nature portfolio

Peer Review File

Gut Microbiota and Brain-Resident CD4$^+$ T Cells Shape Behavioral Outcomes in Autism Spectrum Disorder

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors present an in vivo and ex vivo study focused on the role of gut microbiota and its metabolites in shaping neuroinflammation and ASD behavior by modulating brain-resident T cells.

What are the noteworthy results?
The results obtained in mouse model of ASD (BTBR), describe the mechanisms involved in gut microbiome-metabolome-immune-brain axis and specifically demonstrated:
- the role of gut microbiota (GM) in modulating neuronal activation and ASD behavior
- the role of GM in immune modulation and neuroinflammation. Authors found a reduction of brain-resident CD4+ and CD8+ T cells and their functional activity in GF-BTBR mice
- the role of CD4+ T cells in ASD. By depleting brain resident CD4+ T cells in BTBR mice the authors showed decrease of neuroinflammation and improving of some ASD social behavior
- the role of selected bacteria (especially L. murinus spp) and their derived metabolites, in inducing ASD behavior. L. murinus spp. colonization of GF-BTBR mice recapitulates ASD symptoms by increasing the glutamate/GABA ratio as well as IFNgamma in CD4+ and CD8+ T cells, with respect to GF-BTBR.
- the role of healthy gut microbiota transplantation in preventing or reducing neuroinflammation (it may manage brain resident T cells phenotypes), in modulating metabolite imbalance thus resulting in mitigation of ASD behavior
- the role of L. reuteri IMB015 in reducing glutamate/GABA ratio (demonstrated both by in silico and by targeted metabolomic analysis), inflammation, neuronal hyperactivation and some ASD symptoms
The results are also well described in singular paragraph each including the hypothesis, the results obtained and the conclusion

The work is of significance to the field and related fields since it highlights potential therapeutic strategies, including healthy GM transplantation and supplementation with L. reuteri IMB015, to alleviate ASD symptoms through modulation of the gut-metabolite-immune-brain axis.

Compared to the consolidated literature, one part of the study confirms previous results, another brings new ones but above all it delves into the GM-metabolite-immune-brain axis in a fairly complete mechanistic approach.

The work supports the conclusions and claims. The project design, analyses, results and conclusions are very well done, completed and clearly reported and do not require revision

Methodology
All sections of M&M except one (see below) are well described and meet expected standards.

M&M, section "Targeted antibiotics treatment"
To provide enough details to reproduce the work, the authors should specify:
- whether the antibiotics administered are time- or concentration-dependent. Time-dependent antibiotics require regular administration to maintain adequate concentrations.
Concentration-dependent antibiotics can be administered less frequently, as long as a high peak is achieved.
- if the mice were in individual cages so they could calculate the amount of antibiotics taken by each individual animal.

Reviewer #2

(Remarks to the Author)
In this manuscript, Park et. al provides new evidence for the gut microbiota and CD4 T cells to regulate the behavior outcomes relevant to ASD. The datasets provided are rich and helps understand better the relationship between the microbiota and genetic backgrounds in the context of ASD. Although interesting, some points need to be addressed.
- Why was the BTBR model chosen? While this strain presents behavioral phenotypes relevant to ASD, the used of genetic models of ASD risk genes (such as Shank3) would strengthen the paper.
- In figure S1A there is a preference for the empty chamber in the BTBR model, however this is not observed in the SPF BTBR model in figure 1A. Is there any reason on why these differences are present?
- The flow analysis on immune cells in the brain were conducted on the whole brain. Why is that the case? Authors do analysis of c-fos on specific brain areas (hippocampus and amygdala), why not choose the same areas or others that are relevant to the behavioral phenotypes?
- Authors use the mean velocity in the open field as a measure of hyperactivity. Why was this metric chosen and not the distanced travelled? Having both measurements would be beneficial.
- Why L. murinus was chosen following the culturing of bacteria? It is not clear why this specific colony was chosen.
- Authors colonized GF-BTBR mice with L. murinus and show that these animals develop the same behavioral impairments as the SPF-BTBR mice. How was the colonization process done? In which point of development was the bacteria introduced? Since ASD is a neurodevelopmental disorder, it is important to note when the bacteria was introduced.
- The authors note that the changes in gut microbiota in BTBR mice results in an imbalance of Glutamate/GABA in the faces and try to relate that to the changes in immunity in the brain. How would an imbalance of these metabolites in the gut influence brain function? Is there also a change in these metabolites in the brain that is recovered in the GF model of BTBR?
- Introduction of VNM microbiota leads to impairments in the marble burying test, was social behavior also assessed?
- L. murinus colonization leads to behavior impairments in GR-BTBR mice. Are these impairments specific to this bacteria? Using other colonies and heat-killed bacteria, would also be beneficial in demonstrating specificity and that the bacteria is required to be alive and therefore have a metabolic output to exert its effects.
- The colors in figure 5 seems to be switched, in A the B6 mice is blue and the SPF-BTBR is red, while in the other graphs the BTBR-BTBR is blue and the B6-BTBR is red.

