Reviewer Comments:

Reviewer #1
Scientific Quality: Grade C (Good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Major revision
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.
The section on SCFA has been reorganized so that information on interaction of SCFA with GPCRs is now grouped together from line 266 to line 289 to read as follows:

"The major microbial fermentation products following microbial degradation of fiber are the SCFAs butyrate, propionate, and acetate. The body utilizes about 10 % of energy supply from the microbially derived SCFAs, meaning that 90 % are stored in the white adipose tissue[63]. Several studies reveal that gut microbial dysbiosis is associated with chronic liver disease such as NAFLD or ALD[64, 45]. In a metabolomic study in children with NASH, serum levels of 2-butanone and 4-methyl-2-pentanone were found to be elevated compared to healthy individuals[65]. Adults with NAFLD were found to have higher levels of faecal propionate and isobutyric acid which are part of the fecal SCFA family[66]. Obese patients with NAFLD were also found to have high levels of propanoic acid and butanoic acid[67]. SCFAs such as acetate and butyrate modulate the host immune response by dampening the LPS-induced hepatocellular inflammatory response and restore the mucosal and systemic immunologic homeostasis thus minimizing liver injury[68,69.] SCFA's can act like hormonal molecules by binding to G-protein-coupled receptors (GPCRs), which leads to activation of the GPCR pathway and this in turn slows gut motility and increases energy harvest[70–72]. Upon activation, glucagon-like peptide-1 (GLP-1) is secreted from epithelial L-cells, enters circulation and induces insulin release from the pancreas[70]. GPCR pathway activation also limits insulin-mediated hepatic and muscular fat accumulation and stimulates energy expenditure[71]. In adipocytes, SCFAs activate GPR41 and GPR43, to inhibit lipolysis and activate adipocyte differentiation[70]. SCFAs also regulate immune cell functions through the GPR43 which is widely expressed in most immune cells[73–75]. SCFAs have also been shown to inhibit histone deacetylases (HDAC) which downregulates gene expression and reduces production of inflammatory cytokines, particularly in macrophages and blood mononuclear cells during acute inflammatory hepatitis[69]. Therefore, it can be argued that dysbiosis that reduces microbial SCFA generation will result in a dysregulated inflammatory response and thus contributing to the progression of liver disease."

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).
Overall statements have either been removed or added to reflect the content of the references as advised by the reviewer. The hyphenation and the use of the words probiotics and mouse models (eg line 130, 131, 132 and line 140) have also been observed.

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. 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

We thank the reviewer for the comment and we combined the sentences from lines 104-107 (formerly 107-111) and changed the reference accordingly to reference 6 (formerly 4). To now read as follows:

"The neonatal microbiota is also influenced by the mode of feeding where breast fed babies show a more stable microbiota that has a higher copy number of *Bacteroides* and *Bifidobacterium* but a lower abundance of *Enterococcus* and *Streptococcus* species, while formula fed babies have a higher abundance of *Clostridium, Streptococcus* and *Enterococcus*[6]."

In general, consider adding more references to statements that are asserted without references.
We have now added references to statements that previously did not have citations to

What is meant by "stable" microbiota or microbial stability? Please define or consider a reference or measurement method.
This was intended to mean homeostasis or in equilibrium with each other. The phrase microbial stability in line 121 (formerly 115) has now been replaced by microbial homeostasis

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?
We appreciate that there is controversy over the impotance of the Firmcutes to Bacteroidetes ratio as an indicator of dysbiosis. The Milani et al, 2017 https://doi.org/10.1128/MMBR.00036-17 paper supports the idea that the ratio is a significant one. Manor et al https://doi.org/10.1038/s41467-020-18871-1 also supports the idea of a Firmicutes-Bacteroidetes axis as a measure of diversity. We have modified the statement in lines 113-121 (formerly lines 118-121) to indicate the importance of the ratio in homeostasis and to provide the appropriate references for that. We have also acknowledged the opposing argument as well. The statement reads as follows:

"The colon has the highest density of microbes in the gastrointestinal tract harboring about 70% of all gut microbes and are mostly members of the Firmicutes and the Bacteroidetes phyla[9]. The Firmicutes to Bacteroidetes axis is important in maintaining gut homeostasis as members of each phylum have specialized metabolic roles (i.e. metabolism of sugar vs indigestible fibers) that impact the microbiome and the host. It is believed that the role in homeostasis is optimized when

the relative abundance is 15% Firmicutes and 80% Bacteroidetes [8,10] [11]. However, the significance of this value and the actual impact it has on the host has been questioned by some researchers,[12] emphasizing the importance of more research on the role of Firmicutes and Bacteroidetes in gut microbial homeostasis, health and disease.

