nature portfolio

Peer Review File

Humid heat environment causes anxiety-like disorder via impairing gut microbiota and bile acid metabolism in mice

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The gut microbiota impacts multiple aspects of brain function and behaviour via the gut-brain axis, and microbial metabolites are important conduits of this host-microbe dialogue. The gut microbiota also exhibits substantial compositional and functional plasticity in response to a variety of environmental factors. In this study, the authors evaluate the impact of a humid heat environment (HHE) on behaviour, brain function and relevant microbiota-gut-brain axis signalling pathways. Key observations reported include that HHE-induced compositional alterations in the gut microbiota in an animal model is associated with anxiety-like behaviour, an impact potentially mediated by Lactobacillus murinus via impaired bile acid metabolism and enhanced neuroinflammation. Similar observations were recorded in human samples harvested during the humid heat season.

This is an intriguing and very comprehensive study with potentially important translational implications. Notable features include the variety of perspectives from which the hypothesis is evaluated, including the use of FMT and the administration of single bacterial strains. The broad range of behavioural and molecular assessments included is also very impressive. I have the following comments:

(1) I am not entirely convinced about the role of lithocholic acid since it is likely that activation of peripheral inflammatory pathways could also produce similar observations, and the authors report both increases of serum lithocholic acid and increased proinflammatory cytokines in the serum in both the animal and humans. It would be important to either demonstrate that there is also an increase in CNS concentrations of this bile acid, or to demonstrate that it is responsible for the barrier function disruption underpinning the neuroinflammatory phenotype.

(2) Germ-free animals are markedly altered at multiple levels of the gut-brain axis, including many of the behavioural and molecular features reported here. It is thus difficult to parse the impact of the HHE-associated microbiota-induced disruption from such underlying neurodevelopmental consequences of growing up with a gut microbiota. An alternative model of microbiota-disruption, such as FMT following antibiotic-induced knockdown, would be necessary to validate the claims made here.

(3) Additional detail is required for the processing of samples for the faecal transplantation study. Were these samples processed in anaerobic conditions and from fresh or frozen samples for example? I refer the authors to the GRAFT guidelines for additional experimental details that should

be reported (Guidelines for reporting on animal fecal transplantation (GRAFT) studies: recommendations from a systematic review of murine transplantation protocols https://doi.org/10.1080/19490976.2021.1979878).

(4) The details provided for the processing of the microbiota sequencing is very limited. What databases and bioinformatic pipelines were used?

(5) The term 'Gut flora' is obsolete, please use gut microbiota throughout the manuscript.

Reviewer #2 (Remarks to the Author):

In this manuscript, "Humid heat environment causes anxiety-like disorder through impairing gut microbiota and bile acid metabolism," the authors attempted to elucidate the potential mechanisms by which humid heat environments can cause anxiety disorders. They found a decrease in intestinal L. murinus bacteria and an increase in blood lithocholic acid in mice exposed to a humid heat environment and proposed that these could be inflammation-caused in the brain, resulting in anxiety, in a fecal transplantation and L. murinus supplementation experiment. This is a very interesting study, but several issues should be addressed to strengthen the paper.

1. The lack of behavioral abnormalities in heat-exposed female animals is a very important result. Did changes in the microbiome occur? Confirmation of the author's findings of reduced L. murinus and elevated lithocholic acid should be required.

2. Could the denaturation of the diet by humid heat treatment have affected the food intake, body growth, and gut microbiota of the mice? The preference of mice for humid heat-treated food and its effect on gut microbiome needs to be investigated.

3. How did the authors determine that L. murinus was the key bacterium? Could other L. reuteri and Akkermansia be recovered from behavioral abnormalities in a humid heat environment? A more detailed analysis of the gut microbiota is needed after supplementation of L. murinus bacteria. Are there increases in L. reuteri and Akkermansia?

4. Similarly, why did they conclude that lithocholic acid is key among the bile acid components?

5. Where do they think the inflammatory cytokines in the blood come from? As lithocholic acid causes liver damage, could damage in the liver be the origin of inflammation? Has the liver been examined?

6. Or do they believe that lithocholic acid disrupts the intestinal or brain barrier? The authors would like to present the mechanisms they envisage, from elevated blood lithocholic acid to elevated inflammatory cytokines in the blood and inflammation in the brain.

7. Although it is understood that the source of lithocholic acid is in the gut and that L. murinus are responsible for lithocholic acid synthesis, the mechanism for the inverse correlation between blood and fecal lithocholic acid in Fig 6 is not understood. If barrier disruption is the only reason, then all other metabolites would also increase in blood. The mechanism by which secondary bile acid metabolites, including lithocholic acid, characteristically increase in blood needs to be discussed.

Manuscript ID number

NCOMMS-23-54798-T

We sincerely thank the editor and all reviewers for their valuable feedback that we have used to improve the quality of our manuscript. The reviewer comments are laid out below in italicized font and specific concerns have been numbered. Our response is given in normal font and changes/additions to manuscript are given in the blue text.

RESPONSE TO REVIEWERS' COMMENTS

The two reviewers raised a number of constructive criticisms and suggestions. To fully address them, we performed additional experiments as well as implementing considerable changes to the manuscript. As a result, we believe the manuscript is much stronger. We wish to take this opportunity to thank the reviewers for their valuable input. Below, we summarize the reviewers' comments, and describe point-by-point how we have addressed them.

