Peer Review File

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Reviewer A

Comment 1: This is an interesting topic to pursue and I thank the authors for their efforts to investigate the relationship of fecal microbiome and synovial fluid and PBMC transcriptomes in horses with and without OA. However, there seems to be a distinct flaw in the study design with regards to the control group compared to the OA group, namely the difference in diet, age, and BCS between groups. Because of these population differences, it is difficult to attribute any of the differences detected in this study to the subjects' OA status. I commiserate with the authors that finding horses to enroll in a study can be difficult. However, it is the opinion of this reviewer that these differences make the scientific veracity of this study quite poor for achieving the stated objective and many of the stated conclusions (i.e. concluding the fecal microbiome differed between horses with and without OA; lines 342-344) are misleading. The differences detected in the fecal microbiome, synovial joint transcriptome, and PBMC transcriptome could be attributed to differences in diet, BCS, or age of the enrolled subjects rather than their disease status. If subjects were enrolled based on population-based sampling (versus convenience sampling) then the differences in age and BCS would be of interest, however, with the chosen methods the differences between the populations introduces significant biases. To overcome these limitations, additional groups of horses with obesity + no OA and/or healthy BCS + OA would be recommended to enhance the scientific merit of this study.

Reply 1: Thank you to the reviewer for their thoughtful comments and time in reviewing the manuscript. To address the reviewer's concerns, the authors have provided 1) additional data that was previously analyzed including 62 horses (31 OA, 31 healthy controls) and 2) new correlative data demonstrating that while there was significant overlap in microbial taxa associated with OA as well as elevated age and body condition, there were correlations identified with microbial genera and OA status alone (not age or body condition). The 62 horses presented below were initially enrolled based on population-based sampling identified via clinical and radiographic evidence of OA. As the original goal for this manuscript was to assess microbiomic transcriptomic correlations, the 6 OA cases presented in the original manuscript were those identified to have the most severe OA that had undergone arthroscopy and therefore had available synovial fluid analyses in addition to fecal microbiome for correlative analyses. When this larger OA population was examined for relative abundance of microbial taxa by age (old indicated as >15 years of age, young <15 years) and body condition (lean indicated as 1-5/9 point scale) and obese indicated as >5), there was no difference in relative taxonomic abundance or alpha diversity between old vs. young or lean vs. obese horses. Beta diversity was demonstrated to differ by age within the OA group ($p=0.007$). The authors have addressed the point of age further with the additional correlation analyses performed below, providing those microbial clades that were

solely associated with OA and not age or body condition.

Correlative analyses were added to further address both reviewers' comments (presented below in response to reviewer 2 regarding the correlation of specific genera to OA status, age and body condition. ***To summarize:*** LEfSe (Linear discriminant analysis Effect Size) identified 11 genera that are significantly higher in abundance in the OA samples (*Akkermansia, Camplobacter, p75a5, Phascolarctobacterium, Oscillospira, Pseudobutyrivibrio, Dorea, Butyrivibrio, Blautia, Ruminococcus, Mogibacterium*) and 6 additional genera that are significantly higher in abundance in the healthy feces (*Aneroplasma, RFNN20, Epulopiscium, Sarcina, Clostridium, Fibrobacter, BF311*) (**Figure 8F**). Of these, 2 genera were significantly higher in abundance in OA (*Blautia, Phascolarctobacterium*) and 4 were higher in abundance in healthy feces (*Aneroplasma, RFNN20, Epulopiscium, Sarcina, Clostridium, Fibrobacter, BF311)* but were not associated with age or body condition, indicating the differential abundance appeared solely attributed to OA status (**Table 1**)

