Author’s response to reviews

Title: Oral treatment with Lactobacillus Rhamnosus attenuates behavioral deficits and immune changes in chronic social stress

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Author’s response to reviews:

We thank the reviewers for their careful assessment of our manuscript and constructive criticisms. Based on reviewers’ comments we have made an number of significant changes in the revised manuscript including additional data relating to assessment of the hippocampus and detection of L.rhamnosus in fecal samples. We feel the manuscript in significantly improved over our previous submission.

In their general comments reviewers indicated the limited mechanistic insights provided by the study. While we did not delineate mechanism of action in the current manuscript, we feel our study includes several interesting and novel observations that will aid in more direct mechanistic studies related to the microbiota-gut-brain axis, both by ourselves and other investigators and thus merits publication. Amongst these observations we would argue that the fact behavioral effects exerted by JB-1 are independent of the fecal microbiota is in itself an important mechanistic insight and adds to existing evidence, in other model systems, suggesting that ability to restore or protect against disturbance in the gut microbiota is not necessarily an indicator of the efficacy of beneficial bacteria. Additional important information provided by our study includes the lack of effect of JB-1 on baseline behavior of the C57BL/6 mice in contrast to previous observations in the more anxious Balb/c strain. This has potential implications for future translational studies where JB-1 and other potentially beneficial might not be expected to modulate anxiety in non-anxious control subjects.

We include a point-by-point response to specific reviewers comments below.
Reviewer #1 (Peter Holzer):

Specific issues

1. Introduction. The statement that it is not known "whether bottom-up signalling along the gut-brain axis regulates stress-related changes in behaviour and neural function" neglects some of the pertinent findings, including those of the authors of this manuscript themselves (reference # 10), let alone other authors (e.g., Sudo et al. J Physiol 2004;558:263-275; Ait-Belgnaoui et al. Psychoneuroendocrinology 2012;37:1885-1895; Liang et al. Neuroscience 2015;310:561-577; Tarr et al. Brain Behav Immun 2015;50:166-177; Farshim et al. Sci Rep 2016;6:21958).

R1: The reviewer is correct in noting that we and many other investigators have provided evidence of bottom-up regulation of stress related pathways. We have altered this statement in the text of the introduction.

2. The experimental procedures relating to splenocyte isolation and processing need be detailed.

R2: The method section now contains details of the experimental procedure for splenocyte isolation.

3. Figure 3A and 3B. It seems as if the same data are presented twice, in the left panel of Figure 3A without significant difference between VEH and JB-1, but with a significant difference in Figure 3B because here only two groups are compared with each other. Please explain.

R3: The reviewer is correct and figure 3B was unnecessary and has been removed.

4. Figure 4F and related text. In contrast to what is claimed by the authors, this Figure does not show that JB-1 facilitates the recovery of the dysbiotic microbiota. This could only be claimed if there were a significant difference between Defeat/VEH and Defeat/JB-1, but such a difference does obviously not exist.

R4: We thank the reviewer for identifying this misinterpretation of the data. We have altered all text related to this figure and now clearly state that there is no significant effect of JB-1 on stress induced dysbiosis.
5. The authors invested quite some efforts into analyzing the faecal metabolome and found many alterations following exposure to CSD. Unfortunately, these findings are hardly dealt with in the Discussion, although it has to be admitted that alterations in the faecal metabolite concentrations may be of limited relevance to gut-brain communication. The plasma metabolome would provide much more valid information on metabolites that may play a role in gut-brain interaction.

R5: We agree that plasma metabolome may provide valid information on the role of metabolites in gut brain interaction unfortunately we do not have this data available. However, it is also possible that gut lumen metabolites acting at the level of the gut epithelium/enteroendocrine cells and ENS may play a role in microbe-gut-brain signaling that is just as, or even more, important than circulating metabolites. In the revised manuscript we have discussed the results of metabolite analysis in more detail and highlight Tyramine, the only metabolite decreased by stress that is significantly corrected by JB-1 treatment, as meritng further study.

6. JB-1 was without appreciable effect on CSD-evoked faecal dysbiosis. This finding fits largely with the literature showing that probiotic treatment has inconsistent effects on the faecal microbiota profile in humans and mammals. An important question is whether JB-1 was at all detectable in the faecal samples. This touches the issue whether JB-1 was at all able to reach the large intestine. If not, a primary effect in the small intestine may be envisaged, as alluded by the authors in the Discussion.