Reviewer #3

(Remarks to the Author)
In the manuscript, “Gut Microbiota and Brain-Resident CD4 T Cells Shape Behavioral Outcomes in Autism Spectrum Disorder,” the authors studied the changes to the genetic ASD mouse model, namely BTBR mice, when reared in a germ-free state compared to specific pathogen free conditions. While in many cases this comparison is too artificial and simplistic with current progress in the field, in the manuscript, the authors go much further and identify likely candidate bacterial taxa and metabolic functions that can be manipulated to modify the behavioral and immune phenotypes of the model. Overall, it is a well-written and clear story. Many next steps I would hope to see were right around the corner in the next figure or paragraph. I am particularly enthusiastic about the use of in silico methods to predict metabolic capabilities of candidate microbes, and am excited by the results.

Major concerns
In figure 1A, SPF BTBR mice do not display a phenotype in sociability in 3-chamber, but the second sentence of the results section plainly states that they do. This data is inconsistent with Supp Fig 1A. If this mouse line does not display consistent behavioral phenotypes, conclusions cannot be drawn from changing additional factors and measuring behavior. Repeat the experiments to ensure the phenotype is consistent, or clarify the text if I am misinterpreting Fig 1A.

In my experience, BTBR mice are highly prone to grooming (and overgrooming to baldness). In fact, in many behavioral tests, I’ve observed that they score poor social interaction times, exploration, etc, and the results look quite striking but it seems that rather than an avoidance of typical behaviors- there is a compulsion for constant grooming and no other activity is performed. Please observe your videos of behavior tests completed thus far for this phenotype. If this could be a confounding variable in your hands as well, please think about what this could mean for your results. It will likely support your marble burying results. Your hyperactivity phenotype may indicate the opposite of what I have just described!

The lone c-fos data in SPF is orphaned. It will fill the story in nicely if you can please include this analysis for the later experiments as well.

In the abstract and introduction, you tie anxiety into ASD phenotypes as if it is a main characteristic, and you describe BTBR as a good mouse model, yet your data shows that BTBR mice display lower anxiety-like behavior than WT mice. In the text you seem to avoid confronting this by vaguely stating “abnormal anxiety levels” (e.g. line 131). However, this is a bit contradictory. It is not without precedent for BTBR mice to display low levels of anxiety-like behavior, so this should be able to be clarified and explained. Overall, the description of the anxiety-like behavior could be improved. Later, in fig 3g, an increase in anxiety-like behavior is described as “improved” anxiety.

I’d relabel your anxiety graphs. First, it should always be referred to as “anxiety-like” behavior, because we are unable to truly measure anxiety in mice. Second, the label of “anxiety” at the top of the graph will be misleading to anyone less familiar

with the OFT, where it is easy to interpret that higher numbers in this graph=higher anxiety.

Are you measuring hyperactivity or simply locomotion?

Figure 4 data is so exciting. Is ampicillin alone sufficient to induce the altered behavior?

Minor concerns
It might be a good idea to clarify that abnormal social interactions and repetitive behaviors are core features defining ASD, while anxiety is not. Rather, it is a common comorbidity.

Typo line 43, hyperactivity

Rephrase from “prevented the onset of multiple ASD-associated behaviors” and change it to something like “delayed the onset…”

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed all the concerns raised. The addition of new experiments involving heat-killed and another bacterial strain strengthens the findings. Although there remains a missing link between gut glutamate and brain alterations, I believe the manuscript is acceptable for publication.

Reviewer #3

(Remarks to the Author)
My concerns have been sufficiently addressed.

Response to Reviewers

Reviewer #1:

M&M, section "Targeted antibiotics treatment"
To provide enough details to reproduce the work, the authors should specify:
- whether the antibiotics administered are time- or concentration-dependent. Time-dependent antibiotics require regular administration to maintain adequate concentrations. Concentration-dependent antibiotics can be administered less frequently, as long as a high peak is achieved.
- if the mice were in individual cages so they could calculate the amount of antibiotics taken by each individual animal.

