Bouilloud et al. examined the gut bacteriome, intestinal hemlinths, and pathogenic bacteria in the spleen of the bank vole *Myodes rufocanus* 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. 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. 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.

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.

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.

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).

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.

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.

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.

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.

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".

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.

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".

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?

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.

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.

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.

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.

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 degredation to an extent that could be impacting your results. 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.

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.

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

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.

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.

Lines 377-378: I am not familiar with using Tukey post-hoc on a GLM, but do you mean that Mont-sous-Vaudrey and Coraranche were the only two sites that differed from each other rather than from the other localities (based on Tables S2A & S2B).

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.

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.

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.

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.

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.

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.

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."

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?

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 the 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?

Lines 725-727: This is opposite to what you found and discussed on lines 692-699. 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?

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.

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). 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.

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.

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.

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.