generation sequencing of the bacterial 16S rRNA gene have allowed detailed surveys of skin bacterial populations (microbiota) of the skin.

Hypothesis/objectives – This study evaluated temporal changes in the skin microbiota in a canine model of atopic dermatitis.

Animals – Eight atopic dogs previously sensitized with house dust mites (HDM).

Methods – The dogs were topically challenged on the right groin with HDM allergens. Swabs were collected from the challenged and the contralateral nonchallenged sites prior to provocation (pre-challenge; baseline sample) and on days 1, 7, 14, 21 and 28 after allergen challenge. The 16S rRNA gene was amplified, sequenced and analysed. *Staphylococcus* spp. and *Staphylococcus pseudintermedius* were quantified with quantitative PCR (RT-qPCR).

Results – Skin lesions developed in all dogs on the challenged sites. Differences in bacterial groups were observed on the challenged site over time. Relatively lower abundances of Fusobacteriaceae on Day 7, and, based on LEfSe, increased abundances of Corynebacteriaceae on Day 1, and Staphylococcaceae on days 7, 14 and 21, were observed on the challenged site, compared to the contralateral site. Results of RT-qPCR correlated with those of next-generation sequencing, with significantly increased numbers of *Staphylococcus* spp. and *S. pseudintermedius* on Day 21, and days 7 and 21 on the challenged site compared to the contralateral site, respectively.

Conclusions and clinical importance – This study demonstrates that an allergen challenge in sensitized dogs leads to bacterial dysbiosis with increased abundance of *S. pseudintermedius* at the site of lesion induction.

Introduction

Disturbances of the skin microbiota in canine atopic dermatitis (CAD) have focused mainly on pathogenic bacterial species, most commonly those due to *Staphylococcus pseudintermedius*.[1] In humans, atopic dermatitis (AD) is also associated with alterations in the skin microbiome, with most studies focusing primarily on *Staphylococcus aureus*.[2] It remains unclear if some skin lesions are initiated by imbalances in the residing cutaneous microbiota (dysbiosis), or whether alterations in the skin barrier, as well as an enhanced inflammatory response, result in cutaneous dysbiosis.[3]

The advent of next-generation sequencing (NGS) of the bacterial 16S rRNA gene has allowed for a more detailed analysis of the bacterial populations inhabiting the skin of humans[3,4] and dogs[5,6] in health and during cutaneous diseases. A metagenomic evaluation of shifts in the skin microbiome of children with AD revealed a marked reduction in microbial diversity during AD flares, with increased relative abundances of cutaneous *S. aureus*.[4] These authors proposed that increases in the proportion of *Staphylococcus* and reductions in microbial diversity might even precede AD flares. Furthermore, the lower bacterial diversity that occurred during flares was reversed even before clinical improvement was observed. This resolution of signs was accompanied by significant increases in proportions of the genera *Streptococcus*, *Corynebacterium* and *Propionibacterium* after treatment.[4]

We have described the skin microbiota of healthy dogs and dogs with allergic skin diseases without active

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[1]Present address: Department of Clinics and Surgery, Veterinary School, Universidade Federal de Minas Gerais, Pampulha, Belo Horizonte, Minas Gerais 31270-901, Brazil.

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lesions; we demonstrated that the nonlesional skin of allergic dogs showed significant reductions in bacterial species richness when compared to the same skin sites (i.e. axilla, groin and interdigital skin) of healthy dogs.[6] Another study also showed that atopic dogs have a lower diversity of their skin microbiota with a markedly increased abundance of *S. pseudintermedius* during flares, with increased diversity and lower abundances of *S. pseudintermedius* after treatment with antimicrobials.[5] Both studies in humans[4] and dogs[5,6] have demonstrated differences between the skin microbiome of healthy individuals and those with AD.

Acute atopic skin lesions have been induced in a genetically predisposed canine model via epicutaneous applications of house dust mite (HDM) allergens.[7,8] This model reproduces the macroscopic, microscopic and immunological changes seen in spontaneous CAD skin lesions.[7] This canine model is considered relevant to the study of the pathogenesis of acute atopic skin lesions and to test the effectiveness and potency of antiallergy drugs.[7,8]

The aim of this study was to evaluate the temporal changes in the skin microbiota that occur in acute CAD skin lesions through bacterial 16S rRNA gene sequencing. Furthermore, we wanted to validate the use of real time quantitative PCR (RT-qPCR) for quantification of *Staphylococcus* spp. and *S. pseudintermedius* to confirm increases in the relative abundance of this bacterial genus and species.

Material and methods

Study subjects

Eight Maltese–beagle cross-bred dogs were enrolled into this study. Their age ranged from 2 to 14 years (average age: 6.7 years). There were six female dogs (four intact and two spayed) and two intact male dogs. Dogs 2, 3, 5 and 6, and dogs 7 and 8 were closely related. All dogs were kept indoors primarily to limit their exposure to allergens, but they were allowed out into their runs at least twice a day to exercise and interact with the other dogs. The dogs' runs were cleaned twice daily. Some dogs were housed together in the same run (dogs 2 and 3; dogs 7 and 8). The animals were not treated with antibiotics, anti-inflammatory agents or immunosuppressive drugs for at least three months prior to sample collection. The dogs previously had been sensitized and challenged with *Dermatophagoides farinae* (Df) HDM.

Challenges with Df HDM

The atopic dogs were washed with a shampoo with a neutral pH 24 h prior to challenge. Immediately after washing, a small area on the right groin was prepared for application of a solution of Df by clipping the hair with scissors and disinfecting with 70% alcohol. The Df solution contained 500 μg of lyophilized Df whole mite powder (Greer Laboratories; Lenoir, NC, USA) suspended in mineral oil. Twenty microliters of this suspension were painted onto the right groin every 24 h for three days, giving a total of three applications, except for Dog 6 that developed severe local lesions after two mite applications.

Sample collection and assessment of inflammatory skin lesions

Skin swabs from the challenged side (i.e. right groin) and contralateral nonchallenged side (i.e. left groin) were collected prior to provocation (referred to here as "pre-challenge" or "baseline" sample) and at 1, 7, 14, 21 and 28 days (i.e. days 1, 7, 14, 21 and 28) after the second (Dog 6) or third application of Df suspension (all other dogs). Twelve samples were collected from each of the atopic dogs. For each skin side, three swab applicators (Isohelix DNA Buccal Swabs; Harrietsham, Kent, UK) were used. Each swab applicator was rubbed on the skin 20 times (10 strokes per swab side).

Finally, to avoid any contamination of the dog skin with the microbiota contained in the allergens, we evaluated the microbiota of the lyophilized Df whole mite powder. Four swabs were inserted into the Df solution and placed into two separate tubes. All samples were stored immediately at 4°C.

Assessment of inflammatory skin lesions

Skin lesions were scored before allergen challenge (pre-challenge) and on Day 1. Erythematous macules, oedema, papules/pustules and excoriations were scored as 0 (absent), 1 (faint, mild), 2 (moderate) or 3 (strong, severe) for a total maximum lesional score of 12 points.

DNA extraction and next generation sequencing

Genomic DNA was extracted from each set of sterile swabs collected from each skin site from the AD and healthy pet dogs, and from the Df solution, using the MoBio Power soil DNA isolation Kit (MoBio Laboratories; Carlsbad, CA, USA), as recommended by the manufacturer. Negative samples with and without sterile swabs were also included. The V4 region of the 16S rRNA gene was amplified with primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) at the MR DNA Laboratory (Shallowater, TX, USA). Barcodes were added to the forward primers. PCR amplification products were verified on 2% agarose gels and samples were purified using calibrated Ampure XP beads. The Illumina TruSeq DNA Library was used to prepare a DNA library and sequenced at MR DNA on an Illumina MiSeq instrument (Illumina Inc.; San Diego CA, USA). The sequences obtained in this study have been deposited in the NCBI Short Read Archive (accession # SRP057606).

