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PEER-REVIEW REPORT

Name of journal: *World Journal of Gastroenterology*

Manuscript NO: 80270

Title: Microbiome-Liver Crosstalk: A multihit therapeutic target for liver disease

Provenance and peer review: Invited Manuscript; Externally peer reviewed

Peer-review model: Single blind

Reviewer's code: 05120663
Position: Peer Reviewer
Academic degree: MD
Professional title: Doctor
Reviewer's Country/Territory: United States
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Scientific quality	[Y] Grade A: Excellent [ ] Grade B: Very good [ ] Grade C: Good [ ] Grade D: Fair [ ] Grade E: Do not publish
Language quality	[ ] Grade A: Priority publishing [Y] Grade B: Minor language polishing [ ] Grade C: A great deal of language polishing [ ] Grade D: Rejection
Conclusion	[Y] Accept (High priority) [ ] Accept (General priority) [ ] Minor revision [ ] Major revision [ ] Rejection
Re-review	[Y] Yes [ ] No
Peer-reviewer	Peer-Review: [Y] Anonymous [ ] Onymous

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statements	Conflicts-of-Interest: [ ] Yes [Y] No

SPECIFIC COMMENTS TO AUTHORS

The authors wrote a quite interesting review on the microbiota and liver diseases along with other factors. This is generally of high interest. Topics are hot. This covers good amounts of data although it lacks discussion in some areas. Diets certainly influence pathogenic mechanisms. Diet can also interact with other factors, eg, smoking, alcohol, obesity, sleep, exercise, etc. These factors together may influence molecular pathologies in each patient differentially. There are also influences of germline genetic variations on diets (appetite and food preference), immune status, and diseases. Gene-by-environment interactions should be discussed. The authors should discuss such contexts. Research on dietary / lifestyle factors, microbiome, and personalized molecular biomarkers is needed for non-communicable disease research such as liver diseases. The authors should discuss molecular pathological epidemiology research that can investigate diet and other factors in relation to molecular pathologies, microbiome, and clinical outcomes. Molecular pathological epidemiology research can be a promising direction and should be discussed, eg, in Ann Rev Pathol 2019; Gut 2022.

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SPECIFIC COMMENTS TO AUTHORS

Overall, reconsider the structure and organization of content and breakup paragraphs. In particular, the discussion on GPCRs and SCFA is repetitive and circular. Throughout, please doublecheck that all statements reflect the content from the references. Please pay attention to naming of strains and bacteria and specific activities, structure of the paragraphs and organization of the text, use of the word probiotics, use "mouse" instead of "mice" models, and usage of hyphens were appropriate (i.e., SCFA-producing). Specifically: 1. On line 110, reference 3 is cited, which does not support the statement; however, reference 4 supports the statement from 107-110; change to reference 4. Consider combining the statements across 108-111 and modify the statement to refer that it was only "in one study" whereas the information in reference 3 is a review article that doesn't show that multiple studies have demonstrated the same thing, and therefore it may be better to refer to ref 3 and speak consistently according to ref 3. 2. In general, consider adding more references to statements that are asserted without references. 3. What is meant by "stable" microbiota or microbial stability? Please define or consider a reference or measurement method. 4. Although ref 6 describes how to measure the Firmicutes to Bacteroides ratio, it does not ratio itself affects metabolism, and a recent article has called into question the ratio as a relevant marker of obesity Please see https://www.mdpi.com/2072-6643/12/5/1474/htm Magne, F.; Gotteland, M.; Gauthier, L.; Zazueta, A.; Pesoa, S.; Navarrete, P.; Balamurugan, R. The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? Nutrients 2020, 12, 1474. https://doi.org/10.3390/nu12051474 Is there another reference that supports your statement or a revised version of your statement? By-products is one word byproduct or hyphenated; not two words. Line 127 – ref 7 does not show

