« Three-way relationships between gut microbiota, helminth assemblages and bacterial infections in wild rodent populations" by Bouilloud et al.

Dear authors,
Thank you for submitting your article in PCI infections. Your manuscript has been evaluated by two expert reviewers. Apologies for the time it has taken to reach this point. As underlined by both reviewers, I would like to point out the relevance of the questions addressed in this study and commend the authors for the large amount of work and data. This study would unquestionably be an important contribution to the field. However, as rightly pointed out by both reviewers, the main focus/story of the study is lost in the large amount of results and the manuscript should be modified/shortened to improve the readability of the article. Furthermore, several major remarks and comments will have to be considered and addressed before a final decision can be made.

Please address all these points and submit both a revised version of your manuscript and response letter.
I look forward to receiving your revised manuscript.
Yours sincerely
Thomas POLLET
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We thank you for the positive feedback on our work and the constructive suggestions proposed to improve the manuscript. We hope that the new version of this article will be more focused and easier to read.
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Review by Jason Anders, 05 Jul 2022 13:45

Bouilloud et al. examined the gut bacteriome, intestinal hemlinths, and pathogenic bacteria in the spleen of the bank vole *Myodes glareolus* and how they vary geographically and characterize potential interactions between the three. They found that the infra-gut bacteriome and intestinal helminth communities varied geographically, primarily between the most northern site (Mont-sous-Vaudrey) and the most southern site (Cormaranche-en-Bugey) but there was limited geographical variation in the pathogenic bacteria of the spleen.
Associations were also found between the gut helminths and gut bacteriome in terms of both alpha diversity and dissimilarity matrices. There was little evidence to support the claim of geographic differences in the three-way relationship and instead may support the opposite; the associations found may remain relatively consistent regardless of geographic distances or the site specific differences in the gut bacteriome and intestinal helminths which is potentially more interesting.
I commend the authors for the large amount of work that has gone into this manuscript. It is no small feat with a lot packed into a single project. This is an area of study that I myself am particularly interested in and I believe this manuscript has potential to help advance the field. However, the authors get lost somewhere along the way within the results section and lose sight of the main focus of the manuscript as outlined with the clearly stated questions they are addressing in the introduction. Both in the title and introduction the three way relationship between the gut bacteriome, intestinal helminths, and pathogenic spleen bacteria is emphasized as the main point and is arguably the novel (and interesting) aspect of this work.
We thank the reviewer for these encouraging remarks.

Yet only about 25% of the results and discussion are dedicated to this topic.
Furthermore, the discussion of these potential relationships is limited and the authors instead focus more on the impact of each community on the host. From the results section onward this paper reads more as a descriptive study characterizing among site variation in the three communities of interest with some potential interactions included as a sub-topic. This paper has a lot of moving parts which is always difficult to combine into a single story. I think that if the authors decrease the amount of infra- community characterization and instead focus more on apparent associations between the gut bacteriome, helminths, and pathogenic bacteria, it would help to improve the manuscript a lot. Another option of course is to rephrase the overall story of the manuscript so that the apparent associations found are simply a part of the whole story rather than the main point.
We thank the reviewer for his constructive criticism that helped us clarifying more the focus of our manuscript. We agree that the infra- community characterization was making the reading of the ms long and laborious. We shortened this part and focused more on gut bacteriota and its associations with pathogenic bacteria and gastro-intestinal helminth communities.

In addition, I have several concerns regarding the analyses used that the authors must address. I also advise them to be careful when reporting the results of their analyses to avoid misleading the readers. I hope that my comments below addressing these points in more detail among others help the authors in their revisions.
Yes, these comments were very appreciated as they were targeting important weaknesses of our ms and also because they included solutions to write a new version of the ms based on the strong elements of our work. We are very thankful for the time you have dedicated to this review.