By-products is one word byproduct or hyphenated; not two words.
This has been corrected and now reads as "byproduct"

Line 127 – ref 7 does not show 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?

- We admit that proline is not expressly mentioned in reference 12 (formerly ref 7) and have therefore replaced proline with nutrients in that statememnt. Proline is however implicated by Momose et al, 2008 https://doi.org/10.1371/journal.pone.0053957 which is ref 14 in our manuscript. We have explained it in details whithin the text (lines 123-136) which now reads as follows:

"Different animal studies have shown that nutrient competition occurs between metabolically related microbiota members. For example, germ-free mice colonized with three human commensal strains of *Escherichia coli* (*E.coli* HS, *E.coli* Nissle 1917, *E.coli* MG1655) successfully prevented colonization of the caecum by the pathogen enterohaemorrhagic *Escherichia coli* (EHEC) EDL933- an *E.coli* 0157:H7 biotype, due to the three precolonized commensal biotypes outcompeting *E.coli* EDL933 for nutrients[13]. This colonization resistance was further shown to occur by use of multiple sugars as metabolic substrates for probiotic *E.coli* Nissle 1917 and commensal subtype *E. coli* HS, whose rapid growth effectively limited the colonization of EHEC *E.coli* EDL933 in a mouse model[14] Competition for a shared nutritional niche of proline was similarly demonstrated in a gnotobiotic mouse model colonized with early life microbiota where the early-life *E.coli* 1 was shown to outcompete *E.coli* 0157:H7[15]. This colonization resistance was also thought to be attributed to the production of lactate and acetate by bifidobacteria and enterococci which can suppress motility of *E.coli* 0157:H7 under cecal anaerobic conditions [15]."

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
We appreciate the precise comment of the reviewer and applied the term probiotic as defined in the international standards. We have added the bacterial alphanumeric references as indicated above in lines 125-136 and differentiated probiotics from commensals.
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Both strains were used in Ref. 8. Ref 15 cites ref 16 for the statement lines 147-148; please simply use ref 16

Reference 22 (formerly ref 15) in lines 151 -152, has been removed and ref 21 (formerly 16) used instead

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.

Additional bacteria known to produce butyrate have been added to the text. In addition, we explained which bacteria produce which specific SCFAs so that the text in lines 157-162 (formerly lines 151-154) now reads as follows:

"Bacteria enhance the mucus layer in numerous ways, such as through the production of secondary metabolites. SCFAs, such as acetate produced by *Bifidobacterium* or butyrate produced by gram-positive Firmicutes such as *Faecalibacterium prausnitzii, Roseburia sp, and Butyricicoccus pullicaecorum*[23,24], are known to strengthen gut barrier function, normalize permeability, improve intestinal epithelium defense, protect against pathogenic infections, and reduce inflammation [25–28]."

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.

The reviewer raised a good point- activity by SCFAs is not limited to only a colitis mouse. We have thus edited Line 154 (now line 162) to remove the implication that it is, and have made the statement more general. Ref 19 removed and 20 (now reference 27) retained for its relevance on epithelial lining.

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.

Text in lines 156-157 (now lines 164-169) has been modified to include the role of *Lactobacillus* in tight junction protection as indicated in the Rose et al, 2021 paper suggested by the reviewer. The text now reads as follows

"Intestinal epithelial cells are held together by a set of tight junction proteins that are molecules situated at the tight junctions of epithelial cells. The integrity of these tight junctions can be influenced by commensal bacteria and their effects on tight junction proteins. For example, *Lactobacillus rhamnosus* (*L. rhamnosus*) GG induces claudin-3 expression, *L. acidophilus* and *L. plantarum* stimulates expression of occludin, and *Bifidobacterium infantis* preserves claudin-4 and occludin deposition at tight junctions[29,30]."