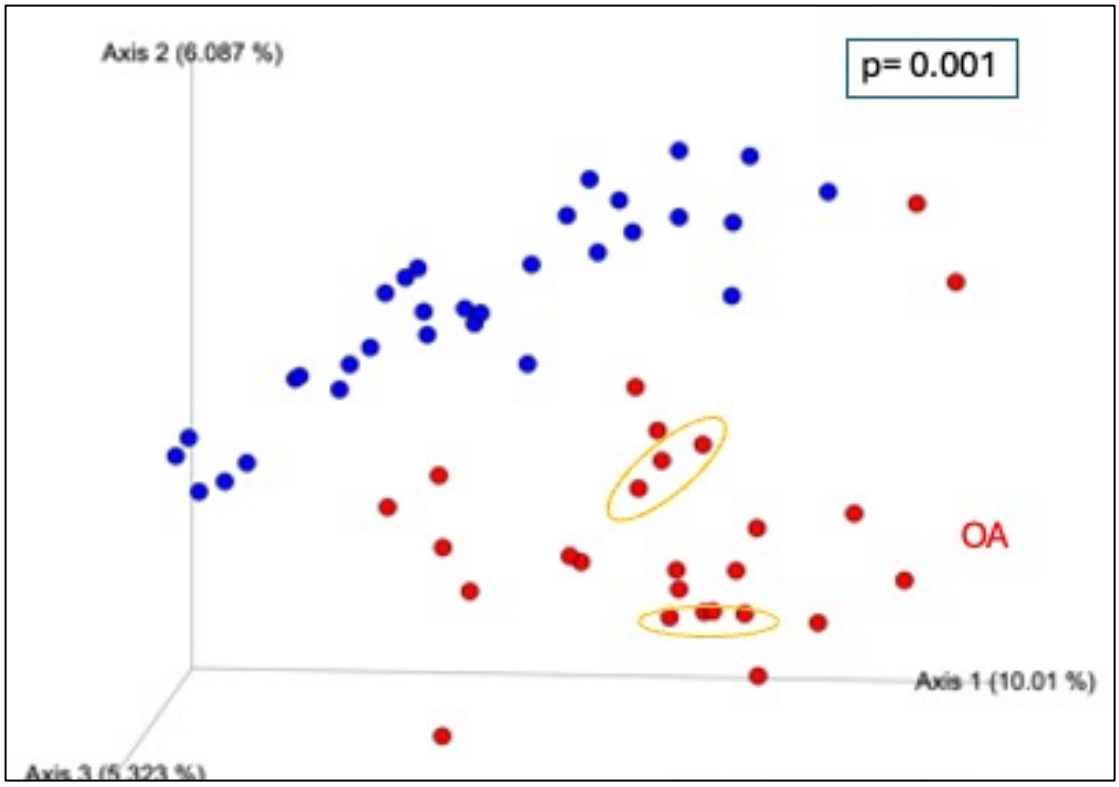
Data shown below include this additional analysis for the reviewer's reference, including **A)** Bray-curtis beta diversity plot of OA versus healthy control horses with expanded data set **B)** average relative abundance on the phyla level of a) old versus young horses and b) obese versus lean horses in the expanded OA group, and **C)** Bray-curtis beta diversity plot of a) old versus young horses and b) obese versus lean horses in the OA group, **D)** Faith alpha diversity phylogenic distance measurement between a) old versus young and b) obese versus lean horses in the OA group.

The reviewer's points regarding the interaction of OA with age and body condition are acknowledged as critically important. The goal of this manuscript was originally to present and focus upon the correlation of fecal microbiome to synovial transcriptome for the most severe OA cases available – further rationale for study design and case enrollment have been added to the results section. The authors are happy to provide this data (summarized below) as a figure or supplemental figure for the manuscript if requested by the reviewer and editors as well.