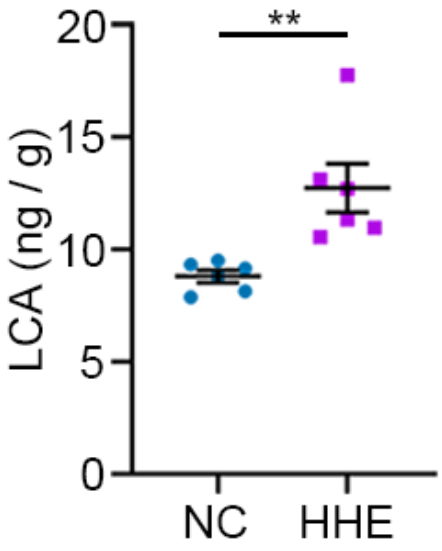
Reviewer 1

- ***The gut microbiota impacts multiple aspects of brain function and behaviour via the gut-brain axis, and microbial metabolites are important conduits of this host-microbe dialogue. The gut microbiota also exhibits substantial compositional and functional plasticity in response to a variety of environmental factors. In this study, the authors evaluate the impact of a humid heat environment (HHE) on behaviour, brain function and relevant microbiota-gut-brain axis signalling pathways. Key observations reported include that HHE-induced compositional alterations in the gut microbiota in an animal model is associated with anxiety-like behaviour, an impact potentially mediated by Lactobacillus murinus via impaired bile acid metabolism and enhanced neuroinflammation. Similar observations were recorded in human samples harvested during the humid heat season. This is an intriguing and very comprehensive study with potentially important translational implications. Notable features include the variety of perspectives from which the hypothesis is evaluated, including the use of FMT and the administration of single bacterial strains. The broad range of behavioural and molecular assessments included is also very impressive.***

Response: Many thanks for the reviewer's positive comments. We tried our best to improve our manuscript according to your constructive comments.

- ***Point 1: I am not entirely convinced about the role of lithocholic acid since it is likely that activation of peripheral inflammatory pathways could also produce similar observations, and the authors report both increases of serum lithocholic acid and increased proinflammatory cytokines in the serum in both the animal and humans. It would be important to either demonstrate that there is also an increase in CNS concentrations of this bile acid, or to demonstrate that it is responsible for the barrier function disruption underpinning the neuroinflammatory phenotype.***

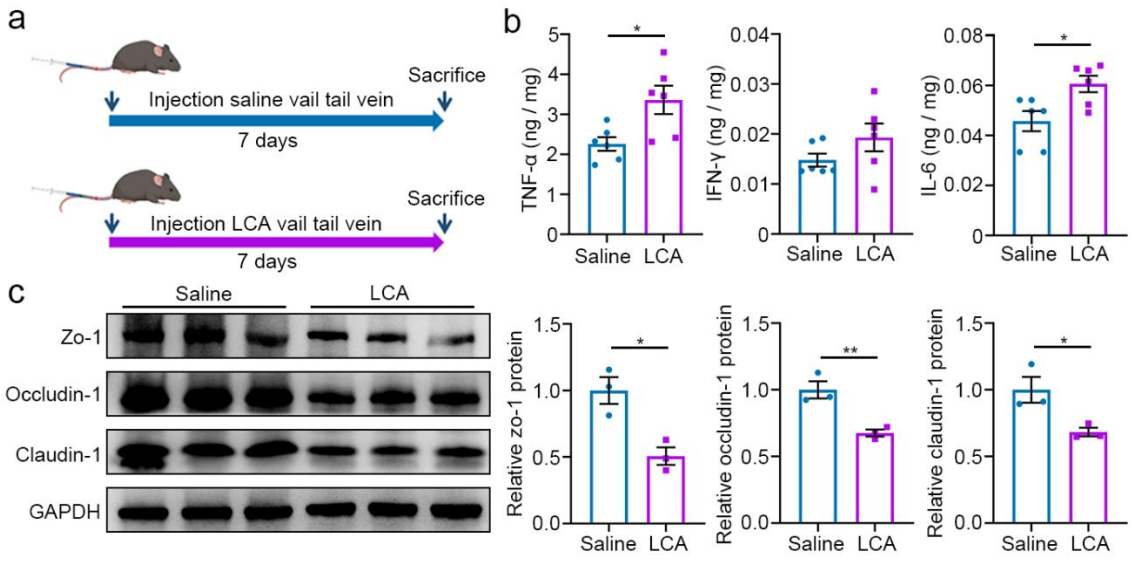
Response: According to the reviewer's comments, we provided the additional information in the revision as below: Firstly, we measured the concentration of lithocholic acid (LCA) in the brain in the NC and HHE groups, showing a significant increase in the HHE group compared to the NC group (new Extended Data Fig. 5). These additional results have been added to the revision (Result section: Page 8, Line 154) and are also shown as below.



Extended Data Fig 5. The level of Lithocholic acid increased in the brain.

LCA concentration measured by targeted mass spectrometry in the brain showed a significant increase in the HHE group compared to the NC group. **, $P<0.01$; Student's *t*-test; n=6 mice/group.

Secondly, to test the impact of LCA on the BBB permeability and neuroinflammation, mice in the NC group were subjected to vein injection of LCA or saline for 7 consecutive days, and we then analyzed the expression levels of the inflammatory factors and tight junction proteins (ZO-1, claudin-1 and occludin-1) in the brain (new Extended Data Fig. 12a). The results showed that LCA treatment significantly increased the expression of TNF-α and IL-6 (new Extended Data Fig. 12b) and downregulated the tight junction proteins (new Extended Data Fig. 14c) compared to the mice treated by saline. The results suggest that LCA is responsible for the BBB disruption and neuroinflammation. These additional results have been added to the revision (Result section: Page 12, Line 273-279) and are also shown as below. We briefly discussed the potential mechanisms in the revision (Discussion section: Page 17, Line 430-438)