Need reference for line 16 – E. coli Nissle 1917 Ref given as
We added Guo et al, 2019 (doi: 10.1155/2019/5796491) which is Ref. 32.

Ref 26 is a study in mice
Ref 26 in lines 188-191 formerly 179-183) was replaced with a new ref Ghosh et al, 2021 doi: 10.1016/j.jcmgh.2021.02.007 (ref 36) which describes microbial metabolites in humans.

Line 181-183 refers to in vitro experiments. Ref 27 doesn't seem to say anything about LPS
Corrected LPS with phosphorylation of EGFR, cited Raimondi et al 2008 (10.1152/ajpgi.00043.2007) which is Ref. 38 in line 194-196.

Ref 28 shows that IL-10, IL-6 and TNF alpha increased. Also, IL-10 is typically considered anti-inflammatory.
There is indeed an increase in the pro-inflammatory cytokines IL-6, and TNF-alpha and the anti-inflammatory cytokine L-10 in Ref 39 lines 196-200 (formerly Ref 28) . Authors believe the net effect is an improved intestinal barrier due to a higher inflammatory counteraction by IL-10 and ZO-1.

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?
We intended to write liver cirrhosis. "Dysbiosis" in the original text has now been changed to cirrhosis in line 207.

Lines 194-196 claims that "ALL mouse models of liver disease include dysbiosis" – is that true?
Lines 208-211 (formerly 194-196) has been changed to "many" as opposed to "all". These changes are now as follows

"Dysbiosis has been noted in many mouse models of liver disease such as secondary biliary fibrosis (common) induced by bile duct ligation, alcoholic liver disease induced by alcohol uptake in drinking water and hepatotoxicity induced liver cirrhosis using carbon tetrachloride (CCL4) treatment [42,43]."

Do refs 31 or 32 include transgenic diabetic models? I didn't see them.
The sentence was intended to mean knockout mouse models. The sentence in line 210-211 has been edited to clarify the use of genetically modified models and the sentence now reads as follows:

"alcohol uptake in drinking water and hepatotoxicity induced liver cirrhosis using carbon tetrachloride (CCL_4) treatment [42,43]"

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

Information corrected to reflect a higher abundance of Ruminococcae in healthy volunteers and a higher abundance of Gram positive bacteria in NAFLD patients. We also included a few of genera that are higher in NAFLDs and two that are higher in healthy volunteers Ref Jiang et al (DOI: 10.1038/srep08096). The modified sence in lines 211-215 (formerly 197-198) now reads as follows:

"In humans, several gram-positive bacteria including members of the genera *Clostridium* XI, *Anaerobacter*, *Streptococcus*, and *Lactobacillus* were found to be more abundant in the gut in NAFLD patient biopsies compared to healthy volunteers[45]. In contrast *Oscillibacter* and *Flavonifractor* of the family *Ruminococcaceae* were abundant in the healthy volunteers relative to the NAFLD[45]."

Line 199 – B. vulgatus (not vulgaris) is in the reference 35 as one of the most abundant in severe fibrosis

Edits in line 216 have been made and it is now *B. vulgatus* (not B.vulgaris as originally written)

Line 202. See Figure 3a of ref 36. Healthy controls have a higher firmicutes to Bacteroidetes ratio than NAFLD

Information in line 217 (formerly Line 202)changed to reflect lower ratio is indicative of disease. The statement in lines 217-219 now reads as:

"Although there has not yet been a general consensus on what microbial ratios of different strains exist in NAFLD patients, many research findings indicate that a lower Firmicutes to Bacteroidetes ratio is associated with liver disease[47,11]."

Line 208 – do you mean worsen dysbiosis (not liver cirrhosis) because that is what ref 31 says?

"liver cirrhosis in line 224 has been changed to "dysbiosis to be in line with what Ref 42 says"

Dysbiosis seems to accompany liver cirrhosis but is it really proven that it causes worse liver cirrhosis ?

