Dysbiosis of the Gut Microbiome Impairs Mouse Skeletal Muscle Adaptation to Exercise.

Taylor R Valentino, Ivan Vechetti, C. Brooks Mobley, Cory Dungan, Lesley R Golden, Jensen Goh, and John Joseph McCarthy
DOI: 10.1113/JP281788

Corresponding author(s): John McCarthy (jjmcca2@uky.edu)

The referees have opted to remain anonymous.

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 17-May-2021

Dear Dr McCarthy and Mr Valentino,

Re: JP-RP-2021-281788 "Dysbiosis of the Gut Microbiome Impairs Skeletal Muscle Adaptation to Exercise" by Taylor R Valentino, Ivan Vechetti Jr., C. Brooks Mobley, Cory Dungan, Lesley R Golden, Jensen Goh, and John Joseph McCarthy

As promised, following appeal, we are pleased to invite your paper for revision.

The ORIGINAL review reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

Scott K. Powers
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EDITOR COMMENTS

Reviewing Editor:

Thank you for submitting your original research manuscript for consideration by The Journal of Physiology. We recruited two Referees with expertise in this area of physiology to participate in the peer review process. Both express enthusiasm for rigorous studies focused on this important topic. However, you will also see that a number of concerns were raised. These include, but are not limited to the following: 1. Background for the study is built on aging, muscle atrophy, and endocrine consequences of muscle loss; however the study is not designed to address aging, atrophy, or endocrine disruptions; 2. Conclusions are unsupported by the study findings; 3. Sequencing data were superficially examined; 4. Functional outcomes are limited which, in turn, limits interpretability of the study findings. We do hope that the detailed feedback provided through this peer review process is helpful for you as you move forward with this work. Thank you again, and we look forward to seeing more from your research group in the future.

Senior Editor:

Thank you for submitting your work to the Journal of Physiology. Your report was carefully reviewed by two referees and a review editor (RE) that is an expert in the field. Unfortunately, both referee #2 and the RE expressed several concerns that limit enthusiasm about the impact of the study. Therefore, given this consensus of referee opinion, your manuscript will not undergo further review. I am sorry to relay this news and hope that this decision will not prevent the authors from submitting future work to the Journal of Physiology.

REFEREE COMMENTS

Referee #1:

Line 129: Skeletal muscle functions as *a* biological motor

Line 371: delete the period after group

Line 456: The symbols for IFN, TNF didn't convert

Line 493: it could *be* plausible

Only female mice were studied-are these data similar in male mice? This should at least be indicated as a study limitation.

This paper is well written, is experimentally sound, and is an advancement in the gut-muscle field.

Referee #2:

This study examined the impact of gut disruption on how skeletal muscle adapts to exercise. Although this relationship has not been well studied, there are several aspects of the study design, data analysis and manuscript organization that limited enthusiasm for this work.

Generally speaking, the manuscript lacks focus and doesn't demonstrate a clear cause and effect relationship in the data interpretation and study conclusions. For example, the first two paragraphs of the introduction focus on the negative health effects of muscle atrophy, including hospitalizations in the elderly and endocrine consequences of skeletal muscle loss. However, the study does not use an aging model, does not measure any endocrine outcomes, and ultimately isn't look at atrophy, but rather reduced hypertrophy. The introduction should focus on the function of the different muscle types as this is ultimately where the effects were observed.

The discussion/conclusions of the study were also discordant with the actual findings. For example, a major conclusion was that the effects observed in muscle hypertrophy and fiber type were due to differences in microbial metabolites between the antibiotic treated and untreated animals. This is completely unsupported by the existing data as no metabolomics were completed nor did the shotgun sequencing data include an analysis of differentially abundant gene pathways. In fact, the sequencing data is only very superficially examined and discussed in this paper, and much more (including functional profiles) could be gleaned from the data.

The authors also fail to report any functional outcomes in the mice. For example, showing whether time to exhaustion was altered with both the exercise regimen (so untrained animals should be included in the functional test) or microbiota manipulation could have increased the impact and interpretation of the study findings. The only function-related outcome that was reported was total running distance and this was not significantly different among groups; calling into question the physiologic relevance of the muscle-level changes that were observed.

There were also several smaller issues with the methods.

- a qPCR of the 16s gene with universal primers would be helpful in determining the degree of microbiota suppression after antibiotics and improve the ability to interpret the microbiome data presented.

-the analysis of sequence data was not well described, including lack of detail on the processing and quality control of reads and the statistical approaches used in the analysis.

- the model is more a model of microbial suppression than true dysbiosis and should be referred to as such in the manuscript.

- what is the justification for using only female mice, the use of the high protein diet, and for the non-standard 10:14 light cycle?