Response to Reviewer #1

- We appreciate the reviewer's suggestions to improve the antibiotic treatment methodology. We have modified the 'Targeted antibiotics treatment" section of the methodology to include the following changes: 'Antibiotic combinations were administered in drinking water *ad libitum* in a concentration-dependent manner, and solutions were changed daily with new antibiotic mixtures to maintain high active concentration peaks. Pregnant BTBR mice were treated from E12.5 until the offspring were 8 weeks of age, and no significant weight change was observed in the groups. Mice were caged in groups of three to four, and the amount of antibiotics consumed was monitored by averaging the amount of solution consumed daily in each cage.' (lines 476-481).

Reviewer #2:

Question #1:
Why was the BTBR model chosen? While this strain presents behavioral phenotypes relevant to ASD, the used of genetic models of ASD risk genes (such as Shank3) would strengthen the paper.

Response to Question #1

- We appreciate the reviewer's question and suggestion to utilize another mouse model (such as *Shank3*) for our manuscript. We chose to use the BTBR mouse model as it has an extensive history in ASD research. Notably, BTBR mice were previously reported to have gut dysbiosis and systemic inflammation, suggesting a robust basis for a gut-immune-brain axis in ASD. The BTBR mice are also known to have several single nucleotide polymorphisms (SNP) tied to ASD behavioral abnormalities. McFarlane et al., reported that among 124 putative autism candidate genes, BTBR mice had SNP mutations in four genes, including *Kmo*, *Slc6a4*, *Smo*, and *Pkd1* (https://doi.org/10.1111/j.1601-183X.2007.00330.x). We believe these genetic mutations in ASD-associated genes might be helpful in classifying the BTBR mice as an acceptable genetic mouse model for ASD. We clarified this point in the main text, under "Introduction" in lines 75-78
We also recognize the importance of testing other genetic mouse models for our study, such as *Shank3*. While we agree that including additional models could strengthen our findings, conducting parallel experiments in another germ-free line would constitute a substantial undertaking, likely requiring additional years. For example, the derivation and utilization of a germ-free BTBR mouse line was critical to our study, and establishing comparable *Shank3* line world be beyond the scope of current study.
According to previous studies, antibiotic treatment in *Shank3*$^{-/-}$ mice exacerbated social behavior deficit without affecting anxiety (10.1016/j.bbi.2023.08.020). Furthermore, numerous prior publications have demonstrated the beneficial effects of *L. reuteri* probiotics on *Shank3*$^{-/-}$ and *Cntnap2*$^{-/-}$ mice. To address this point, we have substantially expanded our 'Discussions' section.

Firstly, we have included additional details of a *Cntnap2* KO study, which describes the effects of gut microbiota manipulation on behavior in lines 366-370. We included a study using *L. reuteri* in *Shank3* KO mice to improve behavior in lines 417-420. Finally, we have addressed this point as a major limitation in our study, and have provided an example of antibiotic treatment on *Shank3* KO mice, and additional *L. reuteri* studies on *Shank3* and *Cntnap2* KO mice in lines 431-437.

We apologize to the reviewer for not being able to experimentally address this point. However, we hope that our additions to the text may sufficiently guide the audience to prior studies that address these concerns.

Question #2:
In figure S1A there is a preference for the empty chamber in the BTBR model, however this is not observed in the SPF BTBR model in figure 1A. Is there any reason on why these differences are present?

Response to Question #2
We thank the reviewer for pointing out this discrepancy. In line with previous publications, we generally perceive the results from the three-chamber sociability assay as being either social or non-social. Low social preference can be defined as showing no preference between chambers and more time spent in the empty chamber. However, we acknowledge that the results from Supplementary Fig. 1A and Fig. 1A display a discrepancy and may cause confusion.

The data presented in Supplementary Fig. 1A data were obtained from initial preliminary experiments, and minor changes to the testing facility and procedures may have occurred since then. To resolve this issue and address the reviewer's comment, we have performed additional experiment for Supplementary Fig. 1A. The text has also been modified to indicate that BTBR mice showed no

preference for the social chamber in Supplementary Fig. 1A, with non-social phenotype described as similar or less time spent in the social chamber. We have added a clarification to our definition in lines 95-96.