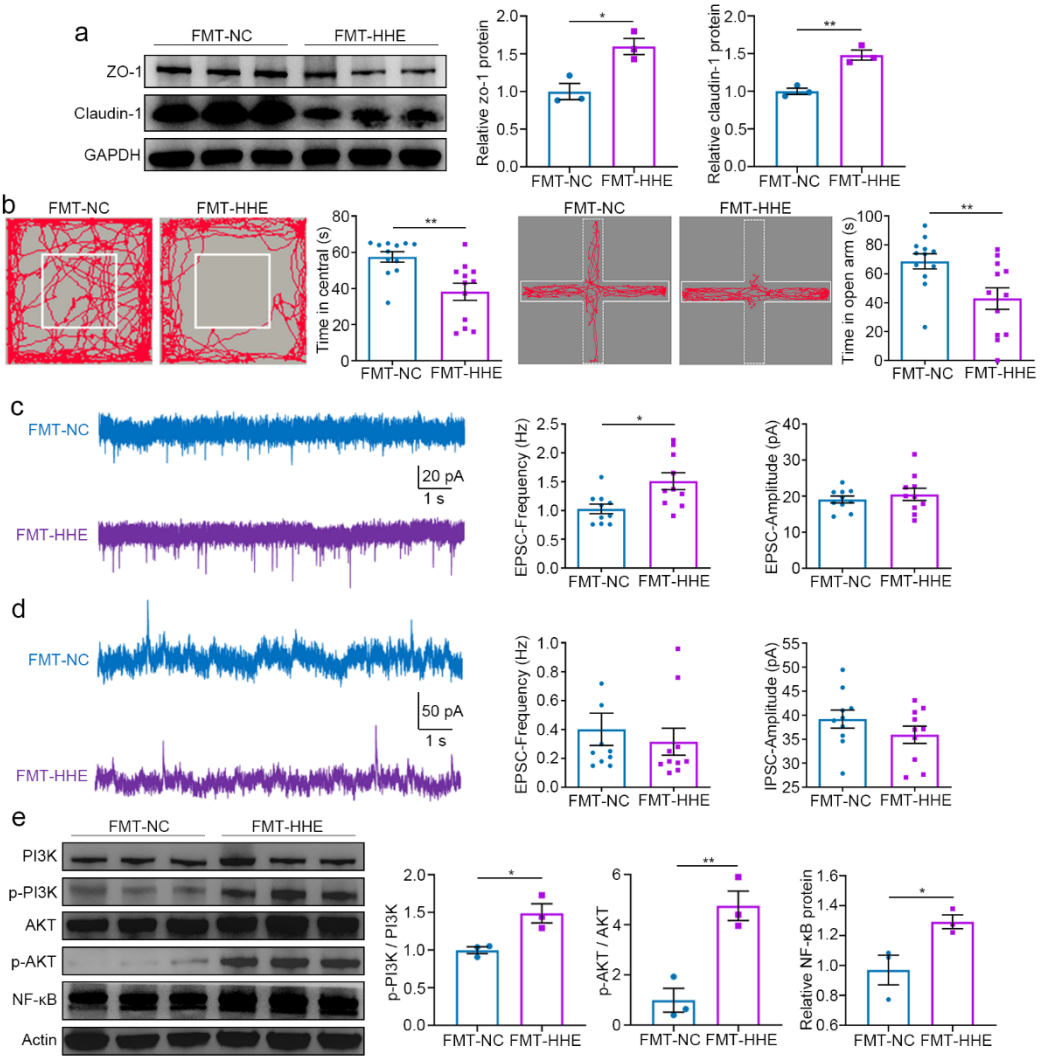
Extended Data Fig 12. Increased lithocholic acid impairs the BBB impermeability and promotes neuroinflammation in mice.

a Illustration of the experimental outflow. **b** Mice received vein injection of LCA for 7 days showed an increase of TNF-α and IL-6 detected by ELISA in the brain compared to the mice treated by saline. **c** Western blots showed a significant decrease of junction proteins including claudin-1, occludin-1 and ZO-1 in the cortical samples in the mice treated by LCA compared to those treated by saline. *, $P<0.05$; **, $P<0.01$; Student's *t*-test; n=6 mice/group for ELISA and mass spectrometry, and n=3 mice/group for

Western blots.

- ***Point 2: Germ-free animals are markedly altered at multiple levels of the gut-brain axis, including many of the behavioural and molecular features reported here. It is thus difficult to parse the impact of the HHE-associated microbiota-induced disruption from such underlying neurodevelopmental consequences of growing up with a gut microbiota. An alternative model of microbiota-disruption, such as FMT following antibiotic-induced knockdown, would be necessary to validate the claims made here.***

Response: Thank the reviewer. We tested the impact of FMT using the microbiota-depleted mice, and the phenotypes in the HHE group was recapitulated as well (please see new Extended Data Fig. 10). Briefly, mice were treated by an antibiotic cocktail (ampicillin, 0.25 mg/mL; neomycin, 0.25 mg/mL; metronidazole, 0.25 mg/mL; vancomycin, 0.125 mg/mL) to deplete the microbiota as described before[1] and then underwent FMT from either the NC group or HHE group. Mice received FMT from the HHE group showed the downregulation of tight junction proteins (new Extended Data Fig. 10a), anxiety-like behaviors (new Extended Data Fig. 10b), excitability increase of pyramidal neurons in the cortex (new Extended Data Fig. 10c, d), the upregulation of phosphorylated PI3K and AKT and increase of NF-kB in the brain (new Extended Data Fig. 10e). These additional results have been added to the revision (Result section: Page 10, Line 216-222) and are shown as below.



Extended Data Fig. 10. HHE mice-derived FMT results in microbiota-depleted mouse recapitulating the phenotypes observed in the HHE group.

Mice were treated by an antibiotic cocktail to deplete gut microbiota and then underwent FMT from the HHE group or the NC group, which were short for the FMT-HHE group or the FMT-NC group respectively. **a** Western blots of colon samples showed a significant increase of claudin-1 and ZO-1 in the FMT-HHE group compared to the FMT-NC group (n = 3 mice/group). **b** The time of travelling the central zone in the open-field test and staying close arms of elevated plus maze was significantly increased in the FMT-HHE group compared to the FMT-NC group (n=12 mice/group). **c, d** Electrophysiological recordings in mouse acute brain slices showed a significant frequency decrease of pyramidal neuron sEPSC, but no differences of sEPSC amplitude, in the FMT-HHE group compared to the FMT-NC group (**c**). There were no differences

of sIPSC recordings in two groups (**d**). Total 10 neurons in each recording and 3 mice in each group. **e** Western blots of cortical samples showed a significant decrease of phosphorylated PI3K and AKT1, total NF-κB proteins in the FMT-HHE group compared to the FMT-NC group. *, $P<0.05$; **, $P<0.01$; Student's *t*-test; n=3 mice/group.