Major Comments

1. For the sequence processing step, I recommend you use amplicon sequence variants (ASVs) rather than OTUs. ASVs are a much more robust method and allow for a more accurate characterization of the microbial community as well as greater among study comparisons (See https://doi.org/10.1038/ismej.2017.119 and https://doi.org/10.1128/mSystems.00163-18). Indeed, ecological gut microbiome studies have largely been moving away from the use of OTUs in favor of ASVs. I do, however, understand that this would require you to redo all statistical analyses which is a significant amount of work. I'm also aware that both the medical and veterinary medicine fields still often use OTUs. Therefore, if you do have a sufficiently valid reason for preferring OTUs, I recommend you to provide such an explanation within the main body of text.

Due to the "ASV vs OTU" debate, the term OTU is currently negatively connoted and creates confusion by suggesting that all methods producing OTUs use greedy, input-order dependent algorithms, with arbitrary selection of global cluster size and cluster centroids (fixed clustering threshold, classically at 97% similarity) and are therefore bad. This is of course not the case: the criticism of fixed threshold methods preceded the use of the term ASV and several previously published tools produce ASV-like clusters, including SWARM, the clustering tool used in FROGS and that was applied in this study.
Furthermore, the (yet to be demonstrated) improvement in accuracy of the ASV approach compared to FROGS/SWARM would have no effect here since the community analyses are done at the genus or family levels.
We therefore advocate for the use of the clustering tool included in FROGS. In the new version of the ms, we provided details about the ASV-like clusters produced by SWARM, and specify that 'fine-scale molecular operational taxonomic units (fine-scale OTUs)' were produced.

> Details about SWARM algorithm: "it is a fast and robust method that recursively groups amplicons with ***d* (usually 3) or less differences**. SWARM produces natural and stable clusters centered on local peaks of abundance, mostly free from input-order dependency induced by centroid selection. Exact clustering is impractical on large data sets when using a naïve all-vs-all approach (more precisely a 2-combination without repetitions), as it implies unrealistic numbers of pairwise comparisons. SWARM is based on a **maximum number** of differences *d* between two amplicons, and focuses only on very close local relationships. For *d* = 1 (default value), SWARM uses an algorithm of linear complexity that generates all possible single mutations and performs exact-string matching by comparing hash-values. For *d* = 2 or greater, SWARM uses an algorithm of quadratic complexity that performs pairwise string comparisons. An efficient kmer- based filtering and an astute use of comparisons results obtained during the clustering process allows to avoid most of the amplicon comparisons needed in a naïve approach."
>
> Also note that FROGS is continuously cited since its release in 2018 (316 citations to date).

2. I'm curious as to why you used SILVA SSU Ref NR 119 (Line 207) instead of the 138 release for classifying the taxonomies of your sequences. 119 was released in 2014 and numerous changes in the taxonomical relationships of bacteria have been made and there have also been descriptions of numerous new bacterial taxa (at all levels) in large part thanks to next generation sequencing. I believe that Firmicutes, a group that you focus on in the manuscript, has also had a significant number of changes within it. Therefore, I believe it is more appropriate to use a more updated reference database such as SILVA SSU Ref NR 138 (released in 2020) as this is likely impacting the results of your analyses and may lead to inaccurate conclusions.
We are sorry about this mistake. We used the SILVA SSU Ref NR128 (and not 119). The bioinformatic analyses had been made in early 2018. Unfortunately, the publication process has taken a long time and we were not able to use the most up-to-date version (at the time of submission/revision) of this reference. Nonetheless, we could expect that (a) new version(s) of such reference is(are) likely to be published regularly over time, even between the resubmission and the publication (if accepted) of our manuscript. This means that this is not possible to ensure using the most current, up-to-date version of such an evolutive resource given the delays inherent to the submission-to-publication process. As long as the version number – and all associated details – are transparently stated in the text, we think that this is enough to allow readers understanding and interpreting the outcomes from the study in that perspective.

3. I recommend you have this manuscript checked by a native English speaker as I will only point out a few things. Try to avoid using "transition words" at the beginning of paragraphs such as "besides" on line 110 or "moreover" on line 116. These words connect ideas while each paragraph should be its own complete idea. If you are using a transition word at the beginning of a paragraph, it indicates that it should be part of the previous paragraph. Also, try to avoid using casual words and phrases such as "besides" (line 110) or "whatever" (line 383).
This has been done.

