Response letter

ONE-D-20-10260  
Description and comparison of the skin and ear canal microbiota of non-allergic and allergic German shepherd dogs using next generation sequencing  
PLOS ONE  
  
Dear Dr. Glaeser,  
  
Thank you for submitting your manuscript to PLOS ONE. After careful consideration, we feel that it has merit but does not fully meet PLOS ONE’s publication criteria as it currently stands. Therefore, we invite you to submit a revised version of the manuscript that addresses the points raised during the review process.

The manuscript has been reviewed by two experts in the field who made specific recommendations for improvements, of statistical analyses in particular but also with regard to the clarity of content and presentation of data in figures and tables. The detailed reports are enclosed.

Please submit your revised manuscript by Oct 02 2020 11:59PM. If you will need more time than this to complete your revisions, please reply to this message or contact the journal office at plosone@plos.org. When you're ready to submit your revision, log on to https://www.editorialmanager.com/pone/ and select the 'Submissions Needing Revision' folder to locate your manuscript file.  
  
Please include the following items when submitting your revised manuscript:

* A rebuttal letter that responds to each point raised by the academic editor and reviewer(s). You should upload this letter as a separate file labeled 'Response to Reviewers'.
* A marked-up copy of your manuscript that highlights changes made to the original version. You should upload this as a separate file labeled 'Revised Manuscript with Track Changes'.
* An unmarked version of your revised paper without tracked changes. You should upload this as a separate file labeled 'Manuscript'.

If you would like to make changes to your financial disclosure, please include your updated statement in your cover letter. Guidelines for resubmitting your figure files are available below the reviewer comments at the end of this letter.  
  
If applicable, we recommend that you deposit your laboratory protocols in protocols.io to enhance the reproducibility of your results. Protocols.io assigns your protocol its own identifier (DOI) so that it can be cited independently in the future. For instructions see: <http://journals.plos.org/plosone/s/submission-guidelines#loc-laboratory-protocols>  
  
We look forward to receiving your revised manuscript.  
  
Kind regards,  
  
Kristin Mühldorfer  
Academic Editor  
PLOS ONE  
  
Additional Editor Comments:  
Please check italic type of bacteria names in the Results section. Taxonomic names above family should not be italicized as in the Discussion.

Journal Requirements:

When submitting your revision, we need you to address these additional requirements.

1. Please ensure that your manuscript meets PLOS ONE's style requirements, including those for file naming. The PLOS ONE style templates can be found at

https://journals.plos.org/plosone/s/file?id=wjVg/PLOSOne\_formatting\_sample\_main\_body.pdf and

https://journals.plos.org/plosone/s/file?id=ba62/PLOSOne\_formatting\_sample\_title\_authors\_affiliations.pdf

2. In your Methods section, please state where the participants were recruited for your study.

 - (line 96-97) we added this information. All samples were taken at the small animal clinic of JLU.

3. We note that you are reporting an analysis of a microarray, next-generation sequencing, or deep sequencing data set. PLOS requires that authors comply with field-specific standards for preparation, recording, and deposition of data in repositories appropriate to their field. Please upload these data to a stable, public repository (such as ArrayExpress, Gene Expression Omnibus (GEO), DNA Data Bank of Japan (DDBJ), NCBI GenBank, NCBI Sequence Read Archive, or EMBL Nucleotide Sequence Database (ENA)). In your revised cover letter, please provide the relevant accession numbers that may be used to access these data. For a full list of recommended repositories, see [http://journals.plos.org/plosone/s/data-availability#loc-omics](about:blank#loc-omics) or [http://journals.plos.org/plosone/s/data-availability#loc-sequencing](about:blank#loc-sequencing).

All the raw sequence reads are available in NCBI, in the Sequence Read Archive (SRA) with BioSample Accession numbers SAMN14565128 to SAMN14565223 in the BioProject PRJNA624030, as stated in the lines 195-197

4. Thank you for stating the following in the Competing Interests section:

"The authors have declared that no competing interests exist."

We note that one or more of the authors are employed by a commercial company: Small Animal Clinic AniCura Kleintierspezialisten Augsburg GmbH.

-> The authors working there wrote that Paper based on their scientific interest, there is no link to the Animal Clinic at all. We can confirm that herewith.

4.1. Please provide an amended Funding Statement declaring this commercial affiliation, as well as a statement regarding the Role of Funders in your study. If the funding organization did not play a role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript and only provided financial support in the form of authors' salaries and/or research materials, please review your statements relating to the author contributions, and ensure you have specifically and accurately indicated the role(s) that these authors had in your study. You can update author roles in the Author Contributions section of the online submission form.