The review reference 39 Albhaisi et al, 2020 doi: 10.1152/ajpgi.00118.2019 (now ref 42) and Trebicka et al, 2020 DOI:https://doi.org/10.1016/j.jhep.2020.11.013 (ref 48) indicate that dysbiosis causes worsening cirrhosis.

Lines .213-214. Very unclear as written. Prevotella and faecalibacterium were at higher abundance in feces from patients with HCV

The sentence in Lines 230-233 (formerly Lines 213-214)has been edited to clarify the nature of the study and the bacterial abundance. Lines 330-233 (formerly Lines 213-215) now reads as:

" In a study examining the gut microbiota of stage 4 hepatitis C virus (HCV) patients, *Prevotella* and *Faecalibaterium* were found to be more abundant in HCV patients compared to healthy controls, while *Rhuminococcus* and some *Clostridium* species were more abundant in healthy controls compared to HCV patients. *Bifidobacterium* was found only in healthy individuals[50]."

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.
Corrected to reflect that the study was mouse FMT where the donors were either hyperglycemic or normoglycemic. The sentence in lines 234- 238 (formerly lines 215-218) now reads as follows:

"Germ-free mice were shown to develop NAFLD following fecal microbial transplantation from donor hyperglycemic mice with systemic inflammation when fed a high fat diet[51]. On the other hand, germ-free recipients that received fecal transplantation from normal donors (i.e. normoglycemic with negligible systemic inflammation) did not develop NAFLD and were normoglycemic when fed a high fat diet [51]."

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.
Reference 45 has been removed as per the reviewer suggestion. The sentence in lines 257-258 (formerly 236-237) has been left since it is common knowledge.

Ref 52 says nothing about short chain fatty acids. Please reconsider lines 246-251.

- Reference 52 in lines 264-265 (formerly Lines 246-251)has been replaced with Bergmann, 1990 DOI: 10.1152/physrev.1990.70.2.567 which is now reference 63.

Ref 56 is not a metabolomic study in children
Replaced by the metabolomics and metagenomics study Del Chierico et al, 2017 doi: 10.1002/hep.28572 which is Ref 65 in Lines 270-272.

Ref 57 did not measure SCFA
Reference 57 has been replaced with Rau et al 2018 doi: 10.1177/2050640618804444, which is now reference 66 in Lines 271-273

Ref 59 discusses SCFA in the colon but not the liver
Ref 59 formerly in line 263 has been substituted with review Correa et al, 2016 doi: 10.1038/cti.2016.17 which is ref 68 in line 277.

Ref 60 is in cells…is this a good model of the liver?
We appreciate the reviewer's comment that the cell line model is not best suited for the liver and Reference 60 has been substituted with a review by Visekruna and Luu, 2021 https://doi.org/10.3389/fcell.2021.703218 which is now reference 69 in line 276

Ref 61 doesn't mention GPRs

Reference 61 has been substituted to van der Hee and Wells 2021
https://doi.org/10.1016/j.tim.2021.02.001 which is now Ref 70 in lines 279-280

Review paper Ref 65 mentions acetate but not the other SCFAs

Review 65 in lines 274-277 (formerly Lines 266-270) removed and replaced with Visekruna and Luu 2021 https://doi.org/10.3389/fcell.2021.703218 which is ref 69

Ref 66 doesn't mention the aryl hydrocarbon receptor
Reference Zelante et al, 2013 doi: 10.1016/j.immuni.2013.08.003 which is ref 76 in line 292 - 294 (formerly Line 275) quoted instead.

It doesn't look like Ref 67 or 68 mention Indole-3-propionate or PXR
The references Zelaneta et al, 2013 doi: 10.1016/j.immuni.2013.08.003 ref 76 and Zhang and Davies 2016 doi: 10.1186/s13073-016-0296-x ref 77 in lines 292-294, which discuss this subject more elaborately, have been cited instead.

The discussion about supplementing with SCFAs and FXR agonists as therapies seem out of place and could go in the therapeutics section later.
The dicussion about SCFAs and FXR agonsists as therapies has been moved to the therapeutic section.

