

Gut microbiome remodeling by antibiotics reduces neuroinflammation in traumatic brain injury

Corresponding Author: Dr Sonia Villapol

This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have presented the manuscript clearly and provided a well-explained account of the ABX treatment. The study is novel and will be of interest to the scientific community. The work is convincing, with appropriately performed statistical analyses, and the results appear to be reproducible.

Authors are requested to address the following comments

1. Can elaborate the abstract in detail addressing all the results.
2. The authors included only male mice, citing reports that males exhibit a higher inflammatory response after TBI. Could the authors provide more supporting references for this choice and discuss how the exclusion of females may affect the generalizability of the findings, as well as whether sex-specific differences in neuroinflammatory responses might influence the study outcomes?
3. Are there any other antibiotic-resistant taxa that contribute to gut microbiome remodeling and reduction of neuroinflammation, aside from those mentioned in this TBI study, including evidence from other neurodegenerative disorders? If so, could the authors include this information in the Introduction and update the citations accordingly?
4. Why was a 38-day interval selected between the first and second CCI in the 2xTBI group, and how does this time point reflect the chronic phase of recovery or the clinical relevance of repeated brain trauma?
5. With the impact site localized 2 mm lateral and 2 mm posterior to Bregma using a 3 mm tip, along with the reported velocity and depth, how do the authors classify the severity of the CCI (mild, moderate, or severe), and is this consistent with established references for injury grading and also cite accordingly.
6. Could the authors clarify why these specific antibiotic concentrations were chosen to achieve microbiome depletion in C57BL/6 mice?
7. The ABX cocktail was administered for 3 days. Could the authors clarify whether this duration is sufficient for gut microbiome remodeling, and whether any validation, such as CFU plating, was performed to confirm the effectiveness of microbiome depletion prior to 16S sequencing?
8. The authors mention that DNA was extracted from fresh-frozen stool samples. Could the authors clarify from which part of the gastrointestinal tract these samples were collected (e.g., fecal pellets, cecal content, colon)?
9. Metagenomic analyses identified *Parasutterella excrementihominis* and *Lactobacillus johnsonii* as potential ABX-resistant taxa post-injury. Could the authors provide evidence of their functional role in modulating neuroinflammation, or cite studies supporting this association?
10. The authors suggest that short-term ABX treatment may offer microbiome-targeted therapeutic potential. Could the authors discuss potential risks or limitations of translating this approach to humans?
11. The study shows heightened neuroinflammation and larger lesion volumes in germ-free male mice. Could the authors comment on whether these effects are consistent across sexes or ages, and how they validate the role of the microbiome in TBI recovery?
12. The discussion highlights *Lactobacillus johnsonii* and *Parasutterella excrementihominis* as ABX-resistant taxa potentially contributing to reduced neuroinflammation. Could the authors provide evidence that these taxa are functionally

responsible for the observed effects, or clarify whether their abundance correlates with specific immune or neuroinflammatory markers?

13. The authors report that ABX treatment reduced neuroinflammation despite a decline in SCFA levels. Could the authors clarify the proposed mechanisms underlying these SCFA-independent neuroprotective effects, and whether systemic immune markers or endotoxin levels were assessed to support this explanation?

14. The discussion suggests modulation of peripheral immune activation and BBB integrity. Could the authors provide more mechanistic insight or cite evidence on how ABX-induced microbiome shifts affect these pathways?

Reviewer #2

(Remarks to the Author)

Overall assessment

This is a well-designed and comprehensive study that investigates the role of gut microbiome modulation by antibiotics (ABX) in the context of Traumatic Brain Injury (TBI). The use of both wild-type and germ-free mice is commendable, as well as the combination of histological, behavioral, microbiome, and metagenomic analyses. Moreover, the techniques employed are diverse and comprehensive, effectively addressing the paper's objectives. The major claim that ABX treatment remodels the gut microbiome and attenuates neuroinflammation following TBI is of interest to the neuroscience and microbiome communities. The work has the potential to significantly influence the field, particularly in its examination of the complex role of antibiotics as both disruptors of the microbiome and potential modulators of neuroinflammation.

Major Comments

-Narrative clarity: At times, the manuscript becomes confusing in presenting ABX as either "protective" or "detrimental." Since antibiotics are typically associated with microbiota depletion and impaired gut-brain communication, the protective effect reported here needs a more fluid narrative, so that readers are not left uncertain about whether ABX are "friends or foes" in this specific context.