Please also include the following statement within your amended Funding Statement.

“The funder provided support in the form of salaries for authors [insert relevant initials], but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the ‘author contributions’ section.”

If your commercial affiliation did play a role in your study, please state and explain this role within your updated Funding Statement.

changed

4.2. Please also provide an updated Competing Interests Statement declaring this commercial affiliation along with any other relevant declarations relating to employment, consultancy, patents, products in development, or marketed products, etc.

Within your Competing Interests Statement, please confirm that this commercial affiliation does not alter your adherence to all PLOS ONE policies on sharing data and materials by including the following statement: "This does not alter our adherence to  PLOS ONE policies on sharing data and materials.” (as detailed online in our guide for authors [http://journals.plos.org/plosone/s/competing-interests](about:blank)) . If this adherence statement is not accurate and  there are restrictions on sharing of data and/or materials, please state these. Please note that we cannot proceed with consideration of your article until this information has been declared.

changed

Please include both an updated Funding Statement and Competing Interests Statement in your cover letter. We will change the online submission form on your behalf.

Please know it is PLOS ONE policy for corresponding authors to declare, on behalf of all authors, all potential competing interests for the purposes of transparency. PLOS defines a competing interest as anything that interferes with, or could reasonably be perceived as interfering with, the full and objective presentation, peer review, editorial decision-making, or publication of research or non-research articles submitted to one of the journals. Competing interests can be financial or non-financial, professional, or personal. Competing interests can arise in relationship to an organization or another person. Please follow this link to our website for more details on competing interests: [http://journals.plos.org/plosone/s/competing-interests](about:blank)

[Note: HTML markup is below. Please do not edit.]  
  
Reviewers' comments:  
  
Reviewer's Responses to Questions

**Comments to the Author**  
  
1. Is the manuscript technically sound, and do the data support the conclusions?  
  
The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.

Reviewer #1: Yes

Reviewer #2: Yes

2. Has the statistical analysis been performed appropriately and rigorously?

Reviewer #1: I Don't Know

Reviewer #2: No

Response: we have now repeated all statistical analysis and considered all our recommendations. An additional author needs to be added to the paper for that reason, Stefan Jannson.

3. Have the authors made all data underlying the findings in their manuscript fully available?  
  
The [PLOS Data policy](http://www.plosone.org/static/policies.action#sharing) requires authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception (please refer to the Data Availability Statement in the manuscript PDF file). The data should be provided as part of the manuscript or its supporting information, or deposited to a public repository. For example, in addition to summary statistics, the data points behind means, medians and variance measures should be available. If there are restrictions on publicly sharing data—e.g. participant privacy or use of data from a third party—those must be specified.

Reviewer #1: Yes

Reviewer #2: Yes

4. Is the manuscript presented in an intelligible fashion and written in standard English?  
  
PLOS ONE does not copyedit accepted manuscripts, so the language in submitted articles must be clear, correct, and unambiguous. Any typographical or grammatical errors should be corrected at revision, so please note any specific errors here.

Reviewer #1: Yes

Reviewer #2: Yes

5. Review Comments to the Author  
  
Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Dear reviewers, thank you for your valuable and helpful corrections/comments. We carefully checked the comments and made respective changes: We gave a respective response to each comment.

Furthermore, while reading the manuscript again we detected an additional mistakes which we corrected. In the lines 343 – 352 it was falsely used the genus *Bacteroides* instead of the phylum Bacteroidetes. We corrected the text. Supplement material as well the pictures did not have this mistake.

We also detected that the use of colors for the allergic and non-allergic dogs was not homogeneous for the graphs of the Figure 3b and Fig 8. We changed Fig3b and now in both figures the non-allergic GSD have yellow color, while the allergic have green. The Figure 2 in S1 had also a mistake due to editing process. The correct version is now uploaded.

Once again thank you for your time and help with your experience in this field, which improved our manuscript.