- antibiotic protocol is not clear. In different places in the manuscript it describes weekly and 2x week replenishment of abx-treated water. This ABX course also frequently results in taste aversion and weight loss in animals. Many groups add things to the water to mask the taste- if this was done, it should be reported.

-why was the water irradiated but the food was not treated?

END OF COMMENTS

Confidential Review 15-Apr-2021

We wish to thank the editor for the opportunity to respond to the reviewers' concerns with the manuscript. We would also like to sincerely thank the reviewers for their comments as they have helped to improve the manuscript which we now hope is acceptable for publication. Below are our responses to The Reviewing Editor, Referee 1 and 2.

Reviewing Editor:

Thank you for submitting your original research manuscript for consideration by The Journal of Physiology. We recruited two Referees with expertise in this area of physiology to participate in the peer review process. Both express enthusiasm for rigorous studies focused on this important topic. However, you will also see that a number of concerns were raised. These include, but are not limited to the following: 1. Background for the study is built on aging, muscle atrophy, and endocrine consequences of muscle loss; however, the study is not designed to address aging, atrophy, or endocrine disruptions; 2. Conclusions are unsupported by the study findings; 3. Sequencing data were superficially examined; 4. Functional outcomes are limited which, in turn, limits interpretability of the study findings. We do hope that the detailed feedback provided through this peer review process is helpful for you as you move forward with this work. Thank you again, and we look forward to seeing more from your research group in the future.

Response: Thank you for your feedback regarding our manuscript. We believe the referees highlighted important issues with our study and feel that each concern is addressable. As you will see we have addressed the concerns of each referees in the responses below.

Referee #1

1. Line 129: Skeletal muscle functions as *a* biological motor

Response: We have edited the text as suggested. Thank you.

2. Line 371: delete the period after group

Response: We have edited the text as suggested. Thank you.

3. Line 456: The symbols for IFN, TNF didn't convert

Response: Thank you for catching this error which has now been corrected.

4. Line 493: it could *be* plausible

Response: We have edited the text as suggested. Thank you.

5. Only female mice were studied-are these data similar in male mice? This should at least be indicated as a study limitation.

Response: We used C57BL/6J female mice for this first study because they are known to be strong runners and were concerned that antibiotic-induced dysbiosis might negatively impact

running performance. We fully agree this is a limitation of the study and have now added a statement addressing this limitation as shown below.

“The findings of the current study are not without limitations. We used only females because they are better runners than males and we were, based on previous studies, concerned the antibiotic treatment might negatively affect wheel running performance [1].”

6. This paper is well written, is experimentally sound, and is an advancement in the gut-muscle field.

Response: Thank you so much for the positive comment. We hope the field will find the results of the study of interest and inspire further research into the role of the gut microbiome in skeletal muscle adaptation to exercise.

Referee #2

This study examined the impact of gut disruption on how skeletal muscle adapts to exercise. Although this relationship has not been well studied, there are several aspects of the study design, data analysis and manuscript organization that limited enthusiasm for this work.

1. Generally speaking, the manuscript lacks focus and doesn't demonstrate a clear cause and effect relationship in the data interpretation and study conclusions. For example, the first two paragraphs of the introduction focus on the negative health effects of muscle atrophy, including hospitalizations in the elderly and endocrine consequences of skeletal muscle loss. However, the study does not use an aging model, does not measure any endocrine outcomes, and ultimately isn't look at atrophy, but rather reduced hypertrophy. The introduction should focus on the function of the different muscle types as this is ultimately where the effects were observed.

Response: Our intent with the first couple of paragraphs of the Introduction was to provide perspective for why the maintenance of skeletal muscle mass is clinically important; however, we fully agree with your comment that the first two paragraphs lack focus and relevance to the purpose of the study. Thank you for pointing out this discrepancy. We have edited the Introduction to focus on exercise induced skeletal muscle adaptions and removed the discussion on aging, atrophy and endocrine contributions to skeletal muscle.

2. The discussion/conclusions of the study were also discordant with the actual findings. For example, a major conclusion was that the effects observed in muscle hypertrophy and fiber type were due to differences in microbial metabolites between the antibiotic treated and untreated animals. This is completely unsupported by the existing data as no metabolomics were completed nor did the shotgun sequencing data include an analysis of differentially abundant gene pathways. In fact, the sequencing data is only very superficially examined and discussed in this paper, and much more (including functional profiles) could be gleaned from the data.

Response: In response to the Referee's concern, we have gone back and re-read the Discussion to edit or remove any discussion that over-interpreted the findings of the study. For example, we have edited the first sentence of the Discussion to read,

"The major finding of the study is that antibiotic-induced gut microbial dysbiosis is associated with an impaired ability of skeletal muscle to adapt to exercise training."