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that proline was the limiting factor. How do you know that the pre-colonized strains were able to prevent EHEC colonization because of proline? Could it have been something else? The ref talks about E. coli strains using different sugars, nutrients, and nutritional niches. In general when discussing strains, it is best to be specific and refer to the alphanumeric identifier. Are you using the same definition of a probiotic that has been internationally and globally recognized? For the definition of probiotic, please refer to Hill C, Guarner F, Reid G, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506-514. doi:10.1038/nrgastro.2014.66 There is only one known E. coli probiotic (E. coli Nissle 1917), correct? E. coli HS is a commensal, not a probiotic. Both strains were used in Ref. 8. Ref 15 cites ref 16 for the statement lines 147-148; please simply use ref 16. Line 152: There are many other bacteria besides Bifidobacterium that produce SCFAs, and Bifidobacterium, to my knowledge, have not been demonstrated to produce butyrate. Please review the references cited and revise the statement to accurately reflect what is known. Line 154 – is all of this activity by SCFA limited to a colitis mouse model? The references indicate otherwise. Ref 19 and 20 may not be needed here; consider reviews on SCFA on this topic. Lines 156-157 – is Bifidobacterium the only bacteria to support intestinal epithelial cell integrity via tight junction proteins? Clearly the other data in the paragraph indicate otherwise. Please reconsider your paragraph. Consider L. rhamnosus GG and/or this reference Rose EC, Odle J, Blikslager AT, Ziegler AL. Probiotics, Prebiotics and Epithelial Tight Junctions: A Promising Approach to Modulate Intestinal Barrier Function. Int J Mol Sci. 2021 Jun 23;22(13):6729. doi: 10.3390/ijms22136729. PMID: 34201613; PMCID: PMC8268081. Need reference for line 16 – E. coli Nissle 1917 Ref 26 is a study in mice. Line 181-183 refers to in vitro experiments. Ref 27 doesn't seem to say anything about LPS Ref 28 shows that IL-10, IL-6 and TNF alpha increased. Also,

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IL-10 is typically considered anti-inflammatory… Page 5 of 12: Ref. 32 line 194: liver disease, specifically cirrhosis, is correlated with the LPS, dysbiosis etc according to the reference; the reference doesn't say dysbiosis is correlated with those things; also, couldn't someone have dysbiosis but not have an intestinal barrier that leaks, so then not all cases of dysbiosis would be associated with increased intestinal barrier permeability? Could this be a simple typo, where cirrhosis was intended instead of dysbiosis on line 193? Lines 194-196 claims that "ALL mouse models of liver disease include dysbiosis" – is that true? Do refs 31 or 32 include transgenic diabetic models? I didn't see them. Lines 197-198: Ref 34: gram positive bacteria Ruminococcaceae was at higher abundance in the healthy group v. NAFLD. Please ensure your statements accurately reflect what is in the references cited. Line 199 – B. vulgatus (not vulgaris) is in the reference 35 as one of the most abundant in severe fibrosis. Line 202. See Figure 3a of ref 36. Healthy controls have a higher firmicutes to Bacteroidetes ratio than NAFLD. Line 208 – do you mean worsen dysbiosis (not liver cirrhosis) because that is what ref 31 says? Dysbiosis seems to accompany liver cirrhosis but is it really proven that it causes worse liver cirrhosis? Lines 213-214. Very unclear as written. Prevotella and faecalibacterium were at higher abundance in feces from patients with HCV… Line 216 claims that fecal microbial transplantation from sick patients was used with ref 39. Ref 39 is a study using mouse FMT, not human FMT to mouse. Ref 45 likely not needed; LPS as endotoxin is likely common knowledge or would be supported by a microbiology reference, better than an alcohol liver related reference. Ref 52 says nothing about short chain fatty acids ... Please reconsider lines 246-251. Ref 56 is not a metabolomic study in children. Ref 57 did not measure SCFA Ref 59 discusses SCFA in the colon but not the liver Ref 60 is in cells…is this a good model of the liver? Ref 61 doesn't mention GPRs Ref 65 mentions acetate but not the other SCFAs Ref 66 doesn't mention the aryl hydrocarbon receptor It doesn't look like Ref 67 or 68 mention Indole-3-propionate or PXR The