RT-qPCR for *Staphylococcus* spp., *S. pseudintermedius* and *S. aureus*

RT-qPCR for the *Staphylococcus* genus was based on SYBR green detection of the *tuf* gene,[9] whereas detection of *S. aureus* was based on the *S. aureus*-specific gene *sa442*.[10] Detection of *S. pseudintermedius* was based on development of a probe that targets a region of the *nuc* gene that is specific for *S. pseudintermedius*.[11] The forward primer for the *Staphylococcus* genus was 5′-TGAGCGCTTGAATCGCATATT-3′, the reverse primer was 5′-CACAGCCATATAGCCGCATTT-3′ and the *S. pseudintermedius* probe was 5′-AATGTCTGTTTCACTTTCA-3′ labelled with fam at the 5′ end and black hole quencher 1 at the 3′ end. All primers were confirmed *in vitro* to be specific for the organisms tested. All samples were run in triplicates in a 25 μL reaction volume using Taqman Fast Universal PCR Master Mix (2×) (Cat. 4352046, Applied Biosystems; Foster City, AC, USA) with each primer used at 500 nM final concentration and the probe at 500 nM final concentration. A standard curve was included in every run to allow quantitation of the number of bacteria present in the original sample. Each standard curve consisted of purified *Staphylococcus* DNA extracted from a 10-fold dilution series of *S. pseudintermedius*, clinical isolate from a dog confirmed by sequencing the 16S rDNA or *S. aureus* (ATCC29213) (depending on the gene target) isolated using the Mo Bio Powersoil DNA isolation Kit, according to the manufacturer's recommended protocol.

Microbiota data analysis

Quantitative Insights Into Microbial Ecology (QIIME, v1.8) software was used to characterize the samples, as described previously.[12] The raw sequence data were demultiplexed by barcodes, screened, trimmed, denoised and chimera-depleted using uCHIME;[13] low quality reads were filtered using the QIIME database's default parameters, as described previously.[6] A total of 3,041,382 (median: 30,279; range 101–96,882 sequences per sample) were obtained.

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For further analysis, each sample from the atopic dogs was rarefied to an even sequencing depth of 5,000 sequences per sample to adjust for uneven sequencing depth across all samples. Sequences were then clustered into operational taxonomic units (OTUs) using a closed reference OTU picking protocol at the 97% sequencing identity level[14,15] against the Greengenes database.[16] Because most datasets did not meet the assumptions of normal distribution, as assessed by the D'Agostino and Pearson normality test, nonparametric statistical tests were used. Only bacterial taxa that were present in at least 50% of the samples for each time point were included in the analysis.

The differences in the proportions of bacterial taxa between the challenged and contralateral sites (% of total sequences) and differences in the abundance of *Staphylococcus* spp and *S. pseudintermedius* were evaluated using the Kruskal–Wallis test. The Friedman test with Dunn's *post hoc* test for repeated measures ANOVA were performed to evaluate changes in the microbiota and differences in the abundances of *Staphylococcus* spp and *S. pseudintermedius* among all time points for the challenged site. The Kruskal–Wallis test was further used for comparing differences between each of the time points for the challenged site (for instance, right pre-challenged versus right Day 1). *P*-values <0.05 adjusted for multiple comparisons were considered statistically significant. For testing the significance of multiple comparisons for the different OTUs, the data were separated by taxonomic level (e.g. phylum, class, order, family, genus) and pre-filtered to include only those that were present in more than 75% of all individuals in at least one of the sites (challenged and contralateral). The Linear discriminant analysis (LDA) effect size (LEfSe: http://huttenhower.sph.harvard.edu/galaxy/)[17] was also utilized to evaluate differentially abundant bacterial taxa between pre-challenged samples and the various time points from the challenged site, and between samples from challenged and contralateral sites for each time point.

Observed species richness, Chao 1 and Shannon indexes[18] were determined using QIIME. Data in graphs are expressed as the mean +/− standard error (SE) or median and 50 percentile of the group. Due to a very low number of sequence reads, one sample (right pre-challenged sample of Dog 5) was excluded from the QIIME analysis. In order to include Dog 5 in the statistical analysis for the different taxa, the baseline values for the left site were also used as baseline values for the right site. Data were analysed using Prism software 5.0 (GraphPad Software; San Diego, CA, USA) and JMP software (SAS Institute; Cary, NC, USA).

Analysis of beta-diversity was performed using weighted and unweighted Unifrac distance metrics. Statistical significance of the resulting distance metric was tested by analysis of similarities (ANOSIM) using the QIIME software.

Results

Allergen challenge resulted in the development of acute skin lesions

Seven dogs developed noticeable skin lesions after three daily applications of Df suspension, whereas Dog 6 developed similar lesions after only two such applications. Prior to challenge, dogs had scores that ranged from 0 to 3. Twenty four hours after the second or third application of the Df suspension the lesion scores increased in all dogs (Figure S1). By 7 days after the last application of the Df suspension, all skin lesions had resolved. In contrast, no skin lesions were observed on the left groin (contralateral unchallenged control site) in any of the dogs at any time point.

Allergen challenge did not alter the microbial bacterial diversity

Diversity analysis was performed on 5,000 randomly selected sequences per sample. We did not observe any significant differences in the Shannon diversity index (an index that considers the richness and evenness of species), Chao 1 metric (an index that considers the richness of the entire microbial community at a complete sequencing depth) or the number of observed species (the number of observed OTUs and the richness of the sample at given sequencing depth), between samples from the challenged (right) versus the contralateral (left) skin site at any of the time points; Figure S2). When only samples from the challenged site were analysed at different time points, the samples from Day 21 had a significantly higher species richness (i.e. number of observed species) when compared to those pre-challenge (median 327 versus 297; $P = 0.05$; Figure S2).

On principal coordinate analysis plots (PcoA plots), we did not determine any significant clustering between the challenged and the contralateral sites for each of the time points. This evaluation was performed both on the unweighted and weighted UniFrac analysis (ANOSIM, $P = 0.83$). We further analysed if the unweighted Unifrac distance between each time point (distances between: days 1 and 7, days 7 and 14, days 14 and 21, and days 21 and 28) differed between the various time points at the challenged site or between the challenged and contralateral site. Again, there were no significant differences between challenged and contralateral samples for any of the time points (Figure S3).

Allergen challenge resulted in bacterial dysbiosis

The skin of this colony of AD dogs harbours a complex bacterial assemblage composed of at least 23 bacterial phyla, with the main dominant phyla being Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria. A total of 256 bacterial families were identified across all samples.

We first compared the bacterial microbiota between challenged and contralateral sites. No significant differences, at any phylogenetic level, were identified when the pre-challenged samples from the right site was compared to the contralateral site based on Kruskal–Wallis tests and LDA effect size (LEfSe). The LEfSe was further utilized to determine differentially abundant bacterial taxa between the challenged and contralateral sites for each time point. Significant differences were observed for a few bacterial groups between days 1 and 21, with no differences identified between pre-challenge samples and those from Day 28 ($\alpha = 0.05$, LDA score > 3.0; Figure 1). These differences in the bacterial microbiota included increases in Corynebacteriaceae on Day 1, and in Staphylococcaceae on days 7, 14 and 21 on the challenged site. The order Bacilli and class Bacillales, which include the families Streptococcaceae and Staphylococcaceae, were increased significantly at the challenged site on Day 21, based on LefSe and Kruskal–Wallis tests, respectively (Figure 1, Table S1). We observed no significant differences in relative proportions of bacteria belonging to the family Staphylococcaceae between challenged and contralateral sites.

