

Small intestinal microbial fiber metabolism dysfunction in celiac disease

Corresponding Author: Dr Elena Verdu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Summary & General Comments

In the present study, Wulczynski et al. set up to investigate a critical gap in knowledge of celiac disease, namely targeting the chronic fiber insufficiencies commonly observed in patients. They studied the microbial fiber metabolism in the small intestine in adult patients with active and treated celiac disease and compared the results with healthy age-matched controls. This manuscript presents an investigation into the relationship between dietary fiber intake, gut microbiota composition, and gastrointestinal symptoms, complemented by an animal model to explore potential mechanisms. They used 16S rRNA gene from low biomass samples to sequence the human aspirates and state that they found impaired microbial fiber metabolism in the small intestine which associates with depletion of the carbolytic taxa *Prevotella* spp. To further investigate this mechanistically, they next sensitized mice with transgenic expression HLA-DQ8 gene in non-obese diabetic background (NOD/DQ8) with gluten to determine timing of mucosal recovery after 2, 6, 10, and 24 weeks of gluten-free diet. After 10 weeks of gluten-free diet (GFD) when sensitized NOD/DQ8 mice normalized histological parameters, the effect of fiber supplementation with either inulin or HylonVII supplemented for 6 weeks.

They found that supplementation with inulin during GFD improved histology versus the no added fiber group, whereas HylonVII did not normalize IELs, but had improved histology versus the no added fiber group. All mice became seronegative after 12 weeks of inulin or HylonVII. More importantly, colonization with *Prevotella* spp increased small intestinal short chain fatty acids (SCFA) with inulin supplementation during GFD, compared with no added fiber. Based on these results, the authors conclude that the small intestinal microbiota in adult patients with active or treated celiac disease patients contains less fiber-degrading bacteria and that certain fibers could be used as adjuvant therapy for the gluten-free diet to promote mucosal healing.

Fiber insufficiency and microbiome dysbiosis are topics of importance in celiac disease, especially if studied prospectively, although this study centers around individuals already with the disease. However, while the introduction raises important points, the study design and evidence presented do not adequately address the key gaps and limitations initially outlined. While a mechanistic model is used, there are challenges in translatability to a clinical context in humans. The central message is a bit unclear and hard to follow and can be improved to strengthen the paper.

Here are some specific comments for the authors to consider when revising their manuscript.

Major Concerns

- The exclusive use of 16S rRNA sequencing and PICRUSt is a critical limitation, especially given the small sample size of both the human and mouse arms of this paper. PICRUSt inherently does not allow robust functional inference. At minimum, shotgun metagenomics should have been used, if not also metatranscriptomics, especially given the small sample size in both the human and mouse arms of this study. This would greatly strengthen the paper. Drawing strong functional conclusions based on imputed gene content, given the tone and scope of this paper (centered around microbial metabolic potential as it relates to fiber), is not sufficient.
- It is notable that some of the reported findings are only marginally significant (e.g., *Prevotella*, $p=0.07$). We appreciate that these samples are challenging to obtain, but with a low n and high inter-individual variability in adults, the authors should be cautious of overinterpretation. Again, metagenomics would help overcome these limitations and make the findings more robust. The rationale for selecting 10 *Prevotellaceae* strains is unclear, especially given that not all were significantly associated with human data.
- The authors report no correlation between fiber intake and gastrointestinal scores in patients with celiac disease (page 6), yet they proceed as though fiber depletion plays a key role in symptoms. This contradiction is not acknowledged or

reconciled in the discussion.

- Regarding the attempt to address potential mechanisms, there are some challenges in the present design. There is no well-validated mouse model for celiac disease. This should be noted as a limitation. The use of histology in germ-free mice is not a sufficient substitute and should be discussed more critically.
- The diet intervention in the mice is undermined by the extreme dosages, which simply cannot scale to human study. The doses of inulin (52.8 g/kg) and Hylon VII (136 g/kg) used in mice are extraordinarily high and would be impractical and unsafe in humans, likely resulting in significant GI distress. The manuscript should estimate human-equivalent doses and discuss feasibility and tolerability (e.g., <https://pmc.ncbi.nlm.nih.gov/articles/PMC4804402/>). Using the formula $HED = \text{animal dose} \times [K_m\text{ mouse}/K_m\text{ human}]$ (or 3/37), the doses used by the authors equate to 299 g/day of inulin and 772 g/day of resistant starch for a 70-kg human. These levels far exceed tolerable human intakes (typically 5–15 g/day for inulin and 20–40 g/day for resistant starch), and would likely cause significant GI distress, making them unfeasible for human consumption and especially problematic for celiac disease patients. To put this in context, 772 g of resistant starch is equivalent to ~1.7 lb of purified starch or >22 lb of cooked green bananas/potatoes. Similarly, 299 g of inulin equals ~1.5 lb of raw chicory root. This exercise underscores a fundamental issue in the design and by extension, translational relevance. While the diets may be commercially available by Envigo (Inotiv), they are not translatable. No dosage response experiments were conducted. The HED calculations call into question the feasibility, safety, interpretability, and real-world relevance of these experiments.
- Bridging the human data and the mouse model more explicitly might enhance the study's overall flow. How do the authors interpret the lack of microbial changes after inulin treatment in mice? Does this undercut one of the core premises? Are microbial dysbiotic shifts seen after supplementation with *Prevotella* in addition to inulin?

Minor Suggestions

- The introduction would benefit from additional references studying celiac disease in adults, as microbiome stability and resilience differ significantly by age group, i.e., the bacterial differences observed in very young children with (or preceding) celiac disease may not be the same as those experienced by adults with new-onset or treated celiac disease.
- The effects of diet + bacterial supplementation on microbial dynamics are warranted, which was not presented.
- Supplementary Figure 2b is missing.
- The authors colonized mice with specific strains of bacteria from the human study. It is important to address in the paper that *Prevotella* is not universally beneficial. For example, *P. copri* has been linked to both pro- and anti-inflammatory roles, including associations with RA and JIA, other autoimmune diseases that are also often comorbid with celiac. The manuscript does not discuss this nuance, nor the delivery challenges associated with strict anaerobes.
- The sample size could be problematic in this study. A power analysis within the methods or beginning of results or addressing this further with a statement on generalizability is therefore warranted.
- Discuss whether any microbial changes were observed after combined supplementation with inulin and *Prevotella*—this is alluded to but not clearly reported.

Other Remarks

- The figures are clear and very well done, for instance Figure 5. They are easy to follow. The panels demonstrating study design (like Figure 3a) are especially helpful to the reader. Colors for Hylon VII and inulin are consistent across figures, which facilitates understanding and flow of the results. The histological findings in this and other figures for gluten immunopathology using brightfield microscopy are a strength of the paper.

