Author's response to reviews

Title: Antioxidative protection of dietary bilberry, chokeberry and Lactobacillus plantarum HEAL19 in mice subjected to intestinal oxidative stress by ischemia-reperfusion

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Author's response to reviews: see over
We would like to thank referees on the helpful and brilliant comments and questions. In the following letter we tried to answer their questions and concerns giving a point-by-point response. Also few changes are made in the manuscript and these are highlighted by red-colored text.

Referee 2. Ignazio Castagliuolo

We tried to re-write “Results” section under Abstract but it could not be changed much since the number of words in abstract is limited.

1. In the para ?experimental diets? the authors indicate that the L. plantarum HEAL19 was mixed in freezing medium and then administered to the animals. Is this treatment able to preserve the probiotic alive or the bacteria are supposed to be dead in the food. If the Authors suppose to keep the microbes alive, did they test the vitality after 24 hours?

Answer: Freezing medium is used in our lab to preserve alive bacterial cultures at -80°C. We have been able to isolate high numbers of alive bacteria kept in freezing medium at -80°C after many years of storage. In this study HEAL19 strain was prepared 2-3 weeks before administered to the animals and was kept at -80°C all the time. The bacteria was divided into the tubes containing daily portion of 10^8 cfu/tube. We didn’t find it necessary to test viability again since our previous experiments and experiences have shown a very good viability and preservation of bacteria in freezing medium prepared according to the recipe described in the study.

2. Can the Authors specify when the animal chow is prepared? Is it prepared daily 8with fresh ingredients and bacteria) or do they make a stock? In the latter case how is the vitality of the probiotic after 10 days? This point in important in view of the limited effects of probiotic supplementation per se or in combination: is it possible that the vital dose is lower than108 cfu day?

Answer: The bacteria was prepared 2-3 weeks before the feeding started and was kept in freezing media at -80°C all the time. As previously mentioned, the viability in this medium at
these conditions is very good even after many years of storage. As it says in manuscript, berries had been picked, freeze-dried and grounded. The powder was kept in the freezer at -20°C.

Every day old feed was thrown away and feed bowls were hand-washed (we were very careful that each mouse got the same bowl through the whole study). New feed was prepared by weighting 1.6 g of berry powder and mixing it with soft standard chow and then adding a daily dose of viable bacteria (10^8 cfu) from stored tubes (see answer 1). The chance is very small that bacteria was much lower than 10^8 cfu/day. All the animals were comparable to each since they got exactly the same portions of bacteria, prepared in the same batch and the fresh diet was prepared every day.

Since we were able to re-isolate HEAL19 (results not shown) from the groups that were administered HEAL19 but not from the controls (without bacteria) it means that HEAL19 survived in the feed and was present in the intestines after administration of 10 days. The fresh feed was given to mice just before 6 p.m. (before the light was turned off) since mice start their active period from 6 p.m.to 6 a.m. The animals started to eat directly.

3. In the para ?experimental groups? please indicate for each group the treatment you used

**Answer:** It was explained in “experimental groups” the treatment i.e. the diet of each group so we don’t really understand what referee wants us to change. Under paragraph “intestinal ischemia-reperfusion procedure” it says that sham was the only group that was not subjected to ischemia-reperfusion injury. Just in case, we added as the last sentence in “experimental groups” that all groups except sham-group were subjected to the intestinal ischemia-reperfusion (see red-colored text in the manuscript).

4. Para ?Malondialdehyde? the Authors should clearly state how they normalize the different samples.

**Answer:** Unfortunately, we don’t really understand what referee means by the normalization of different samples.

5. In the results section, page 12 lane 4, the authors refer to ?other abnormalities?: they should specify these anomalies since they discarded some experimental animals on these parameters.

**Answer:** These abnormalities were seen in one animal that had “anatomical abnormalities” in form of very small organs compared to the all other animals. Everything looked diminished and was not normal so the animal was discarded from the study. We changed this in the text. See red-colored text under this section.
6. the data regarding colonic tissue MDA should be reported in a more clearly, comparing the effect of I/R to sham and then the effect of treatment to I/R.

**Answer:** We tried to change this in the manuscript. I hope we understood correctly what referee wanted for a change. See red-colored text under this section.

7. As the Author state also in their conclusions, would be helpful to few more measurements of the tissue damage and inflammation such as histology and MPO quantification.