-Figure 2: In panels A-B, the increased macrophage infiltration in the cortex of the 1xTBI-ABX is clear but not explicitly discussed in the text. A short comment should be added. The same applies to the differences in P2Y12 expression.

-Figure 3C: It is misleading to state that microbial composition remains relatively stable in the Sh-ABX group over time; on the contrary, a shift is evident ($R^2=0.677$, $p=0.001$), comparable to the 2xTBI-ABX group ($R^2=0.677$, $p=0.001$). This needs clarification.

-Figure 3E-F: Similarly, the text claims that the 2xTBI-VH group shows the greatest shift, while the R^2 values suggest a stronger effect in the 2xTBI-ABX group. This may suggest a synergistic effect between TBI and ABX, although the ABX-driven microbial remodeling appears to induce neuroprotection compared to VH.

-Beta diversity analysis: A multivariate approach that corrects for treatment and injury (e.g., PERMANOVA with both factors, or pairwise analyses) would strengthen the interpretation and clarify whether the observed effects are mainly driven by ABX, by TBI, or their interaction.

-Gut morphology (Figure 6): The data suggest that ABX also worsens intestinal architecture (shorter villi, fewer goblet cells in 2xTBI-ABX). The statement that "repetitive TBI has a greater effect [...] than ABX treatment" may need rephrasing to acknowledge the contribution of ABX to the observed intestinal damage.

-SCFA analysis: It should be made explicit in the Results that SCFA levels were measured in serum, not feces. Measuring fecal SCFAs would add valuable complementary data, as systemic levels do not fully reflect intestinal metabolic output.

-Supplementary Figure 3 caption: The caption incorrectly states that *Akkermansia muciniphila* was exclusively present in the VH group, while the figure shows it present in both VH and ABX groups. This should be corrected.

-*Alistipes muris*: Please clarify the rationale for focusing on *Alistipes muris* in the metagenomic analysis. Was it based on abundance, differential representation, or functional interest?

Additional considerations

-The rationale for including germ-free mice could be strengthened. I recommend emphasizing the rationale for including GF mice and providing a clearer explanation of how their use strengthens or complements your study objectives.

-The SCFA-independent mechanism of ABX protection is intriguing and could be expanded upon, especially given the metagenomic GO-term analysis pointing to SCFA-related functions.

Statistical analysis and reproducibility

The statistical analyses are generally sound and described in sufficient detail. For microbiome analyses, additional multivariate testing (see comment above) would improve robustness. The experimental design, sequencing methods, and code availability on GitHub suggest that the study is reproducible.

Conclusion

This is a valuable and interesting manuscript that will be of broad interest to a wide audience. With improved clarity in the

narrative and addressing the points above, the work will make a strong contribution to the field of microbiota–brain interactions in TBI.

I hope these comments will be constructive and helpful to the authors in improving their manuscript.

Reviewer: Dr. Luigia Turco

(Signed referee report)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Authors have addressed all the comments

Reviewer #2

(Remarks to the Author)

I carefully evaluated the revised version of the manuscript.

The authors have adequately addressed all the concerns raised in my previous review.

In particular, the revisions have improved the clarity of the narrative on antibiotic treatment, corrected figure descriptions, and implemented additional bioinformatics analyses that were previously suggested. These additions substantially strengthen the manuscript and support the authors' conclusions.

Overall, the revised manuscript is clearer, more robust, and methodologically solid.

I have no further major comments and consider the manuscript suitable for publication in Communications Biology.

Signed:

Dr. Luigia Turco

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In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

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Reviewers' comments:

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Reviewer 1's comments:	Authors' responses
1. Can elaborate the abstract in detail addressing all the results.	Page 2: We have expanded the Abstract to provide a more comprehensive overview of the key findings, including microbiome remodeling, lesion volume, neuroinflammatory markers, and SCFA results. These additions more accurately reflect the scope and depth of our study.
2. The authors included only male mice, citing reports that males exhibit a higher inflammatory response after TBI. Could the authors provide more supporting references for this choice and discuss how the exclusion of females may affect the generalizability of the findings, as well as whether sex-specific differences in neuroinflammatory responses might influence the study outcomes?	We thank the Reviewer for emphasizing the need to better discuss mechanisms underlying the sex-specific effects of TBI. We included only male mice to reduce biological variability in this initial mechanistic study and because multiple preclinical reports, including our prior work, indicate that males can exhibit a more robust acute neuroinflammatory response after CCI compared with females, including greater microglial activation and peripheral myeloid cell recruitment (Villapol et al., 2017; Tucker et al., 2021). We have added additional supporting references in the Methods and Discussion. Sex hormones and sex-dependent immune programs influence microglial states, cytokine profiles, BBB dynamics, and gut microbiome composition; therefore, ABX-driven microbiome remodeling may interact differently with injury-induced neuroimmune responses in females. It is possible that the magnitude, timing, or even direction of ABX-associated neuroprotection and the enrichment of ABX-resilient taxa could differ in female mice. We have added these points as a dedicated statement in the Limitations section in the Discussion (Page 32) and note that future studies will systematically examine females and incorporate sex as a biological variable across injury severity and recovery stages.
3. Are there any other antibiotic-resistant taxa that contribute to gut microbiome remodeling and reduction of neuroinflammation, aside from those mentioned in this TBI study, including evidence from other neurodegenerative disorders? If so, could the authors	We thank the Reviewer for this helpful suggestion. We have expanded the Introduction (Page 4) to acknowledge that, beyond <i>Parasutterella excrementihominis</i> and <i>Lactobacillus johnsonii</i> , other taxa with known antibiotic resilience may contribute to microbiome remodeling with downstream immunologic consequences. Notably, <i>Akkermansia muciniphila</i> has been reported to exhibit relative resistance to several

include this information in the Introduction and update the citations accordingly?	antibiotics, including vancomycin, and can become enriched after selective antibiotic exposure. We also note that <i>Bacteroides</i> species are well recognized for β -lactamase-mediated intrinsic resistance to penicillins and broad-spectrum cephalosporins, which may facilitate persistence during ABX-driven remodeling. Because both genera are frequently linked to gut barrier and immune modulation in the broader microbiota–gut–brain axis literature, including neurodegenerative contexts, we now highlight them as additional plausible contributors to ABX-associated neuroimmune effects and have updated the citations accordingly. We emphasize that these taxa represent candidate modulators rather than proven causal drivers in our model, and we explicitly state that future targeted colonization/depletion studies will be needed to establish mechanistic roles.
4. Why was a 38-day interval selected between the first and second CCI in the 2xTBI group, and how does this time point reflect the chronic phase of recovery or the clinical relevance of repeated brain trauma?	We chose a 38-day interval to model repeated injury during the chronic recovery window, when acute inflammation has diminished but sustained neuroimmune and microbiome alterations persist, thereby increasing clinical relevance and reducing confounding from overlapping acute responses. From a clinical relevance standpoint, repeated head injuries in athletes, military personnel, and trauma patients often occur weeks to months after an initial event rather than within hours or days. Thus, a 5–6 week interval in mice is intended to approximate a clinically meaningful repeated-trauma scenario, in which residual neuroimmune and gut–brain axis alterations may shape susceptibility to a second injury. We have discussed this in the Methods.
5. With the impact site localized 2 mm lateral and 2 mm posterior to Bregma using a 3 mm tip, along with the reported velocity and depth, how do the authors classify the severity of the CCI (mild, moderate, or severe), and is this consistent with established references for injury grading and also cite accordingly.	We now explicitly classify our CCI as moderate-severe based on published CCI parameter ranges. We add a sentence in the Methods section.
6. Could the authors clarify why these specific antibiotic concentrations were chosen to achieve microbiome depletion in C57BL/6 mice?	We thank the Reviewer for this important question. The ABX concentrations were selected based on widely used murine microbiome-depletion protocols in C57BL/6 mice that combine ampicillin, gentamicin, metronidazole, and vancomycin at comparable doses to achieve broad coverage of Gram-positive, Gram-negative, and