We then restricted our comparison to samples from the challenged sites between the various time points. We found lower proportions of Fusobacteria, Fusobacteriales,

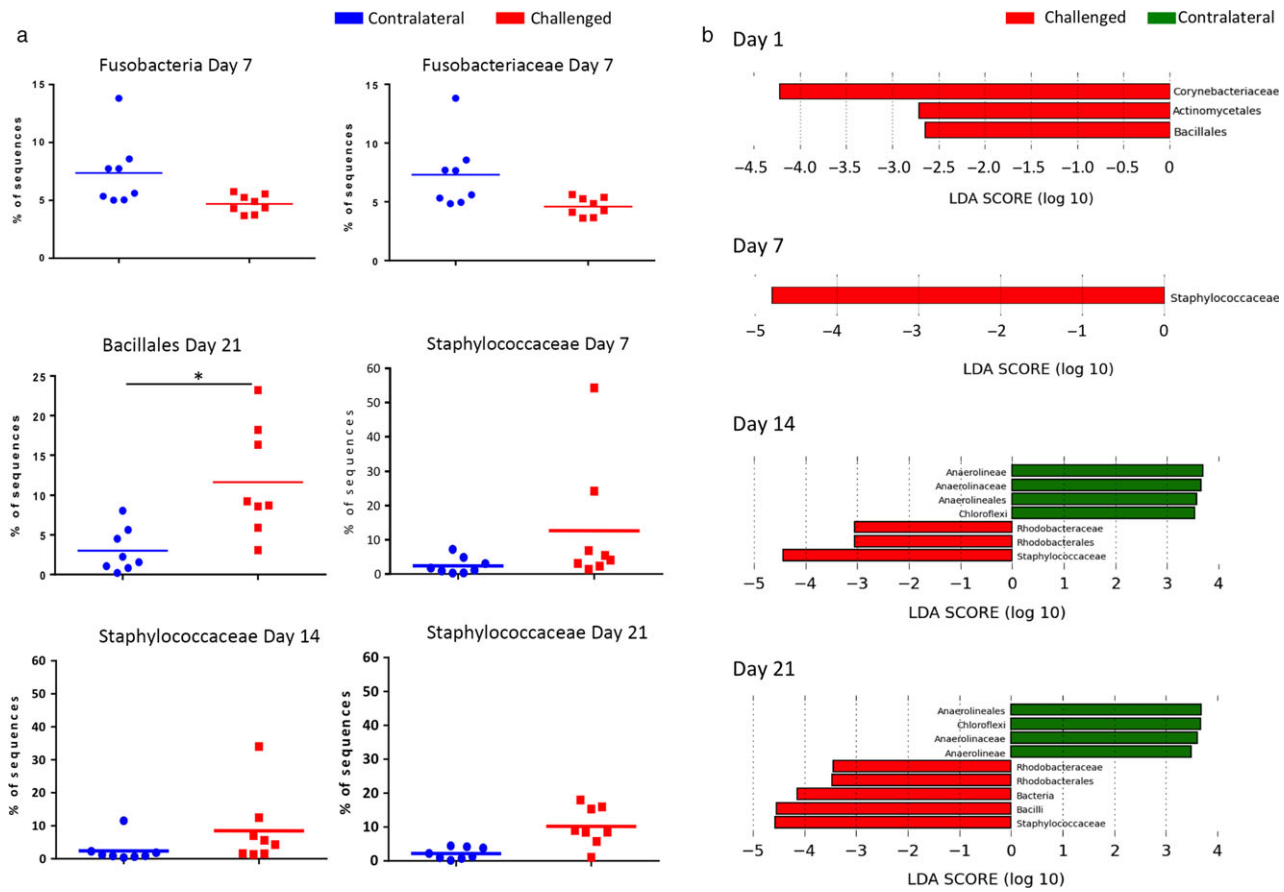

Figure 1. Abundance of microbiota taxa between challenged and contralateral skin. (a) The relative abundance of bacterial groups at different phylogenetic levels (phylum, order and family) at the challenged and contralateral sites, at different time points, based on Illumina sequence analysis. Blue and red lines represent the median of the relative abundance at the challenged and contralateral sites, respectively. The differences in the proportions of bacterial taxa between these two sites were evaluated using the Kruskal–Wallis test with adjustment for multiple comparisons (*$P < 0.05$). (b) Linear discriminant analysis (LDA) effect size (LEfSe) of Illumina sequencing datasets based on 16S rRNA gene sequences. Taxonomic distribution of bacterial groups differentially present in challenged and contralateral sites from dogs with atopic dermatitis collected on days 1, 7, 14, 21 and 28. Fourteen differentially abundant bacterial groups were detected ($\alpha = 0.05$, LDA score > 2.5). Of these, 10 bacterial groups were significantly over represented at the challenged sites (red) and four bacterial groups were over represented at the contralateral sites (green).

Fusobacteriaceae and *Fusobacterium* sp. on Day 7 compared to Day 1 (4.6% versus 7.5%; $P = 0.05$) and for Fusobacteriales on Day 7 compared to Day 21 (4.6% versus 6.7%; $P = 0.013$) (Figure 2; Table S2). On LEfSe analysis, we found relatively increased proportions of Corynebacteriaceae on Day 7, the phylum Firmicutes on days 14, 21 and 28, Staphylococcaceae and six other bacterial groups on Day 21, and Alphaproteobacteria on Day 28, compared to the pre-challenge (baseline) samples (Figure S4).

Allergen challenge resulted in increased numbers of *Staphylococcus* spp. and *S. pseudintermedius*

Copy numbers of *Staphylococcus* spp. and *S. pseudintermedius* were not significantly different before challenge and on Day 1, indicating that no increases in abundances of these bacteria were observed at the time of development of acute skin lesions (Figure 3). However, significantly increased *S. pseudintermedius* copy numbers were observed on Day 7 at the challenged site compared to the contralateral one. On Day 21, copy numbers of *Staphylococcus* spp. and *S. pseudintermedius* were increased markedly at the challenged site compared to samples from the same sites before challenge or on Day 1 after challenge (only *S. pseudintermedius*), and also to the contralateral site. *Staphylococcus aureus* DNA was not amplified from any sample except for that of Dog 2 on Day 21 (350 bacterial copies). The results from *S. pseudintermedius* and *Staphylococcus* spp. also correlated with the increased relative abundance of Staphylococcaceae identified earlier at challenge sites.

House dust mites have their own microbiota

The family Bartonellaceae (phylum Proteobacteria; class Alphaproteobacteria, order Rhizobiales) was the most abundant group, accounting for 72% of the sequences in the samples from the mite solution, followed by the family Leuconostocaceae (phylum Firmicutes; class Bacilli; order Lactobacillales; 25%). Other bacterial groups, including the families Streptococcaceae (2%), Staphylococcaceae (0.2%), Fusobacteriaceae (0.2%), Moraxellaceae (0.2%) and Corynebacteriaceae (0.05%) also were identified in these samples.

Discussion

In the present study, sequencing of the 16S rRNA gene and RT-qPCR for *Staphylococcus* spp. and

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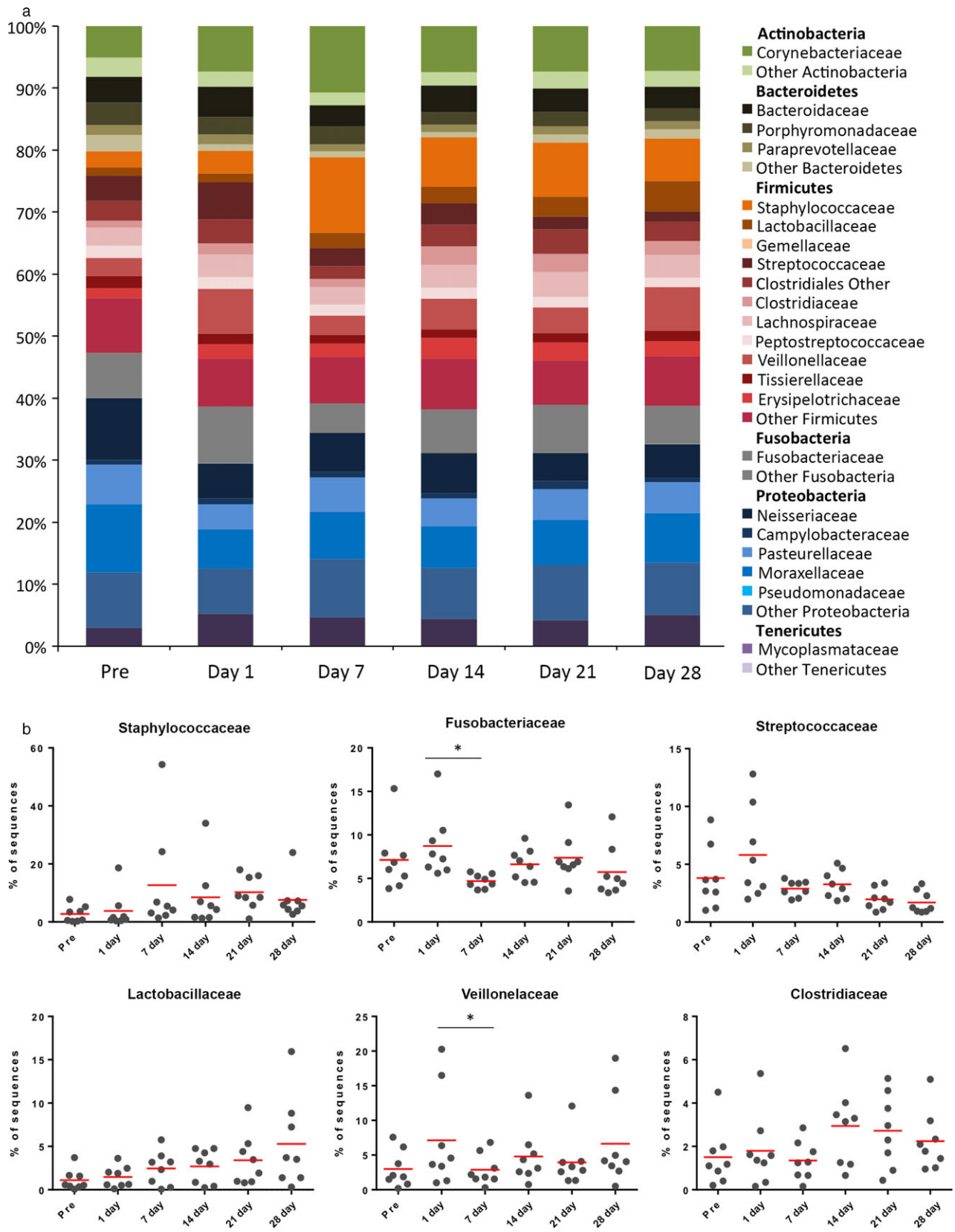