- ***Point 3: Additional detail is required for the processing of samples for the faecal transplantation study. Were these samples processed in anaerobic conditions and from fresh or frozen samples for example? I refer the authors to the GRAFT guidelines for additional experimental details that should be reported (Guidelines for reporting on animal fecal transplantation (GRAFT) studies: recommendations from a systematic review of murine transplantation protocols https://doi.org/10.1080/19490976.2021.1979878).***

Response: Thanks. In the revision, we provided additional details (Methods section: Page 21, Line 537-546) of the sample processing for the faecal transplantation study referred to the guidelines for reporting on animal fecal transplantation (GRAFT) studies.

- ***Point 4: The details provided for the processing of the microbiota sequencing is very limited. What databases and bioinformatic pipelines were used?***

Response: Thanks. The details have been added in the revision (Methods section; Page 19, Line 485-506). Detailed as follow: Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (v1.2.11, http://ccb.jhu.edu/software/FLASH/), a very fast and accurate analysis tool, which was designed to merge paired-end reads when at least some of the reads overlap the read

generated from the opposite end of the same DNA fragment, and the splicing sequences were called raw tags. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME (V1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) quality control process. The tags were compared with the reference database (Silva database https://www.arb-silva.de/) and using UCHIME Algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences, and then the chimera sequences were removed. Then the Effective Tags finally obtained. Sequences analysis were performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/). Sequences with ff97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. Amplicon sequence variant (ASV) were analysised by Deblur, which uses error profiles to obtain putative error-free sequences from Illumina MiSeq and HiSeq sequencing platforms. In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignment was conducted using the MAFFT (v7.490, https://mafft.cbrc.jp/alignment/software/). OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data.

- ***Point 5: The term 'Gut flora' is obsolete, please use gut microbiota throughout the manuscript.***

Response: Thank you for the suggestion. In the revision, "gut flora" has been replaced by "gut microbiota".

Reviewer 2

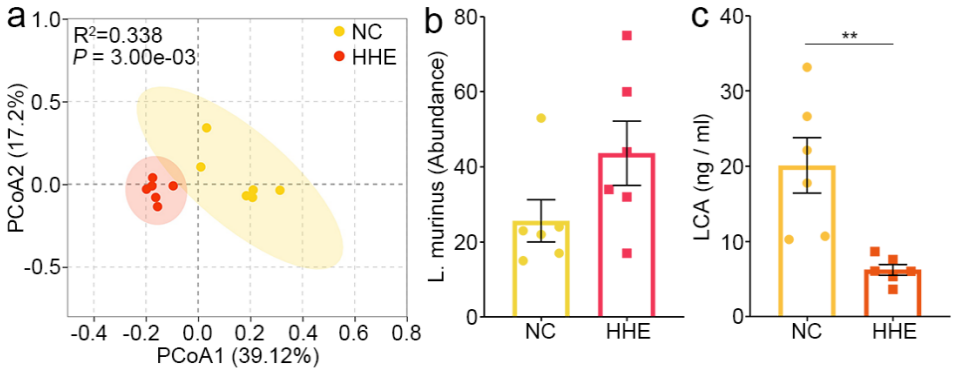
- ***In this manuscript, "Humid heat environment causes anxiety-like disorder through impairing gut microbiota and bile acid metabolism," the authors attempted to elucidate the potential mechanisms by which humid heat environments can cause anxiety disorders. They found a decrease in intestinal L. murinus bacteria and an increase in blood lithocholic acid in mice exposed to a humid heat environment and proposed that these could be inflammation-caused in the brain, resulting in anxiety, in a fecal transplantation and L. murinus supplementation experiment. This is a very interesting study, but several issues should be addressed to strengthen the paper.***

Response: We are very grateful for your constructive and helpful comments.

- ***Point 1: The lack of behavioral abnormalities in heat-exposed female animals is a very important result. Did changes in the microbiome occur? Confirmation of the author's findings of reduced L. murinus and elevated lithocholic acid should be required.***

Response: Thank the reviewer for this important comment. We collected the faecal samples from female mice and performed 16S rRNA gene sequencing. Although the clustering of gut microbiota was different in the NC and HHE groups (Attached Fig. 1a), the abundance of *L. murinus* showed no significant differences between two groups (Attached Fig. 1b). In addition, we compared the expression of LCA in the female mice between the NC and HHE groups and found that serum LCA was significantly decreased in the HHE group compared to the NC group (Attached Fig. 1c). The finding is different from the observation in the male mice in which the abundance of *L. murinus* was significantly decreased and LCA was significantly increased in the HHE group. Thus, humid heat environment imposes different effect on the gut microbiota and

metabolism in males and female mice, and the potential mechanisms are required for further study in the future. In the work, we focused on the study using male mice. As suggested by the Editor, we changed our title as 'Humid heat environment causes anxiety-like disorder through impairing gut microbiota and bile acid metabolism in male mice', and the related explanation was also provided in the text.

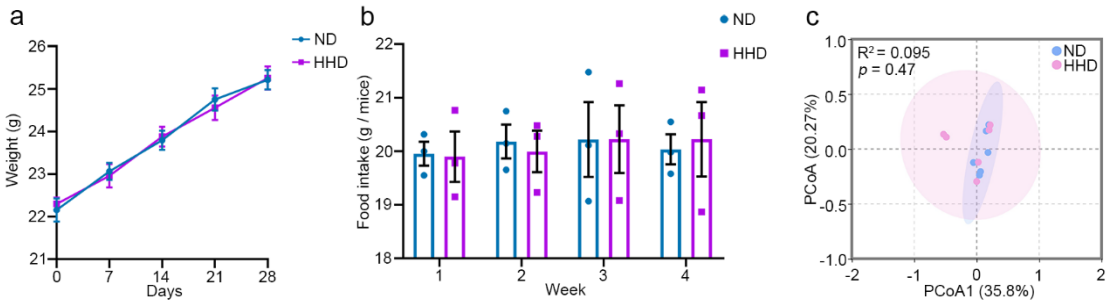


Attached Fig. 1. Impact of humid heat environment on gut microbiota and serum LCA in female mice. Female mouse faecal samples were collected from the HHE and NC groups for 16S rRNA sequencing, showing the significant differences of microbial composition in the PCoA (beta diversity) (a), and the abundance of *L. murinus* was comparable in the HHE and NC groups (b). LCA concentration in serum of female mice was significantly decreased in the HHE group compared to the NC group (c). **, $P<0.01$; Student's *t*-test; n=6 mice/group.