	anaerobic taxa. This cocktail has been repeatedly shown to induce rapid and robust microbiome depletion within a short treatment window while maintaining acceptable tolerability in adult mice. The 3-day regimen was intentionally designed as a short, acute depletion/remodeling paradigm to minimize prolonged systemic or intestinal toxicity while still producing measurable, reproducible reductions in bacterial load and community restructuring. We have clarified this rationale in the Methods.
7. The ABX cocktail was administered for 3 days. Could the authors clarify whether this duration is sufficient for gut microbiome remodeling, and whether any validation, such as CFU plating, was performed to confirm the effectiveness of microbiome depletion prior to 16S sequencing?	We appreciate the Reviewer's comment and the opportunity to clarify our antibiotic regimen. Our goal was to acutely and robustly remodel, rather than completely sterilize, the gut microbiota while minimizing systemic toxicity and weight loss. In our cohort, we confirmed effective remodeling by a substantial reduction in total bacterial DNA concentration (Fig. 1b) from fecal pellets collected before versus after ABX treatment showing >80% reduction in microbial load, and 16S rRNA gene sequencing showing a pronounced shift in community composition compared with untreated controls. In addition, we added 16S qPCR which detected an increase in bacterial DNA possibly from lysed and extracellular fragments as well as viable and non-viable bacteria. We did not perform CFU plating because all fecal samples were collected directly into tubes for freezing and subsequent DNA extraction. We explicitly state in the Methods that we used pre- and post-ABX bacterial DNA quantification and 16S rRNA profiling as our validation strategy and acknowledge in the Discussion that the absence of CFU plating is a limitation of the current study. Although we did not perform CFU plating, depletion was supported by marked reductions in fecal bacterial DNA, sequencing microbiome, and taxonomic richness post-ABX, consistent with established short-course depletion paradigms.
8. The authors mention that DNA was extracted from fresh-frozen stool samples. Could the authors clarify from which part of the gastrointestinal tract these samples were collected (e.g., fecal pellets, cecal content, colon)?	We thank the Reviewer for this comment. The samples used for 16S and metagenomic analyses were fresh fecal pellets collected from the distal gastrointestinal output (i.e., stool/feces). Pellets were collected non-invasively from individual mice at the indicated time points, immediately flash-frozen, and stored at -80°C until DNA extraction. We have clarified this in the Methods.
9. Metagenomic analyses identified <i>Parasutterella excrementihominis</i> and <i>Lactobacillus johnsonii</i> as potential ABX-resistant taxa post-	We thank the Reviewer for this important point. Our metagenomic data identify <i>Parasutterella excrementihominis</i> and <i>Lactobacillus johnsonii</i> as taxa

injury. Could the authors provide evidence of their functional role in modulating neuroinflammation, or cite studies supporting this association?	that persist or are enriched following short-term ABX in the post-TBI context. For <i>Lactobacillus johnsonii</i>, emerging evidence indicates robust anti-inflammatory properties at the gut-immune interface. In particular, <i>L. johnsonii</i>-derived extracellular vesicles promote anti-inflammatory macrophage polarization and improve barrier integrity in colitis models, in part through suppression of pro-inflammatory signaling pathways. These findings support a mechanism whereby <i>L. johnsonii</i> enrichment could reduce peripheral inflammatory tone and secondarily dampen CNS neuroinflammation after TBI. Publications also summarize broader immunoregulatory and gut-brain axis-relevant roles for <i>L. johnsonii</i> strains across disease contexts. For <i>Parasutterella</i>, colonization studies indicate that this genus is a core gut member capable of reshaping host-relevant metabolic outputs, including bile acid and purine-related metabolites, which are known to influence immune homeostasis. Murine <i>Parasutterella</i> colonization has been associated with selective shifts in microbial-derived bile acid and purine pathways, supporting its capacity to modulate systemic inflammatory tone. We have added these studies to the Discussion to support the biological plausibility that ABX-resilient enrichment of <i>L. johnsonii</i> and <i>P. excrementihominis</i> may contribute to reduced neuroinflammatory outcomes after TBI, while clarifying that functional validation in the TBI setting will require targeted colonization or supplementation experiments.
10. The authors suggest that short-term ABX treatment may offer microbiome-targeted therapeutic potential. Could the authors discuss potential risks or limitations of translating this approach to humans?	We thank the Reviewer for this important translational point. We have expanded the Discussion with the Limitations section to emphasize that, although short-term ABX can reshape the microbiome and attenuate neuroinflammation in our mouse model, translation to humans carries important risks. We also note that trauma and neurocritical care guidelines generally favor short, indication-specific prophylaxis and caution against extended courses without clear benefit. Accordingly, we now frame ABX in our study as a proof-of-concept tool that motivates safer, more targeted microbiome-based approaches rather than advocating empirical ABX use for neuroprotection.
11. The study shows heightened neuroinflammation and larger lesion volumes in germ-free male mice. Could the authors comment on whether these effects are	These findings, obtained in young adult male GF mice, suggest that the absence of gut microbiota exacerbates lesion volume and neuroinflammation following TBI, characterized by increased microglial and astrocytic activation and larger tissue loss, and support a protective