Taste of SCFAS can easily be masked with proper formulation and special softgels. There are some on the market. And what about butyrate enemas?
Details on the use of microencapsulation and butyrate enemas have been added under the therapeutic approaches in lines 434-443 so that the statement now reads as follows

"Although SCFA supplements could be an attrative therapeutic approach in liver disease, their taste is normally not well tolerated. However, methods like microencapsulation[125], either as soft gels or liquid capsules are available that mask the taste of bitter medications, and could be used for oral delivery of SCFA, which has the added benefit of being slow release and helps prevent evaporation of some volatile SCFAs, like butyrate. Butyrate enemas have been used in a rat model with the treatment group showing improved mucosal repair and reduced colonic damage compared to the untreated control groups[126]. However, butyrate enemas did not show any improvement in clinical studies with ulcerative colitis patients [127]. There is potential for use of SCFA as a therapeutic approach but more research is required to develop an optimal approach"

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?
The whole section from Lines 289-292, formerly in the microbial metabolites section, has now been moved to the therapeutic approach section and can be found at lines 434-443.

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?
The discussion on TMAO which was formerly in lines 299-303 was moved from diet and xenobiotics to the previous Microbial metabolites section so that the entire discussion now in lines 290-307 now reads as follows:

"Indole and its derivatives are microbial metabolites of tryptophan breakdown. Indole upregulates tight junction proteins in the gut and downregulates colonic epithelium inflammatory

genes through the aryl hydrocarbon receptor[76]. Indole-3-propionate activates pregnane X receptor (PXR) to downregulate proinflammatory cytokine production and has been associated with protection against injury through oxidative stress signaling[76,77]. Indole-3-acetate has been shown to modulate hepatocyte lipogenesis thus playing a protective role against NAFLD[78]. Microbial metabolism of dietary choline and L-carnitine produces trimethylamine (TMA) which is oxidized to trimethylamine N-oxide (TMAO) during the hepatic detoxification of the blood through the catalysis of the liver enzyme hepatic flavin monooxygenases[79]. TMAO is excreted in urine and recent findings in animal NAFLD models fed with a high fat diet have shown to increase urine levels of TMAO[80]. In a Chinese cohort study, the severity of NAFLD was closely associated with circulatory TMAO[81]. Bacteria are essential for the conversion of dietary choline to trimethylamine (TMA) which is oxidized in the liver through the catalysis of hepatic flavin monooxygenase to generate trimethylamine-N-oxide whose accumulation has been associated with both cardiac and renal disease[82,83]. Phosphatidylcholine is also metabolized by gut microbes to generate TMA whose oxidation in the liver yields TMAO, and as earlier described, may lead to kidney and cardiac disease[84,85]. It is now thought that accumulation of TMAO in the liver causes NASH through the inhibition of Farnesoid X Receptor (FXR) and alteration of bile acid homeostasis[86]."

ref 96 doesn't seem to address insulin sensitivity
Added the Graham 2006 DOI: 10.1056/NEJMoa054862 paper that discusses retinoic acid and Insulin resistance. This is now reference 105 in Line 356

Spell check thioacetamide
Spelling has been checked and corrected

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
Ref 108 has been substituted with Ref 117- the Ratziu et al 2020 doi: 10.1002/hep.31108 paper which has the actual outcome of CVC phase 2b clinical trial . The text has also been altered in lines 401-403 to read as follows:

"This outcome has since been replicated in phase 2 clinical trials with a remarkable reduction in fibrosis[117]."

Line 408 – please specify L. rhamnosus GG – not all lactobacilli are the same. Please be aware of probiotic strain-specificity
L. rhamnosus GG has been added to specify the strain in Line 453.

Line 409 Clostridiales Incertae Sedis XIV
The nomenclature in line 454-455 was corrected

Lines 413-415 there seems to be a reference missing for the study in children
Famouri et al, 2017 DOI: 10.1097/MPG.0000000000001422 which is ref 133 in Line 461 was added

Line 415 – was ALT affected?