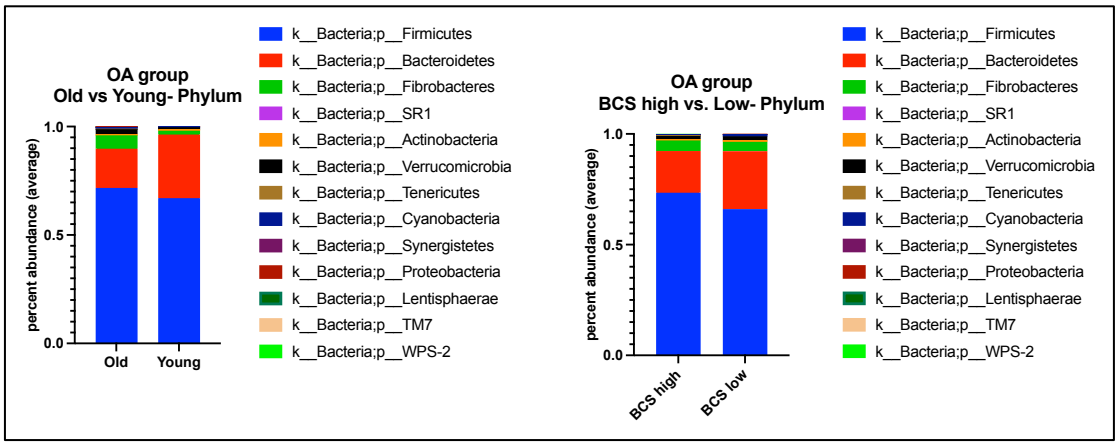
Changes in the text: Additional information has been added to the results section. Correlative analyses have been added as Table 1.

Additional data provided:

A) Bray-curtis beta diversity plot of OA versus healthy control horses with expanded data set (n=62 total, n=31 healthy controls (blue) and n=31 OA) (red).

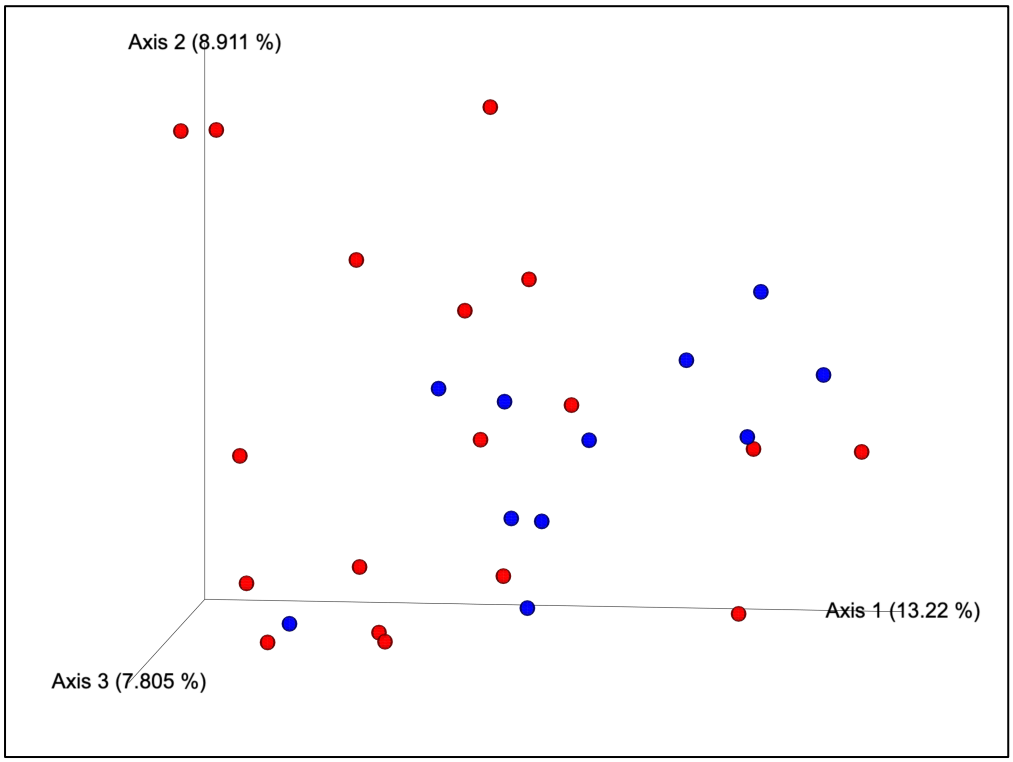


B) Average relative abundance on the phyla level of a) old versus young horses and b) obese versus lean horses in the expanded OA group.