Likewise, we have removed the following sentence from the final paragraph of the Discussion because, as pointed out by the reviewer, is not supported by the findings of the study,

"The current evidence also indicates how the gut microbiome interacts via metabolites through a common mechanism unique for slow-twitch and fast-twitch muscles."

The shotgun sequencing data was provided to show that antibiotic treatment was effective at suppressing the composition of the gut microbiome from 75 bacterial species to 17 bacterial species. We apologize for the lack of details on how the shotgun sequencing was performed and have now provided greater detail in the Methods. If the reviewer found the heatmap to be distracting in the sense that it begs for an in-depth analysis, we can remove it and just report the change in bacterial species with antibiotic treatment. An in-depth analysis of the metagenomic data is currently ongoing and is the focus of a separate study seeking to determine the impact of exercise on the composition and function of the gut microbiome.

3. The authors also fail to report any functional outcomes in the mice. For example, showing whether time to exhaustion was altered with both the exercise regimen (so untrained animals should be included in the functional test) or microbiota manipulation could have increased the impact and interpretation of the study findings. The only function-related outcome that was reported was total running distance and this was not significantly different among groups; calling into question the physiologic relevance of the muscle-level changes that were observed.

Response: We completely agree with the Referee's comment regarding the lack of functional data except for wheel running performance. This an important limitation of the study which we have now acknowledged in the Discussion as shown below:

"…Beyond wheel running performance, we did not perform any skeletal muscle functional analyses to determine if the morphological differences between the PU and PT groups was associated with any change in function. Finally, there is a wide variety of rodent diets used in pre-clinical research which have been reported to significantly alter the composition of the gut microbiome and the production of short-chained fatty acids [2]. Importantly, all mice in the current study consumed exactly the same high protein diet for the duration of the study to insure adequate protein intake to support skeletal muscle growth in response to PoWeR training."

Although additional function data would enhance the findings of the study, its absence does not lessen the significance of the primary finding of the study that disruption of the gut microbiome is associated with an impaired ability of skeletal muscle to adapt to exercise.

As mentioned in response to comment #2, we are currently analyzing metagenomic data to determine if exercise alters the composition and function of the gut microbiota. We hope this analysis will provide further insight for how we might manipulate the microbiota to regulate skeletal muscle mass; however, we think the manipulation of the microbiota is at this time beyond the scope of the study.

The finding that antibiotic and untreated mice had the same running performance was an important finding because it was the training stimulus responsible for inducing myofiber hypertrophy and the fiber-type shift. Had there been a difference in running performance, it would have complicated interpretation of the data as such differences could account for the impaired skeletal muscle adaptations observed in antibiotic-treated mice.

4. There were also several smaller issues with the methods.

4-1. a qPCR of the 16s gene with universal primers would be helpful in determining the degree of microbiota suppression after antibiotics and improve the ability to interpret the microbiome data presented.

Response: Respectfully, we think the metagenomic data clearly demonstrated that antibiotic treatment was effective at suppressing the composition of the gut microbiome and do not think 16S qPCR provides important new information regarding the effectiveness of the antibiotic treatment. As mentioned above, we are currently performing an in-depth analysis of the metagenomic data to determine how exercise (weighted wheel running) might alter the composition and function of the gut microbiome. However, if the Referee thinks there is a better way to present and/or analyze the metagenomic data to show the effectiveness of the antibiotic treatment to suppress the gut microbiome, we welcome the opportunity to perform such an analysis and/or presentation of the data.

4-2. the analysis of sequence data was not well described, including lack of detail on the processing and quality control of reads and the statistical approaches used in the analysis.

Response: We apologize for not providing sufficient information regarding the processing and quality control for the analysis of the sequencing data. We have edited the appropriate section in the Methods by providing more details for how the sequencing data was processed.

4-3. the model is more a model of microbial suppression than true dysbiosis and should be referred to as such in the manuscript.

Response: We have edited the title, text and figure legends to replace any reference to “dysbiosis” with “suppression”.

4-4. what is the justification for using only female mice, the use of the high protein diet, and for the non-standard 10:14 light cycle?

Response: We used C57BL/6J female mice for this first study because they are known to be strong runners and were concerned that antibiotic treatment might negatively impact running performance. We fully agree this is a limitation of the study and have now added a statement addressing this limitation as shown below.

“The findings of the current study are not without limitations. We used only females because they are better runners than males and we were, based on previous studies, concerned the antibiotic treatment might negatively affect wheel running performance [1]”.

A high protein diet was used to insure mice received adequate protein to support skeletal muscle growth in response to weight wheel running. Importantly, all mice were feed exactly the same diet throughout the study to insure a consistent influence on microbiota composition.