Conclusion

Although the study presents interesting findings with therapeutic potential and potential novel insights in the etiopathology of celiac disease, it also has some methodological limitations and ambiguous results. Although it is noteworthy that soluble fiber inulin accelerated mucosal healing by supporting carbolytic activity of the small intestinal microbiota in the mouse, a limitation lies in studying patients either at diagnosis with active celiac disease or in clinical remission after prolonged gluten-free diet, as both states complicate interpretation of causality in microbial alterations. It remains unresolved whether impaired microbial fiber metabolism is a contributing factor to disease pathogenesis or a consequence of mucosal injury, raising the fundamental question of causality.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

Dear Authors,

It was with great interest that I've read your manuscript, dedicated to a disease that increasingly more people worldwide are facing, as well as its highly social and financially impairing treatment. Understanding and demonstrating the pitfalls of a lifelong gluten-free diet is noteworthy in order to propel research towards other treatment options, apart from the highly concerning fact that for some patients, current treatment does not suffice. However, I've missed the groundbreaking novelty of your work. It appears not to deliver information that it is not already widely known and recognized as important to all people, regardless their illnesses: the importance of fiber intake for health, which is, indeed, the main focus and conclusion of your work. In fact, there are already recent published work that demonstrate the lack of fiber-foraging bacterial species in the gut of celiac disease patients, and there are several papers addressing the importance of fiber intake to our health,

regardless our comorbidities. The food wheel is a worldwide acknowledged graphic that greatly highlights the importance of fiber intake and, as already mentioned, several published work has already demonstrated the importance of fiber intake for all people, including celiac patients. Thus, although technically sounding, your work does not appear to contribute with significant insights to the disease pathophysiology or to specifically scope for novel therapies. Maybe your goal was to emphasize the importance of fiber intake to the patients from a specific region of the world, as it is also well known that geography greatly determines the diet. If so, I believe it is best that you publish your work in a journal targeting that geographical region. Also, you lack to discuss your gut microbiota results with those from previously published work, apart from the fact that you analyzed a very small sample of celiac patients. Regarding the mice work, as technically sounding as it may be, results again showed what is already known by the scientific community, that *Prevotella* species are fiber-foraging bacterial species and that fiber intake helps reduce inflammation, with *Prevotella* contributing to that, again lacking a prominent novelty in your work, as you did not demonstrate any specific and distinct association to celiac disease and its pathophysiology, i.e., if you had conducted the same experiments with a different mice model the results would have been the same as those obtained with the HLA-DQ8 models. Moreover, as already acknowledged by the scientific community, the use of axenic animal models places the obtained results farther from reality than other models, as it is unclear how much the lack of natural microbiota in the animal influences the experiments conducted in them, particularly regarding the immune system, highly impaired in these animals. In this experiment you demonstrated the obvious association of *Prevotella* and SCFAs production. In HLA DQ8 mice you showed the positive effect of inulin supplementation, which is already known, but did not tested *Prevotella* contribution. Considering all this, it would have been more pertinent to conduct a clinical trial focused on the diet of celiac patients, controlling their fiber intake and/or supplementing them with *Prevotella* probiotics, or commercially available ones, and evaluate serum and gut biomarkers of inflammation and you would have had a study system closer to reality with similar conclusions being obtained. In fact, you suggest that as future work, indicative that you hadn't had the opportunity to conduct a clinical trial. However, as said, available literature already indicates the conclusions you obtained with your work, thus failing in the required groundbreaking novelty. Considering all this, I believe the work does not suffice to be considered for Nature Communications. Perhaps you may want to consider a journal focused on celiac disease or nutrition.

Reviewer #4

(Remarks to the Author)

The manuscript from Wulczynski et. al. aims to define the metabolic capacity of the gut microbiota in the context of Celiac disease (CeD). Using human fecal samples and duodenal aspirate samples, the authors show that microbiota composition, function, and metabolic profile differ between patients with CeD (including Treated CeD) and healthy individuals. The authors also show that colonization with *Prevotella* species as well as dietary supplementation with inulin increases SCFA production in the small intestine enhances mucosal healing in a mouse model of CeD.

This work addresses an important and understudied topic—the potential benefit of dietary fiber to improve CeD outcomes. The authors also show that *Prevotella* species may be an important genus for SCFA production during CeD. Unfortunately, the manuscript has some major limitations that limit enthusiasm for its publication—please see below for details.

Introduction

- First paragraph: using both “prevalence and incidence” is redundant and needs to be revised
- The authors used the term “carbolytic” in the introduction and throughout the manuscript to describe fiber-degrading bacteria—the more appropriate terminology for describing fiber-degrading bacteria is saccharolytic

Figure 1 Results

- The authors need to revise this section for readability by combining the text describing the demographic data and microbiota characterization for CeD patients into a more cohesive narrative that starts off with a clear scientific rationale for why the study was conducted. Specifically, the authors need to emphasize why fecal samples were collected—to identify fiber-degrading taxa potentially lacking in CeD patients.
- When describing samples/data from humans, please consider using the term “healthy individuals” instead of “controls”.
- Include a brief description of the population demographics in the Results section. It would also be helpful to compare these characteristics to larger CeD cohorts reported in the literature either in the introduction or the discussion.
- Clarify the meaning of T-CeD—does “T” denote “treated,” and is the treatment a gluten-free diet?
- There is inconsistency in the methods reported: this section begins by stating 16S sequencing was used, but later references metagenomic data (e.g., Fig. 1e). Please clarify whether both 16S rRNA gene sequencing and shotgun metagenomics were performed (or if it was one or the other).
- Replace “carbolytic” with a more appropriate term
- Faith's Phylogenetic Diversity (PD) is the sole metric presented for alpha-diversity. It is important to explain why this metric was chosen over others and to clarify whether additional metrics were analyzed (e.g., Shannon, Simpson). Faith's PD accounts for phylogenetic breadth but not abundance, which may limit interpretability of the results.

- Did the authors see changes in the abundance of *Prevotella* when assessing compositional changes in patient microbiomes?
- Confirm the spelling of “*Prevotella nanciensis*” in the Figure 1 legend.

Figure 2 Results

- A citation is needed when referencing daily fiber intake recommendations.
- The comparison between CeD and controls regarding fiber intake needs improvement in flow. The authors make the conclusion that there is no difference but it’s not clear until the end of that paragraph what question the authors are asking; it’s also not clear which treatments are being compared to arrive at that conclusion
- The sentence “Although a similar trend was observed for total fiber, there were no statistical differences between the calculated soluble and insoluble fiber fractions between groups (Fig. 2b)” is awkward. Please revise for clarity and flow.
- Why was Shannon diversity used as the sole alpha-diversity metric in this section? How does this choice relate to earlier use of Faith’s PD? The authors should consider using multiple complementary metrics throughout the paper.
- “No statistical difference” should be changed to “no significant difference.”
- Again, the authors need to not use the term “carbolytic”. Also, the authors need to recognize that fiber degradation is not the sole pathway for SCFA production— gut proteolytic bacteria can ferment protein to produce SCFA.
- The sentence “The results suggest T-CeD partially recovers microbial carbolytic capacity, despite the sub-optimal amount of fiber ingested by these patients, which may be related to histological amelioration” is vague. Amelioration of what—villous atrophy, inflammation? Please clarify the intended meaning.
- The terminology “fecal plant DNA” is confusing. Please make it more clear that you are detecting plant DNA from the diet in the human fecal samples.

Figure 3 Results

- The choice of Hylon VII as a source of dietary fiber is a major limitation. In particular:
 - o Hylon VII—although 70% RS2—is only 12% fiber (at least according to the technical sheet that I found on Ingredion’s website), which significantly limits the ability to ask if dietary fiber can improve CeD outcomes. This choice of “fiber” also has significant implications for how the authors are interpreting their results. This results section and the corresponding discussion need to be re-written to reflect what the authors are actually feeding to mice.
 - o The authors also need to provide justification for the selected dosages of Hylon VII and inulin—for instance, the HylonVII dose is nearly 3X the inulin dose—why? Calculating the human dose equivalent would also be helpful.
 - o Provide vendor information for the inulin and HylonVII.
- The sentence “Mice with transgenic expression HLA-DQ8 gene in non-obese diabetic background...” is missing a word; likely “of the.”
- The schematic timelines in Figures 3A and S3A should indicate the 3-week immunization period.
- The transition into the Figure 3 results is abrupt. The authors need to reorder the paragraph to begin with the question about fiber intervention and then integrate the time-course data as justification for evaluating the 6-week timepoint for healing.
- There is a discrepancy between the text and figure: the fiber intervention is described as lasting 10 weeks in the text, but the figure indicates 6 weeks. Please address.
- Current microbiome analyses appear to be limited only to endpoint analyses, which is disappointing. More information about the microbiomes at baseline before fiber interventions and prior to disease induction would provide a more thorough analysis.