Furthermore, to better define our interpretation, we have defined our parameters for 'social' or 'non-social', as well as 'social memory', in the methods section: "Neurotypical social preference was defined as more time spent in the social chamber, while ASD-associated non-social behavior was defined as either no preference between chambers or greater preference for the empty chamber. We defined neurotypical social memory as more time spent in the novel chamber, whereas impaired social memory was defined as no preference between chambers or more preference for the familiar chamber" (lines 496-500).

Question #3:

The flow analysis on immune cells in the brain were conducted on the whole brain. Why is that the case? Authors do analysis of c-fos on specific brain areas (hippocampus and amygdala), why not choose the same areas or others that are relevant to the behavioral phenotypes?

Response to Question #3

We appreciate the reviewer's insightful and relevant question.

The roles of brain-resident immune populations are an emerging focus in both immunology and neurobiology. Many aspects still need to be elucidated, mainly their functions within specific brain regions, where data remain remarkably limited.

In wild-type B6 mice, the total number of $CD4^+$ T cells in the entire brain is known to be typically fewer than 2,000, and in our facility, we could detect approximately 250 $CD4^+$ T cells by flow cytometry in healthy B6 mice. Since these numbers represent the whole brain, isolating and analyzing specific regions such as the hippocampus or amygdala would be technically challenging, as the cell yield would be insufficient for reliable flow cytometric analysis. Our previous efforts to visualize and quantify $CD4^+$ T cells in discrete brain regions using immunofluorescence imaging were also unsuccessful, largely due to the extremely low cell numbers and the technical limitations of our imaging facility. Conducting region-specific analyses at this resolution would require advanced single-cell technologies, which, while highly valuable, fall beyond the scope of the present study. The primary objective of our work was to address $CD4^+$ T cell-mediated neuroinflammation at the level of the whole brain rather than delineating their roles in individual compartments. We agree that a more detailed, region-specific exploration of T cell dynamics would be an important avenue for future research and best addressed in dedicated follow-up studies.

To address this concern, we have explicitly acknowledged these points as a major limitation of this study in the "Discussions" section, lines 438-444. While we regret being unable to verify the brain regions for T cells in ASD experimentally, we hope clear recognition and explanation of this limitation may suffice and help guide and promote future in-depth studies on this topic.

Question #4:

Authors use the mean velocity in the open field as a measure of hyperactivity. Why was this metric chosen and not the distanced travelled? Having both measurements would be beneficial.

Response to Question #4:

We initially only included Mean Velocity graphs from the open field maze to depict hyperactivity, because the trends between the mean velocity and distance traveled were usually similar (ie, higher mean velocity generally resulted also in higher distance traveled).

However, to better clarify the results, we have included both Mean Velocity and Distance Traveled as the reviewer has suggested in Supplementary Fig. 1E and Fig. 1E.

Question #5:
Why L. murinus was chosen following the culturing of bacteria? It is not clear why this specific colony was chosen.

Response to Question #5:

We appreciate the reviewer's question and would like to clarify the selection of *L. murinus*.

In Figure 4, we performed an antibiotic selection process to narrow down which group of gut microbes are causative for ASD-associated behaviors in the BTBR mice. We narrowed down the 'VNM' (vancomycin, neomycin, and metronidazole) antibiotic combination, which recapitulated high repetitive behavior (Fig. 4B). We then performed 16s rRNA sequencing of feces from BTBR mice treated with VNM to determine the identity of bacteria present after this antibiotic combination treatment. The VNM microbiota was enriched for *Esherichia/Shigella* and *Lactobacillus* genera (Fig. 4C) (the resolution of the 16S rRNA sequencing could only accurately specify up to the genus level). To avoid any infectious and pathogenic potential, we targeted the *Lactobacillus* genera instead of using *Esherichia/Shigella*, which are more commonly associated with their pathogenic properties. This rationale is included in the main text (lines 242-244).

We performed single-cell colony picking under *Lactobacillus* enrichment media conditions, which is listed in the methods section. Over 100 single colonies were picked and sequenced, and almost all of them were identified as *Ligilactobaillus murinus spp*., which suggests that this is the majority dominant *Lactobacillus* microbe in the VNM-treated mice. We have clarified this rationale by modifying the text to: "Single colony picking under Lactobacillus enrichment conditions and 16S rRNA sequencing resulted in the identification of *Ligilactobacillus murinus* spp., which composed the majority of single colonies (Fig. 4H)." (lines 244-246)

Finally, we found that various *L. murinus* strains carry the genes encoding enzymes involved in glutamate metabolism pathways. Previous studies have also discussed *L. murinus*'s glutamate-producing potential (citation within main text). Because our results indicate a pathogenic role of glutamate signaling in BTBR mice, *L. murinus*'s glutamate-producing capacity further validated its candidacy. We have included this point in the main text in lines 246-247 to clarify this point better.