The 10:14 light:dark cycle is the standard light:dark cycle used in the animal facilities at the University of Kentucky. Kim and colleagues reported that changes in the light:dark cycle can influence the composition of the gut microbiome; importantly, the 10:14 light:dark cycle was constant for the duration of the current study [3].

4-5. antibiotic protocol is not clear. In different places in the manuscript it describes weekly and 2x week replenishment of abx-treated water. This ABX course also frequently results in taste aversion and weight loss in animals. Many groups add things to the water to mask the taste- if this was done, it should be reported.

Response: We apologize for the confusion regarding the administration of antibiotics. We have edited the manuscript to clarify how antibiotics were administered. As mentioned in the Discussion, we used a dose of antibiotics that was 10-100 times lower than previous studies such that we saw no difference in water consumption between treated and untreated non-runners (5.28 ± 0.28 ml/day and 4.56 ± 0.09 ml/day, respectively) or runners (5.78 ± 0.45 ml/day and 5.29 ± 0.12 ml/day, respectively) without the need to add sweetener to the water.

4-6. why was the water irradiated but the food was not treated?

Response: We apologize for the confusion. The water was autoclaved and the food irradiated as part of the standard operating procedure at the University of Kentucky. We edited the Methods section to accurately describe these details.

References

1. Murach, K.A., et al., *Making Mice Mighty: recent advances in translational models of load-induced muscle hypertrophy.* J Appl Physiol (1985), 2020. **129**(3): p. 516-521.
2. Tuck, C.J., et al., *Nutritional profile of rodent diets impacts experimental reproducibility in microbiome preclinical research.* Sci Rep, 2020. **10**(1): p. 17784.
3. Kim, Y.M., et al., *Light-Stress Influences the Composition of the Murine Gut Microbiome, Memory Function, and Plasma Metabolome.* Front Mol Biosci, 2019. **6**: p. 108.

1st Revision - Editorial Decision 03-Aug-2021

Dear Dr McCarthy,

Re: JP-RP-2021-281788R1 "Antibiotic Suppression of the Gut Microbiome Impairs Skeletal Muscle Adaptation to Exercise." by Taylor R Valentino, Ivan Vechetti Jr., C. Brooks Mobley, Cory Dungan, Lesley R Golden, Jensen Goh, and John Joseph McCarthy

Thank you for submitting your revised Research Paper to The Journal of Physiology. It has been assessed by the original Reviewing Editor and Referees and has been well received. Some final revisions have been requested.

Please advise your co-authors of this decision as soon as possible.

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To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Word, or similar, file and respond to each point in colour or CAPITALS and upload this when you submit your revision.

I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

Scott K. Powers
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-You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

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-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

-If n {less than or equal to} 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

-If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

-'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

-All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

-The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

-Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

-Statistics Summary Document completed appropriately upon revision

-A Data Availability Statement is required for all papers reporting original data. This must be in the Additional Information section of the manuscript itself. It must have the paragraph heading "Data Availability Statement". All data supporting the results in the paper must be either: in the paper itself; uploaded as Supporting Information for Online Publication; or archived in an appropriate public repository. The statement needs to describe the availability or the absence of shared data. Authors must include in their Statement: a link to the repository they have used, or

a statement that it is available as Supporting Information; reference the data in the appropriate sections(s) of their manuscript; and cite the data they have shared in the References section. Whenever possible the scripts and other artefacts used to generate the analyses presented in the paper should also be publicly archived. If sharing data compromises ethical standards or legal requirements then authors are not expected to share it, but must note this in their Statement. For more information, see our Statistics Policy.

-Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

EDITOR COMMENTS

Reviewing Editor:

The authors used an antibiotic treatment and a weighted wheel running intervention to assess whether changes in muscular hypertrophy, fiber type conversion, and satellite cell accumulation induced by exercise are reliant on the gut microbiota. The study design is novel and has the potential to describe the gut microbiota as a key regulator of skeletal muscle responses to exercise. Whereas Reviewer 1 has only relatively minor comments, Reviewer 2 points to the absence of data specifically dealing with muscle function. This could change data interpretation and limit the scope of the manuscript. The authors should convincingly address this issue. The fact that the study was carried out on mice should be mentioned in the title.

Senior Editor:

Thank you for submitting your work to the Journal of Physiology. Your revised manuscript has been carefully reviewed by two new reviewers and a new review editor (RE). I am happy to report that the referees and RE agree that your work has merit but please note that some minor revisions are suggested prior to acceptance. Please consider review comments carefully in revising your report. We look forward to receiving your revised manuscript.

REFEREE COMMENTS

Referee #1:

Why were only female mice used? It should be included as a limitation that it is unknown if these effects are also present in male mice.