Figure 4 Results

- Were changes in the abundance of Prevotellaceae observed in the mouse study? These results are not shown and would be relevant given the experiments to come in Figure 5.
- Is the “SCFA” reported in Fig 4A the total SCFA? Or just the sum of the three reported SCFAs (acetic, propionic, butyric)?
- Why was Aitchison distance used here? Bray-Curtis is commonly seen in microbiome assessments.

Figure 5 Results

- This section opens with SCFA data from inulin-fed GF and SPF mice, but this is not novel nor is it the main point of this results section. This choice for order also interrupts the flow of the paper. The authors should reorganize so that they lead with the important research question and then present these data later as confirmation that SCFA production after inulin consumption is microbiome-dependent.
- The justification, design, and interpretation of the Prevotella experiments in Figure 5 represent a major limitation of this work:
 - o The rationale for introducing Prevotella species into GF mice is not clearly explained. The authors need to expand on why this experiment was conducted and how it relates to the overall hypothesis.
 - o The use of a cocktail of 10 Prevotellaceae strains should also be justified. How were these strains selected? Did the authors confirm that they all colonized the mice? Table 3 includes taxonomic info, but no such detail appears in the main text or methods.
 - o The authors write that strain selection was “based on their reduced abundance in CeD” but it is not clear what this means. Did the authors select Prevotella strains for the mouse studies that were decreased in CeD patients? Or did the selected strains possess the fiber-metabolizing genes that are depleted in CeD patients. If this is a key assumption, it should be explicitly stated and supported.
 - o Why not evaluate disease parameters and mucosal healing in the Prevotella-associated mice? This is a missed opportunity.
- Fig5B: need to define AA, PA, BA in the figure legend
- Throughout the study, it's not always clear in the results sections which mice were used for which studies, i.e., GF or SPF. Please specify this throughout.

Discussion

- The statement “Our results are consistent with previous descriptions that duodenal microbiota profiles differ between active CeD, T-CeD and healthy subjects” needs a citation(s) to support it.
- The statement “It is possible that inulin undergoes more rapid partial digestion in the small intestine compared with HylonVII, a type-2 resistant starch made of linear α -1,4 and branching α -1,6 linkages” is not correct. Neither inulin nor RS2 are digested in the small intestine, save for some RS2 fermentation in the terminal ileum. Please see excellent work in swine models on this topic:
 - o Tan, F.P.Y., Beltranena, E. & Zijlstra, R.T. Resistant starch: Implications of dietary inclusion on gut health and growth in pigs: a review. *J Animal Sci Biotechnol* 12, 124 (2021). <https://doi.org/10.1186/s40104-021-00644-5>
 - o Haenen D et al. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. *J Nutr* 143, 3 (2013). <https://doi.org/10.3945/jn.112.169672>
- The explanation for why inulin led to more mucosal healing than RS2 is underdeveloped and needs work. As was mentioned above, there is a lot of digestible starch in the HylonVII that the authors chose to feed the mice as a source of “fiber,” and there is a lot of literature describing microbiome changes that occur when feeding diets rich in digestible carbohydrates.
- For the statement “We did not perform direct measurement of small intestinal SCFA from human aspirates because it is technically challenging,” please explain why this was challenging? Not enough material?

Methods

- Need to define anaerobic conditions for human sample collection (what ratio of hydrogen, nitrogen, and/or CO₂ was used?)
- Need to describe how the bacteria were prepared for administration to the mice. What media? Anaerobically? How was cell count quantified?
- Was bioavailability/composition of the diets affected by autoclaving? Non-chow diets are most commonly irradiated to avoid affecting non-temperature stable compounds that could be found in the diet.
- How was the small intestine washed out for the necropsy?
- Were the GF mice used in this study confirmed to be germ-free at the end of the study? This information should be provided.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

We are very pleased with the revisions, and the manuscript has improved a lot in its clarity and message. Overall, their responses to our comments are thorough and thoughtful. In particular, the newly added qRT-PCR analyses (of two genes from the *Prevotella* strain) using RNA substantially strengthen the manuscript by providing transcript-level validation of the functional predictions, and we commend the authors for incorporating this experiment in response to feedback.

Still, we have just a few, minor comments that need to be addressed below to clarify the message to readers.

Minor comments:

1. Please include sample size consideration in limitations of the discussion.
2. Line 75 page 4: consider adding "independent of GFD" to subheading.
3. Line 107 page 5: consider adding "nor gastrointestinal symptoms" to subheading, as this point is notable.
4. Lines 233-235 page 11 are ****critical in the premise of the study**** and should be further highlighted (abstract). Now with all the major revisions and new experiments, the title and abstract could align better if they are revisited in the lens of the new findings.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

Dear authors,

You have greatly improved your manuscript accordingly to the reviewers comments.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

We would like to thank the editors and reviewers for their thorough and thoughtful comments. Considering the suggestions and criticisms raised, we hereby submit an extensively revised manuscript.

Detailed responses to each of the reviewers' comments and suggestions are provided below. We are grateful for the opportunity to strengthen the manuscript, which we hope will now be acceptable for publication in Nature Communications.

Point-by-point answer to Reviewers' comments:

REVIEWERS 1&2

“The exclusive use of 16S rRNA sequencing and PICRUSt is a critical limitation, especially given the small sample size of both the human and mouse arms of this paper. PICRUSt inherently does not allow robust functional inference. At minimum, shotgun metagenomics should have been used, if not also metatranscriptomics, especially given the small sample size in both the human and mouse arms of this study. This would greatly strengthen the paper. Drawing strong functional conclusions based on imputed gene content, given the tone and scope of this paper (centered around microbial metabolic potential as it relates to fiber), is not sufficient.”

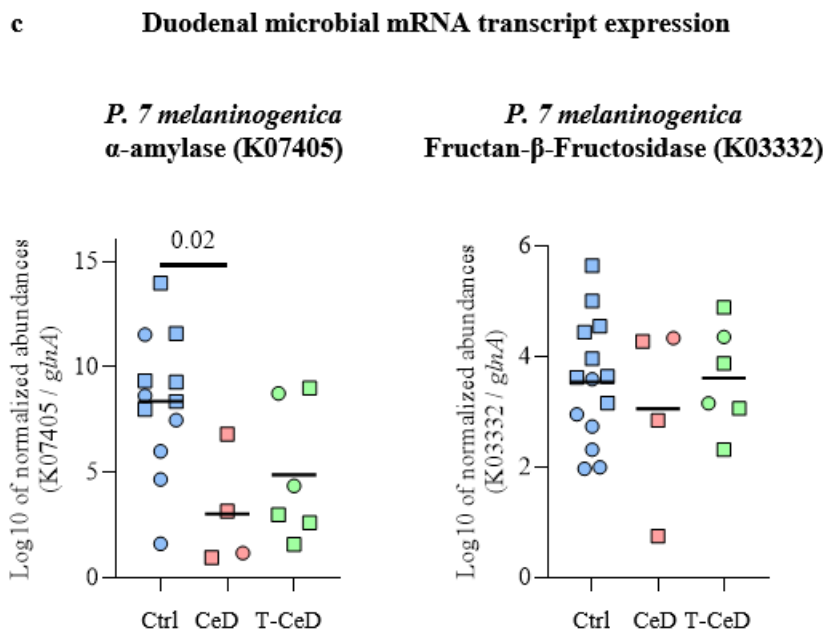
Response:

We agree that PICRUSt2 is mainly focused on prediction of bacterial core genomes and has lower resolution for functional inference compared with shotgun metagenomics. However, 16S rRNA gene sequencing and functional predictions are still relevant (Jovel et al., 2016), especially for low biomass samples. On the other hand, metagenomics or metatranscriptomics analysis of the duodenum predominantly captures host-derived DNA and transcripts. Duodenal aspirates have low bacterial biomass and a high host-to-microbe DNA ratio, making metagenomic approaches difficult, less reliable, and more costly (Caminero et al., 2025; Jervis-Bardy et al., 2015; Machado et al., 2024). Removing the host DNA from low biomass samples remains a challenge for small intestinal microbiota research. New techniques are being proposed but, we will need to wait to have those validated specifically for proximal small intestinal biopsies from the duodenum and jejunum, as well as luminal aspirates. Thus, we employed 16S rRNA gene sequencing, as our study targeted a low-biomass site and utilized a method that was previously validated for this condition in humans (Constante et al., 2022). We however acknowledge these limitations and raise the methodology constraints of shotgun metagenomics and metatranscriptomics in low yield ecosystems in the discussion (Pg 14, lines- 297-300). We agree that while PICRUSt is most valuable as a data mining tool (Jovel et al., 2016), ideally predictions should be confirmed using other methods. Our group has previously used PICRUSt to guide the analysis of bacterial stool transcripts to confirm how the presence of the gene and the mRNA expression (similar to what one may obtain in a metatranscriptomics analysis) affect microbial function and result in disease activity (Cholet et al., 2019; Santiago et al., 2023). Thus, to address the reviewers' point we completed a similar experiment to validate the expression of glycoside hydrolases in the aspirates using targeted quantitative reverse-transcriptase PCR. The quantification of bacterial RNA demonstrates not only the relevance of the PICRUSt predictions but also, as suggested by the reviewer, it goes one step further by quantifying RNA expression

instead of DNA levels. We thank the reviewer for this comment as we believe it made the results stronger by including this transcript-level data. This result is included in Supplementary Figure 2c, and we also briefly discuss it in limitations paragraph (Discussion, page 14).

Page 5 (line 102 – 106):

“To confirm the PICRUSt2-predicted results, we performed qRT-PCRs for the highest abundance bacterium identified at the species level (*Prevotella 7 melaninogenica*) for genes K07405 and K03332. We found that K07405 had a similar RNA expression profile as the predicted gene content with reduced expression in CeD compared with controls. No statistical differences were observed for K03332 (Supplementary Figure 2c)”.



Supplementary figure 2c:

“**c** Duodenal microbial mRNA transcript expression of α -amylase (K07405) and fructan- β -fructosidase (K03332) normalized to glnA transcript levels in healthy controls (blue, $n = 12-14$), CeD (red, $n = 4$) and T-CeD (green, $n = 6$). Samples with low RNA integrity were excluded from analysis. Multiple differences were compared using one-way ANOVA followed by Tukey’s test, and sex was represented by females (squares) and males (circles).”

Page 14 (line 297 –300):

“For microbiome analysis, next-generation sequencing such as shotgun metagenomics may provide more robust functional analysis than 16S rRNA gene sequencing, especially if they include jejunum or ileum, but could not be completed in our low-biomass duodenal aspirates due to high levels of host contamination (Caminero et al., 2025; Jovel et al., 2016). We used a previously validated 16S rRNA gene sequencing protocol (Constante et al., 2022, and performed qRT-PCR for targeted measurement of gene expression.”

“The rationale for selecting 10 Prevotellaceae strains is unclear, especially given that not all were significantly associated with human data.”

Response:

We have now revised the discussion to expand on the rationale for the selection of Prevotellaceae strains. The 10 species were selected from our large biobank of human derived bacteria (Lau et al., 2016; Whelan et al., 2020). We centered on species belonging to *Prevotellaceae*, which is a family of bacteria that are at high abundance in the oral cavity and colonize the small intestine (Könönen & Gursoy, 2022; Tett et al., 2021). They are known to be a fiber-degrading taxa with capacity for SCFA production (Chen et al., 2017; Prasoodanan P. K. et al., 2021) due to the many glycoside hydrolase enzymes they carry in addition to the two identified in our study (α -amylase and fructan- β -fructosidase). Our observation that the relative abundance of *Prevotella* spp is decreased in CeD compared with healthy controls is supported by our previous paper (Constante et al. 2022), and other independent cohorts (Nistal et al., 2012; Roque et al., 2024). Thus, given that our mouse SPF colony contained a low relative abundance of *Prevotellaceae*, we designed a mechanistic experiment in gnotobiotic mice using well characterized *Prevotellaceae* strains from Surette’s lab collection at McMaster to functionally validate their role in fiber metabolism and SCFA production in the small intestine. This is now better explained in the discussion of the revised manuscript on page 12.

Page 12 (lines 266-273)

“The relative abundance of Prevotella spp was low in the mouse small intestine and was not significantly affected by either fiber diet. Thus, we designed a mechanistic experiment by colonizing germ-free mice with a collection of Prevotella spp from our biobank of human derived bacteria (Lau et al., 2016; Whelan et al., 2020). These species were all confirmed to carry the genes of interest for α -amylase (K07405) and fructan- β -fructosidase (K03332), and in colonized mice, they increased small intestinal SCFA upon inulin supplementation.”

“The authors report no correlation between fiber intake and gastrointestinal scores in patients with celiac disease (page 6), yet they proceed as though fiber depletion plays a key role in symptoms. This contradiction is not acknowledged or reconciled in the discussion.”

Response:

Thank you for this important comment and the opportunity to clarify it. Our study was not designed to investigate symptoms in celiac disease in response to fiber challenge or supplementation or due to differences in fiber intake. This would require a different design, such as a clinical study with dietary interventions, and longitudinal tracking of symptom responses and dietary evaluations. The results of our study could help better design such an intervention or longitudinal study by providing valuable mechanistic insight and microbial (structure and function) targets. In our cohort, the estimated fiber intake did not correlate with gastrointestinal symptoms rating scores patients were reporting at a cross-sectional level. There was no fiber challenge; patients were on their usual diet and only T-CeD were on a GFD. Many participants were below the recommended fiber intake levels, and this was more evident in the GFD group. We do not claim that fiber plays a role in symptoms in this study, although there is literature that suggests some IBD patients can be intolerant to some fibers (Armstrong et al., 2023), and these studies are cited as general background to highlight beneficial and detrimental effects of fiber as

shown by others. We were intrigued though by the finding that dietary fiber intake *did not* fully explain the lower saccharolytic activity observed in CeD patients. This suggested a primary microbial functional deficiency, rather than lack of dietary substrate, motivating the mechanistic studies. We have explained this better in the manuscript on page 10.

Page 10 (lines 203-208)

“There was no relationship between estimated fiber consumption and symptoms in our cohort, although many participants were already consuming lower than recommended fiber. Our study was not designed to investigate fiber intolerance in humans with CeD, as has been done previously in inflammatory bowel disease (Armstrong et al., 2023; Kuang & Binion, 2022), but to investigate the impact of diet on microbial saccharolytic function in the small intestine.”