Reviewer #1: General comments:  
  
This paper describes the skin microbiota of 6 non-allergic and 6 allergic German shepherds dogs. I am not a bioinformatician and therefore I cannot assess the appropriateness of the methods and (statistical) analyses used in this study. It seems rather thorough, but a bioinformatician is needed to check this.

reviewer #1

We would like to point out that our study included 12 allergic and 12 non-allergic GSD. But although our population is 24 dogs (2x 12) it is still not a large population (especially in comparison with human medicine studies). We emphasize this matter in line 607 (referred to as sample size). However, our study includes more patients than other previous studies (see citations line 608 and sentence line 608 to 610), especially including one breed only. One of the studies cited there is also a study published in PlosOne with a population of 12 healthy and 6 allergic dogs. (Hoffmann AR, Patterson AP, Diesel A, Lawhon SD, Ly HJ, Stephenson CE, et al. The Skin Microbiome in Healthy and Allergic Dogs. PLOS ONE. 2014; 9: e83197. doi: 10.1371/journal.pone.0083197.). Unfortunately, in veterinary medicine most of NGS skin studies have a small number of individuals due to several factors (costs, recruiting animals etc.). Given these limitation factors the above studies have been published with very much smaller sample size and as far as we know many of them do not discuss the population issue in their discussion. Therefore, we believe that our study with the current number of studied dogs is in a range for statistical analysis in comparison to other studies and deserves to be published adding with some valuable information to the veterinary medicine as well to the global allergy and microbiology community. Of course, in the discussion section we discussed this limitation factor to be clear to the readers.

Detailed comments:  
  
Material and Methods  
- line 99: please omit 'non-allergic' as this is explained in the same sentence 'without any .....any sampling'. You might choose to mention that these dogs will be referred to as 'non-allergic' in the rest of the study.

Response: We changed the text as proposed.

- line 103-105: by specifically mentioning the need for 2 non-allergic GSDs per household I was triggered. Why 2 and not more? Maybe better to replace 'required' by 'included'? And if this is required in non-allergic dogs, why not in allergic dogs? I realize that it will not be feasible to include two allergic dogs per household, but this sentence triggered me.

Response: We changed the text as you proposed. Indeed, to include more than two non-allergic GSDs per household or two allergic GSDs per household is not feasible at least at the area where we recruited the dogs.

´required` was replaced by ‘included’

- lines 165-170: the bacterial community composition are strongly impacted by the different V regions. Please argue why you chose for V4-V5 and discuss the potential effect on your results.

Response: We added now a paragraph to the discussion about primer selection. 632-647

- line 215: check spelling of 'performed'

Response: done.

- line 222: a closing parenthesis is missing after 'distributed'

Response: done.

- line 226-227: do you mean 'a body site' or 'body sites'?

Response: done. We mean ‘body sites’.   
  
  
Results  
- line 250: replace 'sprayed' with 'spayed'

Response: done.

- line 370: 'changes' implies changes over time. I would suggest to omit 'changes in the' in this title.

Response: The title is changed as recommended.  
  
Discussion:  
In general, the discussion is way too long and some parts are rather speculative/non-informative.

Response: We shortened the discussion.

- line 507-518: Authors write themselves that the influence of pH was not assessed in this study and then expose different inconclusive studies and finish with the need for measurement of pH in future studies. To my opinion lines 509 - 516 can be omitted.

Response: omitted as suggested

- line 543 - 571: please specify in more detail the body sites, animal species and clinical condition of the studies that are mentioned. It is evident that this can influences the results rather strong, so this information is needed to make sure that these comparisons are justified. Without this information the comparison is flawed.

Response: We added these information (species: canine; condition: healthy skin; and in parentheses the skin sites sampled) for the studies as you requested.

In the part on Macrococcus the discussion directs towards the potential pathogenicity of Macrococcus sp. whereas you can also speculate on the potential protective role of this species in the normal microbiota. Any bacterial species can be an opportunistic pathogen (in the right circumstances), but the limited number of reports on the clinical significance of Macrococcus rises the question on its pathogenicity.

Response: We agree to the given statement and changed the text as recommend.

- line 627: do you mean 'counties' or 'countries'?

Response: Corrected.

- line 634: please omit 'is the first'

Response: omitted  
  
Figures:  
- Please reconsider the number of figures, they are numerous.

Response: We removed Fig. 1 c and Fig. 4 (no significant differences) and Fig. 6 to the supplement.

- The captions and legenda are not always completely clear. Especially in Fig. 1c, 2 and 6 it is hard to figure out what are the allergic and non-allergic dogs. The codes/numbers beneath the figures are not immediately clear.

-> we made it more clear and added recommendations

Reviewer #2: This manuscript by Apostolopoulos et al. compares the skin microbiome of healthy German Shepherd dogs (GSD) to that of atopic GSD, at several body sites. Differences between body sites in healthy dogs are discussed, and atopic dogs receiving apoquel are compared to those not receiving apoquel. Significant differences in community composition and richness were found between healthy and atopic dogs, but there was no significant effect of apoquel exposure.  
  