Figure 2. Abundance of microbiota taxa on challenged skin. (a) Bacterial phyla and families inhabiting the skin of the challenged site at different time points. Average of most common bacterial phyla and families identified in pre-challenge samples and samples collected on days 1, 7, 14, 21 and 28 post-challenge. (b) The relative abundances of the most prevalent bacterial families at the challenged sites at different time points, based on the Illumina sequence analysis. Changes among all time points were evaluated using the Friedman's test with Dunn's *post hoc* test for repeated measures ANOVA with adjustment for multiple comparisons. $*P < 0.05$.

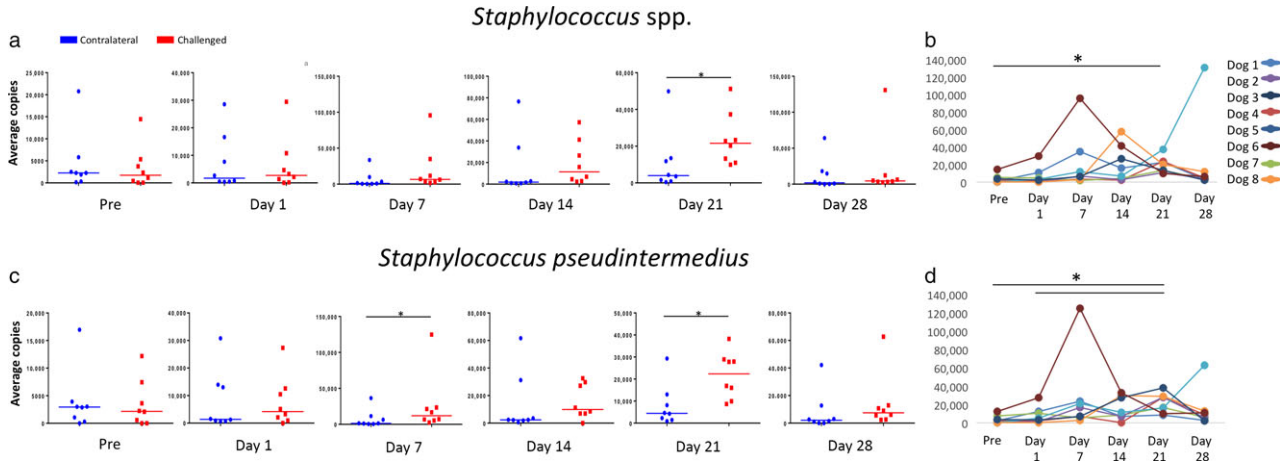

Figure 3. Average copy numbers of *Staphylococcus* spp. and *Staphylococcus pseudintermedius*. (a) Average number of bacterial copies of *Staphylococcus* spp. at the challenged and the contralateral skin sites at different time points, based on real time quantitative PCR. (b) Average number of bacterial copies of *Staphylococcus* spp. on the challenged sites across different time points. (c) Average number of *S. pseudintermedius* copies at the challenged and the contralateral sites at different time points. (d) Average number of *S. pseudintermedius* on the challenged skin sites across different time points. The differences in number of bacterial copies at the challenged and the contralateral skin sites were evaluated using a Kruskal–Wallis test. Changes among all time points at the challenged sites were evaluated using a Friedman's test with Dunn's *post hoc* tests for repeated measures ANOVA. $*P < 0.05$.

S. pseudintermedius were used to evaluate changes in the bacterial skin microbiota in acute experimental lesions of CAD following challenge with Df HDM allergens. Our results demonstrate bacterial dysbiosis in allergen challenged (i.e. lesional) skin areas compared to contralateral nonchallenged areas (i.e. nonlesional) in atopic dogs. These observations suggest an association of experimentally induced lesions of AD with increased proportions of *Staphylococcaceae* and the persistence of these changes for three weeks after mite challenges, with relative abundances returning to those of pre-challenge samples only after four weeks.

In children with AD, microbial alpha and beta diversity are altered during flares, and diversity might recover even before resolution of clinical lesions in children undergoing treatment for skin lesions.[4] Likewise, dogs with chronic CAD presented during a flare appear to have low diversity of the skin microbiota, which recovered after treatment with antimicrobials.[5] No significant changes in microbial diversity were observed across the different time points of the challenged skin or between challenged and contralateral sites in this canine experimental model of AD. This is likely to be due to the fact that most lesions were considered mild, developed acutely and only affected the area of HDM challenge. Conceivably, changes in microbial diversity could also have occurred within a different interval than that evaluated in our sampling schedule, for instance, between days 2 and 6. Day 7 post-challenge coincided with recovery of AD skin lesions and, based on a previous study in atopic children,[4] the microbial diversity would be expected to recover and be similar to pre-challenge even before the resolution of lesions.

The results of RT-qPCR for *Staphylococcus* spp. and *S. pseudintermedius* paralleled those of the 16S rRNA NGS, thereby establishing that these bacteria were an important factor of microbial dysbiosis at the site of allergen challenge. Furthermore, these results also demonstrated that an increased proportion of *Staphylococcaceae* identified with NGS was due to increased numbers of bacterial copies of *S. pseudintermedius*. *Staphylococcus aureus* was not identified in baseline controls or associated with the development of lesions, as most samples did not show any copies and a single sample showed a very low number of copies of *S. aureus*. Our findings mirror those of culture-based and molecular studies, which demonstrate that *Staphylococcus* spp. are associated with AD lesions in both humans and dogs.[1,2,4–6,19]

Staphylococcus aureus and *Corynebacterium bovis* were reported to likely contribute to eczematous inflammation in an ADAM17-deficient mouse model of AD.[20] These knockout mice developed bacterial dysbiosis and this was found to be capable of inducing eczematous lesions.[20] In our canine model, based on LefSe analysis, the abundance of bacteria of the family *Corynebacteriaceae* was increased significantly at the challenged sites compared to the contralateral sites on Day 1, and even before increased relative abundances of *Staphylococcaceae* were observed. In another study evaluating the microbiota in dogs with chronic AD, authors also reported that *Corynebacterium* spp. were more abundant in the groin area during flares of AD, compared to those of control dogs.[5] These findings demonstrate that the bacterial communities inhabiting the skin have very complex relationships; additional studies should focus on interactions between these communities and the development or remission of skin lesions.

In a previous study, we found that the betaproteobacteria *Ralstonia* spp. predominated in several skin samples from different body regions in dogs, with healthy dogs having increased relative abundances of these bacteria compared to allergic dogs.[6] In this current study and other studies including one using healthy pet dogs currently ongoing in our laboratory, *Ralstonia* spp. was only present in very low relative abundances across all samples and no

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significant differences between samples from atopic dogs and those from healthy pet dogs (data not included) were observed. This could be due, in part, to use of different supplies for the collection of samples, modifications in sample storage, extraction methods and/or changes in the high throughput sequencing platform used.