4. Reporting the results of so many statistical analyses is never an easy task. This is something I've also struggled with when working with large datasets. To help simplify things a little and make it smoother for the reader, it is always a good idea to keep the reporting of statistical results consistent. For example, when reporting the results of your GLMs for alpha diversity analyses of the gut microbiome (Lines 372 – 393) you only provide the *p*-value on Line 378 but you provide both the estimate and *p*-value elsewhere (e.g.

Lines 387 & 389). Sometimes you provide both the 95% confidence intervals and *p*-values (Line 381), sometimes only *p*-values (Line 387), and sometimes only the 95% confidence interval (Line 379). Furthermore, sometimes you provide the name of the test used (Line 386), but often times you don't (Line 380). This makes it fairly confusing and difficult to understand.
We have modified the results to make them easier to follow. We have removed delta AIC and confidence intervals. We have indicated the name of the corresponding tests and associated p-values. All details about our results are provided in the supplementary files.

5. To add to my previous comment, to help improve the reporting of your analyses, I recommend you to leave both the delta AIC values and 95% confidence intervals within the supplementary material. Reporting both the CI and *p*-value is a bit redundant and most readers will be more familiar with *p*-values. Also, please provided the standard error as well and the name of the test used in each case.
We have homogenized the statistical results and we have kept the information recommended above in the main text.

6. I have one more comment regarding the structure of the paper. You use the same three sub-title headings (Gut bacteriome, Pathogenic bacteria, & Gastro-intestinal helminths) multiple times throughout the results and discussion. If you instead provide more meaningful sub-titles such as "No geographical change in pathogenic bacteria diversity" on Line 395, it will be easier for the reader to follow as well as find their place again if they go look at one of your nice figures.
We have provided more 'meaningful' sub-titles. We hope that it makes the ms easier to follow.

7. Lines 383-385: This statement is misleading. It implies that your GLM results indicate that the gut microbiome (family level) at all localities differed significantly for both specific richness and Shannon index. But looking at table S2B, for specific richness only at Cormaranche was there a significant effect while for Shannon index there was not significant effect for Chauz-des-Crotenay. Please be careful in reporting your results.
We have modified the text to make these results clearer. The first sentence referred to the significant effect of the 'locality' factor in the GLM performed. The next sentences referred to post-hoc tests that next enabled to determine which pairs of localities showed significantly different levels of diversity.

Minor comments

Line 89: "obvious" is a very strong word with an aggressive nuance. I recommend toning down the language a little and use something more along the lines of "It is important to understand".
Done
Line 91: I'd argue only some studies put an emphasis on this as numerous other aspects are studied in regards to the gut microbiome.
Modified
Lines 91-92: Please be careful with your terminology. "Microbiota" refers to the organisms while "microbiome" refers to their genetics, the same applies to "bacteria" and "bacteriome".
We included the term 'bacteriota' throughout the text to refer to bacteria taxa.
Line 103: What do you mean by "favor"? Do you mean these helminths promote higher abundance of these bacteria or that they are simply associated with higher abundances?
Modified: They may be associated with higher abundances of these bacteria.
Line 121-122 & 132-134: I agree that field studies are an important necessity for understanding real world situations.
Lines 128-129: While I agree that there has been increased interest in the role of co-infections on the host, I would argue that parasitologists have known that helminth co-infections are the norm for as long as they have been collecting and describing parasites.
Done
Line 137: This statement about zoonotic agents is tacked on and requires elaboration to be included. You already have so much packed into this manuscript that I recommend omitting the zoonotic angle as it doesn't add much to the story.
We have removed this part from the ms.
Lines 157-159: Similar to my previous comment, you aren't explicitly studying zoonotic viruses or immune gene expression, so I'd leave out such a statement.

We have removed this sentence.

Lines 183-185: What do you mean by "this part of the digestive tract"? Are you referring the lower gastrointestinal tract (Cecum to rectum)? If so, according to Suzuki & Nachman 2016 that you cite, the cecum has slightly higher microbiome diversity than the colon but it is fairly similar throughout the lower gastrointestinal tract.