**Answer:** We agree but unfortunately we don’t have more tissue to perform these analysis. That is definitely something that we will have in our minds in the future studies.

8. How do the Authors explain the large variations in anthocyanin composition and concentrations, taking into account that mice are kept in similar conditions and receive an identical diet? How relevant is this aspect in light of administration to humans that already present an important variability in gut microbiota?

**Answer:** Well, just like humans also mice have individual microflora that differs between different individuals. So that may be the one reason for the differences in the anthocyanin variation. Another reason may be that animals had free access to feed and water. They may have eaten at different time points and also in different amounts. It may also play a certain role how long before the sample-taking they stopped eating. We agree that it’s a very complex system and the individual responses may be very different.

9. is it possible to correlate the level and type of anthocyanin and phenolic acids formed from microbial degradation to the level of lipid peroxidation?

**Answer:** We didn’t try to the correlation of the level and type of anthocyanin and phenolic metabolite with MDA since we were more interested to see how everything works together i.e. complex polyphenolic composition in food (here in aronia and bilberry) rather than what individual phenolics have for the effect. When we eat, we ingest a mixture of all different polyphenols and food components rather than single compound.

10. In the Para ?conclusions? the authors indicate that chockberry did not have anti-inflammatory effects in their study, however, as stated above, the Authors did not measure any parameter of inflammation and this statement should be refrased.

**Answer:** We agree that we must rephrase this statement. The changes are made in the manuscript where “anti-inflammatory” is exchanged with “anti-oxidative” since MDA is the
only parameter measured. We also deleted phrase “anti-inflammatory effects” in few places. Changes are marked with red-colored text.

Referee 1. Hossein Hosseinzadeh

1. Reviewer's report The MDA content should be express as nmol/protein not /g tissue.

**Answer:** There are studies that use both nmol/protein and nmol/g tissue. Our references use both units. Muiá C et al (ref 7), Håkansson Å et al (ref 28), Jakesevic M et al (ref 35), Osman N et al (ref 36) express MDA as nmol/g tissue. Bengt Jeppsson, surgeon and one of the co-authors, explained that it shouldn’t make difference if we express MDA as nmol/g tissue or nmol/g protein if we don’t expect a lot of edema in the tissue, which we did not expect in this model with the given parameters. Since we used up all the tissue for MDA analysis, it is unfortunately impossible now to measure the protein content in the tissue, so we must keep the present units.

2. In this study the anti oxidant effect was studied and not anti-inflammatory effect. Thus, emphasis on anti-inflammatory effect is not correct.

**Answer:** We agree, and the changes are made in the manuscript where “anti-inflammatory” is exchanged with “anti-oxidative” since MDA is the only parameter measured. Changes are marked with red-colored text.

3. Why positive control for example an antioxidant such as vitamin E was not used?

**Answer:** This model with ischemia-reperfusion is already used in many studies and some groups used pure antioxidative compounds to examine the effects. Özkan et al (Tohoku J Exp Med 2009, 218, 251-258) used resveratrol to examine the effects on intestinal I/R-injury, Tsuda et al (Archives of biochemistry and biophysics 1999, 368, 361-366) used pure cyanidin-3-glucoside in an I/R-model and Günel et al (J. Ped. Surg. 1998, 33,1536-1539) used among others vitamin E in an intestinal I/R-injury in rabbits. In that study they didn’t see any significant changes in mucosal injury between vitamin E administration and ischemia-reperfusion control.

Objective of the present study was not to compare the berry and probiotic diets to the diet of an already known antioxidant, such as vitamin E. We wanted to compare how the antioxidative status of the animal is changed between administration of bilberry diet and chokeberry diet and also what happens if we add a probiotic bacterium. We also wanted to compare animals fed antioxidant-rich diet with controls that were not fed antioxidant-rich diet.
4. As only one dose was used in this study, the relationship of dose-response is not clear.

**Answer:** In the present study we were not focused to examine dose-response relationship but to see what happens with oxidative-stress in animals treated with the same dose of a certain diet during 10 days period before oxidative-stress injury. The next step could be to change the dosage and see if that may change the outcome of the study.

5. *Balb/c* should be changed to *BALB/c*.

**Answer:** This change was made in the manuscript and marked with red-color.