Dear Dr Ze-Mao Gong,

Science Editor, World Journal of Gastroenterology

Please find enclosed the revised version of the manuscript Manuscript NO.: 36534: **"Rifaximin ameliorates hepatic encephalopathy and endotoxemia without affecting the gut microbiome diversity"** by Kosuke Kaji, Hiroaki Takaya, Soichiro Saikawa, Masanori Furukawa, Shinya Sato, Hideto Kawaratani, Mitsuteru Kitade, Kei Moriya, Tadashi Namisaki, Takemi Akahane, Akira Mitoro, Hitoshi Yoshiji for publication as an article in ***World Journal of Gastroenterology***.

We carefully evaluated the concerns raised by the Reviewers, performed the requested analyses, modified the text and added new data (please refer to new Table.1) as suggested. Detailed responses to each of the Reviewers' comments are provided in the attached pages.

We would like to extend our thanks to the Reviewers for providing helpful and constructive comments on our work and to you for a chance to resubmit our manuscript.

Additionally, we would like to ask you to add two authors Hideto Kawaratani (0000-0002-4361-0592) and Mitsuteru Kitade (0000-0001-7592-7589).

I hope that we satisfactory addressed yours and Reviewers concerns and the revised manuscript is now acceptable for publication in ***World Journal of Gastroenterology***.

Sincerely,

Kosuke Kaji, M.D., Ph.D.
Assistant professor
Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, Japan.
TEL:81-744-22-3051(ex:2314)
E-mail: kajik@naramed-u.ac.jp

Reply to the points raised by the Reviewers.

We thank all Reviewers for his/her positive evaluation of our work.

Reviewer: 03253490

The study is investigating 'the impact of rifaximin on the endotoxin activity and gut microbiota identified by 16S ribosome RNA (rRNA) gene sequencing in patients with decompensated cirrhosis. New studies /reviews suggest that ' Rifaximin may have beneficial actions beyond its direct antibiotic activity. Rifaximin's clinical activity may be attributed to effects on metabolic function of the gut microbiota, rather than a change in the relative bacterial abundance'. The discussion part may be updated by adding new reviews and studies focused on this topic.

We appreciate for the Reviewer's kind suggestion. Bajaj et al. demonstrated that the effects of rifaximin on metabolic function of the gut microbiome by metabolome analysis. We added the description with their study (new ref 19) and review (new ref 40) in the revised discussion (page 16, line 4-6, underlined).

Reviewer: 00182114

Antibiotics have been administered in the treatment of HE, usually employed as second-line therapy. The mechanism is thought to relate to decreased colonic deaminating bacteria that produce nitrogenous compounds (i.e. ammonia) by metabolism of urea. The most common antibiotics used on a historic basis for HE include neomycin and metronidazole. Neomycin is approved as adjuvant therapy in hepatic coma. Metronidazole is not approved for HE. Neomycin is better studied than metronidazole. There are several controlled trials involving neomycin. Most studies,

including those with metronidazole, are small and uncontrolled. Few support the use of either antibiotic for treatment of HE. Furthermore, long-term use of neomycin is limited by ototoxicity and nephrotoxicity Rifaximin is a poorly absorbed, oral antibiotic. It is derived from rifamycin and has a broad spectrum of activity against Gram-positive and Gram-negative, aerobic and anaerobic, enteric bacteria. It is thought to diminish deaminating enteric bacteria to decrease production of nitrogenous compounds that are subsequently absorbed and cause HE. I ask some questions to author. 1. Therefore, please comment the mechanism for the decreased NH3 with Rifaximin from the point of Gram-positive and Gram-negative, aerobic and anaerobic, enteric bacteria. 2. I think decreased endotoxin was due to improvement of intestinal tight junction by Rifaximin. Therefore, please comment intestinal barrier function parameter with Rifaximin. 3. How about amino acid level with Rifaximin?

1. We appreciate for the Reviewer's kind comments. Although the causative bacteria for the development of HE has been unidentified yet in detail, rifaximin could exert antimicrobial activity against ammonia-producing enteric bacteria including i) Gram-positive aerobic bacteria such as *Streptococcus* and *Bacillus*, ii) Gram-negative facultative anaerobic bacteria such as *E-coli*, *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Proteus*, iii) Gram-positive obligatory anaerobic bacteria such as *Clostridium*, iv) Gram-negative obligatory anaerobic bacteria such as *Bacteroides* (new ref 17). In current study, however, endotoxin-generating Gram-negative bacteria were unchanged by treatment with rifaximin. Therefore, it will be required further investigation to explore additional effects of rifaximin other than the action as antibiotics, including the effect on metabolic status in gut microbiome (as described in new ref 19) and intestinal barrier function. We add the description in the revised introduction (page 6, line 5-10, underlined) and discussion (page 17, line 3-9, underlined).

2. We strongly agree with the Reviewer's suggestion. We also think it important to elucidate the effect of rifaximin on intestinal tight junction. As described in discussion part, recent *in vitro* study has demonstrated that rifaximin could improve intestinal barrier function via activation of PXR (new ref 42), and we observed that poorly absorbed antibiotics, which exert similar function to rifaximin in this aspect, markedly attenuated endotoxin-induced tight junction damages determined by the expression of

ZO-1 (new ref 43). Since we assume that same biological phenomenon could be observed in the clinical practice, we will examine the effect of rifaximin on intestinal tight junction protein in the clinical practice in near future after the approval of ethical committee.

3. We actually measured Branched chain amino acid & Tyrosine Ratio (BTR) in all subjects of current study. In current analysis, there was no significant difference in BTR between before and after treatment with rifaximin. This data is shown in the new Table 1.

Reviewer: 03567380

The case control study by Kaji et al. describes the use of rifaximin for the management of HE that results from decompensated liver cirrhosis. The authors describe an improvement in ammonia, endotoxin and neurocognitive assessments in subsets of patients without a significant change in the composition of the gut microbiome. The authors perform the study well and acknowledge a significant area of concern due to the low number of patients. That being said, there are a few areas the authors should address to improve this report which are listed below: 1) How many patients were included in the analysis with the high endotoxin activity (>0.4), delayed NCT and the high ammonia groups? Are these the same patients? The number of patients of each group should be included in the results and figure legends. This information (regarding the split of the groups) should be included in the methods or results. 2) Table 1 should include the scores after the 4 weeks of rifaximin treatment (or it could be included as a second table). Any significant differences should be discussed in the text.

1. We apologize for insufficient description about the number of patients in the results part and figure legends. The numbers of patients in each group are 11/10/16 (high endotoxin activity/delayed NCT/high ammonia). All of the patients with high endotoxin activity were included in the high ammonia group. 7 patients with delayed NCT were included in the high ammonia group. We add these description in the revised results and figure legends (page 11, line 2-6, underlined).

2. We appreciate for the Reviewer's kind suggestion. According to the Reviewer's comment, we modified Table 1 including the score after treatment of rifaximin and *P*

values and add the description in the revised results (page 10, line 22-page 11, line 2, underlined). Also, we add the score of Branched chain amino acid & Tyrosine Ratio (BTR) in new Table 1 according to other reviewer's comment.