We meant 'in the lower segment of the digestive tract'. We have modified the text.

Lines 185-187: How much time passed from the collection of the samples in 2014 to when the extractions occurred? As far as I know, microbiome studies have only investigated the efficacy of 95% ethanol preservation on microbial community analysis for up to 6 months of storage. If the extractions occurred years after collection, there may be DNA degradation to an extent that could be impacting your results.

Extractions were performed in 2016. We agree that storage conditions may affect the composition of microbial community. Here, all samples were collected at the time of rodent dissection and immediately stored in 96° ethanol at -4°C. As such, they have all been stored in similar conditions and along the same time duration – which means that they should have been affected (if any) in the same way by these factors. Moreover, some studies that analysed the impact of storage on gut microbiota have shown that the variation between samples remained greater than that related to differences in storage (eg Nel Van Zyl et al., 2020). We understand that our storage conditions may limit the possibility to compare this study with other ones based on samples stored in different conditions. We have included few sentences and references about this issue in the discussion.

Also, was the intestinal tissue included within the DNA extraction or was it only the gut content? If the tissue was included, it may have impacted the effectiveness of the bead beading step on the microbes themselves. It will also have caused a high proportion of host DNA within the extractions that could impact the PCR amplification step. Both of these things need to be addressed when interpreting the results.

We have removed the gut content to limit the detection of bacteria associated with recently ingested food, and we have extracted DNA from colon tissue only. Indeed, the digestive content can induce several important biases in the analyses due to (1) the presence of PCR inhibitors, (2) the presence of bacterial DNA from plant foods and (3) the presence of chloroplastic DNA that can be amplified in the PCR. In contrast, we did not detect any problem due to the presence of host DNA, probably because the digestive tract contains a high bacterial biomass on the wall. The DNA of these bacteria is therefore present in sufficient quantity to be amplified optimally and without the presence of non-host specific amplification for all samples.

Regarding the bead beating step, it is not affected by the presence of tissue. In our protocol, the tissue is completely digested with proteinase K before the bead beating step. The beads can therefore circulate and impact the bacterial cell walls without any difficulty during grinding on TissueLyzer.

Finally, as all samples were processed under the same conditions, any bias related to the DNA extraction step should likely be equivalent for all samples.

Some of these details are now added to the discussion.

Lines 213 – 215: This is a good way to account for false positives/ contamination, but did you control for the number of sequence reads per sample? More reads inherently leads to more bacterial taxa identified and needs to be controlled for. This could especially be an issue if there is a large discrepancy in the number of reads between the two technical replicates from the sample individual. If one of the replicates has half the number of sequence reads as the other, you could potentially be unnecessarily removing important microbial taxa from your analyses.

At this step, we did not control for the number of sequence reads per sample (this was done later during the community ecology statistical analyses).

But note that at this step, for a given OTU, a single read is enough to consider that a technical replicate is positive. We therefore do not think that this step removes accidentally important microbial taxa.

Lines 227-229: This belongs in the statistics section of your methods.

We have moved this part in the 'material and method section.

Lines 258-260: How did you use Shannon index for measuring alpha diversity of pathogenic bacteria if only their presence / absence was considered (stated on Lines 240-241)? An important factor in the Shannon index calculation is the proportional abundance of each species (evenness). The same reasoning applies to your bray-curtis dissimilarity matrix as that also includes abundances in the calculation.

We are sorry, we thought that Shannon index could also be estimated from 0/1 data. We have only kept the specific richness of helminth and pathogenic bacteria communities.
For beta diversity, we corrected the distances of pathogenic bacteria and helminth communities with the jaccard distance index (presence/absence).

Lines 261-263: Why was capture month not considered as a variable? Seasonal differences, especially in relation to diet, are not uncommon in the gut microbiome.
We have not designed the sampling to test for seasonal variations in gut bacteriota composition and diversity. We have performed the sampling during summer, and sampling localities have been performed during different weeks during the summer. It is therefore not possible to discriminate the influence of environmental/abiotic parameters (eg altitude, forest composition, meteorological variates) vs temporal parameters (date of sampling). As such, we have not including the 'capture month' in our models.