In the Famouri paper (Ref 133) DOI: 10.1097/MPG.0000000000001422 for children ALT was reduced but it was not in the Kobyliak adult NAFD paper doi: 10.15403/jgld.2014.1121.271.kby (Ref 134) (Lines 457-463)

AST was reduced-
corrected to show the reduction in AST. The paragraph in lines 449-463 was modified so that it reads as follows

"Treating dysbiosis and restoring homeostasis is complicated due to the wide range of associated factors that lead to a loss of important microbial populations or diversity in the first place. In most cases, treating dysbiosis with a single approach usually gives discouraging outcomes. However, studies involving probiotics have shown encouraging results in terms of safety, tolerance and efficacy[130]. In a Phase 1 clinical trial, *Lactobacillus rhamnosus* GG administered to cirrhotic patients resulted in reduced *Enterobacteraceae*, and increased relative abundance of *Clostridiales incertae* Sedis XIVand *Lachnospiriceae* with reduced endotoxemia and decreased pathogenic bacterial growth indicative of improved health[131]. In another study using multiple probiotic strains, reduction in inflammatory cytokine flares in cirrhotic patients was observed[132]. In obese, sonographically identified NAFLD children, treatment with a probiotic combination of *Bifidobacteria (B.bifidum, and B. lactis)* and two *Lactobacilli* (*L.rhamnosus* DSMZ 21690, and *L. acidoph*ilus) strains significantly lowered intrahepatic fat content and alanine aminotransferase (ALT) levels as well as aspartate amino transferase (AST) relative to the placebo treatment[133]. This reduction in hepatic steatosis was replicated in NAFLD patients treated with a multistrain probiotic[134]."

Please consider including the probiotics from the World Gastroenterology Guidelines 2017 for NASH/NAFLD in your discussion on probiotics
Details added with corresponding citations . The modified section in lines 474-489 after addition of the probiotics information in the guidelines now reads as:

In rats fed a high fat diet, treatment with *Bifidobacteria longum* or *Lactobacillus acidophilus* significantly reduced hepatic fat accumulation[138]. There was also a strong negative correlation between fat liver content and probiotic concentration in the stool [138]. In addition hepatic steatosis was markedly reduced after 12 weeks of treatment with *B. longum* but this was not the case with *L.acidophilus* treatment[138]. In a diabetic rat model, treatment with *Akkermansia muciniphila* led to a decreased inflammatory response and improved liver function[139]. In hepatic encephalopathy, a mixture of *Lactobacillus plantarum*, *L. casei, L. delbrueckii* subsp. Bulgaricus, *Bifidobacterium infantis , B. longum, B, breve and Streptococcus salivarius* subsp. Thermophilius have been associated with both primary and secondary prophylaxis[140,141]. Yogurt containing *L. bulgaricus, S. thermophilus, L. acidopilus* La5 and *B. lactis* Bb12 as well as a prebiotic mixture of fruco-oligosaccharides and *L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B.longum,* and *L. bulgaricus* have been shown to improve aminotransferase in NAFLD patients[142–144]. In NASH patients, probiotics containing *L. bulgaricus* and *S. thermophilus* have also shown improvement in aminotransferase[145]. A combination of *B. longum* W11 and fruco-oligosaccharides on the other hand has shown improvement in aminotransferase and the histological score activity of NASH patients[146]."

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?

We have now cited the Castillo et al, 2021 paper https://doi.org/10.3390/foods10081719 (Ref 136) which has better details on prebiotics, probiotics, and combined therapies. Castillo et all indicates that there is value in combining prebiotics, probiotics and synbiotics. We have included some of the details so that lines 467-473 read as follows

"When probiotics are mixed with compatible prebiotics, better outcomes have been achieved in clinical trials but more studies are needed to determine the most effective combinations[136,137].Hepatic steatosis has, for example, been reported to decrease in patients with NASH following a symbiotic and prebiotic treatment. Serum alkaline phosphatase (ALP) was decreased following a treatment with probiotic, prebiotics and synbiotics[136] It is however noteworthy that the outcomes are dependent on the composition of probiotics, the exposure time and the dosage[136]."

