March 12, 2023

Professor Andrzej S Tarnawski, DSc, MD, PhD

Editor-in-Chief

*World Journal of Gastroenterology*

Dear Dr. Tarnawski:

Thank you for your careful examination and kind consideration of our manuscript titled "**Effects of ethanol and sex on propionate metabolism evaluated via a faster $^{13}$C-propionate breath test in rats**," (Manuscript NO.: 83637).

We appreciate the invaluable comments that the reviewers provided, which we are confident have helped us improve the manuscript. We have provided point-by-point responses to each of the reviewers' comments and described the related revisions below. As per your instructions, we highlighted in yellow and underlined all changes in the revised manuscript.

We look forward to any further comments regarding the revised manuscript.

Sincerely yours,

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Response to comments from Reviewer #1:

Comment 1.

It is not very clear why propionate metabolism is associated with a deficiency of B12 only, when a large number of factors can affect the level of propionate

**Response:**

**Thank you for the important comment. We agree with your opinion. In the present study, we focused to the association between propionate metabolism and $VB_{12}$ deficiency based on a previous study. However, as you pointed out, various factors could affect propionate level. Thus, various evaluation methods combined with invasive evaluation of propionate metabolism using faster PBT may provide new findings on propionate metabolism under various condition, which can be a novel point of this study. To explain this, we have added the following sentences in the last section of the Discussion with new citation (#23 Biotechnol Lette 2017;39:635-45):**

> **"Moreover, the present study only focused on the association between propionate metabolism and $VB_{12}$ deficiency based on a previous study on PBT[3]. However, considering the complexity of intestinal propionate production due to the variety of propionate-producing bacteria, including *Clostridium* spp., *Veillonella* spp., *Fusobacterium* spp., *Salmonella ruminantium*, and *Propionibacterium* spp., and the complexity of substrates[23], the findings obtained herein, including the promoted propionate metabolism in male ERs and sex-related difference, may have potential clinical utility and provide a basis for future research into propionate metabolism and intestinal microbiota under various conditions.**

**For instance, comparison of findings between faster PBT and the composition or changes in gut microbiota may provide interesting information on the association between gut microbiota and their products."**

Comment 2.

Additionally, it would be important to evaluate the composition of the gut microbiota as a factor that can change the level of propionate

**Response:**

**Thank you for the very interesting comment. We agree with your opinion. Considering the complexity of intestinal propionate production due to the variety of propionate-producing bacteria and the complexity of substrates, comparison of findings between faster PBT and changes in the composition of the gut microbiota associated with alcohol consumption may provide interesting information. To explain this, we have added the following sentence in the last section of the Discussion:**

**"For instance, comparison of findings between faster PBT and the composition or changes in gut microbiota may provide interesting information on the association between gut microbiota and their products."**

Comment 3.

It is unclear how long the rats took alcohol. If not for long, it is difficult to expect the effect of alcohol on ALT and B12 levels

**Response:**

**We apologize for the lack of information on the duration of alcohol intake in**

**ERs. Because we started feeding with ethanol after 4 weeks of weaning, ERs consumed ethanol for 23–27 weeks. We have added this in the Material and Methods section as "ERs continuously consumed alcohol for 23–27 weeks."**

Comment 4.

It is not clear whether a C13 propionate breath test is eventually proposed to diagnose B12 deficiency or intestinal microbiota disorders.

**Response:**

**Thank you for the important comment. We initially intended to evaluate faster PBT as a diagnostic modality for VB12 deficiency associated with alcoholism but we eventually failed to prove this. However, we found unexpected changes in propionate metabolism associated with alcohol consumption. To explain this, we have added the following sentence in the last section of the Conclusion:**

> **"Although we could not evaluate the usefulness of faster PBT as a diagnostic modality for $VB_{12}$ deficiency as initially intended because we failed to create a rat alcoholism model with $VB_{12}$ deficiency,"**

Comment 5.

It is recommended to describe in detail of alcohol intake.

**Response:**

**We apologize for the unclear description of alcohol intake. We have added the following two sentences in the Material and Methods section:**

1. **"… by replacing the content of water bottles with ethanol solution in all**

**cages of ERs."**

2. **"…; ERs continuously consumed alcohol for 23–27 weeks."**

Response to comments from Reviewer #2:

Comment 1.

Novelty of the research could be discussed more in the discussion section.

**Response:**

**Thank you for the comment. As per your suggestion, we have added the following sentences at the end of the discussion:**

**"Moreover, the present study only focused on the association between propionate metabolism and $VB_{12}$ deficiency based on a previous study on PBT[3]. However, considering the complexity of intestinal propionate production due to the variety of propionate-producing bacteria, including *Clostridium* spp., *Veillonella* spp., *Fusobacterium* spp., *Salmonella ruminantium*, and *Propionibacterium* spp., and the complexity of substrates[23], the findings obtained herein, including the promoted propionate metabolism in male ERs and sex-related difference, may have potential clinical utility and provide a basis for future research into propionate metabolism and intestinal microbiota under various conditions. For instance, comparison of findings between faster PBT and the composition or changes in gut microbiota may provide interesting information on the association between gut microbiota and their products."**

**We have also added the following sentence in the Conclusion:**

**"Although we could not evaluate the usefulness of faster PBT as a diagnostic modality for $VB_{12}$ deficiency as initially intended because we failed to create a rat alcoholism model with $VB_{12}$ deficiency, our study suggests that chronic**

**consumption of 16% ethanol changed the composition of fatty acids produced by the intestinal flora"**

Comment 2. English language needs to be revised.

**Response:**

**We apologize for this. Although our manuscript had received professional English editing before first submission, we have asked the English editing company (Editage) to carefully check the revised manuscript for language.**