Line 456-IFN-*gamma* and TNF-*alpha* have squares instead of the starred words.

Line 586: The current evidence also indicates how the gut microbiome interacts via metabolites through a common mechanism unique for slow-twitch and fast-twitch muscles.

-No metabolites were measured in this study. Measuring gut bacterial metabolites under these same experimental conditions should be proposed as a future study.

Referee #3:

The authors use a 2 x 2 design with antibiotic treatment and a weighted wheel running intervention (PoWER) to assess whether changes in muscular hypertrophy, fiber type conversion, and satellite cell accumulation induced by exercise are reliant on the gut microbiota. This is a novel study design that has the potential to describe the gut microbiota as a key regulator of skeletal muscle responses to exercise.

A primary concern (also brought by the previous reviewers) is that the manuscript contains no outcomes directly assessing muscular function. There are a number of outcomes (treadmill performance, grip strength, etc) that could have been assessed. It is unclear why these tests were omitted by the authors. Such data would provide much more context as to the significance of CSA and fiber type data presented by the authors. Wheel running does not constitute a performance measure as it is voluntary and submaximal. If the authors are to present the data as is (without functional outcome measures), the title of the paper needs to be changed. As is, reporting that muscle adaptations are 'suppressed' is a bit speculative and misleading. Title and discussion should instead focus on specific outcomes that authors reported in the paper (e.g. CSA deficits by Abx).

The authors have no biochemical data of mitochondria adaptations outside of fiber type staining. A measure of mitochondria activity (citrate synthase and/or succinate dehydrogenase) would greatly strengthen the manuscript. Especially in the plantaris muscle, where authors argue a blunted increase in type 2a fibers in Abx treated mice.

It appears that satellite cells are increased in Control-Abx treated animals (CT vs CU; Fig 8). The authors should discuss this as it a potential limitation to their interpretation of the data. Because there is no significant differences exist between PU and PT, a claim that Abx suppresses satellite cell accumulation is not supported by these figures. Throughout the manuscript, authors should report post-hoc P values to compare differences between PU and PT. This is the most important comparison.

Minor Weaknesses:

The authors should reconsider their group notation. "T" and "U" reminds me of Trained vs. Untrained instead of Treated (Abx) vs. Untreated. Please reconsider notation.

In the introduction lines 130-132 authors state that previous data suggests germ free mice are more reliant on fatty acid oxidation yet much of the discussion refers to the role of gut microbes in mediating the increase in muscle oxidative fibers, this discrepancy is not addressed in the discussion.

Lines 137-142 - These sentences seem out of place, don't really seem relevant to the data reported.

Lin 199- Actimetrics appears to be misspelled.

Lines 457-459- This sentence is not clear. Please revise.

Lines 566-576 - This paragraph probably isn't necessary, since no measurement of metabolites was conducted.

Line 471- Authors should remove statement that their data supports the findings that microbiota regulates skeletal muscle "function". This is not supported by the data presented in this manuscript.

Line 278, 475 and 586 - ensure, not insure

No sources cited to justify the relevance of the cytokine profile.

Given the only performance based outcome they have is voluntary, I would refrain from using words like "performance" or "capacity" when referring to this data, as it is misleading. Authors should consider words like "activity" (see lines 480-482 and 511).

Line 530-532 - this sentence needs to be revised

END OF COMMENTS

1st Confidential Review **24-May-2021**

Reviewing Editor
The authors used an antibiotic treatment and a weighted wheel running intervention to assess whether changes in muscular hypertrophy, fiber type conversion, and satellite cell accumulation induced by exercise are reliant on the gut microbiota. The study design is novel and has the potential to describe the gut microbiota as a key regulator of skeletal muscle responses to exercise. Whereas Reviewer 1 has only relatively minor comments, Reviewer 2 points to the absence of data specifically dealing with muscle function. This could change data interpretation and limit the scope of the manuscript. The authors should convincingly address this issue. The fact that the study was carried out on mice should be mentioned in the title.

Response. We thank the Reviewing Editor and Referee 1 and 2 for the opportunity to respond to their concerns with the revised manuscript. We have tried to respond fully to each of the concerns and hope this revised manuscript is now acceptable for publication in the *Journal of Physiology*.

Referee #1
1. Why were only female mice used? It should be included as a limitation that it is unknown if these effects are also present in male mice.