Finally, we also evaluated the microbiota inhabiting the Df HDM used in this study; this is an important evaluation to assess for any potential transfer of their microbiota to the skin of the dogs. Interestingly, Df HDM was colonized mainly by Bartonellaceae (72%) and Leuconostocaceae (25%) and no changes in the proportions of these bacterial groups were observed in the skin of atopic dogs, indicating that there was no bacterial contamination of the skin of dogs by the HDM allergens.

Conclusions

The present study, in spite of its inherent limitation due to a small sample size, demonstrates that allergen challenge in HDM sensitized dogs is associated with bacterial dysbiosis during the development of skin lesions, with alterations of several bacterial groups persisting for two weeks after the recovery of skin lesions. This study also demonstrates that *S. pseudintermedius* plays an important role in this bacterial dysbiosis. The lack of changes of bacterial diversity of the skin after allergen challenge might be due to the mild and focal nature of these experimental lesions, compared to the often severe and chronic changes seen in natural CAD. Future longitudinal studies using this model and dogs with natural AD should focus on microbial interactions during and after development of skin lesions, on relationships between the immune system and the cutaneous microbiome, as well as how different treatment regimens might influence the skin microbiome in atopic dogs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Skin lesion scores. Scores obtained for skin lesions before allergen challenge (pre-challenge; baseline) and on Day 1 (post-challenge d1). Scores were based on the development of erythematous macules, oedema, papules/pustules, and/or excoriations scored as 0 (absent), 1 (faint, mild), 2 (moderate) or 3 (strong, severe), for a total maximum lesional score of 12.

Figure S2. Alpha diversity analysis. (a) Rarefaction curves for 16S rRNA gene sequences obtained from the skin of the right (challenged) and left (contralateral) groin of dogs with AD at different time points. The analysis was performed on a randomly selected subset of 5,000 sequences per sample. No differences of the Chao 1 index (species predictor estimator), the number of observed species nor the Shannon index (diversity index that accounts for species abundance and evenness) were observed between the challenged and contralateral sites at different time-points. Lines represent the mean for each group, whereas the error bars represent the standard deviations. (b) Comparisons of alpha diversity measured at 5,000 sequences per sample from the challenged and contralateral sites (*x*-axis). The *y*-axis represent the data points for the Chao 1 index, number of observed species and Shannon diversity index for the

samples of the two sites at different time points. Challenged samples from Day 21, had a significantly higher species richness (number of observed species; median 327) when compared to the pre-challenged samples from the right groin (median 297; $*P < 0.05$). Blue and red lines and boxes represent the median and 50% percentile for the samples from challenged and contralateral sites, respectively.

Figure S3. Beta diversity analysis. Effect of allergic skin lesions on the community structure of the skin microbiota. Principal coordinates analysis plots of unweighted Unifrac distances of 16S rRNA genes from challenged (a) and contralateral (b) sites. ANOSIM revealed no clustering between samples from the challenged or contralateral sites. Dashed lines connect the samples of each dog, following the time points, from the sample pre-lesion (light green circle) to the sample of last day of collection (28 days after development of the lesion). (c) Median, 50% percentile and standard deviation of the unweighted Unifrac distance of samples from the challenged and contralateral sites at different time points.

Figure S4. Differences in abundances of bacterial taxa on challenged side. Linear discriminant analysis (LDA) effect size (LEfSe) of Illumina sequencing datasets based on 16S rRNA gene sequencing. Taxonomic distribution of bacterial groups in pre-challenged and samples collected on days 1, 7, 14, 21 and 28. A total of 13 differentially abundant bacterial groups were detected ($\alpha = 0.05$, LDA score > 2.0). Of these, three bacterial groups were significantly over-represented in baseline pre-challenged samples (green) and 10 bacterial groups were over-represented in samples collected after challenge (red).

Table S1. Relative percentages of the most abundant bacterial groups at various phylogenetic levels (phylum, class, order, family or genus) on the challenged and contralateral skin sites at different time points based on Illumina sequencing.

Résumé

Contexte – Les études portant sur le séquençage de dernière génération du gène bactérien ARNr 16S ont permis de détailler les surveillances des microbiotes de la peau.

Hypothèses/Objectifs – Cette étude évalue les changements temporaires de microbiote cutané dans un modèle de dermatite atopique canine.

Sujets – Huit chiens atopiques précédemment sensibilisés aux acariens de poussière de maison (HDM).

Méthodes – Les chiens ont été provoqués par voie topique sur le pli axillaire droit avec des allergènes HDM. Les écouvillons ont été collectés sur les chiens provoqués et sur les sites controlatéraux non provoqués avant provocation (pré-challenge; échantillon de base) à jours 1, 7, 14, 21 et 28 après provocation allergénique. Le gène ARNr 16S a été amplifié, séquencé et analysé. *Staphylococcus* spp. et *Staphylococcus pseudintermedius* ont été quantifiés par PCR quantitative (RT-qPCR).

Résultats – Les lésions cutanées se sont développées chez tous les chiens des sites testés. Les différences dans les groupes bactériens ont été observés sur les sites provoqués au cours du temps. Une relative faible abondance de Fusobactériacées aux jours 7 et basé sur LEfSe, une augmentation de Corynebacteriacées à jour 1 et de Staphylococcies à jours 7, 14 et 21, ont été observés sur les sites provoqués et comparés aux sites controlatéraux. Les résultats de RT-qPCR corrélaient avec ceux de séquençage de dernière génération avec des nombres significativement augmentés respectivement de *Staphylococcus* spp. et *S. pseudintermedius* à jour 21 et jours 7 et 21 sur le site testé comparé au site controlatéral.

Conclusions et importance clinique – Cette étude démontre qu'une provocation allergénique chez des chiens sensibilisés mène à une dysbiose bactérienne avec augmentation importante de *S. pseudintermedius*.

Resumen

Introducción – Los estudios centrados en la secuenciación de próxima generación del gen 16S rRNA bacteriano han permitido evaluaciones detalladas de las poblaciones bacterianas de la piel (microbiota).

Hipótesis/objetivos – En este estudio se evaluaron los cambios temporales en la microbiota de la piel en un modelo canino de dermatitis atópica.

Animales – Ocho perros atópicos previamente sensibilizados con ácaros del polvo doméstico (HDM).

Métodos – Los perros fueron expuestos por vía tópica en la zona inguinal derecha con alergenos de ácaros del polvo. Los hisopos se obtuvieron de la zona de exposición y de la zona contralateral no expuesta antes de la inducción (pre-exposición; muestra basal) y en los días 1, 7, 14, 21 y 28 después de la exposición al alérgeno. El gen 16S rRNA se amplificó, secuenció y analizó. *Staphylococcus* spp. y *Staphylococcus pseudintermedius* se cuantificaron con PCR cuantitativa (RT-qPCR).

Resultados – Lesiones cutáneas se desarrollaron en todos los perros en las zonas expuestas. Se observaron diferencias en los grupos bacterianos en la zona expuesta con el paso del tiempo. Hubo una frecuencia relativa menor de Fusobacteriaceae en el día 7, y, basándonos en LEfse, un aumento de la abundancia de Corynebacteriaceae en el día 1, y de Staphylococcaceae en los días 7, 14 y 21, en el sitio expuesto, en comparación con el sitio contralateral. Los resultados de RT-qPCR se correlacionaron con los de secuenciación de nueva generación, con un aumento significativo de los números de *Staphylococcus* spp. y *S. pseudintermedius* en el día 21 y días 7 y 21, respectivamente, en la zona expuesta en comparación con el sitio contralateral.

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Conclusiones e importancia clínica – Este estudio demuestra que la exposición al alérgeno en perros sensibilizados conduce a disbiosis bacteriana con una mayor abundancia de *S. pseudintermedius* en el sitio de la inducción de la lesión.