The study is well designed, adequately sized, and the manuscript is well written. In particular I appreciated the attention to detail in recruitment and exclusion of study subjects, and the display of all data in figures where appropriate. My major recommendations for improvement of the manuscript center around the statistical approach and some stylistic choices in figure formatting and labeling. I found the authors’ approach of testing variables (disease state, body site, etc.) singly rather than in a multi-factor model an uncommon choice, and I think the paper needs either a revised statistical approach or a strong a priori explanation and justification of this choice. I was also expecting a comparison of body sites in allergic dogs, paralleling that performed by the authors for healthy dogs, and think this would be an interesting addition to the manuscript. Overall my recommendation is for acceptance pending revisions.

Response: The statistical analysis of the dataset was proofed by a bioinformatician now included as new co-author. Important adds were done, e.g. forward step redundancy analysis.

The comparison of body sites in allergic dogs was done as you suggested. See lines 397 – 400 & 427 – 432.

Major comments:  
  
For the alpha diversity metrics and microbial community composition, I am not convinced that the approach of testing factors singly (body site, allergic state) is the most appropriate choice. Why not a more traditional 2-factor or 3-factor test or a linear mixed model (to account for samples from the same animal), followed pairwise testing as appropriate? As written, it is difficult to compare the relative importance of body site and allergic status in alpha diversity or community composition, which in my opinion is an interesting question.  
  
For differential abundance of specific taxa, it appears that not all taxa were tested, i.e., there was some selection process applied to the taxa. I think this section needs at minimum a more explicit explanation and justification for how the taxa for testing were selected, or (preferably) a method such as ANCOM (Mandal et al 2015, Microbial Ecology in Health and Disease), gneiss (Morton et al 2017, mSystems), corncob (Martin et al 2020, Annals of Applied Statistics), or similar, that accounts for the characteristics of microbiome data and tests all taxa.

Response: All taxa were tested, only most abundant once shown and t-test determination was adjusted to that muli tet

Also, there is no language here (lines 242-244) about multiple test correction where multiple taxa were tested, was this applied? If not, it absolutely should be.

Response: text removed/corrected   
  
In the initial section of results, the microbiome of healthy dogs is discussed; I would have liked to see a similar section discussing patterns within the allergic dogs. Do allergic dogs also have similar alpha diversity and overall microbial composition across body sites? From this, the next section about differences between allergic and non-allergic dogs would flow nicely.

In lines 397 – 400 & 427 – 432 we added this information for the allergic dogs as you suggested. We did not perform a comparison haired vs non-haired for the allergic dogs. Reason for that is that it is unknown if all the body sites are in the same allergy state (also subclinical…we did not perform histology). Thus comparing haired vs non-haired (ear canal…other anatomy) skin in allergic dogs only will be only statistic analysis but without a stable biological/medical reasoning and meaning. We hope you agree with us and the new changes are adequate.   
  
Minor comments:  
  
General readers who are less familiar with atopy in dogs would likely appreciate some discussion about why these body sites were chosen, and some indication (for example in a supplemental table) of which body sites were most the symptomatic for which individual dogs.

In line 145-148 we have stated why we sampled and analyzed these body sites. “These body sites appear to be most commonly affected by atopy in this breed [15]”.

Furthermore, we added in the suppl. Material Table 1, which body sites where affected for each allergic GSD individually, so our readers can have an overview of the clinical picture of each dog. Thank you for this recommendation.   
  
In the discussion section, there is discussion of the key phyla and genera that were detected in this study, and comparison of these taxa with previous studies. The authors explain differences between studies as due to different environmental exposures, daily habits, etc. This may be the case, but I would have liked more discussion of the implications of this claim. Is there evidence that skin microbiome studies are replicable across time in the same animal, either in dogs or other species? What have studies in other animals shown about the consistency of skin microbiomes between different studies, using different techniques? Is the level of disagreement between studies that is discussed here typical of skin microbiome studies, or is it unique to dogs?

Response: To the author`s knowledge there are not studies comparing the results between different NGS studies and their methodology in animals. Only in humans. Information added in the discussion.  
  
Regarding the significant difference in the age of healthy vs. allergic dogs, I appreciate this being discussed as a caveat. It might be useful to add more detail: when does this transition to an “older” skin microbiome start to happen, based on the literature? Is this a similar life stage to either group of dogs in the study For example, are skin microbiome changes only observed in geriatric humans? Dogs in the healthy group are not yet (on average) geriatric.

Age has an effect on microbiota in humans either during the first years of life or with sexual maturation. Thus, it is not a similar age group of our study. But to be more clear we added (line 659-660 ) this information.  
  
I liked that the authors overlaid all data points on boxplots so that distribution of data is more apparent.  
  