consistent across sexes or ages, and how they validate the role of the microbiome in TBI recovery?	role of the microbiome in constraining acute neuroinflammatory damage. Future studies including females are planned. We clarified that our GF cohort is limited to adult males and expanded the interpretation in the Discussion.
12. The discussion highlights <i>Lactobacillus johnsonii</i> and <i>Parasutterella excrementihominis</i> as ABX-resistant taxa potentially contributing to reduced neuroinflammation. Could the authors provide evidence that these taxa are functionally responsible for the observed effects, or clarify whether their abundance correlates with specific immune or neuroinflammatory markers?	We thank the Reviewer for this insightful comment. We agree that our study design, which did not involve monoassociation, targeted supplementation, or depletion experiments, does not allow us to claim causal functional responsibility for <i>Lactobacillus johnsonii</i> and <i>Parasutterella excrementihominis</i>. However, we have revised the Discussion to better contextualize these findings within the existing literature, which supports the plausibility of these taxa acting as neuroprotective candidates. Specifically, we have added references discussing the functional roles of these species with relevance to the gut-brain axis and inflammatory phenotypes: 1. <i>L. johnsonii</i>: Previous studies have demonstrated that <i>L. johnsonii</i> can prevent memory dysfunction and reduce hippocampal neuroinflammation (e.g., decreasing TNF-α and IL-6) by modulating the gut-brain axis and tryptophan metabolism (Zhao et al., 2025, Wang et al., 2021; Marcial et al., 2017). 2. <i>P. excrementihominis</i>: Emerging evidence identifies <i>P. excrementihominis</i> as a key modulator of tryptophan availability and succinate production. Recent work by Galley et al. (2024) has shown that supplementation with <i>P. excrementihominis</i> can ameliorate stress-induced inflammatory phenotypes and behavioral deficits, suggesting a potential neuroprotective role. We have amended the text to avoid implying definitive causality while highlighting that these ABX-resistant taxa are consistent with a "protective" functional profile described in broader literature.
13. The authors report that ABX treatment reduced neuroinflammation despite a decline in SCFA levels. Could the authors clarify the proposed mechanisms underlying these SCFA-independent neuroprotective effects, and whether systemic immune markers or endotoxin	Thank you for asking, here is the clarification. For the figure 4E we used serum SCFA, and for the Figure 4F we use the metagenomics data from the fecal samples. We did not directly measure systemic cytokines or LPS. We expanded the mechanistic discussion. We edited the Results and Discussion.

levels were assessed to support this explanation?	
14. The discussion suggests modulation of peripheral immune activation and BBB integrity. Could the authors provide more mechanistic insight or cite evidence on how ABX-induced microbiome shifts affect these pathways?	We appreciate this comment. We added mechanistic citations and context to the Discussion (pages 26-27).

Reviewer #2	
Overall assessment This is a well-designed and comprehensive study that investigates the role of gut microbiome modulation by antibiotics (ABX) in the context of Traumatic Brain Injury (TBI). The use of both wild-type and germ-free mice is commendable, as well as the combination of histological, behavioral, microbiome, and metagenomic analyses. Moreover, the techniques employed are diverse and comprehensive, effectively addressing the paper's objectives. The major claim that ABX treatment remodels the gut microbiome and attenuates neuroinflammation following TBI is of interest to the neuroscience and microbiome communities. The work has the potential to significantly influence the field, particularly in its examination of the complex role of antibiotics as both disruptors of the microbiome and potential modulators of neuroinflammation.	
Major Comments	
Reviewer 1's comments:	Authors' responses
-Narrative clarity: At times, the manuscript becomes confusing in presenting ABX as either "protective" or "detrimental." Since antibiotics are typically associated with microbiota depletion and impaired gut-brain communication, the protective effect reported here needs a more fluid narrative, so that readers are not left uncertain about whether ABX are "friends or foes" in this specific context.	Thank you for pointing this out; post-ABX neuroprotection is indeed a concept that must be approached with caution. We have done so in this revised manuscript. We have also added the corresponding edits to the discussion (Page 26).
-Figure 2: In panels A-B, the increased macrophage infiltration in the cortex of the 1xTBI-ABX is clear but not explicitly discussed in the text. A short comment should be added. The same applies to the differences in P2Y12 expression.	Thank you for the comment. We have added explicit statements in the Results section on Figure 2 (Pages 8-9).
-Figure 3C: It is misleading to state that microbial composition remains relatively stable in the Sh-ABX group over time; on the contrary, a shift is evident ($R^2=0.677$, $p=0.001$), comparable to the 2xTBI-ABX group ($R^2=0.677$, $p=0.001$). This needs clarification.	Thank you very much for pointing out this error. We have recalculated the statistics to obtain the correct values and have also reformatted the beta diversity analyses. The revised Figure 3 now includes the updated graphs and statistics, demonstrating a significant short-term ABX-induced remodeling of the gut microbiota. This correction is now included in the Results.
-Figure 3E-F: Similarly, the text claims that the 2xTBI-VH group shows the greatest shift, while the R^2 values suggest a stronger effect in the 2xTBI-ABX group. This may suggest a synergistic effect between TBI and ABX, although the ABX-driven microbial remodeling appears to induce neuroprotection compared to VH.	Correct, as explained in the previous question. These graphs have been redone, and the new data discussed and added in the methods section. We have replaced the description in the Results section (Pages 14-15).