Zusammenfassung

Hintergrund – Studien, die sich auf die Next-Generation Sequenzierung der bakteriellen 16S rRNA Gene konzentrieren, haben detaillierte Untersuchungen von Bakterienpopulationen (Mikrobiota) der Haut ermöglicht.
Hypothese/Ziele – Diese Studie evaluierte zeitliche Veränderungen bei den Microbiota der Haut in einem Hundemodell von atopischer Dermatitis.
Tiere – Acht atopische Hunde, die schon früher mit Hausstaubmilben sensibilisiert worden waren (HDM).
Methoden – Die Hunde wurden topisch in der rechten Inguinalgegend mit HDM Allergenen provoziert. Tupfer wurden von den provozierten und von den nicht provozierten Hautstellen der kontralateralen Seiten vor der Provokation (vor-Provokation; Basisprobe) und an den Tagen 1, 7, 14, 21 und 28 nach der Allergenprovokation entnommen. Das 16S rRNA Gen wurde amplifiziert, sequenziert und analysiert. *Staphylococcus* spp. und *Staphylococcus pseudintermedius* wurden mittels quantitativem PCR quantifiziert (RT-qPCR).
Ergebnisse – Hautveränderungen entstanden bei allen Hunden an den provozierten Stellen. Es wurden an den provozierten Stellen mit der Zeit Unterschiede bei den Bakteriengruppen beobachtet. Es wurde auf den provozierten Stellen ein relativ geringeres Auftreten von Fusobakterien am Tag 7 und – basierend auf LEfSe – ein zunehmendes Auftreten von Corynebakterien am Tag 1 und Staphylokokken an den Tagen 7, 14 und 21 im Vergleich zur kontralateralen Seite beobachtet. Die Ergebnisse des RT-qPCR korrelierten mit jenen der Next-Generation Sequenzierung mit signifikant höheren Zahlen an *Staphylococcus* spp. und *S. pseudintermedius* an den Tagen 21 und an den Tagen 7 bzw 21 auf der provozierten Seite im Vergleich zur kontralateralen Seite.
Schlussfolgerungen und klinische Bedeutung – Diese Studie zeigt, dass eine Allergenprovokation bei sensibilisierten Hunden zu einer bakteriellen Dysbiose mit zunehmendem Auftreten von *S. pseudintermedius* an den Stellen der induzierten Veränderungen führt.

要約

背景 — 次世代シーケンシングを用いた細菌の16S rRNA遺伝子解析によって、皮膚の細菌叢の詳細な調査が可能になった。
仮説/目的 — 本研究の目的は、犬アトピー性皮膚炎モデルにおける皮膚細菌叢の一過性の変化を評価することである。
供与動物 — ハウスダストマイト(HDM)にあらかじめ感作させたアトピー犬8頭。
方法 — 実験犬の右側内股にHDM抗原を塗布し、感作させる。暴露前(感作前；基準サンプル)および感作後1，7，14，21，28日に、感作部位および反対側の非感作部位より綿棒にてサンプルを採取した。16S rRNAを増幅し、シーケンスと解析を行った。Staphylococcus spp.およびStaphylococcus pseudintermediusについては、定量PCR(RT-qPCR)にて定量した。
結果 — すべての実験犬において、感作部位に病変が出現した。時間の経過とともに、感作部位における細菌群に変化が認められた。感作7日後には、Fusobacteriaceaeがやや減少した。LEfSe解析により、感作部位では非感作部位と比較して、感作1日後にCorynebacteriaceae、感作7，14，21日後にStaphylococcaceaeの増加がそれぞれ認められた。RT-qPCRと次世代シーケンシングの結果は相関し、感作21日後にStaphylococcus spp.、感作7，21日後にはS. pseudintermediusの有意な増加が感作部位にてそれぞれ認められた。
結論および臨床的な重要性 — 本研究により、抗原暴露により感作された犬は、病変部位においてS. pseudintermediusの増加を伴う細菌叢バランス失調をきたすことが示された。

摘要

背景 — 研究目的是使用细菌16S rRNA基因新一代测序技术,对皮肤细菌种群(微生物区)进行详细调查。
假设/目的 — 研究评估犬异位性皮炎模型的皮肤微生物,其动态变化。
动物 — 八只对屋尘螨(HDM)敏感的异位性皮炎患犬。
方法 — 使用HDM过敏原刺激犬右侧腹股沟。动物接受刺激前(刺激前、基础样本),对侧未受刺激腹股沟相应位点处,分别进行棉拭子取样,并且在刺激后第1、7、14、21和28天分别棉拭子取样。对16S rRNA进行扩增、测序和分析。通过量化聚合酶连反应(RT-qPCR),量化葡萄球菌和假中间型葡萄球菌。
结果 — 所有犬刺激位点均出现病变。位点接受刺激后的不同时间,细菌组成有所不同。相对于对侧位点,刺激位点刺激后第7天,梭杆菌相对较少;相对于LEfSe,刺激后第1天棒状杆菌数量增长;在刺激后的第7、14和21天均在刺激位点发现葡萄球菌。结合新一代测序技术分析RT-qPCR结果发现,刺激位点的葡萄球菌和假中间型葡萄球菌分别在第21天以及第7、21天数量明显增多。
总结和临床意义 — 这项研究表明,敏感动物接受过敏原刺激后,病变位点出现假中间型葡萄球菌大量繁殖,进而导致菌群失调。

Resumo

Contexto – Estudos focados no sequenciamento de nova geração do gene bacteriano 16S tem permitido avaliação detalhada da população bacteriana (microbiota) da pele.

Hipótese/Objetivos – Este estudo avaliou as mudanças temporais na microbiota cutânea de cães, em um modelo experimental canino de dermatite atópica.

Animais – Oito cães atópicos previamente sensibilizados com ácaros da poeira doméstica (APD).

Métodos – Os cães foram desafiados topicamente na virilha direita com APD. Hastes de algodão estéril formam coletadas da virilha no lado desafiado e no lado contralateral, antes do desafio (amostra basal), e nos dias 1, 7, 14, 21 e 28 após o desafio alergênico. O gene 16S foi amplificado, sequenciado e analisado. *Staphylococcus pseudintermedius* foram quantificados por PCR quantitativa (RT-qPCR).

Resultados – As lesões cutâneas foram desenvolvidas em todos os cães nos locais desafiados. Diferenças nos grupos bacterianos foram observadas na região desafiada, ao longo do tempo. Relativamente menor abundância de Fusobacteriaceae no dia 7, e, baseado em LEfSe, abundância aumentada de Corynebacteriaceae no dia 1, e Staphylococcaceae nos dias 7, 14 e 21, foram observadas na região desafiada, comparada com a contralateral. Resultados da RT-qPCR correlacionaram com os encontrados no sequenciamento de nova geração, com aumento significativo nos números de *Staphylococcus* sp. *S. Pseudintermedius,* no dia 21, e nos dias 7 e 21, no local desafiado comparado com o contralateral, respectivamente.

Conclusões e importância clínica – Este estudo demonstra que o desafio alergênico em cães sensibilizados gera disbiose bacteriana com aumento da abundância de *S. pseudintermedius* no local de indução de lesão.