Lines 377-378: I am not familiar with using Tukey post-hoc on a GLM, but do you mean that Mont-sous-Vaudrey and Cormaranche were the only two sites that differed from each other rather than from the other localities (based on Tables S2A & S2B).
Yes, this is what was observed at the phylum and family levels for the Shannon index (gut bacteriota).
For the specific richness, at the family level, significant differences were also found between Cormaranche vs Chaux-des-Crotenay and Cormaranche vs Chatillon.

Lines 388-399: Shannon index doesn't exactly correct for rare taxa if that is what you mean here. It is a different type of alpha diversity that takes into account species abundances as opposed to richness which only looks at the number of species present. Shannon index is more sensitive to rare species than Simpson's index, but in this study that distinction is not so important as you removed rare taxa (<500 sequence reads, Line 217) before you conducted your diversity analyses. That being said, the fact that richness and Shannon index exhibited opposite trends at the family level is interesting.
We modified this sentence to better reflect the specifity of Shannon index.

Lines 465-466: Perhaps I missed it, but I couldn't find the use of Tukey on Betadisper described within the methods section. Please add this.
We have added this in the 'material and methods' section.

Lines 541-544: This is quite interesting and indicates very different associations. What about individuals that harbor both Bartonella sp. and H. mixtum? Focusing more on co-infections like this as the introduction emphasizes would really help to strengthen this manuscript and make it stand out from all the other descriptive papers.
We have checked whether the infections and co-infections of the 3 significant pathogens presented particular points. We just noted that H. mixtum being present only in Corm and Chau, co-infections between H. mixtum and Bartonella sp.

Lines 604-606: Both Anders et al. 2021 and Gu et al. 2013 as well as Suzuki and Nachman 2016 that you cite earlier all found that while the cecum, colon, and feces had some differences in the microbial communities, they were relatively similar, especially in regards to taxa identified.
We have removed this part of the discussion.

Lines 607-608: While you did confirm that it is common for *M. glareolus* to harbor helminths, there have been numerous parasites studies that have done the same (also when it was known as *Clethrionomys glareolus*). For examples, those from Jerzy Behnke (e.g. https://doi.org/10.1017/S0031182001008605 , https://doi.org/10.1017/S0031182008004393 , etc.) and Voitto Haukisalmi & Heikki Henttonen (e.g. https://doi.org/10.2307/5353 focusing on co- infections and should probably be mentioned in your manuscript as that is one of your focuses) along with those that you have cited in the next sentence. I recommend toning down this statement a bit.
We absolutely agree. The authors you cite are major scientists in the fields of parasitology and disease ecology. We have even to admit that we admire them (Heikki has been an important person for the career of one of the co-authors of our paper !). We have therefore added some references to cite their work and emphasize their input in the discipline.

Lines 614-618: Which bacteria or helminths that you found are zoonotic? There was no previous characterization or indication of which are zoonotic or not. Similar to my other comments above, while this is certainly important from a public health perspective, it is outside the scope of this manuscript and feels added on because it is trendy.
We agree and we have deleted this part of the intro/discussion.

Lines 622-626: I recommend paraphrasing this so that it leads directly into the discussion as this is just repeating your results. For example, this could be reduced to something along the lines of, "We found significant inter-individual variation in the gut bacteriome composition
although intrinsic factors such as sex and age played little role. Interestingly, we found that all individuals were clustered within two distinct enterotypes."
We have followed this recommendation.

Lines 641-643: I find this quite interesting. Were these enterotypes associated with specific field sites or did both enterotypes occur at all four sites? Were these four sites ecologically similar? You state that the capture of these animals was conducted in June and September. Are the enterotypes associated with capture date? If so, that could potentially strengthen your argument here if the diets differ between the months. Also, were either of these enterotypes associated with specific helminths or pathogenic bacteria?
Unfortunately, while this analysis enables to define enterotypes, this does not allow to assign individuals to particular enterotypes. It is thus not possible to analyze how these enterotypes are distributed among the four sites studied, or to look for associations between helminths, pathogens and enterotypes.

