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Editor-in-Chief

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Title: Procyanidin B2 protects against diet-induced obesity and non-alcoholic fatty liver disease via the modulation of the gut microbiota in rabbits

Dear Editor:

Thank you for your letter concerning our manuscript entitled "Procyanidin B2 protects against diet-induced obesity and non-alcoholic fatty liver diesease via the modulation of the gut microbiota in rabbits". Those comments are all valuable for improving our paper. We have studied the comments carefully and made correction which we hope meet with approval. According to the suggestion of the editor, we have revised the format problems and made audio core tipe. The following is our point-by-point response to the reviewers'comments. The revisions have been highligted in the manuscript.

#Reviewer 1 (Number ID 02885976):

Question 1: *Language: the manuscript needs to be revised, there are some grammatical mistakes, misspellings and typos.*

RE 1: Thank you and in response to your valuble suggestions, we have carefully reviewed the manuscript and made modifications, including grammatical mistakes, missspellings and typos. The langue certificate (Editage) of this article has been submitted with the revised manuscript.

Question 2: *Methods: the authors mentioned in page 7, line 4: "Thirty New Zealand White rabbits"……and after in lines 8 and 9: "The rabbits were randomly divided into three groups of 8 animals". Why the authors use three groups of 8 animals? What happen with the rest of the rabbits?*

RE 2: Thank you. At the beginning of the experiment, thirty New Zealand white rabbits were recruited. After two weeks of a high-fat, cholesterol diet, some rabbits were excluded because of very high (>2mmol/L) or low (< 0.5mmol/L) cholesterol levels. Thus, eight remained in each of the two groups on the high-fat, cholesterol diet. The CHOW group also retained eight rabbits for matching. The aim was to reduce interspecific variation and to ensure that subsequent results were more feasible.We have restated the problem in detail in the manuscript now. Experimental methods refer to previous literature [1]. We have replaced this as "After two weeks of a high-fat, cholesterol diet, some rabbits were excluded because of very high (> 2mmol/L) or low (<0.5mmol/L) cholesterol levels".

Question 3: *The authors mentioned in the introduction section that PB2 is a natural plant compound with low bioavailability in humans. Can the author provide the corresponding percentage of bioavailability? Is this low bioavailability also observed in rabbits?*

RE 3: Thank you for you carefulness. It was reported that the plasma concentration of procyanidin was 10.6 +/- 2.5 nmol/l 2 hours after 2.0g of procyanidin was administrated in human body [2]. In rats, the concentration of procyanidin was 20.57 μM 2 hours after the ingestion of 1.0 g procyanindin extract *per* kilogram of body weight [3]. Although, the bioavailability of procyanidin in rabbit has not been reported until now, procyanidin is still thought to be poorly absorbable in mammal. We have also inserted these references into the manuscript now.

Question 4: *Rabbits received a daily dose of PB2 of 150 mg/kg. Please indicate how PB2 was administrated (route of administration).*

RE 4: Thanks for your corrects. 150mg/kg procyanidin B2 were dissolved in 20ml normal saline and administered by gavage everyday. And the control group was given 20ml normal saline every day. We have made modifications as "Group PB2 (fed a high-fat-cholesterol diet, n=8) received daily doses (150 mg/kg, dissolved in normal saline) of PB2 by gavage, whereas the other two

groups (groups CHOW and HCD, fed a chow or high-fat-cholesterol diet, n=8) received the vehicle (normal saline) by gavage."

Question 5: *Is PB2 dose equivalent to the average consumption of this compound in a human regular diet? This issue should be considered in the discussion section.*

RE 5: Thank you. This dose is based on previous studies and is obtained by conversion factor, which we have cited the reference in the corresponding section in the manuscript.

Question 6: *Taking into consideration that PB2 has low bioavailability and its possible benefits through modification of gut microbiota, a Control plus PB2 group must be included in order to determine the effect of PB2 on control healthy gut microbiota and metabolic parameters.*

RE 6: Thank you for your valuable advice.The low bioavailability of procyanidins refers to the low plasma concentration after absorption. The amount of procyanidin entering the intestinal tract by gavage is the amount of drugs given by gavage. When we set up a group, the main purpose was to compare the effects under high fat. However, there is no study on the effect of procyanidins on normal gut microbiota, and we will include the normal dietary procyanidin B2 group when we further explore the possible mechanism.

Question 7: *Please provide the composition of the high fat cholesterol diet in materials and methods section (Supplementary 1 file is missed or not available for the reviewer).*

RE 7: Thank you for your consideration. When we uploaded the file, we also found that supplementary file could not be uploaded. We have now listed the ingredients of the high fat cholesterol diet (10% lard, 0.5% Cholesterol, 5% Sucrose, 1% Maltodextrin, FBSH Biotechnologhy Inc.) in the material section of the manuscript.

Question 8: *The author should explain in the manuscript the concept of "liver index".*

RE 8: Thank you. Liver index was calculated as the ratio of liver to body

weight. We have now explained it in the manuscript.

Question 9: *Introduction section should be shortened.*

RE 9: Thank you for your advice. We have read the text carefully, and then deleted some repeated sentences. So that the introduction section looks more concise now.

Question 10: *Is it plasma lipid or serum lipid? Please check and correct it.*

RE 10: Thank you. The criterion should be written as serum lipid. We have replaced plasma lipid in the manuscript as serum lipid. Thanks again for your correction.

Question 11: *Given that PB2 was administered at the beginning of the experiments, all the effects regarding PB2 group must be referred as preventive. In this way, authors must replace terms like "increased" "decreased" "downregulated" "upregulated" to describe the PB2 effects along the entire manuscript.*

RE 11: Thank you. Yes, we added procyanidin at the beginning of the experiment. The effect of PB2 in the experiment should be preventive. We are deeply sorry for the mistakes by our poor English. We have replaced all these words in the manuscript with "prevent", "improve", "alleviate". We carefully checked the full text to avoid such mistakes again.