“Regarding the attempt to address potential mechanisms, there are some challenges in the present design. There is no well-validated mouse model for celiac disease. This should be noted as a limitation. The use of histology in germ-free mice is not a sufficient substitute and should be discussed more critically.”

Response:

No animal model will ever reproduce human disease completely and there is no human disease for which there is a single animal model. For instance, in IBD, T cell transfer, DSS, TNBS, IL-10 KO models (and other genetic models) are used, depending on the scientific question. There is no reason why there should be only one model for CeD, rather a large toolbox of *in vitro* and *in vivo* models should complement clinical interventions and samples, including organoids.

We would like to share a quote from a commentary from the Scientific Advisory Board for AGA Center for Gut Microbiome Research and Education that specifically addresses the role of animal models in GI disease:

“Preclinical models of disease ... aim to reproduce specific features of the disease being studied, leading to new insights that require validation in humans. The three key aspects traditionally used to evaluate preclinical models relate to (1, face validity) the features of the disease being modelled, (2, construct validity) whether the features of the model arise for biologically relevant reasons, and (3, predictive validity) how well the model predicts expression of those pathways or responses to treatment in humans...”
(Britton et al., 2024).

We agree that there is no perfect model for any human disease (including celiac disease) and, that models are used based on the 3 criteria above and depending on the question under study. For celiac disease, most current animal models are based on MHC class II transgenic mice expressing HLA-DQ2 or HLA-DQ8, which can, or not, include transgenic expression of IL-15 to study particularly that pathway, but no single mouse model recapitulates all features of human disease. With respect to the NOD-DQ8 model, it expresses the human *DQ8* gene in the absence of endogenous mouse MHC-II and it has extensively been characterized for the study of gluten-specific immune responses at the Mayo Clinic (Black et al., 2002; Marietta et al., 2004; Verdu et al., 2008). The model was further validated by our group and others for the past 15-20 years to study the influence of microbial colonization and composition on gluten-induced immunopathology (Bodkhe et al., 2021; Caminero et al., 2016, 2019; Galipeau et al., 2015; Lamas et al., 2020), demonstrating face and to certain extent, construct validity. More recently,

the group of Detlef Schuppan (U of Mainz) has used this model to study mechanistic pathways, and reproduce effectiveness of the TG2 inhibitor ZED1227, showing promising *predictive* validity (unpublished, presented at UEGW 2025). Thus, in our opinion the model is useful to complement our clinical observations and to study microbial pathogenesis as the model shows microbial colonization affects and modulates gluten immunopathology.

We believe the strength of our paper is the use of a variety of experimental tools including gnotobiotic mouse models for direct interrogation of specific microbial function (*Prevotellaceae*), functional assays, molecular biology and biochemistry and validations in clinical cohorts, truly encompassing what *translational research* should represent. This is now discussed in limitations on Page 14.

Page 14 (lines 302-308)

“ ... the NOD/DQ8 mouse employed here requires sensitization and the use of a microbial adjuvant and is well suited for the study of environmental triggers or modulators of disease severity (Caminero, McCarville, Galipeau, et al., 2019) and fulfills validity criteria of animal models (Britton et al., 2024) such as ability to produce mechanistic insight, for clinical trial validation. This translational study provides unique insight into the relevance of fiber supplementation in improving gluten-specific immunity through microbial metabolism, a finding that warrants further evaluation in clinical trials using human-equivalent doses.”

“The diet intervention in the mice is undermined by the extreme dosages, which simply cannot scale to human study. The doses of inulin (52.8 g/kg) and Hylon VII (136 g/kg) used in mice are extraordinarily high and would be impractical and unsafe in humans, likely resulting in significant GI distress. The manuscript should estimate human-equivalent doses and discuss feasibility and tolerability (e.g., <https://pmc.ncbi.nlm.nih.gov/articles/PMC4804402/>). This exercise underscores a fundamental issue in the design and by extension, translational relevance. While the diets may be commercially available by Envigo (Inotiv), they are not translatable. No dosage response experiments were conducted. The HED calculations call into question the feasibility, safety, interpretability, and real-world relevance of these experiments.”

Response:

Our study was not intended to determine human doses of fiber supplementation in future studies. The value of our study is the mechanistic demonstration that some fibers can influence the function of the small intestinal microbiome differentially. This concept will be important to better design future challenge studies in humans, for instance for the choice of biogeographic location of fiber-microbiome interactions and taxa of interest as outcomes. Our study did not evaluate effective doses in humans, which we clarify now in the revised Discussion.

We would like to further explain how our calculations for the mouse feed were done. As reviewers suggest, doses based on mass, are well suited for calculations of “human equivalent doses” of pharmacological intervention or drugs. However, these conversions are less appropriate for studies like ours incorporating nutritional substrates in mouse feeds. The formulation of the animal diets used in our study is based on allometric scaling which was calculated to consider how the metabolic and biological processes scale with the size of the

animal (Rucker, 2022). When using direct mass conversion, the assumption is that the human would require 2500-2800 times more nutrients than the mouse. However, when using metabolic calculations based on resting energy expenditure, humans only require 200-400 times more energy than mice. Therefore, we calculate that the equivalent dose for humans could range from 40-80g inulin daily, which is slightly higher than the currently recommended doses by Health Canada for females (25g/d) and males (38g/d). The transition to the fiber-supplemented diets though was performed progressively not all at once; allowing the mice in our study to acclimate to a reduced dose of fiber over the first week, ensuring they were well adapted to the higher doses. Moreover, as the fiber was incorporated in the feed (not by a challenge), ingestion was physiological.

An unknown factor to consider is the relative contribution of the microbiota to metabolism; and how disease-associated alterations in microbiota can further impact metabolism *in vivo* (Caminero et al., 2016; Caminero, McCarville, Zevallos, et al., 2019). This is now explained in methods:

Page 13 (lines 284-289)

“Our dietary intervention was designed to investigate the effect of fiber on the small intestinal microbiome and how that related to intestinal healing in genetically susceptible sensitized mice, not to determine the relevant dose of fiber sufficient to increase microbial activity, promote healing, or impact symptoms in human CeD patients. Our study should encourage future clinical investigation with prospective trials to further elucidate the relationship between fiber and gastrointestinal symptoms in CeD.”

Page 14 (lines 306-308)

“This translational study provides unique insight into the relevance of fiber supplementation in improving gluten-specific immunity through microbial metabolism, a finding that warrants further evaluation in clinical trials using human-equivalent doses.”

Page 18 (lines 363-366)

*“The total fiber content in the supplemented diets was increased to approximately 15% from a grain-based chow formula with approximately 7.5% dietary fiber. Diet composition is listed in **Table 2** and was calculated based on allometric species nutritional requirements and not direct mass-to-mass conversions (Rucker, 2022).”*

It is relevant to point out for context, that the dose of supplemented fiber (+5% inulin or HylonVII = 15% total fiber from all sources) *is lower* in our study than what has used to demonstrate health benefit in other inflammatory mouse models (Corrêa et al., 2023; Le Leu et al., 2009; Niu et al., 2024), including delaying spontaneous diabetes onset in the NOD mouse (Zou et al., 2021).

Finally, during our study, the mice were monitored for potential signs of distress, food consumption, weight gain, fecal output, and at sacrifice, for presence of gas in the bowels. None of these were observed in any of the groups. We now indicate this in the Results section manuscript on page 7, and in the Methods on Page 17.