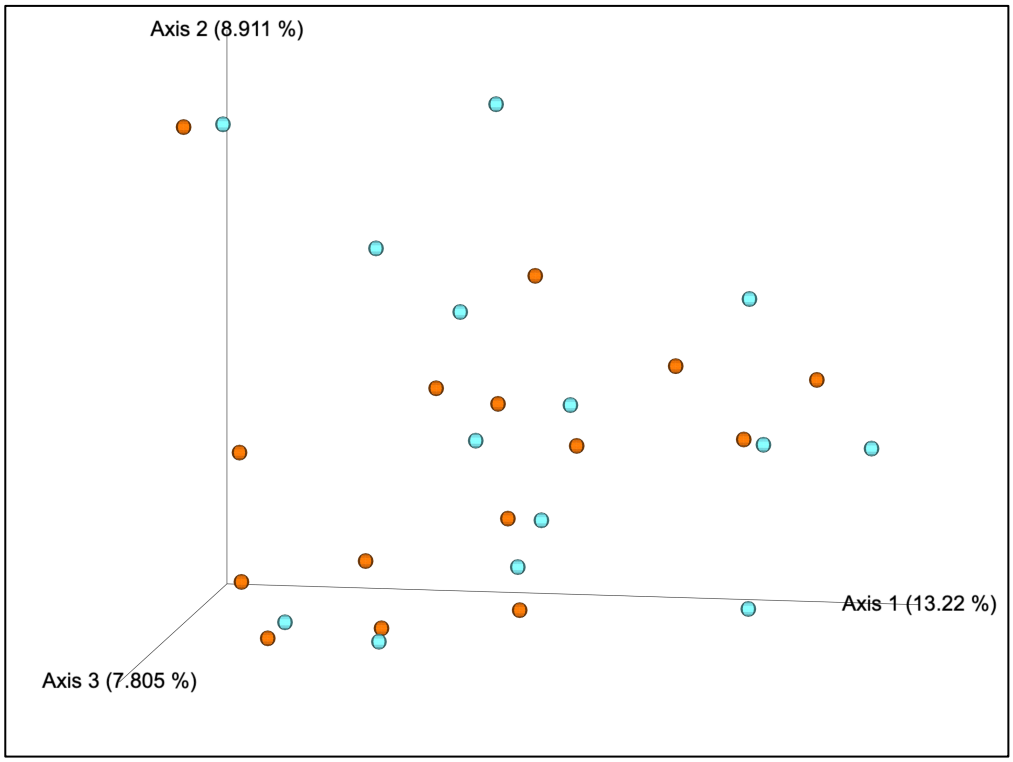


C) Bray-curtis beta diversity plot of a) old (red) versus young (blue) horses (p=0.007) and b) obese (orange) versus lean (light blue) horses in the OA group (p=0.2).