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discussion about supplementing with SCFAs and FXR agonists as therapies seem out of place and could go in the therapeutics section later. Taste of SCFAS can easily be masked with proper formulation and special softgels. There are some on the market. And what about butyrate enemas? Lines 287 – That would be an engineered analog of FGF19 and an Fc-FGF21 fusion protein to be more precise. But what does this have to do with microbial metabolites? Connecting the concepts to lines 300-301 would make more sense, but in the therapeutics section. Discussion on TMAO in diet and xenobiotics section – why not combine with TMAO discussion earlier? ref 96 doesn't seem to address insulin sensitivity. Spell check thioacetamide Ref 108 has some human biopsies but is an animal study and doesn't seem to show phase 2 clinical trials with close to 90% reduction in fibrosis… Line 408 – please specify L. rhamnosus GG – not all lactobacilli are the same. Please be aware of probiotic strain-specificity Line 409 Clostridiales Incertae Sedis XIV Lines 413-415 there seems to be a reference missing for the study in children Line 415 – was ALT affected? AST was reduced. Please consider including the probiotics from the World Gastroenterology Guidelines 2017 for NASH/NAFLD in your discussion on probiotics. Lines 420-422 – if it is true that combining probiotics with prebiotics results in better outcomes, then why are none of those trials mentioned in this review? Where is the data to support that probiotics combined with compatible prebiotics "always" results in better outcomes? Lines 424-425 – B. longum was shown to be superior to L. acidophilus in this study; L. acidophilus did not reduce liver fat Lines 431-435 – check. More than 5 words in a row that is lifted from another reference should either be rephrased or used with quotation marks for proper citation I was taught. References 115 and 120- did either of these mention FMT and weight loss changes in humans? Lines 486-488 – the reference says the rLa vaccine was not able to induce "long-term alterations in the intestinal microbial community diversity…" which could potentially suggest resistance to colonization…

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RE-REVIEW REPORT OF REVISED MANUSCRIPT

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Conclusion	[ ] Accept (High priority) [ ] Accept (General priority) [Y] Minor revision [ ] Major revision [ ] Rejection
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SPECIFIC COMMENTS TO AUTHORS

I appreciate the changes made. There are massive improvements. I appreciate the addition of diet and lifestyle as therapeutic changes; this was an essential addition. There are still some mismatches between claims made and references cited. (I am unable to see line references in the copy I have for some reason so I was unable to cite the line references). Perhaps the journal editor can correct the spacing issues, but please be aware that there are some words throughout that are combined together, missing a space between them, or a period missing after a sentence in a few spots. Reference 11 states that the relative abundances of Bacteroidetes and Firmicutes were 15% and 80% respectively; these are reversed in your manuscript (please change to 15% Bacteroidetes and 80% Firmicutes if you want to keep this citation). Further, this is about maximizing Shannon diversity at this ratio; how can it be claimed that maximizing Shannon diversity is equivalent to optimal homeostasis? If that is your belief, you are free to state "We believe" instead of "It is believed…" Please note that the ref: Ma ZS, Li L, Gotelli NJ. Diversity-disease relationships and shared species analyses for human microbiome-associated diseases. ISME J. 2019 Aug;13(8):1911-1919. doi: 10.1038/s41396-019-0395-y. Epub 2019 Mar 20. PMID: 30894688; PMCID: PMC6775969.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6775969/ states "So far, published studies have generated inconsistent results: the microbiome diversity of diseased individuals may be higher, lower, or no different than the microbiome diversity of healthy individuals." There is no consensus on what "optimal homeostasis" is, or what the best Shannon diversity is. Given that later in the paper you cite a study in China that shows "significant rise in diversity as the liver condition advanced from cirrhosis to HCC with cirrhosis119." (meaning fecal microbial diversity), yet at the same time, healthy controls had higher diversity than those with cirrhosis, reference 119