Question 12: *Figure 2H: there are not significant differences between the groups?*

RE 12: Thank you. Our results showed that there were no significant difference between the HCD and the PB2 group. There did exist significant difference between the HCD and the CHOW group. The HCD group was significantly increased than the CHOW group. We have revised and made it clear in the manuscript that there is no significant difference between PB2 and HCD group.

Question 13: *Figure 2I: page 11, lines 11 to 14: "….which was fully prevented by PB2……" According to figure 2I, the effect elicited by PB2 partially prevented the increase in circulating LPS of HCD group.*

RE 13: Thank you for your collection. We are deeply sorry for the impropriety usage of word. In fact, according to our experiment, PB2 partially prevented

the increase in the LPS and partially improved HCD induced endotoxemia. We have replace the "fully" with "partially" in the manuscript now.

Question 14: *Figure 3A: please add arrows or head arrows in the images to point out the histological findings. Also, bar line measure is missing.*

RE 14: Thank you. We have added arrows in the images to point out the steatosis and added bar line measure to the images. We have uploaded the new image to the manuscript now.

Question 15: *Figure 4E and 4D is unreadable.*

RE 15: Thank you. We have gave a more detailed explansion in the figure legend now. (D) Heatmap: Community hierarchical clustering analysis of major taxonomic groups at genus level, the red represent high relative abudance and the blue represent low relative abundance. (E) Venn diagrams described the number of OTUs that are distinct and shared across the groups.

Question 16: *Page 12, lines 5 to 11: Results about the effects of PB2 on the expression of genes that regulate lipid metabolism are repeated. These sentences should be re-written.*

RE 16: Thank you. We have read it carefully and removed some duplicates. We have replace it as "Moreover, in rabbit liver, the procyanidin B2 treatment prevented the upregulation of the steroid response element binding protein 1c (SREBP- 1c) and the downregulation of carnitine palmitoyltransferase (CPT) compared to the HCD group ($P < 0.05$). The expression of fatty acid synthase (FASN) was also decreased to the HCD group but the difference was not significant" in the manuscript.

Question 17: *Figure legend 3: (F) correspond to FASN and (G) to CPT.*

RE 17: Thank you for your carefulness. We reversed the order of figure F and G in the Figure legend. We have revised this in the manuscript now.

Question 18: *Figure 3F and page 12, line 9 of the manuscript: "…CPT was significantly increased compared to the HCD group…." According to the figure 3F, PB2 treatment was not able to prevent the upregulation of CPT expression by HCD. Please correct as well as in discussion section.*

RE 18: Thank you. We reversed the order of Figure 3F and 3G in the figure legend 3. Actually Figure 3G represented the expression of CPT. Our results showed that the HCD induced a downregulation of CPT and the PB2 treatment could partially prevent this trend. We have revised the order in the figure legend 3 now.

Question 19: *Figure 3G and page 12, line 10 of the manuscript: "The expression of FASN was also downregulated but the difference was not significant…." According to the figure 3G, PB2 treatment partially prevent the downregulation of FASN expression by HCD and it was significant compared to HCD and Chow groups. Please correct it as well as in discussion section.*

RE 19: Thank you. We reversed the order of Figure 3F and 3G in the figure legend 3. Actually Figure 3F represented the expression of FASN. Our results showed PB2 treatment partially prevent the upregulation induced by HCD. We have revised the order in the figure legend 3 now. We are deeply sorry for the confusion by our mistake.

Question 20: *Discussion section: please revised, some sentences and phrases are repeating the same concept.*

RE 20: Thank you. We have carefully read the entire discussion section and corrected the repetition and grammatical errors. The changes have been highlighted in the manuscript.

Question 21: *Page 15, lines 5 to 7: "Thus, SREBP1 may be emerges as a PB2 target gene…..triggered by PB2 in hepatic cells". This sentence should be revised. What do the authors want to explain? Are the authors suggesting that this is a direct effect of PB2 on SREBP1 gene? Given the low bioavailability of PB2, how could it be a direct effect of PB2 at gene level?*

RE 21 : Thank you. After careful consideration, we have replaced this with "Our results showed PB2 significantly prevent the upregulation of SREBP-1c compared to the HCD group. The inhibition of SREBP1 upregulation may be included in the mechanisms of PB2 to alleviate the steatosis in hepatic cells." in the disscusion section.

Question 22: *Along the manuscript, "a" and "b" are used to refer p value, however in Figure legends appears as # and *.*

RE 22: Thank you. The magzine requires "a" and "b" used to refer p value. We have corrected the Figure legends according the requirement. Thank you again for your careful work.

Reference

1 Luo F, Guo Y, Ruan GY, Long JK, Zheng XL, Xia Q, Zhao SP, Peng DQ, Fang ZF, Li XP. Combined use of metformin and atorvastatin attenuates atherosclerosis in rabbits fed a high-cholesterol diet. *Sci Rep* 2017; **7**(1): 2169 [PMID: 28526884 PMCID: PMC5438352 DOI: 10.1038/s41598-017-02080-w]

2 Sano A, Yamakoshi J, Tokutake S, Tobe K, Kubota Y, Kikuchi M. Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Biosci Biotechnol Biochem* 2003; **67**(5): 1140-1143 [PMID: 12834296 DOI: 10.1271/bbb.67.1140]

3 Blade C, Arola L, Salvado MJ. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol Nutr Food Res* 2010; **54**(1): 37-59 [PMID: 19960459 DOI: 10.1002/mnfr.200900476]

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

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