In general, I find it easier to look at figures where more of the key information is present in the figure itself rather than in the legend, for example if there are 4 panels of 4 different body sites, I think it’s easier for the reader to have the body site listed in each panel than to have to refer to the letters in the legend. There are several stylistic recommendations to this end in the more detailed comments below.  
-> key information are given to figures

Line 36-38: This sentence could be more clear.

Response: done, now line 40-42

Line 146-147: It’s not clear what is meant by “rotating one-quarter of the swab’s site”.

Response: Done, we added in parentheses the 90°. We were rotating the swab 10 times per quarter of the circle (90°). (line 153)

Line 154: Typo: “swaps”.

Response: Done (line 161)

Line 216-217: Is count data meant by “absolute abundance data”? It seems like the absolute abundance of microbes is not possible to measure with the methods described, because there is no normalization of read counts to amount of DNA, biomass, etc. Please clarify.

Response: As you read/interpreted our statement it is misunderstood, so we removed it here. We just meant that either read counts per taxon or for other analyses directly rel. abundance data of each taxon were used for analysis. data are (line 222-225).

The capter was rewritten with respect to the misunderstanding.

Line 250: Correct “sprayed” to “spayed” (also correct in Table S2).

Response: Corrected.

Line 265-267: This sentence is very unclear. Were only 27.9% of sequences clustered into OTUs? What does the 5.9% denote? Are “replicates” identical sequences or experimental replicates?

Thank you for emphasizing that. We changed the text (new lines 274-280) in order to be more clear. Briefly all sequences (beside the rejected) were assigned in clusters (OTUS). The unique reads clustered OTUs and then the sequences with 98% identity (clustered) and 100% identity (replicates) were assigned to the clusters (as described in the section Material and Method; dereplication and clustering from line 203 to the end of the paragraph). The % numbers is the % of the reads in relation with the total seq. reads, respectively. (new line 274 – 279)

Line 274-276: Because chloroplasts and other removed taxa made up ~20% of the reads, I’d like to see how many reads there were per sample (or at least a min and max) after filtering out these taxa and no-relative reads. In Table 3 S1 you can find all sequences as well as min and max/sample for all groups.   
  
Line 334: Should this be Fig 3a?, changed (line 346)  
Line 339: Should this be Fig 3a? changed (line 351)  
Lines 407-409: Was sample a18O also excluded from the PERMANOVA and ANOSIM analysis? Yes it was excluded from the PERMANOVA and ANOSIM analysis. However, we performed both analysis with a18O and the significance did not changed.  
Lines 412-421: My personal preference would be to see the relative abundance of these taxa in a figure rather than a table, but this is a stylistic choice. Indeed, a picture would also be good but as the reviewer #1 said we already have numerous pictures, so if you agree we will leave the table. But thank you for the recommendation. We will take it into account for our future publications.   
Line 429: Clarify or use generic name, not all readers may know that apoquel = oclacitinib. We missed that mistake. Now we use the generic name (new line 441)  
Line 486-488: Does this figure include data from across all body sites? Please clarify. Yes. We added that information. (line 498)

Line 497: Subject and verb do not agree: “any of those results is useful”. We changed the verb. Line 509  
  
Table S2: I found this table strangely formatted and hard to read as a result. Do you mean S1 Table 2? We agree the format was not nice and we changed it as you proposed.   
Figure S1: There should be color, a separate facet/sub-panel, or some other method to help the reader distinguish allergic from non-allergic dogs in these panels. Dear reviewer, in the figure 1 in S2, are displayed the plots of alpha diversity index Chao 1, only for the non-allergic dogs and therefore there is not a separate panel or color.   
  
Figure 1: The legend should clearly state that panels and b are only for healthy dogs. Panel c needs a better organization of the sample legend, or ideally to have the lines representing different body sites distinguished in some way other than color (weight, dashed, etc.). Alternatively, the authors could facet panel c by body site.  
Figure 2: Because home and sex were significant factors, it would be useful to have these indicated in the figure legend or key. A second facet that shows these factors but not body site might be a good option.  
Figure 3a: It would be helpful to the reader to enlarge the color key to make colors easier to discern.  
Table S6: Typo in table caption: “cCutaneous”. Sampling location and technique for lip commissure body site shown in this table is not described in methods, and this body site is not mentioned anywhere else in the text. Please either remove or explain in the text. changed  
Figure 4: This figure is never cited or discussed in the text. cited  
Figure 7: Sample labels are too small to read. Legend indicates panels should be labeled “A”, “Int”, “L”, “O” but instead labels are a, b, c, d.