Question #6:
Authors colonized GF-BTBR mice with L. murinus and show that these animals develop the same behavioral impairments as the SPF-BTBR mice. How was the colonization process done? In which point of development was the bacteria introduced? Since ASD is a neurodevelopmental disorder, it is important to note when the bacteria was introduced.

Response to Question #6:

We appreciate the reviewer's suggestion, and we have better specified the *L. murinus spp.* colonization procedure in the text.

Under the "Bacteria Treatment" methods section, we have included the protocol for colonizing GF BTBR mice with *L. murinus spp.* (lines 578-586). Briefly, pregnant GF BTBR females were colonized with $1x10^9$ CFU/mouse *L. murinus spp.* or DW control at E12.5. As ASD is a neurodevelopmental disorder, we performed the colonization early on to address any developmental effects of *L. murinus spp*. Successful colonization was checked in the feces using *Lactobacillus* genus-specific PCR primers. In the main text, we have included specifications of the amount of bacterium, delivery route (oral gavage), and timepoint of treatment in GF BTBR mice.

Question #7:
The authors note that the changes in gut microbiota in BTBR mice results in an imbalance of Glutamate/GABA in the faces and try to relate that to the changes in immunity in the brain. How would an imbalance of these metabolites in the gut influence brain function? Is there also a change in these metabolites in the brain that is recovered in the GF model of BTBR?

Response to Question #7:

We thank the reviewer for addressing this critical point.

We acknowledge that a limitation of this study is the assessment of only fecal metabolites and not those within the brain. Thus, it is unclear whether gut microbiota-derived metabolites directly affect metabolite pools within the brains of BTBR mice. While we have looked for solutions to address this point, our facility currently does not harbor capacities to detect glutamate and GABA within the brain. Additionally, such an in-depth analysis comparing fecal and brain metabolite pools may be outside the scope of this study, as it encompasses significantly further correlative analyses. Currently, only a few studies directly correlate specific metabolite levels within the feces with those of the brain. Future studies that address this point can be greatly helpful in understanding whether gut microbiota-derived metabolites directly pool within the brain, affecting its function.

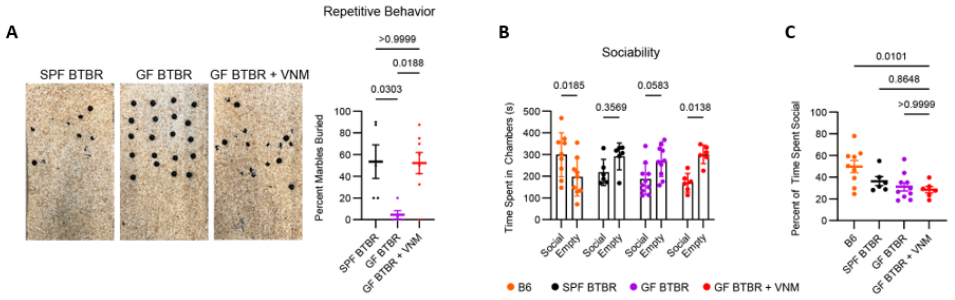
While we could not experimentally answer this question, we have provided additional details within the "Discussions" section of the main text and have identified this point as a limitation of this study.
The BBB harbors glutamate transporters, and glutamate can also directly cause BBB permeability, so it may be hypothesized that elevated peripheral glutamate will result in higher glutamate levels in the brain, although it is yet to be experimentally tested (lines 445-448). ASD patients have been characterized by high levels of glutamate in both the blood and the brain, suggesting that glutamate levels within the serum and brain may be parallel (lines 449-450). Finally, a study in rats demonstrated that feeding poly-gamma-glutamic acid could directly elevate both serum and brain concentrations of glutamate and GABA (lines 451-453).
While we regret that we could not experimentally assess brain levels of glutamate in our GF BTBR mice, we hope our acknowledgment and explanations of this limitation in the "Discussions" section can help fuel future studies into this matter.

Question #8:
Introduction of VNM microbiota leads to impairments in the marble burying test, was social behavior also assessed?