- ***Point 2: Could the denaturation of the diet by humid heat treatment have affected the food intake, body growth, and gut microbiota of the mice? The preference of mice for humid heat-treated food and its effect on gut microbiome needs to be investigated.***

Response: Thanks for this important comment. Actually, we replaced the mice with fresh food every 3 days to avoid the denaturation of the diet caused by humid heat treatment. We performed additional experiments to confirm whether the diet exposed

in the humid heat environment for 3 days impose an effect on mouse food intake, body growth, and gut microbiota. Mice received humid heat environment-exposed diet (The HHD group) or normal diet (the ND group) for 4 weeks, and mouse weight and food intake were monitored every week showing no differences in the ND and HHD groups (Attached Fig. 2a, b). At 4 weeks, 16S rRNA gene sequencing of mouse faecal samples showed that the microbiome composition was comparable in the ND and HHD groups (Attached Fig. 2c). Thus, the diet is not one important cause to account for the phenotypes observed in the HHE group.

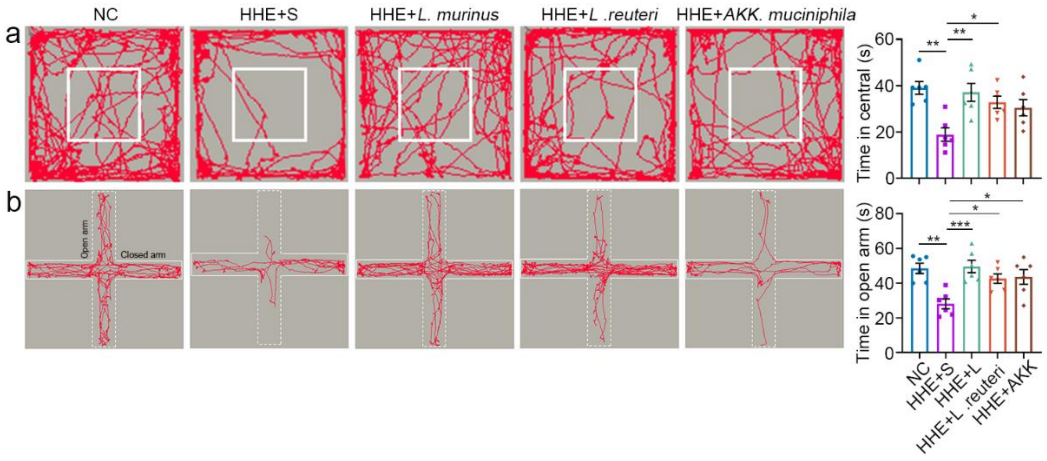


Attached Fig. 2. The diet is not the cause of the phenotypes observed in the HHE group. Mice received humid heat environment-exposed diet (The HHD group) or normal diet (the ND group) for 4 weeks. There were no differences of their weight (a) and food intake (b) in two groups. N = 3 cages per group, 6 mice per cage, two-way repeated ANOVA followed by Bonferroni's multiple comparisons. In addition, the microbial composition showed comparable in the ND and HDD groups (c; n=6 mice/group).

- ***Point 3: How did the authors determine that was the key bacterium? Could other L. reuteri and Akkermansia be recovered from behavioral abnormalities in a humid heat environment? A more detailed analysis of the gut microbiota is needed after supplementation of L. murinus bacteria. Are there increases in L. reuteri and Akkermansia?***

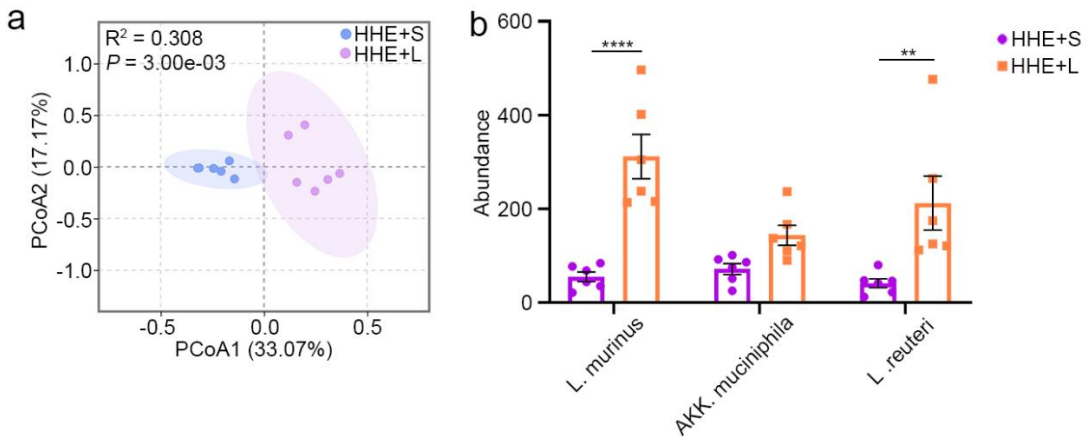
Response: Thank the reviewer. We supposed that *L. murinus* was the key bacterium to account for the phenotypes in the HHE group, and this conclusion was supported by the following information. (i) Among ten altered bacterial groups, the decrease of *L. murinus* was most significant in the HHE group compared to the NC group (Fig. 2f). (ii) Ecological network interaction analysis showed that the reduction of *L. murinus* was synergistically associated with the reduction of protective bacteria (e.g., *L. reuteri*), and that *L. murinus* was the dominant species that dominates interactions and interacted closely with other protective bacteria (Fig. 2g). (iii) The reduction of *L. murinus* abundance was also identified in the GF mice received FMT from the mice in the HHE group and the human subjects in the humid heat season (Fig. 3d, Extended Data Fig. 6d). (iv) *L. murinus* administration reversed mouse abnormalities in the HHE group. (v) Our finding was also in line with the previous reports: *L. murinus* significantly alleviated the anxiety like behaviors[2] and possessed the ability to modify bile acids[3].