R6: We have now included the results from a sample of the mice used in the study for which we assessed fecal levels of L.rhamnosus in vehicle and JB-1 treated mice (new figure S1D.) These results suggest that JB-1 does reach the colon, of course this fact does not alter the possibility that the major site of action is the small intestine.

7. The bibliographic information for references # 20, # 32, # 61, # 62 and # 64 is incomplete or in need of amendment.

R7: We have corrected the references indicated.

Reviewer #2 (Antonio Teixeira):

Specific comments:

Introduction
"The critical role of this community [gut microbial community] in the regulation of diverse physiological functions, including immunity, is well established, as is its bidirectional influence on the central nervous system." Consider rephrasing this statement as there are controversial issues regarding the cross-talk between gut microbiota and CNS/physiological systems.

R1: We have rephrased this to statement to read: “The critical role of gut microbial community in the regulation of diverse physiological functions, including immunity, is well established, as and there is growing evidence of its influence on the central nervous system”.

"Given microbial regulation of host signalling [correct to 'signaling'] at the mucosal interface between microbiota and host, disruptions in this community may lead to systemic changes in peripheral signals [15,16]. Such systemic immune dysregulation has also been implicated in psychological stressors and psychiatric disorders [12,17]."  Consider rephrasing this statement as peripheral signals are not limited to immune ones.

R2: This has been rephrased to “Given microbial regulation of host signaling at the mucosal interface between microbiota and host, disruptions in this community may lead to systemic changes in peripheral signals [15,16]. For instance, systemic immune dysregulation has also been implicated in psychological stressors and psychiatric disorders [12,17]."

Methods:

Why did the authors wait 2 days after behavioral assessment end to euthanize the animals?

R3: We waited for 2 days to give mice time to recover from potentially confounding effects of the stress of behavioural tests, especially aggressor avoidance, which was the final test.

Results:

"Neither stress nor treatment altered the expression of corticotropin-releasing factor receptor type 1 or type 2, or the glucocorticoid receptor in the frontal cortex." The lack of effect of stress on CRH is somehow unexpected. Do the authors have any explanation for this?

R4: We do not currently have an explanation for the lack of change in CRH receptors in the frontal cortex, however while CRH receptor function is clearly required for the response to chronic social defeat, there is very limited information in the literature regarding changes in expression.
Why did the authors choose the frontal cortex instead of other brain areas like hippocampus?

R5: We initially focused on the frontal cortex because of our previous studies showing changes in neurotransmitter (including GABA) levels in the frontal cortex with JB-1 treatment (ref 20). However, in the revised manuscript we have included data from the hippocampus that demonstrates a significant decrease in GR expression with stress exposure but no changes in CRH or GABA receptors.

Besides CRH and GABA, other neural pathways should have been investigated.

R6: We now recognize that the study would have benefited from a broader investigation of neural pathways. However, in the current study we focused examination of pathways determined, a priori, based on our previous studies with JB-1. A more detailed analysis of specific brain regions and multiple neural pathways will be the subject of future study.

Regarding the influence of stress and treatment on immune cells phenotype, why did the authors evaluate only two cell lineages?

R7: We focused on dendritic cells and T cells as previous studies by ourselves and other investigators have demonstrated that they were influenced by stress and, importantly for this study, we have extensive information in relation to the effects and mechanisms of action of JB-1 in targeting and modulating these 2 cell types (REF 23, 24) Furthermore, T regulatory cells in particular have been suggested to mediate anxiolytic effects in response to bacterial challenge (ref 11). However, we acknowledge that there are many additional phenotypes of immune cell that could be investigated in future studies.

Clarify what fecal metabolites and related pathways were altered by Lactobacillus treatment in mice subjected to chronic stress.

R8: We have expanded the results and discussion sections related to fecal metabolites and clarified those modulated by stress exposure and JB-1 treatment.

Discussion

Some parts of the Discussion are highly speculative and not supported by the provided data. For instance, when discussing the findings on T regs, the authors mention "a counteractive response to such pro-inflammatory shifts". Empirical data on enhanced inflammation in the current
protocol (even simple measures of inflammatory mediators) could give more support to authors' hypothesis.

R9: We have rephrased various statements in the discussion to make it less speculative while still highlighting areas that we feel deserve further investigation based on the data presented in the current study. While we do not include measures of inflammation other investigators have described inflammation associated with chronic social defeat. We have now addressed this in the discussion.