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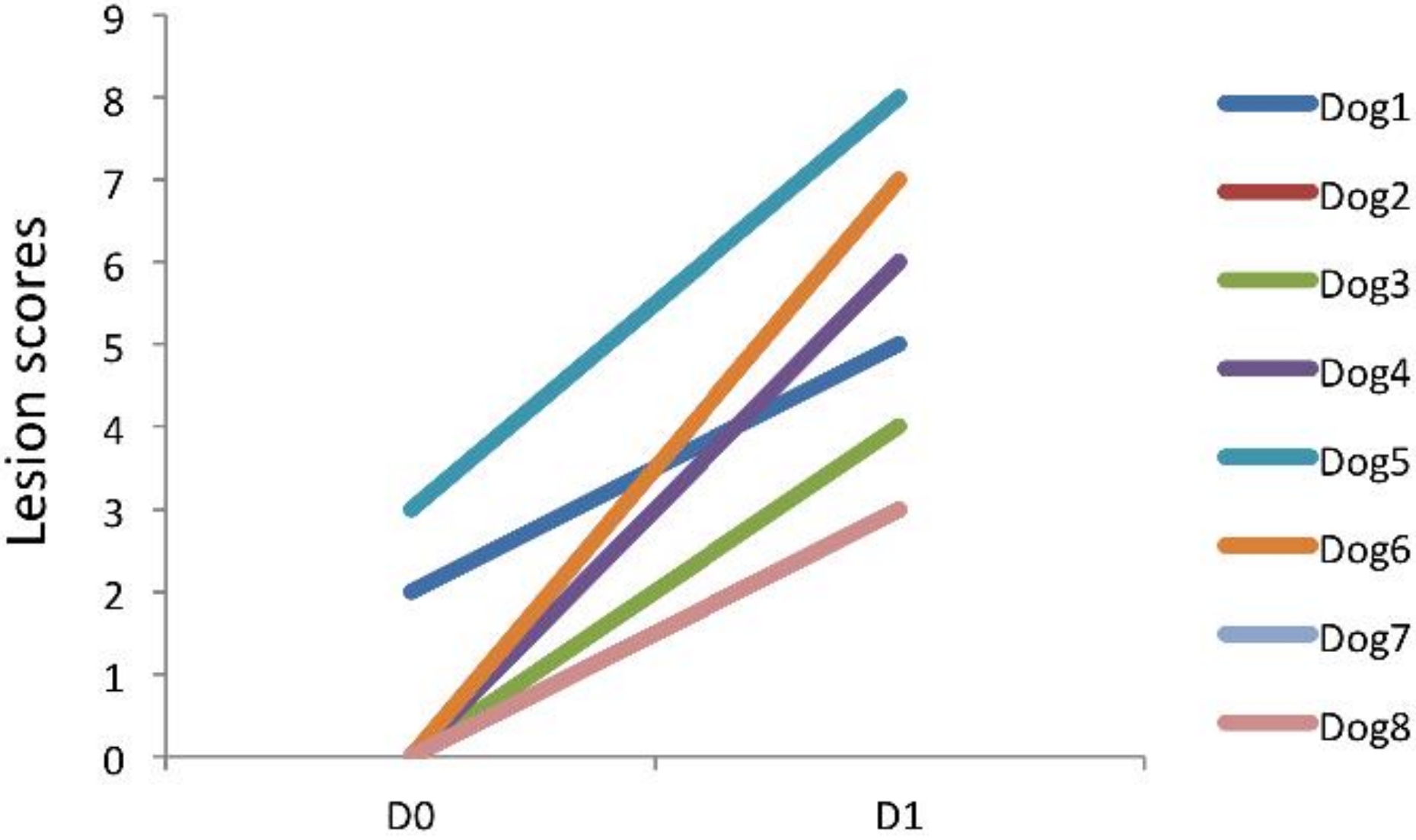
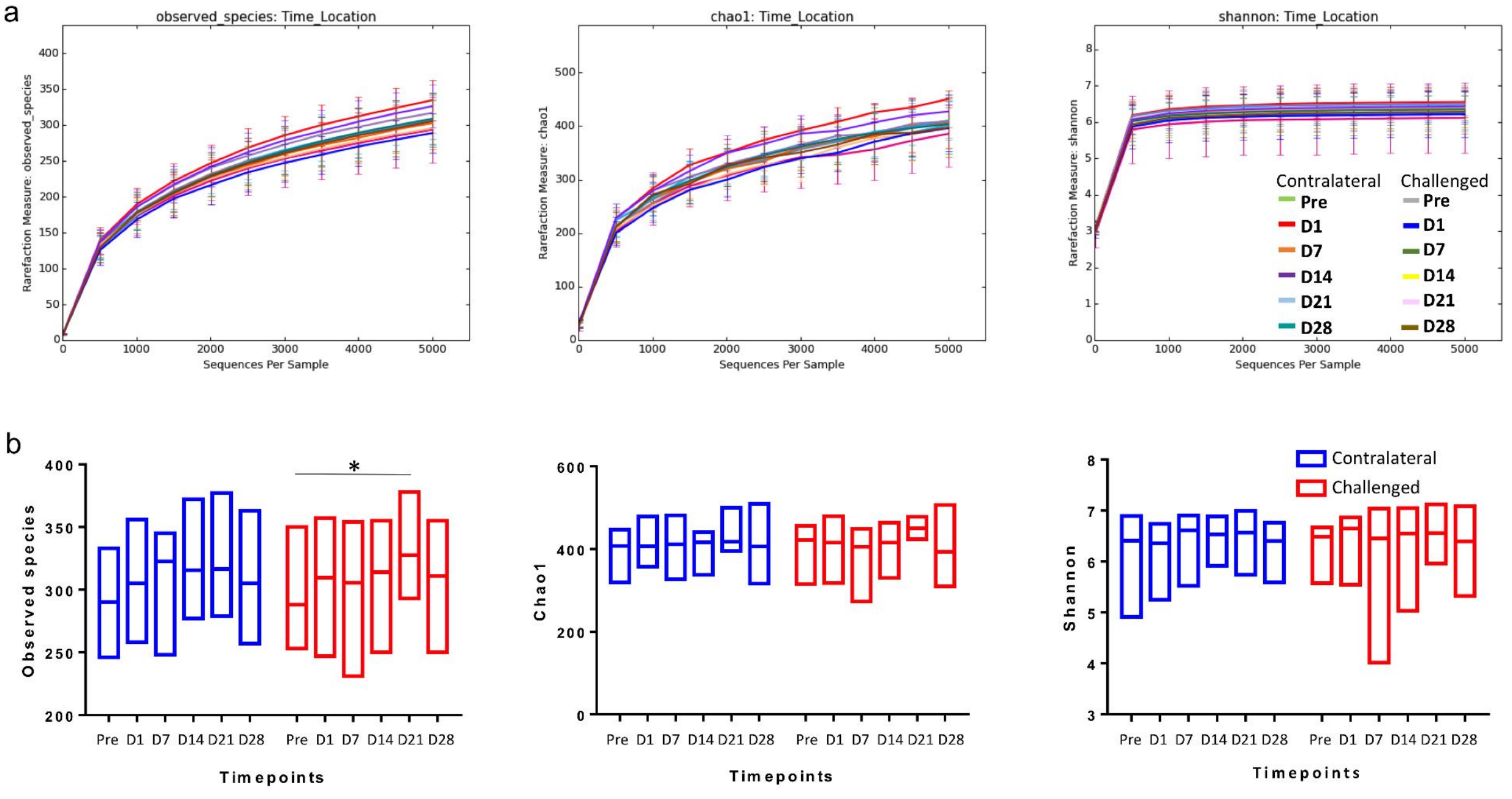
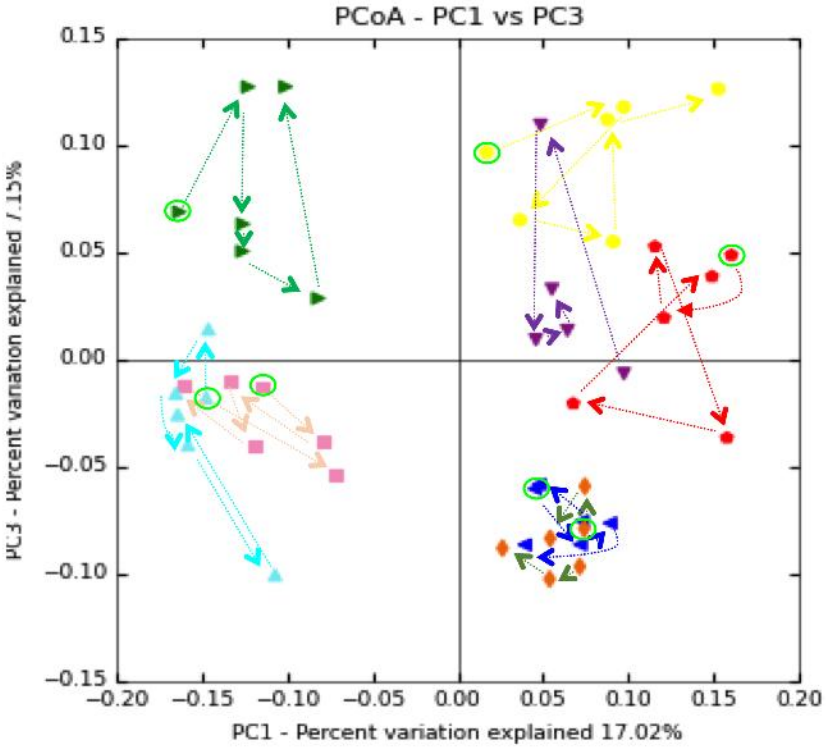
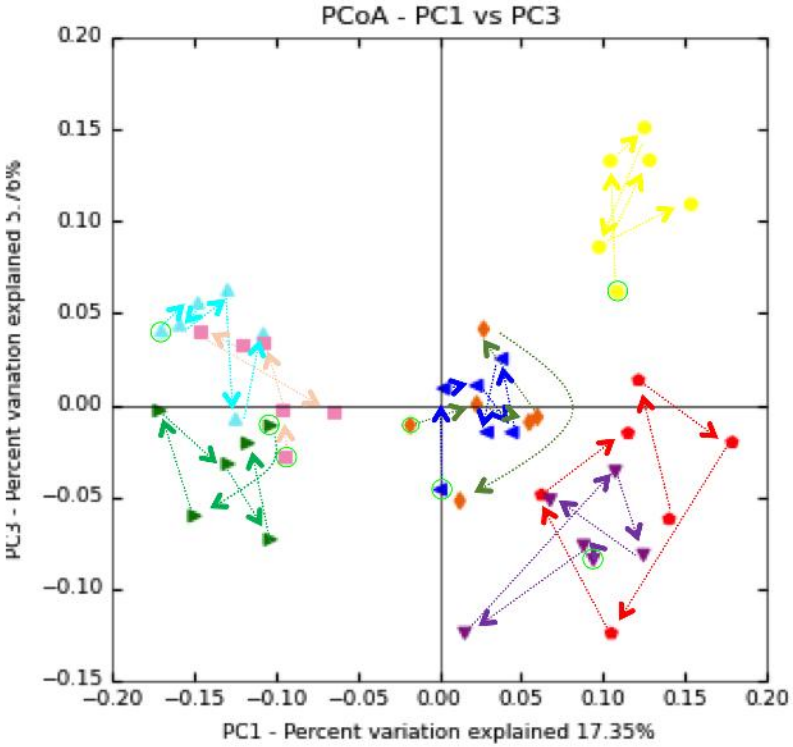