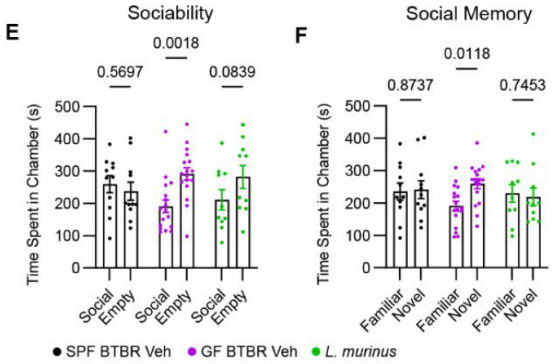
Response to Question #8:

We appreciate the reviewer's question.
We had previously assessed social behavior following colonization of GF BTBR mice with VNM microbiota and found that colonization did not affect sociability. The data is presented below for the reviewer. However, at the time of the experiment, we had not performed a social novelty test for the VNM colonization. Hence, we did not include the VNM sociability data in the manuscript to avoid confusion.
Instead, we have performed a social behavior test in GF BTBR mice colonized with VNM-derived *L. murinus spp*. We believe this data is more relevant to the narrative and better defines how a specific gut microbe can elicit ASD phenotypes. *L. murinus spp.* did not affect sociability, which is concurrent with our BTBR data. However, it did impair social memory compared to GF BTBR mice (similar to SPF BTBR mice), which suggests that VNM-derived bacteria (*L. murinus spp*) affects both repetitive behavior and social memory. We have included the *L. murinus spp.* social behavior data in Supplementary Fig. 6E, F and the main text (lines 251-252). While we could not directly address

whether VNM inoculation in GF BTBR mice affects social memory, we hope that the *L. murinus spp*. data can help address this question more specifically.



Supplementary Figure for the Reviewer | A. After colonizing GF BTBR mice with VNM microbiota, we confirmed that VNM microbiota recapitulate repetitive behavior. **B, C.** We then tested sociability in the three-chamber assay. While we observed that VNM microbiota maintained non-social phenotype in BTBR mice, at the time, we did not check social memory during in the social novelty test. For this reason, we omitted the sociability data for VNM-colonization in the manuscript.



Supplementary Figure 6 (Partial) | E. F, We have included sociability and social memory data for VNM-derived *L. murinus spp.* colonization in GF BTBR mice. We believe this data more specifically addresses whether a harmful bacterium can recapitulate ASD-associated behavior, including sociability and social memory, in BTBR mice.

Question #9:

L. murinus colonization leads to behavior impairments in GR-BTBR mice. Are these impairments specific to this bacteria? Using other colonies and heat-killed bacteria, would also be beneficial in demonstrating specificity and that the bacteria is required to be alive and therefore have a metabolic output to exert its effects.

Response to Question #9:

We thank the reviewer for this comment. We have included additional bacterium data to demonstrate that *L. murinus* spp. recapitulates ASD-associated behaviors in GF BTBR mice rather than any general bacterium.

GF BTBR mice were colonized with *Bifidobacterium bifidum* under the same conditions as *L. murinus spp*. However, unlike *L. murinus spp.*, *B. bifidum* could not induce high levels of marble burying in colonized mice. This suggests that *L. murinus spp.* is particularly responsible for ASD phenotypes in BTBR mice, rather than the phenotype being a nonspecific result of bacteria colonization. This data has been added to Supplementary Fig. 6G, and discussed in the main text (lines 253-257).

We have also treated heat-killed (H.K.) *L. murinus spp.* into GF BTBR mice. H.K. *L. murinus* failed to induce ASD-associated repetitive behavior, suggesting that live bacteria and their metabolites may be essential for ASD development in BTBR mice. We have included this data in Supplementary Fig. 6H and in the main text (lines 257-261).

Question #10:
The colors in figure 5 seems to be switched, in A the B6 mice is blue and the SPF-BTBR is red, while in the other graphs the BTBR-BTBR is blue and the B6-BTBR is red.

Response to Question #10:

We thank the reviewer for pointing out this mistake.
We have adjusted the color in Fig. 5A so that the "BTBR-BTBR" group is blue and the "BTBR-B6" group is red. This will make the colors for groups consistent across the figures.

Reviewer #3:

Question #1:
In figure 1A, SPF BTBR mice do not display a phenotype in sociability in 3-chamber, but the second sentence of the results section plainly states that they do. This data is inconsistent with Supp Fig 1A. If this mouse line does not display consistent behavioral phenotypes, conclusions cannot be drawn from changing additional factors and measuring behavior. Repeat the experiments to ensure the phenotype is consistent, or clarify the text if I am misinterpreting Fig 1A.