Lines 692-699: You talk about the helminths ability to immunomodulate the host and how it can impact the gut bacteriome composition indirectly. Is it possible that these bacteria within the spleen can do the same? What about the hosts immune-response to these pathogens, could that effect the gut bacterial composition if it effects the immune system function in the intestine as well?
Indeed, infections with these bacteria induce host immune responses, with potentially local and systemic impacts on the host. However, there are major differences between gastro-intestinal helminths and these bacteria that may suggest that the impact of these latter on helminth's infections may be far weaker that the impacts of helminths on pathogenic bacteria.
- GI helminths induce chronic infections, so that their impact on host immunity can be seen not only at the time of initial infection but also on the long term, while the bacteria described here do not lead to chronic infections
- Most of the bacteria detected using 16S metabarcoding from spleen are not known to be pathogenic for rodents (except Mycoplasma species). As such, we can expect that they may only induce weak and short impact on host immune responses.
Some of these points have been added in the discussion.

Lines 725-727: "First, some infections are significantly associated with the global composition of the gut bacteriome, but have only little impact on specific gut bacterial taxa. This pattern concerns Heligmosomum mixtum, Bartonella and Aonchothecha murisylvatici." This is opposite to what you found and discussed on lines 692-699 "While we did not detect any relationship between pathogenic bacteria and gut bacteriome diversity or composition, we found evidence for strong association between helminth community and gut bacteriome". Why did your mantel tests find no association between the pathogenic bacterial communities and the gut bacteriome but your db-RDA models found associations with specific pathogens? This is an interesting trend that should be discussed further. For example, are the individual helminths or pathogens more important than the whole community?
The results provided by Mantel tests and Rd-DBA are not opposite but complementary, as they do not exactly test the same hypothesis.
Briefly, Mantel test is appropriate for testing hypotheses concerning the variation in beta diversity among groups of sites, but it is less relevant and has less statistical power for questions related to the variation in the raw community composition data among sites, compared to redundancy analyses (Legendre et al. 2005). These latter are more appropriate to partition the β diversity among sites and to test hypotheses about the origin and maintenance of its variation.
This information has been included in the 'material and methods' section.
In addition, the weak relationships observed between the pathogenic bacteria and gut bacteriota communities is discussed in a paragraph of the discussion. The importance of particular taxa compared

to the whole community (helminths and pathogenic bacteria) is also discussed in another paragraph of the discussion.

Lines 726-729: This is a very general statement that could be applied to almost any association found between organisms. Please provide more specific ideas. You go on to discuss the relationship of *Bartonella* and *H. mixtum* with the host, but not with the gut bacteriome as is the focus of this manuscript as well as this section of the discussion. What could potentially lead to *Bartonella* being associated with higher relative abundance of Bacteroidetes or *H. mixtum* with higher relative abundance of Firmicutes? Please elaborate on this aspect instead.
This general statement has been removed. Based on the (scarce) literature available on these infectious agents, we have proposed some scenario in the discussion to explain the relationships between the gut bacteriota, the helminths and Bartonella observed in the ordination analysis Rd-Dba. We have also included the few evidence found between H. mixtum infection and firmicutes abundance. However, due to the lack of literature on these helminths and bacteria, and their link with gut bacteriota, we preferred not to go too far in the discussion of these results.

Lines 736-738: Similar to my previous comment, the relationship between host fitness and their helminths / pathogens, while interesting, is not one of the two main questions outlined in the introduction (Lines 141-144).
We agree and we have removed this statement.

Although you don't necessarily need to discuss all associations that you found, you can explore what is known about *A. murisylvatici* that may lead to apparent associations found with changes in the gut microbiome, other helminths, or pathogenic bacteria.
We agree and we really looked deeply in the literature to find some arguments that could explain the associations observed between Aonchotheca sp and the gut bacteriota. However, this nematode is not very well studied and the few existing studies are descriptive (distribution, taxonomy…).