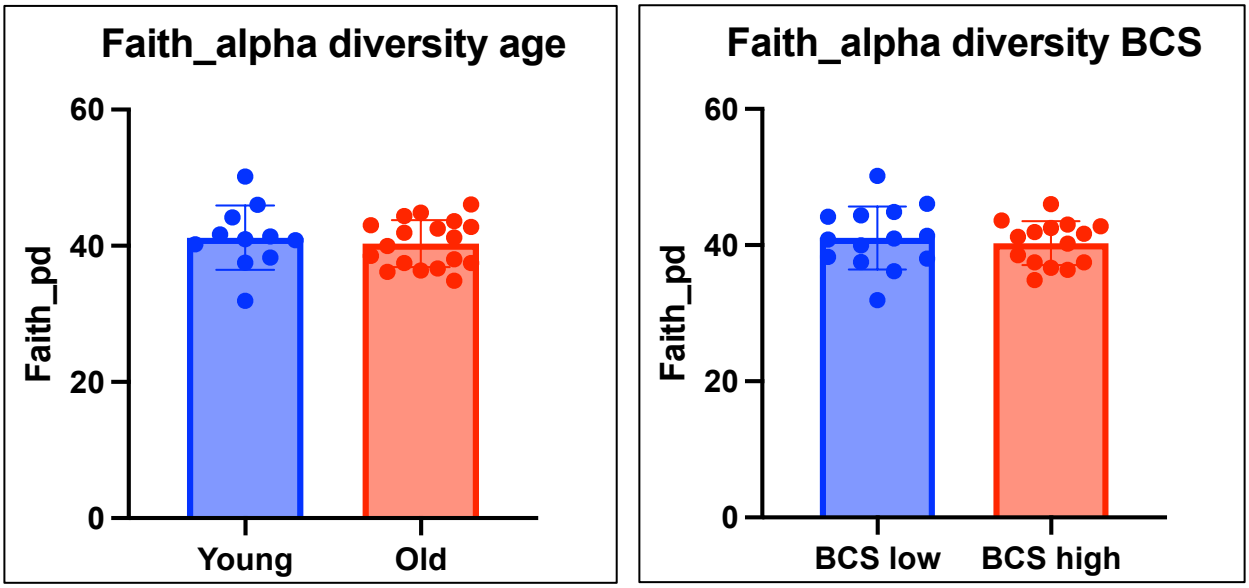
a)



b)



D) Faith alpha diversity phylogenic distance measurement between a) old versus young (p=0.62) and b) obese versus lean (p=0.38) horses in the OA group.



Reviewer B

Comment 1: In this article, the authors sought to compare the fecal microbiome in a naturally occurring equine model of obesity-associated OA and correlate microbiome changes to differential gene expression in cells from synovial fluid, circulating white blood cells, and cytokine levels in plasma and synovial fluid. The study is overall well designed. Power is likely a bit lacking with n=12 healthy and n=6 OA (2 females and 4 males). The OA diagnostic criteria were rigorous. The gut microbiome analysis workflow is rigorous and well described.

Reply 1: Thank you to the reviewer for their time in reviewing and the constructive comments below. As noted in the response to reviewer A and to address the concern related to power noted here, the authors have added back in the analysis of 62 horses (31 healthy control, 31 with OA) from which the 6 most severe cases of OA (those horses undergoing arthroscopy with arthroscopic evidence of OA in addition to clinical and radiographic evidence as originally described) were then selected for further correlative analyses to synovial fluid and PBMC transcriptome. That initial analysis is provided above and has been further described in the methods section as rationale for study design.

Changes in the text: The additional analysis has been described in the methods and results sections.

Questions/concerns:

Comment 2: The study states that horses were fed a consistent diet of grass hay at the time of sample collection, do the authors know how long this diet was fed? It is unclear from mouse studies, for example, how plastic the gut microbiome is /

how long it would take for dietary changes to have a strong effect on the gut microbiome, but data regarding the length of similar-diet treatment in horses would be helpful.

Reply 2: Thank you for requesting this information. The horses had been fed a consistent diet of grass hay at the time of sample collection for a minimum of two months. This has been further discussed in the discussion limitations section to reflect the reviewer's comment that it is unclear from previous studies how plastic the gut microbiome is or how long it would take for dietary changes to have a strong effect on gut microbiome.

Changes in the text: This has been added to the methods section and discussed further in the discussion section.