We agree with the reviewer that other altered bacteria might be also involved in the abnormalities observed in the HHE group. To test this, we performed the additional experiments. HHE-treated mice were subjected to the administration of *L. murinus*, *L. reuteri* (the HHE+*L. reuteri* group), *Akkermansia muciniphila* (the HHE+ Akk group), or saline (the HHE+S group). In the open-field test, the time that mice travelled the central area was increased in the HHE+L, HHE+*L. reuteri* group, but not in the HHE+Akk group, compared with HHE+S group (new Extended Data Fig. 14a). In the elevated plus maze, the time spent in the open arms was increased in the HHE+L group, HHE+L. reuteri group and HHE+Akk group (new Extended Data Fig. 14b). However, *L. murinus* treatment induced most significant behavioral improvements. The results have been added to the revision (Result section: Page 13, Line 312-324) and are shown as below.



Extended Data Fig. 14. *L. murinus* have the better improvement for HHE-induced anxiety disorder compared to *L. reuteri* and *Akkermansia muciniphila.* a, b There was a significant increase of travelling the central zone of the open field and staying open arms of elevated plus maze in the both HHE+L group and HHE+*L. reuteri* group compared to the HHE+S group. HHE+Akk group spent more time stay open arms of elevated plus maze compared to the HHE+S group. n=6 mice/group; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; one-way ANOVA and Tukey's multiple comparisons test.

We also collected the faecal samples from the HHE+L and HHE+S groups and performed 16S rRNA gene sequencing. In the HHE+L group (treated with *L. murinus*), the gut microbiota composition in beta diversity was significantly different from that in the HHE+S group (treated by saline) (new Extended Data Fig. 15a). The abundance of *L. murinus* and *L. reuteri*, but not of *Akkermansia muciniphila*, was significantly increased in the HHE+L group compared to the NC group (new Extended Data Fig. 15b). These results have been added to the revision (Result section: Page 14, Line 327-333) and are shown as below.



Extended Data Fig. 15. *L. murinus* treatment alters the gut microbiota in the HHE group. Mice in the HHE group were treated with *L. murinus* (the HHE+L group) or saline (The HHE+S group), and their faecal samples were collected for 16S rRNA gene sequencing. **a** Unweighted UniFrac distance-based analysis showed the significant differences of microbial composition in the PCoA (beta diversity) in two groups. **b** The abundance of *L. murinus* and *L. reuteri*, but not of *Akkermansia muciniphila* was significantly increased in the HHE+L group compared to the HHE+S group. **, P<0.01; ****, P<0.0001; Student's *t*-test; n= 6 mice/group.

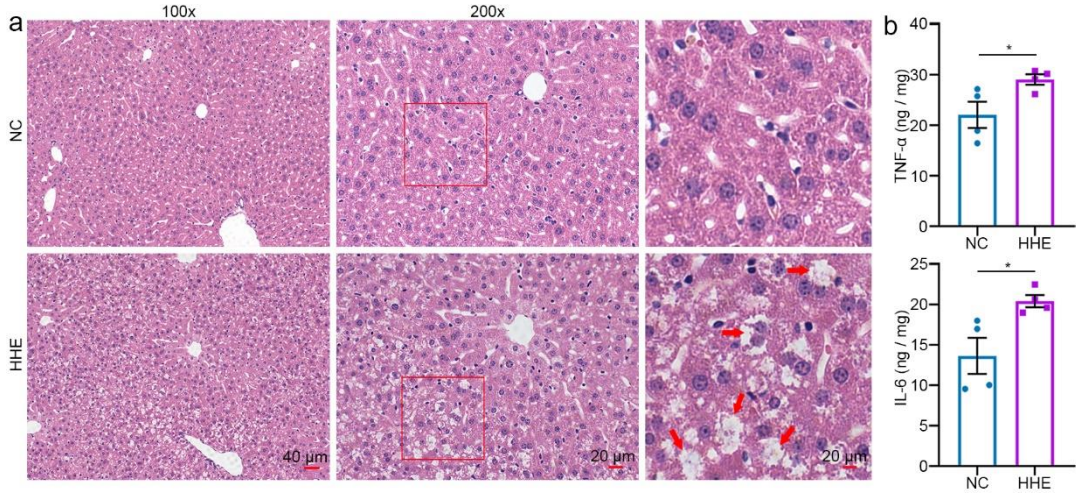
- ***Point 4: Similarly, why did they conclude that lithocholic acid is key among the bile acid components?***

Response: Thanks for the reviewer's question, and the related question was also mentioned by the first reviewer. Our detailed response is as follows: (i) Among the altered secondary bile acids in the HHE group (Fig. 2j), lithocholic acid (LCA) showed the highest toxicity [4] and was known to be closely associated with anxiety disorders [5]. (ii) The concentration of serum LCA was negatively correlated with the abundance of *L. murinus* in faecal sample (Fig. 2l). (iii) Our FMT experiment demonstrated that gut microbiota from the HHE group caused the elevation of serum LCA using germ-free mice (Fig. 3g). Upregulation of serum LCA was also identified in the human subjects

during humid heat season and accompanied with the decrease of *L. murinus* abundance in the faecal samples (Fig. 8d, e). (iii) Our additional experiments as described above showed that LCA was significantly increased in the brain in the HHE group compared with the NC group, and that LCA treatment caused the permeability increase of the BBB and neuroinflammation (new Extended Data Fig. 12 a-c). The additional results have been added to the revision (Result section: Page 12, Line 273-279).