Lines 745-749: Why would bacterial infections be associated with lower relative abundance of Erysipelotrichaceae especially if this bacterial family is associated with viral infections in humans? Why would the opposite trend be found with pathogenic bacteria in mice? Please discuss.
Unfortunately, we have not found any study that would allow us to understand the reasons why Neoehrlichia sp, Orientia sp, Rickettsia sp or P. omphaloides infections are associated with a decreased abundance of Erysipelotrichaceae in rodents. Studies revealing changes in Erysipelotrichaceae abundance following infections concern human data and tuberculosis or viral infections, with a positive association detected between the level of inflammation and the abundance of Erysipelotrichaceae. We have cited these results, but we have not found any information to explain the pattern that we observe in our study.

Lines 758-760: The gut microbiome is fairly plastic and changes in it do not necessarily mean it becomes dysbiotic. Please elaborate on this point.
We agree with this comment. Because it is important for the whole manuscript, we have added a sentence about gut microbiome plasticity in the introduction.

Figure 1c: Your figures and tables are quite nice. I just want to point out that the y-axis labels on the graph of figure 1c look slightly smashed. If you manual reduced the height of this figure without the width you could be distorting the graph. But it could also be an artifact of R which does sometimes happen. In either case, please double check this.
Done

Review by anonymous reviewer, 19 Aug 2022 09:34

The authors have studied bank voles (*Myodes glareolus*) from four different sites in Eastern France and they surveyed colon bacteriome (based on V4 region of 16S), spleen bacteriome (as gut microbiome) and gastrointestinal helminths (based on GI extraction and morphological identification). Then they did a substantial number of different statistical analyses, including alpha diversity in each community (with two metrics), the effect of rodent characteristics on these alpha diversities and the effect of alpha diversities on alpha diversities; beta diversity with a single metric, the effect of geographical distance on beta diversities and factors shaping beta diversities; changes in bacterial taxa explaining gut bacteriome dissimilarities; interactions between different communities including correlations of dissimilarity matrices, the effects of infectious statues on alpha diversity indices and changes in bacterial taxa explaining significant variables.
I find the study as an important characterization of gut bacteriomes, spleen pathobacteriomes and GI helminths and it is definitely a more and more trendy approach to combine different within-host communities and explore their associations. I find the methods convincing and well-executed and the deposited raw data and scripts seem to be usable. That being said, I feel that while I feel this manuscript has a substantial promise on this account, it is not very clear, what the actual aim is.
We are pleased that the reviewer is supportive of our manuscript and are grateful for the thorough evaluation made below.

My major issue with the paper is its immense and sheer volume. It takes ages to read and it just has so much information on it that I get lost in the manuscript – even though I actually have to read it closely and to deliver this critique. The main issue is two-fold: there is a huge amount of data and specifically statistical tests without any clear headline results or larger narrative standing out from the results. This would make a difficult writing in any context. Furthermore, it is not very clear to me what is the actual aim or focus here, or whether it is just a fishing expedition in the sea of potential statistical methods.
We have already responded to some similar points raised in that sense by the first reviewer (see above) with consistent modification of the manuscript. We have also considered the points raised below to improve more our article. We hope that s/he will find the revised version more clear and focused.

As there is no actual theory-driven hypothesis tested here, the reader has to wonder what is the take-away message. For example, the discussion outlines in the beginning (line 598-618) three main findings: bank vole gut bacteriome has same taxa as previously found, bank voles have abundant helminth infections and bank voles have zoonotic bacteria. I feel like the listing of trivialities sells short your great data set.
I am not sure what is the best way in improving the readability. Maybe move some of the statistics to supplement to focus the discussion. Not every piece of information needs to be discussed or presented here. At least the structure needs to be strengthened and focused.
With hindsight, and with these detailed reviews, we understand the difficulties that the reviewers have faced while reading this manuscript. Indeed, our submitted manuscript mixed two distinct objectives: (1) the description of three intra-host communities and (2) the analyses of associations between these communities. As both reviewers rightly pointed out, this made the reading of the ms confusing and not straightforward. We are sorry for that.
The comments above have enabled us to shorten the manuscript and modify its structure. We now focus on the gut bacteriota and its associations with two other intra-host communities, as described in the introduction. We have removed the analyses specifically dedicated to pathogenic bacteria and gastro-intestinal helminth communities' diversity and composition.