Comment 3: One of the largest concerns about the study were the significant differences in horse age. In mice and humans age significant alters the gut microbiome; for example, both age and obesity increase levels of Verrucomicrobia (PMID 37946009), the phylum containing Akkermansia that was found in the current study as associated with OA. Interestingly, gut Akkermansia has previously been correlated with better post-traumatic OA outcomes in mice and is enriched in mouse strains protected from OA (PMID 37979958), suggesting species differences in OA risk between mice and horses; a similar story exists for Oscillospira. One potential way to explore this would be to perform correlation analysis of these clades w/ age on an individual horse level and see if strong correlation exists, or if the bulk of differences can be attributed to the OA group effect. The authors should comment on this possibility in the discussion section.

Reply 3: Thank you for making this suggestion – the authors have added correlation analyses on an individual horse level to see whether the differences noted can be attributed solely to OA or if they were also associated with age and body condition. When evaluated by OA status, there were 11 genera associated with OA and 5 with healthy control horses. While many of these were strongly correlated ($r>0.5$) to either age or body condition score, when we eliminate those genera that were correlated to age or body condition, there were 2 genera that were still associated with OA and 4 that were associated with healthy controls. These are presented in the table below with the genera associated with OA specifically highlighted.

Changes in the text: This has been added to the results section with reference to the table. The correlation analyses will be made available as with all other data analyses as per the data sharing section.

Osteoarthritis	Healthy Control
p__Firmicutes.g__Blautia	p__Fibrobacteres.g__Fibrobacter
p__Firmicutes.g__Butyrivibrio	p__Firmicutes.g__Clostridium
p__Firmicutes.g__Epulopiscium	p__Firmicutes.g__RFN20
p__Firmicutes.g__Mogibacterium	p__Firmicutes.g__Sarcina
p__Firmicutes.g__Oscillospira	p__Tenericutes.g__Anaeroplasma
p__Firmicutes.g__p.75.a5	
p__Firmicutes.g__Phascolarctobacterium	
p__Firmicutes.g__Pseudobutyrivibrio	
p__Firmicutes.g__Ruminococcus.	
p__Proteobacteria.g__Campylobacter	
p__Verrucomicrobia.g__Akkermansia	

Comment 4: Similarly, there is concern that the elevation in inflammatory cytokines seen in serum may represent age-associated 'inflammaging' differences rather than bona fide OA-associated differences.
Reply 4: Thank you to the reviewer for bringing up this excellent point. This has been been expanded upon further in the limitations section as an alternative interpretation of the elevation in inflammatory cytokines seen in plasma of OA cases.
Changes in the text: This has been added to the discussion limitations section.

Comment 5: Do the authors know the identity of the synovial fluid cells used for transcriptomic analysis? Presumably these were granulocytes / lymphocytes. Differences in gene expression in a mixed cellular population may have arisen from differences in cell subtype composition within the fluid. Similarly, transcriptomes of PBMCs may have been altered by cell subtype differences although immunophenotying of these mixed cellular populations would likely be beyond the scope of the current study.
Reply 5: Thank you for requesting this information. Although the authors have previously evaluated the identities of synovial fluid cells from horses with and without OA at a single cell sequencing level (manuscript currently in review to this journal), in this study there was insufficient synovial fluid cells to perform additional flow analyses on the cells obtained for RNA sequencing. The reviewer raises an excellent point regarding alteration of PBMC transcriptome by cell subtype difference. The authors have acknowledged both points further in the discussion as limitations of study design beyond the scope of the current work.
Changes in the text: These points have been expanded upon in the limitations section.

Comment 6: Please name the reference dataset used to generate microbiome taxonomy in QIIME2 (greengenes, silva, etc.), this will help other investigators in the future compare and contrast these findings with other microbiome analyses.

Reply 6: Thank you for requesting this information. The qiime2 version used to align the microbiome data is qiime2-2022.8. the classifier is green genes gg-13-8-99-515-806-nb-classifier.
Changes in the text: This information has been added to the data analysis section of the methods.