-Beta diversity analysis: A multivariate approach that corrects for treatment and injury (e.g., PERMANOVA with both factors, or pairwise analyses) would strengthen the interpretation and clarify whether the observed effects are mainly driven by ABX, by TBI, or their interaction.	We appreciate this helpful suggestion and have now reanalyzed the beta diversity data using PERMANOVA on Bray-Curtis distances, incorporating treatment and injury, as well as timepoint. The updated analyses are presented in the revised Figure 3C-G. First, within each treatment/injury group (Sham-ABX, 1xTBI-ABX, 2xTBI-ABX, 2xTBI-VH), PERMANOVA revealed a robust effect of timepoint on community structure ($\text{Pr}(>F) = 0.001$ for all groups; $R^2 = 0.48\text{--}0.68$), confirming pronounced temporal microbiome shifts following ABX and/or TBI (Figure 3C-F). To directly address the contribution of treatment relative to injury, we then focused on the 2xTBI cohort and fit a PERMANOVA model including treatment (ABX vs VH), timepoint, and their interaction. As shown in Figure 3G, this analysis identified a significant treatment x timepoint interaction ($\text{Pr}(>F) = 0.011$, $R^2 = 0.054$), indicating that ABX exposure modifies the trajectory of post-TBI microbiome changes rather than acting as a purely additive main effect. We have updated the Methods to describe the PERMANOVA models used and revised the Results section to clarify that timepoint is the dominant driver of beta diversity shifts across groups and ABX treatment significantly alters the temporal pattern of microbiome remodeling after repeated TBI.
-Gut morphology (Figure 6): The data suggest that ABX also worsens intestinal architecture (shorter villi, fewer goblet cells in 2xTBI-ABX). The statement that “repetitive TBI has a greater effect [...] than ABX treatment” may need rephrasing to acknowledge the contribution of ABX to the observed intestinal damage.	Thank you, correct. We revised both Results (Page 21) and Discussion (Pages 32-33) to explicitly acknowledge ABX effects.
-SCFA analysis: It should be made explicit in the Results that SCFA levels were measured in serum, not feces. Measuring fecal SCFAs would add valuable complementary data, as systemic levels do not fully reflect intestinal metabolic output.	We have clarified this in the Results and added a text in the section of the limitations in the Discussion.
-Supplementary Figure 3 caption: The caption incorrectly states that Akkermansia muciniphila was exclusively present in the VH group, while	We have corrected the caption.

the figure shows it present in both VH and ABX groups. This should be corrected.	
-Alistipes muris: Please clarify the rationale for focusing on Alistipes muris in the metagenomic analysis. Was it based on abundance, differential representation, or functional interest?	We added a short explanation to the Results.
Additional considerations	
-The rationale for including germ-free mice could be strengthened. I recommend emphasizing the rationale for including GF mice and providing a clearer explanation of how their use strengthens or complements your study objectives.	A new addition in the Discussion (Page 26) the rational that GF mice provide causal insight into microbiome-CNS interactions, complementing ABX models.
-The SCFA-independent mechanism of ABX protection is intriguing and could be expanded upon, especially given the metagenomic GO-term analysis pointing to SCFA-related functions.	This is addressed in the expanded SCFA paragraph under Reviewer 1 comment 13 and in the GO-term discussion you already have (we now explicitly link these in the Discussion). Together, these changes clarify that ABX protection likely involves reduced peripheral immune activation and enrichment of immunomodulatory taxa, rather than increased SCFA production.
Statistical analysis and reproducibility The statistical analyses are generally sound and described in sufficient detail. For microbiome analyses, additional multivariate testing (see comment above) would improve robustness. The experimental design, sequencing methods, and code availability on GitHub suggest that the study is reproducible.	We have clarified the additional multivariate PERMANOVA analyses and continue to provide full code and pipelines via GitHub and SRA accessions already noted in the manuscript.
Conclusion This is a valuable and interesting manuscript that will be of broad interest to a wide audience. With improved clarity in the narrative and addressing the points above, the work will make a strong contribution to the field of microbiota–brain interactions in TBI.	Thank you so much for the kind words, we heightened this relevance in the Conclusion section.
I hope these comments will be constructive and helpful to the authors in improving their manuscript.	Thank you very much.