Response to Question #1:

We thank the reviewer for pointing out this discrepancy. We would like to note that this issue has already been addressed in our response to Question #2 from Reviewer #2.

In line with previous publications, we generally perceive the results from the three-chamber sociability assay as being either social or non-social. Low social preference can be defined as showing no preference between chambers and more time spent in the empty chamber. However, we acknowledge that the results from Supplementary Fig. 1A and Fig. 1A display a discrepancy and may cause confusion.

The data presented in Supplementary Fig. 1A data were obtained from initial preliminary experiments, and minor changes to the testing facility and procedures may have occurred since then.

To resolve this issue and address the reviewer's comment, we have performed additional experiment for Supplementary Fig. 1A. The text has also been modified to indicate that BTBR mice showed no preference for the social chamber in Supplementary Fig. 1A, with non-social phenotype described as similar or less time spent in the social chamber. We have added a clarification to our definition in lines 95-96.

Furthermore, to better define our interpretation, we have defined our parameters for 'social' or 'non-social', as well as 'social memory', in the methods section: "Neurotypical social preference was defined as more time spent in the social chamber, while ASD-associated non-social behavior was defined as either no preference between chambers or greater preference for the empty chamber. We defined neurotypical social memory as more time spent in the novel chamber, whereas impaired social memory was defined as no preference between chambers or more preference for the familiar chamber" (lines 496-500).

Question #2:
In my experience, BTBR mice are highly prone to grooming (and overgrooming to baldness). In fact, in many behavioral tests, I've observed that they score poor social interaction times, exploration, etc, and the results look quite striking but it seems that rather than an avoidance of typical behaviors- there is a compulsion for constant grooming and no other activity is performed. Please observe your videos of behavior tests completed thus far for this phenotype. If this could be a confounding variable in your hands as well, please think about what this could mean for your results. It will likely support your marble burying results. Your hyperactivity phenotype may indicate the opposite of what I have just described!

Response to Question #2:

We appreciate the reviewer's thoughtful suggestion.
BTBR mice are well-characterized to have high levels of self-grooming, which might cause concern on whether grooming might interfere with other behavioral tests. The concern may arise due to the longer immobile times during grooming, especially during tests like the three-chamber assay and open field maze, which are dependent on the duration of time each mouse spends in a location. However, as the

reviewer has noted, BTBR mice demonstrate significantly higher distance traveled and mean velocity during the open field maze than the B6 mice. This suggests that even with greater self-grooming, BTBR mice still moved more actively than B6, thus alleviating concerns of immobility.

We have addressed this concern by calculating self-grooming behavior between B6 and BTBR mice during the open field maze (Supplementary Fig. 1F) and during the three-chamber assay (Supplementary Fig. 1G). In both tests, BTBR mice show greater self-grooming than B6 mice, in accordance with previous publications. In addition, we added the distance traveled and mean velocity for the three-chamber assay (Supplementary Fig. 1H), in which BTBR mice had greater locomotion.
These results suggest that despite more self-grooming, the participation of BTBR mice in these assays did not significantly hinder the results. This rationale has been added to the main text (lines 108-114).

Question #3:
The lone c-fos data in SPF is orphaned. It will fill the story in nicely if you can please include this analysis for the later experiments as well.

Response to Question #3:

We appreciate the reviewer's suggestion.
We have added additional c-FOS imaging data into Figure 5, demonstrating that B6 microbiota colonization in GF-BTBR mice results in reduced c-FOS expression compared to that of BTBR microbiota (Fig. 5E, F). Figure 7 also features c-FOS data after t*reating L. reuteri* IMB015 in BTBR mice (Fig. 7I-K).
In total, c-FOS expression data are now presented in Supplementary Fig. 1, Fig. 1, Fig 5, and Fig. 7 to better connect the observed behavioral phenotypes with neuronal activity.

Question #4:
In the abstract and introduction, you tie anxiety into ASD phenotypes as if it is a main characteristic, and you describe BTBR as a good mouse model, yet your data shows that BTBR mice display lower anxiety-like behavior than WT mice. In the text you seem to avoid confronting this by vaguely stating "abnormal anxiety levels" (e.g. line 131). However, this is a bit contradictory. It is not without precedent for BTBR mice to display low levels of anxiety-like behavior, so this should be able to be clarified and explained. Overall, the description of the anxiety-like behavior could be improved. Later, in fig 3g, an increase in anxiety-like behavior is described as "improved" anxiety.