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makes a good point that "Thus, greater richness or diversity in the bacterial community is not a sign of a healthy gut microbiota in our cohort, but likely suggested the overgrowth of various harmful bacteria or archaea in patients with HCC." So that suggests that homeostasis is likely more than just a measurement of diversity but also needs to take into account what the different types of bacteria are doing (harming or helping). Correct Rhuminococcus to Ruminococcus For reference 63, related to the statement: "The body utilizes approximately 10% of the energy supply from microbially derived SCFAs, meaning that 90% is stored in white adipose tissue63." I'm not sure I agree with your interpretation of this reference. I only have access to the abstract, not the full text. What it says in the abstract is, "Current estimates are that VFA contribute approximately 70% to the caloric requirements of ruminants, such as sheep and cattle, approximately 10% for humans…" My interpretation of that statement is that 10% of the caloric requirements for humans, that is, 10% of the calories that are consumed by humans comes from VFAs (which includes SCFAs), so the question is where is the 90% of the caloric requirement coming from (maybe the rest of their food?)? I think the abstract of the article is talking about VFA being 10% of caloric requirements, with 90% of the caloric requirement being non-VFA, whereas your manuscript is a bit unclear. It would be clearer to say, "90% of the energy supply is stored in white adipose tissue" – but is that correct? Glycogen stored in the liver is also a source of energy storage, in addition to triglycerides in the adipose tissue. Does reference 63 breakdown all sources of energy such as adipose tissue, glycogen and VFA and any others? Ref 66 refers to propionate and acetate but I don't see isobutyric acid referenced; please correct the statement. Ref 71 shows in Figure 7 as well as the title of Figure 4 that GPR43 suppresses insulin signaling in the adipose tissues but not in muscles or liver"; please revise your statement accordingly: "GPCR pathway activation also limits insulin-mediated hepatic and muscular fat accumulation and stimulates energy

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expenditure71" Ref 70 cites a paper about GPR43 inhibiting lipolysis but doesn't seem to include information on how GPR41 inhibits lipolysis and doesn't seem to include information on activating adipocyte differentiation. Please remove, modify or find a reference to support the statement: "In adipocytes, SCFAs activate GPR41 and GPR43 to inhibit lipolysis and activate adipocyte differentiation70" Reference 76 demonstrates that "Tryptophan degradation to indole derivatives activates AhR for IL-22 production" but not downregulation of inflammatory genes; please revise: "Indole upregulates tight junction proteins in the gut and downregulates colonic epithelium inflammatory genes through the aryl hydrocarbon receptor76." Remove Ref 76 therefore from the statement: "Indole-3-propionate activates pregnane X receptor (PXR) to downregulate proinflammatory cytokine production and has been associated with protection against injury through oxidative stress signaling76,77." Reference 105 shows that retinol-binding protein 4 plays a role in insulin resistance and does not discuss lipid metabolism, RXR or FXR. The previously used reference, though it did not directly discuss all these items does weakly support part of the statement so both Wan et al. 2000 doi: 10.1128/mcb.20.12.4436-4444.2000 and current ref 105 would be better than just one to reference this statement, unless you have another reference that is more direct and comprehensive. "Retinoic acid not only regulates bile acid homeostasis but also shares with it the receptors retinoid X receptor (RXR) and farnesoid X receptor (FXR) and therefore shares the functions of lipid metabolism and insulin sensitivity105." Reference 119 states that the butyrate-producing bacteria was high in controls relative to early HCC (not cirrhosis) and LPS-producing bacteria high in HCC relative to controls (please correct your statement: "There was also a high level of butyrate-producing bacteria in healthy controls relative to early cirrhosis patients and a notable rise in LPS-producing bacteria in HCC patients119.") A comment: While I appreciate the inclusion of potential biomarkers in the gut microbiome for liver disease, what may be difficult is the

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discrimination of one disease from another. In addition, not only is liver disease influenced by diet, genes, age, lifestyle, environment but also the gut microbiota can be influenced by all those, and drugs, other comorbidities, etc. While not necessary to include in your manuscript, I would like to draw your attention to the gut microbiome health index published in 2020 (https://www.nature.com/articles/s41467-020-18476-8) and a 2022 attempt to identify a universal dysbiosis index as well as a disease-specific set of markers https://genomebiology.biomedcentral.com/articles/10.1186/s13059-022-02637-7

In the Therapeutic approaches section, the introductory paragraph ends with "which will be highlighted below." Therefore, it seems appropriate to have a title such as "Short Chain Fatty Acid supplements" or maybe "Small Molecule Therapies" to head the section of the SCFA supplements/FGF discussion before the "Probiotic interventions" section. And some introductory/conclusive transition statements would be nice. Oral microencapsulated butyrate might be useful as add-on in ulcerative colitis, small study, doi: 10.3390/jcm9123941 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7762036/) and may change the microbiota, see IBD study https://doi.org/10.1093/ecco-jcc/jjy222.779 https://academic.oup.com/ecco-jcc/article/13/Supplement_1/S446/5301152 For the probiotics section, citing reference 133, it is inconsistent to specify one strain (DSMZ 21690 and not mention the other strain names). Since these are not well-known strains and it's a combination, the reader could look up the strains if interested, and I suggest you leave out the DSMZ21690 from the statement. Suggestion – replication typically suggests similarity in study design or intervention, so I suggest not using "replicated" when referring to the NAFLD patients in reference 134 since the multistrain probiotic used is completely different, the length of intervention was different, the population being treated was different, and the outcomes were different. How about "Changes in