Response: Previous studies investigating the influence of the gut microbiome on exercise performance, using either antibiotic-treated or germ-free mice, found that disruption of the gut microbiome had a negative impact on exercise performance (Hsu *et al.*, 2015; Nay *et al.*, 2019). These findings were a major concern for us as we designed the study because, if wheel running performance (*i.e.,* training stimulus) was different between untreated and antibiotic-treated mice, it would limit our ability to make any conclusions regarding the influence of the gut microbiome on skeletal muscle adaptation to exercise. We knew from our experience using progressive weighted wheel running (PoWeR) that C57BL/6J female mice are much better runners than males so we decided to use females for this initial investigation with the hope the running activity was similar between the two groups. Since there was no difference in the running performance between the two groups, thus an equivalent training stimulus, we were able to draw stronger conclusions about the influence of the gut microbiome on skeletal muscle adaptation to exercise. To address this concern, we have edited the manuscript to acknowledge this important limitation of the study as shown below:

“The current study has some important limitations that need to be acknowledged. For this initial study, we chose to use C57BL/6J female mice because they are known to be better runners than their male counterparts (Murach et al., 2020) and we were concerned the disruption of the gut microbiome might negatively impact exercise activity as previously reported (Hsu et al. 2015; Nay et al. 2019). Since the running activity was the same between the antibiotic-treated and untreated groups, thus having an equivalent training stimulus, we were able to draw stronger conclusions about the influence of the gut microbiome on skeletal muscle adaptation to exercise. Given the reported sexes differences in host-gut microbiome interactions, it will be important for a future study to determine is dysbiosis in males also blunts muscle adaptation to exercise training as observed in females (Rizzetto et al., 2018; Razavi et al., 2019).

Cho DS & Doles JD. (2017). Single cell transcriptome analysis of muscle satellite cells reveals widespread transcriptional heterogeneity. Gene **636,** 54-63.

Hsu YJ, Chiu CC, Li YP, Huang WC, Huang YT, Huang CC & Chuang HL. (2015). Effect of intestinal microbiota on exercise performance in mice. J Strength Cond Res **29,** 552-558.

Murach KA, McCarthy JJ, Peterson CA & Dungan CM. (2020). Making Mice Mighty: recent advances in translational models of load-induced muscle hypertrophy. J Appl Physiol (1985) **129,** 516-521.

Nay K, Jollet M, Goustard B, Baati N, Vernus B, Pontones M, Lefeuvre-Orfila L, Bendavid C, Rue O, Mariadassou M, Bonnieu A, Ollendorff V, Lepage P, Derbre F & Koechlin-Ramonatxo C. (2019). Gut bacteria are critical for optimal muscle function: a potential link with glucose homeostasis. Am J Physiol Endocrinol Metab **317,** E158-E171.

Razavi AC, Potts KS, Kelly TN & Bazzano LA. (2019). Sex, gut microbiome, and cardiovascular disease risk. Biol Sex Differ **10,** 29.

Rizzetto L, Fava F, Tuohy KM & Selmi C. (2018). Connecting the immune system, systemic chronic inflammation and the gut microbiome: The role of sex. J Autoimmun **92,** 12-34.

2. Line 456-IFN-*gamma* and TNF-*alpha* have squares instead of the starred words.

Response: Thank you for catching this typo. We have replaced the “square” with the appropriate Greek symbol.

3. Line 586: The current evidence also indicates how the gut microbiome interacts via metabolites through a common mechanism unique for slow-twitch and fast-twitch muscles.
-No metabolites were measured in this study. Measuring gut bacterial metabolites under these same experimental conditions should be proposed as a future study.

Response: We agree with the Referee that this statement was not supported by the findings of the study since we did not measure microbial-derived metabolites. To address this concern, we have removed the statement from the revised manuscript and made the suggested edit as shown below:

“Additionally, the findings from this study add to the growing body of evidence supporting a gut microbiome-skeletal muscle axis [7, 58-60]. Future studies will seek to identify the bacterial species and associated metabolites that play a critical role in facilitating skeletal muscle hypertrophy and the fiber-type shift that occur in response to exercise with the expectation they will be unique for each of these processes”.

Referee #3:
The authors use a 2 x 2 design with antibiotic treatment and a weighted wheel running intervention (PoWER) to assess whether changes in muscular hypertrophy, fiber type conversion, and satellite cell accumulation induced by exercise are reliant on the gut microbiota. This is a novel study design that has the potential to describe the gut microbiota as a key regulator of skeletal muscle responses to exercise.

1. A primary concern (also brought by the previous reviewers) is that the manuscript contains no outcomes directly assessing muscular function. There are a number of outcomes (treadmill performance, grip strength, etc) that could have been assessed. It is unclear why these tests were omitted by the authors. Such data would provide much more context as to the significance of CSA and fiber type data presented by the authors. Wheel running does not constitute a performance measure as it is voluntary and submaximal. If the authors are to present the data as is (without functional outcome measures), the title of the paper needs to be changed. As is, reporting that muscle adaptations are 'suppressed' is a bit speculative and misleading. Title and discussion should instead focus on specific outcomes that authors reported in the paper (e.g. CSA deficits by Abx).