Page 7 (lines 141-144)

*“Another cohort of sensitized NOD/DQ8 mice (**Fig 3a**) were given GFD with no added fiber (NAF) or supplemented with either inulin (52g/kg) or HylonVII (136g/kg) supplemented diets (described in **Table 2**) for 6 weeks and monitored for signs of distress.”*

Page 18 (lines 366-368)

“Mice were monitored for signs of distress, changes in food consumption, weight gain, and fecal output through the duration of the diet intervention study.”

“Bridging the human data and the mouse model more explicitly might enhance the study’s overall flow. How do the authors interpret the lack of microbial changes after inulin treatment in mice? Does this undercut one of the core premises?”

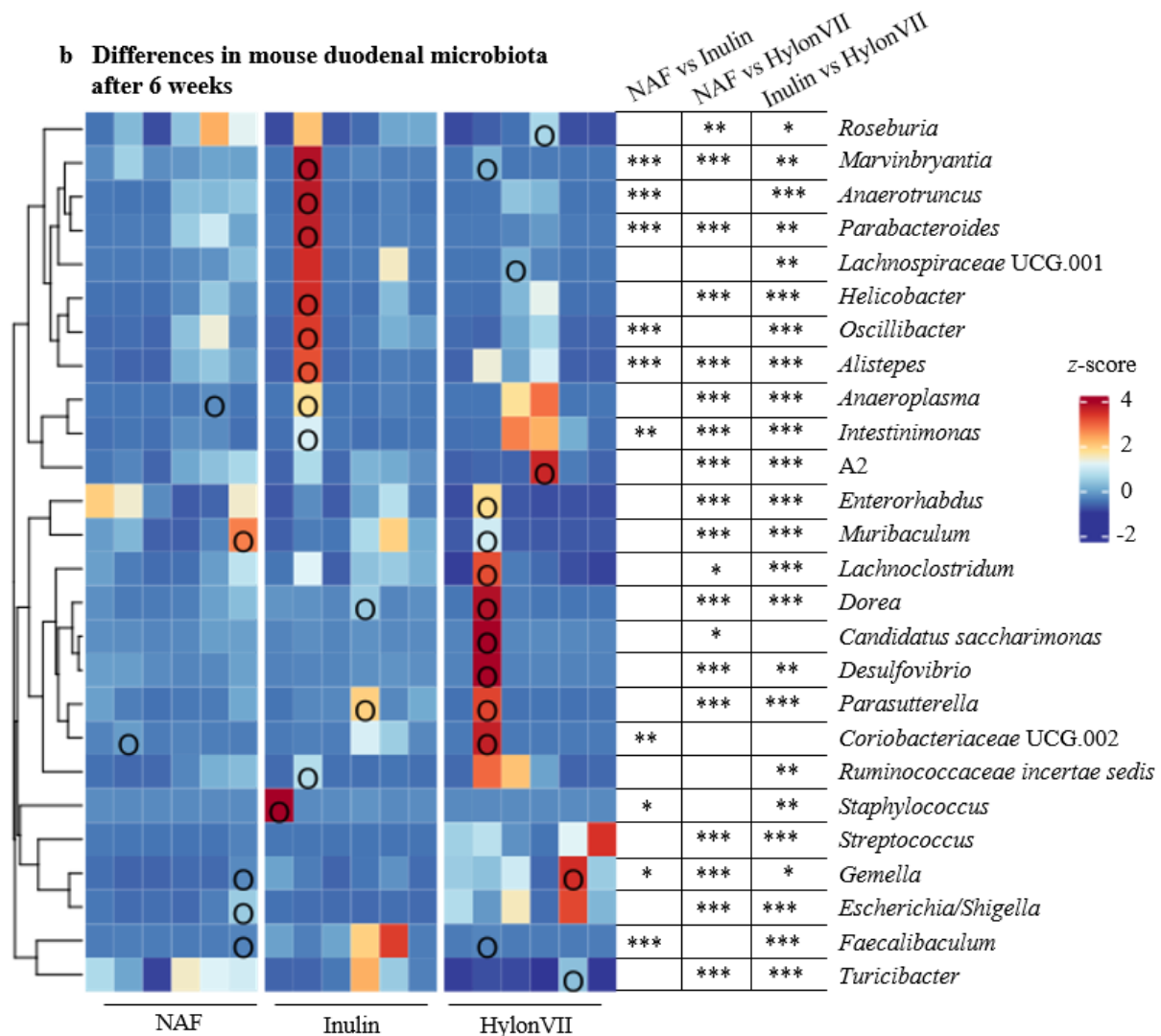
Response:

Thank you for this comment. We now better explain these findings. Changes in the relative abundance of different taxa (see new supplementary Fig 6b, included below as well for the reviewer) were observed following inulin supplementation, however, overall clustering according to β -diversity was not affected when inulin and Hylon VII-fed mice were compared. We do not see this as “undercutting any premise”, as composition and diversity reflect different aspects of ecology. For example, diversity metrics summarize overall richness and evenness, so specific taxa can change without affecting total diversity. This occurs when shifts in some microbes are balanced by opposing changes in others. Due to the inherent inter-variability of the microbiome, we agree that it is more appropriate to describe the observed changes or shifts after an intervention.

Supplementary figure 6b displays the microbial taxa that were significantly affected by the diet intervention at endpoint. Changes in the relative abundance of several taxa were observed between the different groups, suggesting distinct effects of fiber interventions on the small intestinal microbiome. In the revised version we summarize these changes in the text as follows:

Page 7 (lines 154-167)

*“There was no difference in microbial α -diversity between no added fiber, inulin, and HylonVII fed mice after 6 weeks (**Supplementary Fig. 6a**). Duodenal microbial β -diversity was significantly affected by HylonVII, which clustered separately from the no added fiber and inulin groups (**Fig. 4b**). Specifically, HylonVII had higher *Helicobacter*, *Alistepes*, *Aneroplasmata*, *Intestinimonas*, *Candidatus saccharimonas*, *Streptococcus*, *Gemella* and *Escherichia/Shigella* and lower abundance of *Roseburia*, *Turicibacter*, *Parasutterella*, *Desulfovibrio*, *Dorea*, *Lachnospirillum*, *Muribaculum*, *Enterorhabdus*, *A2*, *Parabacteroides*, *Aerotruncus* and *Marvinbryantia*. Inulin, which did not affect β -diversity compared with no added fiber diet, had higher *Faecalibaculum*, *Gemella*, *Staphylococcus*, *Coriobacteriaceae* UCG.002 and lower abundance of *Intestinimonas*, *Alistepes*, *Oscillibacter*, *Parabacteroides*, *Aerotruncus* and *Marvinbryantia* versus no added fiber GFD (**Supplementary Fig. 6b**).”*



Supplementary figure 6d:

“b Heatmap summary of differentially abundant bacterial taxa grouped by diet. Each row represents the z-score for one bacterium and **O** indicates statistical outlier defined as a value more than three times the interquartile range.

“Are microbial dysbiotic shifts seen after supplementation with *Prevotella* in addition to inulin?”

Response:

We prefer not to refer to “dysbiosis”, as this is a term that is not easy to define, in the same way, it is difficult to define “eubiosis” (Britton et al., 2024). Instead, we describe the significant shifts after interventions. In experiments using SPF mice, the relative abundance of *Prevotella* in the mouse duodenum was less than 1% and did not significantly expand when fed inulin compared with the no added fiber GFD. We have clarified this in the discussion on page 12.