- ***Point 5: Where do they think the inflammatory cytokines in the blood come from? As lithocholic acid causes liver damage, could damage in the liver be the origin of inflammation? Has the liver been examined?***

Response: To answer the reviewer's question, we did H&E staining of liver sections and detected the expression of TNF-α and IL-6 in liver samples from the HHE and NC groups. We found numerous vacuoles (red arrows) indicating lipid deposition and elevated pro-inflammatory cytokines (TNF-α and IL-6) in the HHE group compared with the NC group (new Extended Data Fig. 13a, b). The result indicates that the damaged liver may be one origin of the inflammatory cytokines in the HHE group. These results have been added to the revision (Result section: Page 12, Line 279-284) and are shown as below.



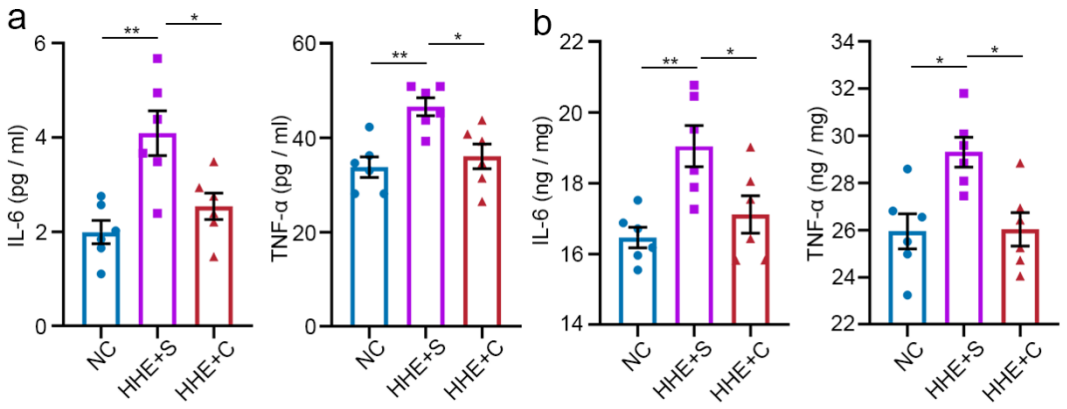
Extended Data Fig. 13. HHE induces mouse live damage and inflammatory cytokine secretion.

a. H&E staining showed lipid deposition (red arrows) in the HHE group but rarely in the NC group. **b.** ELISA of liver samples showed a significant increase of TNF-α and IL-6 in the HHE group compared to the NC group. *, $P<0.05$; Student's *t*-test; n=4 mice/group.

- ***Point 6: Or do they believe that lithocholic acid disrupts the intestinal or brain barrier? The authors would like to present the mechanisms they envisage, from elevated blood lithocholic acid to elevated inflammatory cytokines in the blood and inflammation in the brain.***

Response: We agree with the reviewer that LCA may disrupt the brain and/or intestinal barrier. Our additional experiment as described above demonstrated that LCA treatment resulted in downregulation of junction proteins (ZO-1, Oculudin-1 and Claudin-1) and upregulation of pro-inflammatory cytokines (TNF-α and IL-6) in the brain (new Extended Data Fig. 12). In addition, mice in the HHE group treated by colestyramine (a bile acid binding resin) to reduce bile acid accumulation in the blood showed a significant decrease of inflammatory factors (TNF-α and IL-6) in the serum and brain (Attached Fig. 3a, b). This finding is in line with the previous reports[6]. We briefly discussed the potential mechanisms in the revision (Discussion section: Page 17, Line 430-438) as: Elevated serum LCA may cause the liver to produce inflammatory cytokines and impair the BBB, leading to neuroinflammation in the brain. In addition, the upregulation of serum LCA was positively correlated with the phosphorylation of PI3K and AKT in the cortical samples. Previous studies have shown that phosphorylation of PI3K/Akt plays an essential role in microglial activation by stimulating NF-κB activity[7], following with the increase of inflammatory cytokines release[8-9]. Therefore, we speculate that the inflammatory factors increase in peripheral

induced by LCA disrupts the blood-brain barrier, and the neuroinflammation increased in the brain induced by LCA causes the brain to produce more inflammatory factors into the blood, finally resulting inflammatory cascading response.



Attached Fig. 3. Colestyramine treatment inhibits elevation of TNF-α and IL-6 in the HHE group. Mice in the HHE group were treated with colestyramine (the HHE+C group) or saline (the HHE+S group), and blood and cortical samples were collected for ELISA. The samples from the NC group were used as the reference. In the serum, the expression of TNF-α and IL-6 was higher in the HHE+S group than the NC group and the HHE+C group (a), and the similar changes were found in the cortical samples (b). *, $P<0.05$; **, $P<0.01$; one-way ANOVA and Tukey's multiple comparisons test; n=6 mice/group.

- ***Point 7: Although it is understood that the source of lithocholic acid is in the gut and that L. murinus are responsible for lithocholic acid synthesis, the mechanism for the inverse correlation between blood and fecal lithocholic acid in Fig 6 is not understood. If barrier disruption is the only reason, then all other metabolites would also increase in blood. The mechanism by which secondary bile acid metabolites, including lithocholic acid, characteristically increase in blood needs to be discussed.***

Response: We found an inverse change of LCA levels in the serum and fecal samples after *L. murinus* treatment of mice in the HHE group (see Fig. 6c, d). LCA is one unconjugated bile acid and enters the blood through passive absorption in the colon, and the absorption is highly dependent on the impermeability of the intestinal barrier[10]. HHE-induced gut microbiota dysbiosis (e.g., *L. murinus* decrease) could impair the permeability of the intestinal barrier allowing more passive absorption of bile acids such as LCA into serum[11], which subsequently resulted in the decrease of LCA excretion in the fecal samples. After *L. murinus* treatment, the permeability of the intestinal barrier was reduced; LCA absorption into blood was decreased and LCA excretion in fecal samples was increased. In line with this, we found that the abundance of *L. murinus* was negatively correlated with LCA concentration in serum (Fig. 2l). We added a brief explanation to the revision (Discussion section: Page 16, Line 408-415).