Response: We completely agree with the Referee that the lack of any functional data does limit the conclusions we can make regarding the influence of the gut microbiome on the ability of skeletal muscle to adapt to exercise. The PoWeR training component of the study was just finishing up (March, 2020) as the COVID-19 pandemic caused the University of Kentucky to shutdown which significantly restricted our ability to do research as well as interact with other labs (such as the Fry and Butterfield labs) on campus that have the equipment and expertise to perform muscle function analyses. As pandemic restrictions were gradually lifted over the next 9 months, we were able to focus on assessing the muscle phenotype by immunohistochemistry while compiling with university social distancing requirements. To address this important concern, we have edited the text to acknowledge this limitation of the study as shown below:

“Beyond wheel running activity, we did not perform any muscle function analyses to determine if the observed phenotypic differences between the PoWeR-trained groups was associated with any change in functional

characteristics of the muscle. Thus, future work will need to determine if the changes in fiber size and composition induced by dysbiosis have an impact on maximum and specific force and fatiguability of the muscle".

To address the concern regarding the title, we propose the following title:

"Dysbiosis of the Gut Microbiome Impairs Mouse Skeletal Muscle Adaptation to Exercise".

2. The authors have no biochemical data of mitochondria adaptations outside of fiber type staining. A measure of mitochondria activity (citrate synthase and/or succinate dehydrogenase) would greatly strengthen the manuscript. Especially in the plantaris muscle, where authors argue a blunted increase in type 2a fibers in Abx treated mice.

Response: While we agree the addition of biochemical data would further enhance the findings of the study, we previously reported the increase in Type 2a fibers in the mouse plantaris muscle in response to wheel running were closely paralleled by an increase in succinate dehydrogenase staining intensity. Although not a direct measure of mitochondrial activity, this finding does provide further support for the general consensus that changes in myosin heavy chain isoform expression often reflects a change in the mitochondrial content of the myofiber. To address this well-founded concern, however, we have carefully reviewed the revised manuscript to insure we have not suggested or implied the observed changes in fiber-type composition affected by dysbiosis reflect alterations in mitochondrial content or activity.

3. It appears that satellite cells are increased in Control-Abx treated animals (CT vs CU; Fig 8). The authors should discuss this as it a potential limitation to their interpretation of the data. Because there is no significant differences exist between PU and PT, a claim that Abx suppresses satellite cell accumulation is not supported by these figures. Throughout the manuscript, authors should report post-hoc P values to compare differences between PU and PT. This is the most important comparison.

Response: The effect of dysbiosis on satellite cell abundance, as assessed by Pax7+ staining, was somewhat ambiguous. Looking at the individual data points for CT and PT groups, it appears the response to dysbiosis and exercise was highly variable with some mice showing a clearly higher abundance of satellite cells while other mice showing no real change in stem cell abundance. So, while there may appear to be a numerical increase in satellite cell abundance of the two dysbiotic groups, statistically there is no difference in the abundance of satellite cells between these groups as well as the CU (Control Non-Runner Untreated). To address the Referee's thoughtful concern, we have added the following statement to the Results section and edited the Discussion section by removing any suggestion that dysbiosis and/or exercise may have suppressed satellite cell accumulation.

"There was no difference (p = 0.2782) in satellite cell abundance between the CU and CT groups. Finally, there was no difference (p = 0.6546) when comparing the PU and PT groups in satellite cell abundance (Fig. 8G)".

"Satellite cell and myonuclei abundance of the plantaris muscle were higher in response to PoWeR training in mice with an intact gut microbiome. The effect of dysbiosis on satellite cells was more ambiguous because some mice in both groups, sedentary and runners, showed higher abundance of satellite cells while other mice appeared to be unaffected by dysbiosis. This variability led to there being no difference in satellite cell abundance between the two PoWeR trained groups. This variable response to dysbiosis may reflect the inherent molecular heterogeneity of satellite cells as revealed by single-cell RNA-sequencing to changes in microbial-derived metabolites, though confirmation of such a mechanism awaits further study (Cho & Doles, 2017).

Cho DS & Doles JD. (2017). Single cell transcriptome analysis of muscle satellite cells reveals widespread transcriptional heterogeneity. *Gene* **636,** 54-63.

Minor Weaknesses

4. The authors should reconsider their group notation. "T" and "U" reminds me of Trained vs. Untrained instead of Treated (Abx) vs. Untreated. Please reconsider notation.

Response. Although we fully appreciate the Referee's concern regarding group notation, the Editor and other Referees did not consider this an issue. Respectfully, we request to keep the group notation the same.

