Peer Review File

Reviewer A

Qiu and colleagues presented a research article aimed at elucidating the anti-depressive potential of Lactobacillus delbrueckii subsp. Bulgaricus (Lac) tested in mice with LPS-induced depression. Overall, the research idea is interesting and the experimental approach used could be appropriate. However, the experimental design and the entire study are described in a very confusing manner and some aspects have to be clarified before considering the manuscript suitable for publication. In addition, the entire manuscript needs extensive English revision. Below are reported some minor/major comments that will improve the quality of the manuscript:

1) In the title the authors should indicate the specific strain used in their experiments and not the term "Lactobacillus";

Reply 1: Specific strain has longer name. It is *lactobacillus delbrueckii subsp. Bulgaricus*. We changed it into *lactobacillus delbrueckii* in the title.

Changes in the text: Please see the revisions in the Title section. (Page 1, line 1.)

2) In the Introduction section the authors state "Although, the pathogenesis of depression remains unclear, the role of many immunoreactive cells in the occurrence and development of depression in the central nervous system, especially microglia, which affects neuroplasticity, has been gradually discovered." However, they do not provide references supporting this notion. In addition, the authors should also mention the cytokine alterations induced by alcohol abuse or physio-pathological condition, like pregnancy, often associated with depression. For this purpose, see:

- 10.3892/etm.2020.8410
- 10.3892/etm.2019.7774
- 10.3389/fncel.2015.00476
- 10.3389/fnbeh.2017.00207

Reply 2: We rewrote the Introduction and added some relevant literature.

3) Please provide references supporting the following statement "Lactobacillus delbrueckii subsp. Bulgaricus (Lac), as the most widely used probiotics in dairy products, has attracted more and more attention in alleviating mental illness.";

Reply 3: We have revised it and added references.

Changes in the text: Please see the changes in the Abstract, Introduction and References sections. (Page 2, line 20-24; Page 4, line 79-83; Page 21, line 441-458; Page 22, line 459-462.)

4) Mice were fed with 18% protein, 58% carbohydrate, 4% fat. What are the macronutrients constituting the remaining 20% of food composition? Please clarify;

Reply 4: 15% Water and 5% others including minerals, vitamins and salts. We added it in the Mice model section.

Changes in the text: Please see the changes in the Mice model section. (Page 5, line 95-96.)

5) How long after obtaining successful depressed mice they were treated with the saline or Lac?

Reply 5: Depression-like behavior was detected 24 hours later, and the mice showed obvious depression-like behavior.

6) In section 5, please indicate the genes analyzed in qPCR. Same comment for the proteins analyzed in section 6;

Reply 6: The specific gene names and primer information for qPCR were shown in supplementary table 1 (Page 27, line 556-558). The specific protein names of Western Blot were shown in section 6 (Page 9, line 175-177). We also added specific gene names in the QPCR Section.

Changes in the text: Please see the changes in the QPCR section. (Page 7, line 150-151.)

7) In section 8 it is not clear if the authors assess the MIC concentration of Lac and its fermentation products towards other bacteria or if other types of tests were performed.

For section 8 the authors have to obtain MIC concentration. Please clarify and revise if necessary;

Reply 7: Yes. We identified the MIC of lac, and basically determined that the number of Lac was three times or more than three times that of pathogenic bacteria, which could completely inhibit the growth of several common pathogens we tested. We have added this in the results section and supplemented Figure 1.

Changes in the text: Please see the supplements in the Results section. (Page 13, line 283；Page 14, line 284-292.)

8) The authors should start the Discussion section describing the beneficial effects of probiotics, and in particular Lactobacilli, for the treatment of different pathologies ranging from tumors to inflammatory diseases and from neurological disorders to autoimmune disease. For this purpose, see:
- 10.3389/fphar.2017.00603
- 10.3390/cancers11010038
- 10.3390/nu10101537
- 10.1097/MD.0000000000013792
- 10.1186/s12967-016-1058-7
- 10.1186/s12991-017-0138-2

Reply 8: Thank you very much for the valuable comments. The therapeutic effects of *Lactobacillus* on related diseases have been discussed and the references have been supplemented.

Changes in the text: Please see the supplements in the Discussion and References sections. (Page 14, line 305; Page 15, line 306-314; Page 22, line 471-479.)

9) Throughout the manuscript there are several grammar errors (e.g. line 92 "They was"; etc.). Please check and correct all the errors;

Reply 9: We asked native speaker to check and correct the grammar errors in the manuscript. (Please see the manuscript for details.)

10) The experimental design should be described better;

Reply 10: Thank you very much for the valuable comments. We have modified and

improved the description of the experimental design.

Changes in the text: Please see the revisions in the Mice model and Abstract sections. (Page 2, line 20-24; Page 5, line 102-107; Page 6, line 108.)

11) The whole manuscript needs extensive English editing performed by an English native speaker.

Reply 11: Thank you very much for the valuable comments. We have invited an English native speaker to edit and revise the article. (Please see the manuscript for details.)