Lesion scores
9
8
7
6
5
4
3
2
1
0
D0
D1
Dog1
Dog2
Dog3
Dog4
Dog5
Dog6
Dog7
Dog8


a
observed_species: Time_Location
Rarefaction Measure: observed_species
Sequences Per Sample
chao1: Time_Location
Rarefaction Measure: chao1
Sequences Per Sample
shannon: Time_Location
Rarefaction Measure: shannon
Sequences Per Sample
Contralateral
Pre
D1
D7
D14
D21
D28
Challenged
Pre
D1
D7
D14
D21
D28
b
Observed species
Chao1
Shannon
Contralateral
Challenged
*
Pre D1 D7 D14 D21 D28 Pre D1 D7 D14 D21 D28
Timepoints

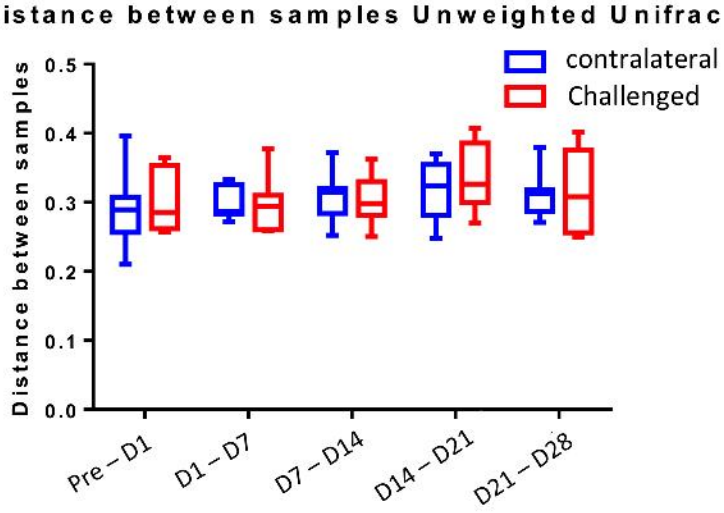
a Challenged

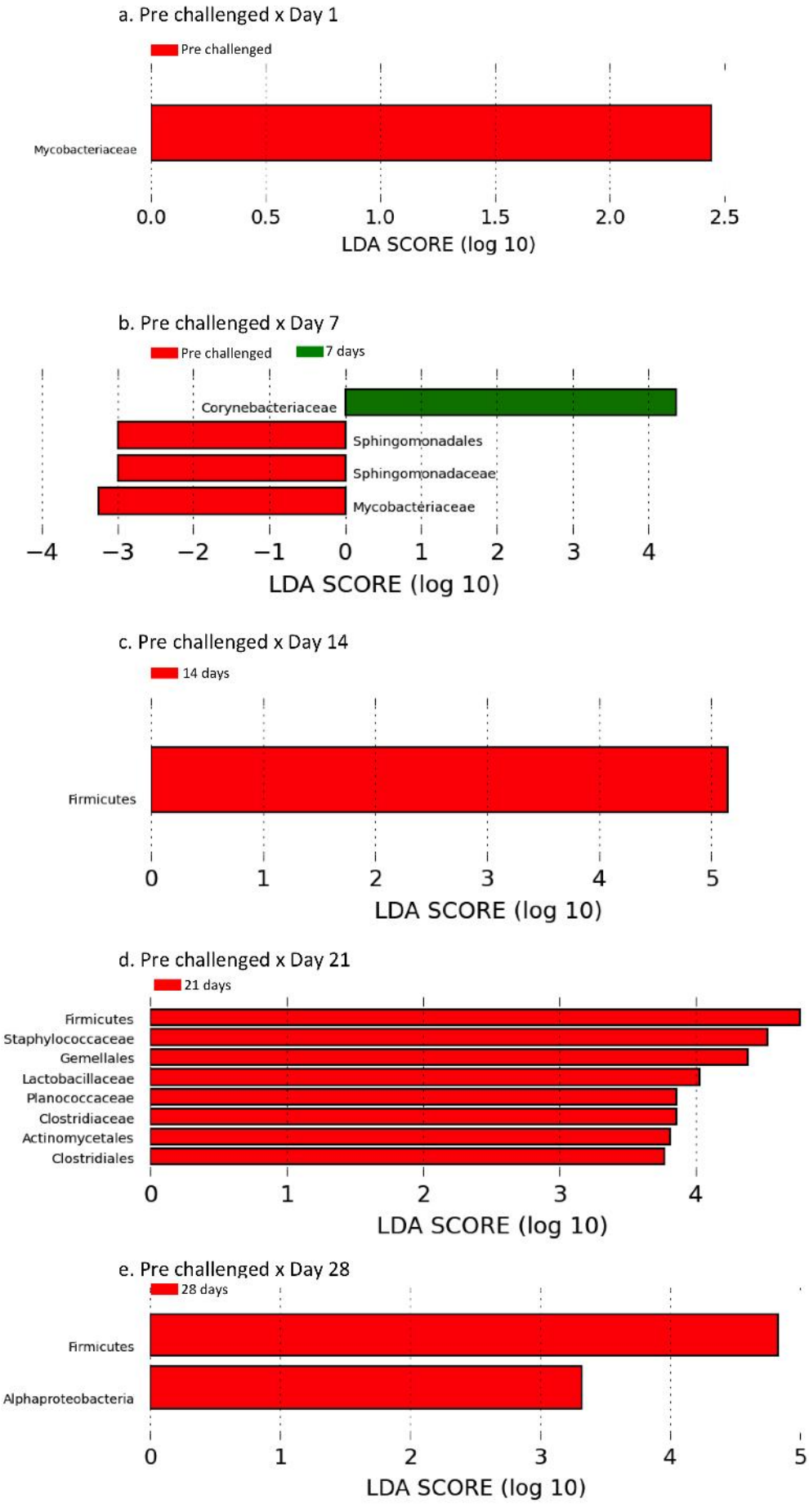


b Contralateral



c




a. Pre challenged x Day 1
Pre challenged
Mycobacteriaceae
0.0
0.5
1.0
1.5
2.0
2.5
LDA SCORE (log 10)
b. Pre challenged x Day 7
Pre challenged
7 days
Corynebacteriaceae
Sphingomonadales
Sphingomonadaceae
Mycobacteriaceae
−4
−3
−2
−1
0
1
2
3
4
LDA SCORE (log 10)
c. Pre challenged x Day 14
14 days
Firmicutes
0
1
2
3
4
5
LDA SCORE (log 10)
d. Pre challenged x Day 21
21 days
Firmicutes
Staphylococcaceae
Gemellales
Lactobacillaceae
Planococcaceae
Clostridiaceae
Actinomycetales
Clostridiales
0
1
2
3
4
LDA SCORE (log 10)
e. Pre challenged x Day 28
28 days
Firmicutes
Alphaproteobacteria
0
1
2
3
4
5
LDA SCORE (log 10)