Prevotellaceae were used exclusively to colonize germ-free mice that were kept gnotobiotically to test a specific role of this taxon in the functional response to fiber (the rationale for using

Prevotellaceae is explained above in an earlier response). *Prevotella histicola* has been previously demonstrated as beneficial in the NOD-DQ8 model (Bodkhe et al., 2021). Supplementation of SPF mice with 10^8 CFU *Prevotella* spp, would not be expected to cause “overgrowth”, as this is usually the probiotic dose administered to mice (for humans daily doses can reach up to 5×10^{10}) which can range between 10×10^8 to 10×10^{11} CFUs (Bodkhe et al., 2021; Caminero, McCarville, Zevallos, et al., 2019; McCarville et al., 2017; Natividad et al., 2012). Moreover, the newly administered taxon would never colonize permanently an SPF mouse and would disappear within 48 hours after stopping administration. This has well been demonstrated in murine and human probiotic supplementation studies (Laursen et al., 2017; Zmora et al., 2018). Furthermore, fiber consumption, *Prevotella* relative abundance, and SCFA are all positively associated with decreased risk of developing food allergy (Goldberg et al., 2020; Vuillermin et al., 2020), supporting the notion of members of this taxon have potential use in microbial therapeutics, that will need to be confirmed in human studies (Itano et al., 2023).

Page 12 (lines 267-273)

“The relative abundance of Prevotella spp was low in the mouse small intestine and was not significantly affected by either fiber diet. Thus, we designed a mechanistic experiment by colonizing germ-free mice with a collection of Prevotella spp. from our biobank of human-derived bacteria (Lau et al., 2016; Whelan et al., 2020). These species were all confirmed to carry the genes of interest for α -amylase (K07405) and fructan- β -fructosidase (K03332), and in colonized mice, they increased small intestinal SCFA upon inulin supplementation.”

REVIEWERS 4&5

Figure 1 Results: “Faith’s Phylogenetic Diversity (PD) is the sole metric presented for alpha-diversity. It is important to explain why this metric was chosen over others and to clarify whether additional metrics were analyzed (e.g., Shannon, Simpson). Faith’s PD accounts for phylogenetic breadth but not abundance, which may limit interpretability of the results.”

Response: Thank you for this comment. We show Faith’s PD figure to account for changes in phylogenetic diversity which reflects changes in function due to evolutionary proximity, more than changes in species richness or evenness alone. We agree with the reviewer that this limits interpretation on evenness analysis or simple richness. We therefore added in the revised manuscript Chao1 and Shannon diversity measures for the microbiota in Supplementary Fig 1, which have similar results. We also complemented those with the microbial Beta-diversity and relative abundance plots in Supplementary Fig 1 and 2.

Figure 2 Results: “Why was Shannon diversity used as the sole alpha-diversity metric in this section? How does this choice relate to earlier use of Faith’s PD? The authors should consider using multiple complementary metrics throughout the paper.”

Response: The analysis for FoodSeq plant DNA was completed independently from the microbiota analysis. Shannon diversity was chosen because it accounts for both species richness and evenness, without relying on phylogenetic relationships. This approach was intentionally chosen as the *trnL* gene region that was amplified is shorter (length ranging from 50-150bp) than the bacterial 16S V3V4 regions, providing less phylogenetic resolution between closely related taxa. Furthermore, the evolutionary distances in plants do not necessarily translate to nutritional

differences relevant in the human diet. Please note that the primary goal of FoodSeq Plant DNA is to estimate dietary plant intake through fecal DNA profiling, rather than to assess ecological dynamics, which is what the metrics used in microbiome studies capture.

Page 11 (Lines 222-225)

“Notably, the FoodSeq analysis was used for the first time as a complementary metric to estimate differences in dietary plant intake between unrestricted diets and gluten-free diets through fecal DNA profiling, not to assess ecological dynamics which is common to microbial DNA profiling.”

Figure 3 Results: “The choice of Hylon VII as a source of dietary fiber is a major limitation. In particular:

- **Hylon VII—although 70% RS2—is only 12% fiber (at least according to the technical sheet that I found on Ingredion’s website), which significantly limits the ability to ask if dietary fiber can improve CeD outcomes. This choice of “fiber” also has significant implications for how the authors are interpreting their results. This results section and the corresponding discussion need to be re-written to reflect what the authors are actually feeding to mice.**
- **The authors also need to provide justification for the selected dosages of Hylon VII and inulin—for instance, the HylonVII dose is nearly 3X the inulin dose—why? Calculating the human dose equivalent would also be helpful.”**

Response:

Thank you for allowing us to clarify this. Our rodent diets are grain-based chow with additional prebiotic supplements. This means that the mice get a variety of different natural fibers, compared to a purified diet where the intervention is all or nothing. Our choice for including HylonVII (Ingredion 03451B00) was based on its potential as prebiotic candidate to investigate alongside the GFD. HylonVII and other type 2 resistant starches are considered prebiotic fibers by North American and European governing agencies for their beneficial properties (EFSA Panel on Dietetic Products and Allergies (NDA), 2010; Sobh et al., 2022). HylonVII contains 70% amylose and is estimated to contain 48% bioavailable RS2 prebiotic substrate (Le Leu et al., 2009). It is difficult to directly compare the amount of different prebiotic substrates for an intervention in an animal model, so the diets were formulated to contain similar amounts of bioavailable carbohydrates and caloric density, which is why there is such a large difference in mass of ingredients (**Table 2**). The total fiber/prebiotic substrate from all sources in both diets was formulated to be 15%. The vendor Envigo (Inotiv) supplied the inulin (TIC Prestested Inulin LV-110 Powder (Ingredion 38910903) and HylonVII for the diets in consultation with us and their specialized technicians. Specifically, we revised the Hylon VII diet with expert Envigo technologists and the values for NDF for the natural ingredients are described in a sheet that can be appended to the manuscript if the editors consider it necessary. Fiber content was calculated using the formulas below:

- Inulin diet fiber: $\text{dietNDF} + \text{dietFiber} - \text{Inulin fiber calc} + \text{Inulin} = \mathbf{158.5 \text{ g/kg (15.8\%)}$

- H7 diet fiber: $\text{dietNDF} + \text{dietFiber} - \text{H7 fiber calc} + (0.48 \times \text{H7}) = \mathbf{155 \text{ g/kg (15.5\%)}$

Calculations for human equivalent doses based on mass are less appropriate for nutritional studies like ours than pharmacological interventions. The formulation of the animal diets used in our study is based on allometric scaling which was calculated to consider how the metabolic and biological processes scale with the size of the animal (Rucker, 2022). Another factor to consider is the relative contribution of the microbiota to metabolism; and how diseased-associated alterations in microbiota can further impact metabolism *in vivo* (Caminero *et al.*, 2016; Caminero, McCarville, Zevallos, *et al.*, 2019). This is now explained better in methods on page 18.

Page 18 (lines 363-368)

“The total fiber content in the supplemented diets was increased to approximately 15% from a grain-based chow formula with approximately 7.5% dietary fiber. Diet composition is listed in Table 2 and was calculated based on allometric species nutritional requirements and not direct mass-to-mass conversions (Rucker, 2022). Mice were monitored for signs of distress, changes in food consumption, weight gain, and fecal output through the duration of the diet intervention study.

Table 2: Composition of mouse diets.