Reviewer comments

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-Figure 2: In panels A-B, the increased macrophage infiltration in the cortex of the 1xTBI-ABX is clear but not explicitly discussed in the text. A short comment should be added. The same applies to the differences in P2Y12 expression.

-Figure 3C: It is misleading to state that microbial composition remains relatively stable in the Sh-ABX group over time; on the contrary, a shift is evident ($R^2=0.677$, $p=0.001$), comparable to the 2xTBI-ABX group ($R^2=0.677$, $p=0.001$). This needs clarification.

-Figure 3E-F: Similarly, the text claims that the 2xTBI-VH group shows the greatest shift, while the R^2 values suggest a stronger effect in the 2xTBI-ABX group. This may suggest a synergistic effect between TBI and ABX, although the ABX-driven microbial remodeling appears to induce neuroprotection compared to VH.

-Beta diversity analysis: A multivariate approach that corrects for treatment and injury (e.g., PERMANOVA with both factors, or pairwise analyses) would strengthen the interpretation and clarify whether the observed effects are mainly driven by ABX, by TBI, or their interaction.

-Gut morphology (Figure 6): The data suggest that ABX also worsens intestinal architecture (shorter villi, fewer goblet cells in 2xTBI-ABX). The statement that “repetitive TBI has a greater effect [...] than ABX treatment” may need rephrasing to acknowledge the contribution of ABX to the observed intestinal damage.

-SCFA analysis: It should be made explicit in the Results that SCFA levels were measured in serum, not feces. Measuring fecal SCFAs would add valuable complementary data, as systemic levels do not fully reflect intestinal metabolic output.

-*Supplementary Figure 3 caption*: The caption incorrectly states that *Akkermansia muciniphila* was exclusively present in the VH group, while the figure shows it present in both VH and ABX groups. This should be corrected.

-*Alistipes muris*: Please clarify the rationale for focusing on *Alistipes muris* in the metagenomic analysis. Was it based on abundance, differential representation, or functional interest?

Additional considerations

-The rationale for including germ-free mice could be strengthened. I recommend emphasizing the rationale for including GF mice and providing a clearer explanation of how their use strengthens or complements your study objectives.

-The SCFA-independent mechanism of ABX protection is intriguing and could be expanded upon, especially given the metagenomic GO-term analysis pointing to SCFA-related functions.

Statistical analysis and reproducibility

The statistical analyses are generally sound and described in sufficient detail. For microbiome analyses, additional multivariate testing (see comment above) would improve robustness. The experimental design, sequencing methods, and code availability on GitHub suggest that the study is reproducible.

Conclusion

This is a valuable and interesting manuscript that will be of broad interest to a wide audience. With improved clarity in the narrative and addressing the points above, the work will make a strong contribution to the field of microbiota–brain interactions in TBI.

I hope these comments will be constructive and helpful to the authors in improving their manuscript.

Final revision of the manuscript “Gut microbiome remodeling by antibiotics reduces neuroinflammation in traumatic brain injury” submitted to *Communications Biology* (Nature Portfolio).

I carefully evaluated the revised version of the manuscript.

The authors have adequately addressed all the concerns raised in my previous review.

In particular, the revisions have improved the clarity of the narrative on antibiotic treatment, corrected figure descriptions, and implemented additional bioinformatics analyses that were previously suggested. These additions substantially strengthen the manuscript and support the authors’ conclusions.

Overall, the revised manuscript is clearer, more robust, and methodologically solid.

I have no further major comments and consider the manuscript suitable for publication in *Communications Biology*.