Reviewer B

This is an interesting paper focused on the beneficial role of Lactobacillus to prevent some depressive-like behaviors and its relationship with peripheral and brain inflammation. Although the topic of research is outstanding, there some major concerns and some minor issues to be addressed.

MAJOR CONCERNS

1. The major flow of the paper is the animal model used to investigate depression. Acute i.p. injection of 1.2 mg/kg of LPS is an extremely high dose that could be used as a model of sepsis instead of depression. If this model is used (acute LPS injection) more physiological doses are recomended, since inducing sepsis may activate additional neuroinflammatory cascades not present in depression. Depressive-like behavior is clearly affected by such a high LPS dose, but this could be due to motor affectation due to the sickness behavior induced by the high dose of LPS. No measures of motor activity were done to discard this hypothesis. There are other models that mimic more adequately depression symptomatology such as chronic mild stress or even chronic injections of moderate doses of LPS.

Reply 1: We have referred to a lot of literatures about the injection dose of LPS, and many studies have shown that the dose of LPS induced depression in mice is usually about 0.5-1.5mg/kg. We conducted a pre-experiment and adopted this concentration. As to whether it can cause sepsis, studies have shown that the dose of LPS induced sepsis in mice is usually 10 mg/kg, and the dose of 1.2 mg/kg will not lead to sepsis (please refer to Oxid Med Cell Longev. 2018; 2018: 5048031. /Peptides. 2014

Nov;61:56-60. /J Biol Chem. 2019 Jan 11; 294(2): 608–622. /Mater Sci Eng C Mater Biol Appl. 2018 Feb 1;83:148-153.).

Changes in the text: The literature on the dose of LPS induced depression in mice has been supplemented in the paper. (please see Page 20, line 422-429.)

2. Statistics are not clearly explained in the paper so it is difficult to see that the results support the hypothesis. Although in the method section is stated that variance analyses were don,e it is unclear which type of ANOVA and which post hoc test have been used. Two-way anova must be performed according to the experimental design, comparing two variables (LPS/control and Lactob/control). The presence of interaction between this two variables is fundamental to understand if the hypothesis are confirmed or not. Additionally, post hoc analyses will help to understand the relationship among different experimental groups. Statistical analysis must be described in the results section (F values, main effects, interactions, etc.) and the p values of post hoc test. It is not clear whether the statistics included in the graphs refer to main effects of the anova, results of post hoc test, etc.

Additionally, the statistical symbols in the graphs are located in a bizarre form that makes difficult to understand it. For instance, when the figure layout says "p<0,05 versus CG" the symbol is located exactly up to the CG graph, which makes no sense and it is harly difficult to understand.

Reply 2: Thank you very much for the valuable comments. We used T-test to compare between groups, and two-way ANOVA was used to compare among multiple groups. In addition, LSD was used for post hoc test.

We are sorry for these incomprehensible descriptions. "* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs CG" means the statistical difference between CG and LPS groups; "+ $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ vs. LPS" means the statistical difference between LPS and LPS+Lac groups; "# $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. CG" means the statistical difference between CG and CG+Lac groups.

Changes in the text: We rewrote the Statistical Analysis section and added the p value in our figures. (see Page 10, line 215-216.)

3. Fig 5 about the mechanism of action is very poor and did not integrate all the components studied in the paper. Main attention has been done to caspases, for example,

when they are not measured or mentioned in the paper. A broader and deeper content in figure 5 is required to help to understand mechanisms explored in the paper.

Reply 3: We revised Figure 5.

MINOR CONCERNS

1.Please, justify the validity of using the HE staining instead Giemsa or other methods. Which is the fiability of this technic for pathological analysis in this context?

Reply 1: HE staining can clearly see the tissue structure of the small intestine, including the integrity of the intestinal mucosa, the shedding of intestinal villi and the infiltration of inflammatory cells.

2. Terminology refering to depression must be softened in Title, Results and along the manuscript, according to the animal model used. For example, instead of "to treat depression" authors may prefer to use something like "ameliorate some symptoms of depression"

Reply 2: Thank you very much for the valuable comments, and we have revised it in the paper. Changes in the text: Please see the revisions at page 4, line 85; page 11, line 226; Page 17, line 360.

3. Indicate the serotype of LPS used in the method section.

Reply 3: It is Escherichia coli 055: B5, we added it in mice model section.

4. Results (page 6) must describe clearly the effects found both at mRNA and protein levels

Reply 4: Thank you very much for the valuable comments, and we have made a supplementary explanation in the text.

Changes in the text: Please see the changes in the Results section. (Page 12, line 240-241, 253-259; Page 13, line 265, 269, 275-278, 283.)

5. Indicate the loading control used for western blot analysis in the method section. Also, correct the name of GADPH (instead of GAPDH) in all figures.

Reply 5: We used GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal control of WB experiment.

6. Please, explain the meaning of "gray value" in the axis of some graphs included in figure 3. Is it refering to the optical densitometry of western blot bands?

Reply 6: Yes, the "gray value" in the axis of graphs refers to the optical densitometry of western blot bands.