5. In the introduction lines 130-132 authors state that previous data suggests germ free mice are more reliant on fatty acid oxidation yet much of the discussion refers to the role of gut microbes in mediating the increase in muscle oxidative fibers, this discrepancy is not addressed in the discussion.

Response: Given that there are few published studies investigating the interaction between skeletal muscle and the gut microbiome, we thought it would be informative to the reader in the Introduction section to provide a brief overview of what is currently know about the skeletal muscle-gut microbiome axis. We cited the Bäckhed *et al.,* (2007) study because it was the first, to our knowledge, study to assess skeletal muscle in a germ-free mouse. While germ-free mice can provide insight into what impact the absence of a microbiome might have on various physiological processes, comparisons to mice with an intact microbiome are not straightforward because the lack of commensal organisms profoundly affects post-natal development. Thus, while comparing germ-free and dysbiotic mice might seem appropriate, it can often give conflicting results as pointed out by the Referee. Similarly, Hsu and co-workers reported that germ-free mice had significantly impaired exercise capacity compared to SPF mice while we found no difference in exercise performance between untreated and dysbiotic mice (Hsu *et al.*, 2015).

6. Lines 137-142 - These sentences seem out of place, don't really seem relevant to the data reported.

Response: The purpose of this statement was to provide the reader with instances in which regular exercise can induce functional changes in the gut microbiome that are beneficial to the host by helping to treat certain pathologies. We think this statement is relevant to the study because we are testing the hypothesis that the gut microbiome is required, is beneficial, for skeletal muscle adaptation to exercise training. Respectfully, we request to keep the statement as part of the Introduction.

7. Lin 199- Actimetrics appears to be misspelled.

Response: We thank the Referee for catching this typo. We have corrected this spelling error in the revised manuscript.

8. Lines 457-459- This sentence is not clear. Please revise.

Response: We apologize for the confusion. We have edited the sentence in the revised manuscript as shown below:

"The major finding of the study is that antibiotic-induced dysbiosis of the gut microbiome impaired the ability of skeletal muscle to adapt to exercise training. Despite a similar training stimulus between untreated and antibiotic-treated groups, dysbiosis resulted in blunted hypertrophy in both the soleus and plantaris muscles following PoWeR training".

9. Lines 566-576 - This paragraph probably isn't necessary, since no measurement of metabolites was conducted.

Response: We agree with the Referee and have removed the paragraph from the revised Discussion section of the manuscript.

10. Line 471- Authors should remove statement that their data supports the findings that microbiota regulates skeletal muscle "function". This is not supported by the data presented in this manuscript.

Response: We agree with the Referee's comment and have removed "function" from the statement.

11. Line 278, 475 and 586 - ensure, not insure

Response: Thank you for pointing this error; we have edited the manuscript accordingly.

12. No sources cited to justify the relevance of the cytokine profile.

Response: We added references in the revised Methods section of the manuscript to justify the cytokines we measured in serum following PoWeR training as shown below:

"We have chosen to measure the serum concentration of the aforementioned cytokines because they are accepted markers of systemic inflammation [36-39]".

13. Given the only performance-based outcome they have is voluntary, I would refrain from using words like "performance" or "capacity" when referring to this data, as it is misleading. Authors should consider words like "activity" (see lines 480-482 and 511).

Response: In response to the Referee's valid point, we have edited the manuscript accordingly to replace "performance" or "capacity" with "activity".

14. Line 530-532 - this sentence needs to be revised

Response: We thank the Referee for catching this typo and have edited the sentence to improve clarity as shown below:

"Given that we observed no difference in muscle phenotype (weight and fiber size and composition) between non-running control groups as well as wheel running activity, we think the relatively low dose of antibiotics administered was able to effectively induces dysbiosis while minimizing any side effects that might have interfered with skeletal muscle adaptation to exercise."

2nd Revision - Editorial Decision 07-Sep-2021

Dear Dr McCarthy,

Re: JP-RP-2021-281788R2 "Dysbiosis of the Gut Microbiome Impairs Mouse Skeletal Muscle Adaptation to Exercise." by Taylor R Valentino, Ivan Vechetti, C. Brooks Mobley, Cory Dungan, Lesley R Golden, Jensen Goh, and John Joseph McCarthy

I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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All queries at proof stage should be sent to TJP@wiley.com

Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

Both referees are satisfied with the revision.

Senior Editor:

Thank you for submitting your work to the Journal of Physiology. Importantly, congratulations on the completion of an excellent study.

REFEREE COMMENTS

Referee #1:

No further comments.

Referee #3:

The reviewers responded thoroughly to each critique. While I disagree somewhat with their conclusions, I still think the data are worth publishing.

2nd Confidential Review 19-Aug-2021