Where is the data to support that probiotics combined with compatible prebiotics "always" results in better outcomes?

We have included the Castillo et al ,2021 https://doi.org/10.3390/foods10081719 review reference 136 which puts together a lot of data showing value for combining probiotics, prebiotics and synbiotics. The Hu et al, 2021 doi: 10.1080/01635581.2020.1767166 Ref 137 paper has data showing that coadministration of Bacteroides longum and Roseveratrol reduced obesity and NAFLD in a moouse model. We have included these details in lines 473- 489 which read as follows

"Studies in animal models have shown similar outcomes as in human studies. In rats fed a high fat diet, treatment with *Bifidobacteria longum* or *Lactobacillus acidophilus* significantly reduced hepatic fat accumulation[138]. There was also a strong negative correlation between fat liver content and probiotic concentration in the stool [138]. In addition hepatic steatosis was markedly reduced after 12 weeks of treatment with *B. longum* but this was not the case with *L.acidophilus* treatment[138]. In a diabetic rat model, treatment with *Akkermansia muciniphila* led to a decreased inflammatory response and improved liver function[139]. In hepatic encephalopathy, a mixture of *Lactobacillus plantarum*, *L. casei, L. delbrueckii* subsp. Bulgaricus, *Bifidobacterium infantis , B. longum, B, breve and Streptococcus salivarius* subsp. Thermophilius have been associated with both primary and secondary prophylaxis[140,141]. Yogurt containing *L. bulgaricus, S. thermophilus, L. acidopilus* La5 and *B. lactis* Bb12 as well as a prebiotic mixture of fruco-oligosaccharides and *L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B.longum,* and *L. bulgaricus* have been shown to improve aminotransferase in NAFLD patients[142–144]. In NASH patients, probiotics containing *L. bulgaricus* and *S. thermophilus* have also shown improvement in aminotransferase[145]. A combination of *B. longum* W11 and fruco-oligosaccharides on the other hand has shown improvement in aminotransferase and the histological score activity of NASH patients[146]."

Lines 424-425 – B. longum was shown to be superior to L. acidophilus in this study; L. acidophilus did not reduce liver fat.

Details corrected to indicate a reduction of the liver fat content with *B. longum* treatment but not in *L. acidophilus*, and the negative correlation of liver fat content with fecal *L. acidophilus* and *B. longum*. The details in lines 473-478 therefore read as follows

"Studies in animal models have shown similar outcomes as in human studies. In rats fed a high fat diet, treatment with *Bifidobacteria longum* or *Lactobacillus acidophilus* significantly reduced hepatic fat accumulation[138]. There was also a strong negative correlation between fat liver content and probiotic concentration in the stool [138]. In addition hepatic steatosis was markedly reduced after 12 weeks of treatment with *B. longum* but this was not the case with *L.acidophilus* treatment[138].

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.

The sentence has been rephrased so that the section from Lines 492-497 (formerly Lines 431-435) now reads as follows:

"Fecal microbiota transplantation (FMT) is the administration of a solution containing fecal material from a "healthy" donor into the intestinal tract of a recipient, in order to modify that recipient's gut microbial composition for targeted health benefits[147]. To date, FMT has been successfully used in the treatment of recurrent *Clostridium difficile* infection, and there is growing evidence that FMT can be used to treat non-infectious diseases such as inflammatory bowel disease, obesity, and other metabolic disorders [147]."

References 115 and 120- did either of these mention FMT and weight loss changes in humans?

The details on weight loss which have not been indicated in either Ref 115 (now Ref 149 or ref 120 now ref 150) have been removed. The sentence in Lines 500-503 (formerly Lines 439-441) now reads as follows.

"There have also been several human clinical trials but with mixed outcomes- with some achieving significant reduction in proinflammatory cytokines and improved gut barrier function and others not responding to the therapy[149,150]."

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…

We appreciate the possibility of colonization resistance as mentined by the reviewer but we also observe difficulty in quantifying Lactobacilli using 16S rRNA PCR because even at its most abundant, Lactobacilli levels are only slightly above noise level. We did not discuss this in our manuscript since it is just an observation.