Response to Question #4:

We appreciate the reviewer's thoughtful comments. The reviewer raised an important point regarding the interpretation of open field maze results, and we fully agree on the concern. In response, we revised the text and figures to improve clarity and accuracy.

In the text, we have clarified our description of the results by removing the phrase "abnormal anxiety levels" (line 105) and rephrasing it as "impaired anxiety-like behaviors."
We tried to avoid using the term "reduced anxiety" as this could be misleading, especially since readers less familiar with behavior experiments might misinterpret the term and consider this phenotype as 'better' than neurotypical mice.
Our aim is to emphasize that although BTBR mice show increased time and frequency in the center zone, this behavior still reflects an atypical, non-neurotypical anxiety-like phenotype. Therefore, we believe the term "impaired" more accurately captures this deviation.

Additionally, we have revised the main text for Fig. 3G to state 'reduced time spent in the center' rather than "improved anxiety" to provide a cleaner and better description of the result. Across the manuscript,

we have also replaced “anxiety” with “anxiety-like behavior” to better reflect the nuances of the behavioral data.

Question #5:
I’d relabel your anxiety graphs. First, it should always be referred to as “anxiety-like” behavior, because we are unable to truly measure anxiety in mice. Second, the label of “anxiety” at the top of the graph will be misleading to anyone less familiar with the OFT, where it is easy to interpret that higher numbers in this graph=higher anxiety.

Response to Question #5:

We appreciate the reviewer’s valuable suggestion. We acknowledge the reviewer’s concern that labeling the graph with “anxiety” may potentially cause confusion, particularly for readers who are less familiar with the open field test and may misinterpret higher values as indicating more significant anxiety.

In response, we have revised the title of all graphs from “Anxiety” to “Open Field Maze” to inform readers of which behavior test the graphs represent, and prevent any potential misinterpretation of the results. Additionally, to provide more comprehensive support for our findings, we have included the data for the “Frequency to Center and Total Distance” graphs (now presented in Supplementary Fig. 1D, E and Fig. 1D, E, respectively).

Question #6:
Are you measuring hyperactivity or simply locomotion?

Response to Question #6:

We have relabeled the figures and text from “hyperactivity” to “locomotor activity” to better describe the phenotype and match the language used in the current literature. Additionally, we have added Distance Traveled during the open field maze in Supplementary Fig. 1 and Fig. 1, alongside the Mean Velocity graph, to better support our findings.

Question #7:
Figure 4 data is so exciting. Is ampicillin alone sufficient to induce the altered behavior?

Response to Question #7:

We appreciate the reviewer’s enthusiasm and insightful question.
Ampicillin treatment alone was indeed sufficient to prevent repetitive behavior in BTBR mice, mirroring the phenotypes observed in GF BTBR mice. These findings suggest that ampicillin-sensitive bacteria may drive the behavior phenotypes of BTBR mice (Supplementary Fig.6 A). Furthermore, colonizing GF BTBR mice with feces from VNM group recapitulated the high levels of repetitive behavior, reinforcing the role of microbiota in driving this effect (Supplementary Fig. 6B, C).

We have added this information to the main text (lines 221-224) and provided the data in Supplementary Figure 6.

Minor concerns #1:
It might be a good idea to clarify that abnormal social interactions and repetitive behaviors are core features defining ASD, while anxiety is not. Rather, it is a common comorbidity.

Response to Minor concern #1:

We thank the reviewer for this comment. We have modified the Introduction part to specify this. The text has been modified to: “Autism spectrum disorder (ASD) is a neurodevelopmental disorder with core behavioral features including social communication deficits and repetitive behaviors, with common comorbidities in neuropsychiatric symptoms such as anxiety and hyperactivity (lines 35-37).”

Minor concerns #2:

Typo line 43, hyperactivity

Response to Minor concern #2:

We thank the reviewer for pointing out the typo. We have corrected the typo.

Minor concerns #3:

Rephrase from “prevented the onset of multiple ASD-associated behaviors” and change it to something like “delayed the onset…”

Response to Minor concern #3:

We appreciate the reviewer’s thoughtful suggestion. We have revised the text to read: “The absence of gut microbiota offset multiple ASD-associated behaviors…” (lines 79-80) to more precisely reflect the lack of behavior manifestation, rather than a mere postponement.