Then there other more tangible major issues:
- I am not sure what is the ecological scale here. The title of the manuscript is a bit misleading. When I think about "small-scale geography", it brings to my mind kilometers, not a hundred kilometer. Why is this the scale? The authors refer to the geographical scale few times, but it is not clear to me what are the implications of this scale.

We agree that we should not have emphasized the spatial scale as an important component of the study. We initially wanted to specify that this research was made at a 'regional' scale and not at a 'large' scale (e.g. between countries) as we had seen in other published studies. We have now removed this from the title, and the geographical scale is detailed in the 'material and methods' section only.

- The authors say in the abstract that they apply the concepts of community and microbial ecology. I am not sure that I agree that they apply the concepts. The introduction mostly deals about tangible physical interactions between host and their symbionts, but I do not really see any, e.g., community ecology framework. The introduction, for example, does not really make clear what counts as an interaction and what is an association and how to tell these apart. These two seem to be used sometimes interchangeably, even though they are not.

We recognize that this study is first and foremost descriptive, and that our analyses enable to detect associations and not interactions. Nevertheless, we think that this study fits well in the context of microbial community ecology as we aim to analyze how biological assemblages are structured and how community structure changes in space.

We have smoothened the text in consequence, and we have made clearer the distinction between associations and interactions throughout the manuscript.

- While the interaction of GI helminths and gut bacterial microbiota is quite understandable, as they are physically in same place and can physically interact, it is not clarified how spleen pathobacteriome relates to this. It is in different place, so there should not be direct interactions? How does one expect the ecological associations to work on this occasion?

There is now strong evidence for interactions between the gut microbiota and extra-intestinal microbiota communities, at least in laboratory mice. This systemic impact of gut microbiota is mediated by host immunity. As such, the gut microbiota produces metabolites (eg bacteriocins, short-chain fatty acids, microbial amino-acids…) that translocate from the intestinal lumen to various organs (e.g., liver, brain, lung) through the circulatory system. This may induce tissue-specific local immune responses, and affect the host's susceptibility/resistance to (non enteric) pathogens. For example, several studies have emphasized the influence of gut microbiota on the inflammasome and induction of proinflammatory cytokines at distal sites. Unfortunately, most of these studies have focused on viruses (eg influenza A, coronaviruses…) and not on pathogenic bacteria.

These elements, and associated references, have been added in the introduction of the manuscript.

- The results are now discussing more statistics than biology. I would rather write out sentences in underlining what is the biological meaning (behind the statistical tests). For example, do not start sentence with a name of the statistical test, but rather the main result.

We have rephrased the 'results' section as recommended, to better reflect the biology and the issues addressed.

- There are so many results on different levels (gut bacteriome, pathogenome, helminths) and different tests, that for example outlining the positive and negative results in a single table would make this very much clearer. Now tracking any significant variables is very difficult and time-consuming.

Because we have strongly simplified the analyses, we do not feel that such table would be useful. We therefore do not have included it in the ms.

Minor issues:

- Table 1: I would use "Full data" as a name for the first set of data. "Total" is now confusing.

Done

Also, the combination of absolute numbers and percentages is confusing.

Absolute numbers of individuals are in grey, and prevalence (percentage) in red. This is detailed in the legend of the table.

- Line 218: "OTUs number of reads" should be something else?

We do not understand this question.

- The in-text references are sometimes misformatted, when they are not in regular type, e.g, line 226, 252, 260,…

We do not understand, references have been formatted using endnote and they are cited as such in the ms.
Author et al., year
Author & Author, year
We have checked carefully the new version of the ms to detect misformats throughout the text.

- Line 310: Should be “absence”?

Yes, sorry, we have modified the text.

- Line 354: “Quality control” rather than “filtration”?

We have modified the text.

- Line 592: What is the intricate thing here?

Sorry. The term ‘relationships’ was enough to describe our objectives and results. We have deleted the word ‘intricate’ in the revised version of the manuscript.

- Line 598: What is the new light here? You frame your study as mainly corroborating previous studies.

We agree. We have modified the text in consequence.