Reviewer #2:
Scientific Quality: Grade A (Excellent)
Language Quality: Grade B (Minor language polishing)
Conclusion: Accept (High priority)

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.
We have briefly discussed the influence of diet, genes, age and lifestyle such as smoking and alcohol and mentioned that their influence make it difficult to identifiy a single microbial signature indicative of good health. The sentence in Lines 423-427 reads as:

"It must however be appreciated that as of yet, a single microbial signature indicative of liver disease does not exist mainly because disease outcome is influenced by multiple factors such as diet, genetic background, age, and lifestyle (such as alcohol consumption), all of which must be considered while interpreting data on the predictive value of fecal microbiota on liver disease[124]."

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.

We have included a discussion on multi-regional studies looking into the dynamics of gut microbiota as a diagnostic biomarker of liver disease that also includes microbial metabolites as biomarkers of liver disease. In our discussion, we note that race, the state of health and geographical locations all influence the microbial signatures. We specifically note that there were differences in microbial signatures between the Asian NAFLD patients relative to the Western NAFLD patients which could not be associated with genetic predisposition known to influence NAFLD but were rather thought to be environmentally driven. The section in line 408-427 reads

"Gut Microbiome as a diagnostic biomarker for liver disease
The dynamics of the gut microbiome could be used as a non-invasive diagnostic tool for liver cirrhosis and hepatocellular carcinoma(HCC)[119]. In a cross regional prospective validation study in China, human fecal samples analyzed for microbial diversity revealed a significant rise in diversity as the liver condition advanced from cirrhosis to HCC with cirrhosis[119]. 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 the HCC patients[119]. In a different experiment, gut microbiota known to originate from the oral cavity were found to be enriched in liver cirrhosis patients relative to healthy volunteers[120]. In an Asian NAFLD cohort, *Ruminococcaceae* and *Veillonellaceae* species were found to be more predominant in NAFLD patients relative to healthy individuals[121]. These microbiome changes could not be associated to genetic predispositions known to influence NAFLD and were thought to be environmentally driven[121].

Bacteroides and *Escherichia* spp have on the other hand been associated with liver fibrosis in NAFLD patients[122]. Overall, these multiregional studies indicate that there is a great potential for gut microbiota as non-invasive diagnostic biomarkers for liver disease with distinct indications of the staging of fibrosis and inflamation[121,123]. There is also great potential for the gut microbiota and associated metabolites to be utilized as therapeutic biomarkers[119–121]. It must however be appreciated that as of yet, a single microbial signature indicative of liver disease does not exist mainly because disease outcome is influenced by multiple factors such as diet, genetic background, age, and lifestyle (such as alcohol consumption), all of which must be considered while interpreting data on the predictive value of fecal microbiota on liver disease[124]."

Molecular pathological epidemiology research can be a promising direction and should be discussed, eg, in Ann Rev Pathol 2019; Gut 2022.

We have included a discussion of Diet and lifestyle changes as therapeutic targets for liver disease. Our discussion in line 557-571 reads as follows"

"*Diet and lifestyle changes as therapeutic targets*
There are many therapeutic options for NAFLD that are being explored, some of which are in advanced levels of clinical trials, however, no treatment is yet available[124]. Diet and lifestyle changes remain the most effective methods of managing liver disease[162]. Low caloric diets, low carbohydrate intake and low protein diets have all been shown to be effective in the management of liver disease [163, 162]. It should however be noted that dietary changes alone cannot achieve the intended long-term weight loss goals to effect reduced liver inflammation. It is rather a combination of correct diet and exercise that is most effective against NAFLD[162]. The response to dietary changes and exercise on both gut microbiota that are negatively associated with liver disease and the amount of fat in the liver is different between individuals and also between races [164] The amount of *Bacteroides* for example is lower in Chinese NAFLD individuals after diet and exercise compared t people from the West, and this is corelated with lower hepatic fat[164] . It has also been noted that Bacteroides increases in obese volunteers but decreases in lean ones following exercise and diet intervention[165]. This is suggestive of personalized intervention approaches of diet and lifestyle changes[164]."

Reviewer respond

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 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 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 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 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 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.