In addition to LCA, other secondary bile acids were also upregulated in serum such as taurochenodesoxycholic acid, glycocholic acid, taurodeoxycholic acid, taurine-α-ratcholate sodium salt, and taurocholic acid in the HHE group compared to the NC group (Fig. 2j). Among them, LCA is a monohydroxylated secondary bile acid formed from the primary bile acid CDCA and is one of the most hydrophobic natural bile acids[12]. Hydrophobic nature of bile acids allows them to enter cells through sodium-independent transporter and passive diffusion[13-14], and this confers LCA with predominance to enter the blood. Our study demonstrated that elevated LCA played a critical role in causing the abnormalities in the HHE group. We added a brief explanation to the revision (Discussion section: Page 17, Line 419-427)

References

1. Zhao, Q.; Dai, M. Y.; Huang, R. Y.; Duan, J. Y.; Zhang, T.; Bao, W. M.; Zhang, J. Y.; Gui, S. Q.; Xia, S. M.; Dai, C. T.; Tang, Y. M.; Gonzalez, F. J.; Li, F., Parabacteroides distasonis ameliorates hepatic fibrosis potentially via modulating intestinal bile acid

metabolism and hepatocyte pyroptosis in male mice. *Nat Commun*. **14** (1), 1829 (2023).

2. Li, L. F.; Zou, H. W.; Song, B. L.; Wang, Y.; Jiang, Y.; Li, Z. L.; Niu, Q. H.; Liu, Y. J., Increased Lactobacillus Abundance Contributes to Stress Resilience in Mice Exposed to Chronic Social Defeat Stress. *Neuroendocrinology*. **113** (5), 563-576 (2023).

3. Jukes, C. A.; Ijaz, U. Z.; Buckley, A.; Spencer, J.; Irvine, J.; Candlish, D.; Li, J. V.; Marchesi, J. R.; Douce, G., Bile salt metabolism is not the only factor contributing to Clostridioides (Clostridium) difficile disease severity in the murine model of disease. *Gut Microbes*. **11** (3), 481-496 (2020).

4. Holtmann, T. M.; Inzaugarat, M. E.; Knorr, J.; Geisler, L.; Schulz, M.; Bieghs, V.; Frissen, M.; Feldstein, A. E.; Tacke, F.; Trautwein, C.; Wree, A., Bile Acids Activate NLRP3 Inflammasome, Promoting Murine Liver Inflammation or Fibrosis in a Cell Type-Specific Manner. *Cells*. **10** (10) (2021).

5. MahmoudianDehkordi, S.; Bhattacharyya, S.; Brydges, C. R.; Jia, W.; Fiehn, O.; Rush, A. J.; Dunlop, B. W.; Kaddurah-Daouk, R., Gut Microbiome-Linked Metabolites in the Pathobiology of Major Depression With or Without Anxiety-A Role for Bile Acids. *Front Neurosci*. **16**, 937906 (2022).

6. McMillin, M.; Frampton, G.; Grant, S.; Khan, S.; Diocares, J.; Petrescu, A.; Wyatt, A.; Kain, J.; Jefferson, B.; DeMorrow, S., Bile Acid-Mediated Sphingosine-1-Phosphate Receptor 2 Signaling Promotes Neuroinflammation during Hepatic Encephalopathy in Mice. *Frontiers in cellular neuroscience*. **11**, 191 (2017).

7. Jayasooriya, R. G.; Lee, K. T.; Kang, C. H.; Dilshara, M. G.; Lee, H. J.; Choi, Y. H.; Choi, I. W.; Kim, G. Y., Isobutyrylshikonin inhibits lipopolysaccharide-induced nitric oxide and prostaglandin E2 production in BV2 microglial cells by suppressing the PI3K/Akt-mediated nuclear transcription factor-κB pathway. *Nutrition research (New York, N.Y.)*. **34** (12), 1111-9 (2014).

8. Cianciulli, A.; Calvello, R.; Porro, C.; Trotta, T.; Salvatore, R.; Panaro, M. A., PI3k/Akt signalling pathway plays a crucial role in the anti-inflammatory effects of

curcumin in LPS-activated microglia. *International immunopharmacology*. **36**, 282-290 (2016).

9. Tarassishin, L.; Suh, H. S.; Lee, S. C., Interferon regulatory factor 3 plays an anti-inflammatory role in microglia by activating the PI3K/Akt pathway. *Journal of neuroinflammation*. **8**, 187 **(**2011).

10. Lennernäs, H., Intestinal permeability and its relevance for absorption and elimination. *Xenobiotica; the fate of foreign compounds in biological systems*. **37** (10-11), 1015-51 (2007).

11. Collins, S. L.; Stine, J. G.; Bisanz, J. E.; Okafor, C. D.; Patterson, A. D., Bile acids and the gut microbiota: metabolic interactions and impacts on disease. *Nature reviews. Microbiology*. **21** (4), 236-247 (2023).

12. Lucangioli, S. E.; Carducci, C. N.; Tripodi, V. P.; Kenndler, E., Retention of bile salts in micellar electrokinetic chromatography: relation of capacity factor to octanol-water partition coefficient and critical micellar concentration. *Journal of chromatography. B, Biomedical sciences and applications*. **765** (2), 113-20 (2001).

13. Van Dyke, R. W.; Stephens, J. E.; Scharschmidt, B. F., Bile acid transport in cultured rat hepatocytes. *The American journal of physiology* 1982, *243* (6), G484-92.

14. Aldini, R.; Roda, A.; Montagnani, M.; Cerrè, C.; Pellicciari, R.; Roda, E., Relationship between structure and intestinal absorption of bile acids with a steroid or side-chain modification. *Steroids*. **61** (10), 590-7 (1996).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have been very responsive to the initial recommendations. This includes the addition of new experimental data which supports the main thrust of the original paper. I have no further comments.

Reviewer #2 (Remarks to the Author):

My comments are appropriately addressed, thus I have no further remarks.