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the fatty liver index and AST were reduced in NAFLD patients treated with a different multistrain probiotic" for ref 134 or something like that? A probiotic strain is designated by an alphanumeric identifier such as DSMZ 21690 or GG. Reference 135 only specifies species, not strains. "six species of bacteria" (not necessary to list all the bacterial species since that didn't seem important when referring to the multistrain probiotic (presumably the company is keeping the strains private/proprietary because they do not specify the strains but only the genera in their paper) in ref 134. If you do list them, it's Lactobacillus rhamnosus (not Lactobacilli rhamnosus) and paracasei not pacasei For reference 135, IL-6 decreased significantly in the placebo group, not the probiotics group. Both the probiotics and placebo groups experienced a reduction in TNF-alpha from baseline to posttreatment. Therefore, it doesn't seem that the probiotics "led to an improvement in proinflammatory cytokines." The same goes for cholesterol, which was reduced in both groups. The main finding of ref 135 was the reduction of intrahepatic fat and triglyceride, but these changes were not different from placebo when adjusting for body weight so it doesn't seem appropriate to make the statement currently written: " In another study, a twelve-week treatment of 30 NAFLD volunteers with six strains of bacteria containing Bifidobacterium breve and B. lactis, Lactobacilli rhamnosus, L. acidophilus and L. pacasei and Pediococcus pentosaceus in a randomized, double-blind, placebo-controlled study led to an improvement in proinflammatory cytokines, a reduction in cholesterol and a decrease in body weight135." While it's a nice hypothesis that combining probiotics with "compatible" prebiotics (do you mean synbiotics?) would result in better outcomes, reference 136 doesn't seem to make this claim with any biostatistical calculation across all the very heterogeneous studies it lists in table 1. It would be appropriate to test this hypothesis with appropriately designed clinical studies evaluating prebiotics v. probiotics v. combinations, with dose differences accounted for (at least a 3-arm study) to determine which ingredients have which effects and if there is

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any synergy. Reference 137 suggests that one combination in a mouse study may support the hypothesis, but it seems far-reaching to make a global overgeneralization such as the statement referenced by 136,137. I think 5 of the 19 studies in table 1 of reference 136 show a reduction in ALP; it seems a gross overgeneralization to say that ALP was decreased following treatment with probiotics, prebiotics and synbiotics; perhaps this could be qualified to "some studies with various probiotics or prebiotics or synbiotics." A conclusive statement or statements would be helpful at the end of the probiotics section to close the section. A transition from the animal models to the clinical studies would be helpful. Suggested correction: "prebiotic mixture of fruco-oligosaccharides and L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B.longum, and L. bulgaricus " to "mixture of fructo-oligosaccharides and L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum, and L. bulgaricus Where does it say a low protein diet has been shown to help with liver disease (looking at refs 163, 162). " The amount of Bacteroides, for example, is lower in Chinese NAFLD individuals after diet and exercise compared to people from the West, and this is correlated with lower hepatic fat164." Ref 164 is a study all conducted in China; Ref 164 did not compare Chinese to people from the West. In the discussion section, they refer to another study that made a comparison between Chinese and western countries. The reference would be: Shen, F. et al. Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease. Hepatobiliary Pancreat. Dis. Int. 16, 375–381 (2017). In the abstract, there is no mention of comparison between Chinese and western countries. I don't have access to this paper. If you keep this statement, it needs to be verified by the content of an appropriate reference. Minor: Please write out acronym first time it is used. For example, you can add (LPS) to lipopolysaccharides in the introduction and MDP (muramyl dipeptide more accurately) for the peptidoglycans so that the acronyms can be used in Figure 1 without writing them out for the first time.