Diets and Nutrient Information		TD.05620 GFD	TD.200056 Wheat diet	TD.210884 Inulin	TD.210885 HylonVII
Protein		20.9	20.9	21.9	20.1
% kcal from	Carbohydrate	65.9	65.9	64.3	67.8
Fat		13.2	13.2	13.8	12.1
Kcal/g		3.2	3.2	3	3.1
Dietary Fiber		5-10%	5-7%	15.80%	15.50%
Ingredients (g / kg)					
Corn		500	N/A	500	364
Soybean Meal		270	265	270	270
Soybean Hull		40	40	40	40
DL-Methionine, FG (99%)		1.5	1.5	1.5	1.5
Maltodextrin		115.73	115.73	62.93	115.73
Lard		25	25	25	25
Mineral Mix, U.S. Rodent (99115)		2.5	2.5	2.5	2.5
Calcium Carbonate, FG (38%)		13	13	13	13
Dicalcium Phosphate, FG, (18.5% P, 21% C)		12	12	12	12
Magnesium Oxide, FD (58%)		2.2	2.2	2.2	2.2
Sodium Chloride, iodized		10	10	10	10
Vitamin Mix, U.S. Rodent (99114)		2.5	2.5	2.5	2.5
Vitamin Mix, U.S. Supplement (99116)		2.5	2.5	2.5	2.5
Choline Chloride, FG (60%)		2.07	2.07	2.07	2.07

Locust Bean Gum w/ gelatin	1	1	1	1
Wheat	N/A	375	N/A	N/A
Corn Starch	N/A	116	N/A	N/A
Cellulose	N/A	1.8	N/A	N/A
Corn Oil	N/A	12.2	N/A	N/A
Inulin *	N/A	N/A	52.8	N/A
High Amylose Corn Starch**	N/A	N/A	N/A	136

Custom diets were based on our mouse gluten-free diet (GFD) formulated by Envigo (Inotiv) to substitute macromolecule sources (e.g. corn vs wheat) or specific nutrients (e.g. inulin or HylonVII).

* TIC Pretested Inulin LV-110 Powder (Ingredion 38910903)

** Hylon® VII (Ingredion 03451B00)

Figure 3 Results: “Current microbiome analyses appear to be limited only to endpoint analyses, which is disappointing. More information about the microbiomes at baseline before fiber interventions and prior to disease induction would provide a more thorough analysis.”

Response:

Our design was limited to endpoint analysis because these *tissue* samples could only be collected at necropsy and cannot be completed longitudinally (paired), unlike feces. The primary outcome was to investigate *in parallel* (cross-sectional), with appropriate controls, the changes in the small intestinal microbiome that accompanied improvements in immunopathology over time with different prebiotic substrates compared to the no added fiber GFD. The longitudinal sampling of the small intestine in mice would require sacrifice at different timepoints and would be comparing different mice as there is not available technique to obtain mucosal biopsies *in vivo* from the mouse duodenum. The NAF GFD group is well suited as a parallel control comparison against the inulin or HylonVII fed mice in the context of recovery from gluten exposure on a gluten-free diet. All the mice were housed in the same room of a well-controlled SPF facility that is regularly monitored for the absence of exogenous contaminations pathogens. Moreover, at the start of the study, and anticipating this, we distributed mice from same litters to all treatment groups, minimizing biases at baseline due to cage effects. This is better explained under methods in the revised manuscript on page 17:

Page 17 (lines 353-354)

“To minimize litter and cage effects, littermates were distributed across treatment groups using random assignment to sex-balanced cages before sensitization with gluten using a previously established protocol (Galipeau et al., 2011).”

- Armstrong, H. K., Bording-Jorgensen, M., Santer, D. M., Zhang, Z., Valcheva, R., Rieger, A. M., Sung-Ho Kim, J., Dijk, S. I., Mahmood, R., Ogungbola, O., Jovel, J., Moreau, F., Gorman, H., Dickner, R., Jerasi, J., Mander, I. K., Lafleur, D., Cheng, C., Petrova, A., ... Wine, E. (2023). Unfermented β -fructan Fibers Fuel Inflammation in Select Inflammatory Bowel Disease Patients. *Gastroenterology*, 164(2), 228–240. <https://doi.org/https://doi.org/10.1053/j.gastro.2022.09.034>
- Black, K. E., Murray, J. A., & David, C. S. (2002). HLA-DQ Determines the Response to Exogenous Wheat Proteins: A Model of Gluten Sensitivity in Transgenic Knockout Mice¹. *The Journal of Immunology*, 169(10), 5595–5600. <https://doi.org/10.4049/jimmunol.169.10.5595>
- Bodkhe, R., Marietta, E. V., Balakrishnan, B., Luckey, D. H., Horwath, I. E., Shouche, Y. S., Taneja, V., & Murray, J. A. (2021). Human gut-derived commensal suppresses generation of T-cell response to gliadin in humanized mice by modulating gut microbiota. *Anaerobe*, 68, 102237. <https://doi.org/10.1016/j.anaerobe.2020.102237>
- Britton, R. A., Verdu, E. F., Di Rienzi, S. C., Reyes Muñoz, A., Tarr, P. I., Preidis, G. A., Tomeo, N. J., Ranallo, R. T., Pimentel, M., & Chang, E. (2024). Taking Microbiome Science to the Next Level: Recommendations to Advance the Emerging Field of Microbiome-Based Therapeutics and Diagnostics. *Gastroenterology*, 167(6), 1059–1064. <https://doi.org/10.1053/j.gastro.2024.05.023>
- Caminero, A., Galipeau, H. J., McCarville, J. L., Johnston, C. W., Bernier, S. P., Russell, A. K., Jury, J., Herran, A. R., Casqueiro, J., Tye-Din, J. A., Surette, M. G., Magarvey, N. A., Schuppan, D., & Verdu, E. F. (2016). Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. *Gastroenterology*, 151(4), 670–683. <https://doi.org/10.1053/j.gastro.2016.06.041>
- Caminero, A., McCarville, J. L., Galipeau, H. J., Deraison, C., Bernier, S. P., Constante, M., Rolland, C., Meisel, M., Murray, J. A., Yu, X. B., Alaedini, A., Coombes, B. K., Bercik, P., Southward, C. M., Ruf, W., Jabri, B., Chirido, F. G., Casqueiro, J., Surette, M. G., ... Verdu, E. F. (2019). Duodenal bacterial proteolytic activity determines sensitivity to dietary antigen through protease-activated receptor-2. *Nature Communications*, 10(1), 1198. <https://doi.org/10.1038/s41467-019-09037-9>
- Caminero, A., McCarville, J. L., Zevallos, V. F., Pigrau, M., Yu, X. B., Jury, J., Galipeau, H. J., Clarizio, A. V., Casqueiro, J., Murray, J. A., Collins, S. M., Alaedini, A., Bercik, P., Schuppan, D., & Verdu, E. F. (2019). Lactobacilli Degrade Wheat Amylase Trypsin Inhibitors to Reduce Intestinal Dysfunction Induced by Immunogenic Wheat Proteins. *Gastroenterology*, 156(8), 2266–2280. <https://doi.org/10.1053/j.gastro.2019.02.028>
- Caminero, A., Tropini, C., Valles-Colomer, M., Shung, D. L., Gibbons, S. M., Surette, M. G., Sokol, H., Tomeo, N. J., Association, =Scientific Advisory Board of the Center for Gut Microbiome Research and Education of the American Gastroenterological, Tarr, P. I., & Verdu, E. F. (2025). Credible inferences in microbiome research: ensuring rigour, reproducibility and relevance in the era of AI. *Nature Reviews. Gastroenterology & Hepatology*. <https://doi.org/10.1038/s41575-025-01100-9>

- Chen, T., Long, W., Zhang, C., Liu, S., Zhao, L., & Hamaker, B. R. (2017). Fiber-utilizing capacity varies in *Prevotella*- versus *Bacteroides*-dominated gut microbiota. *Scientific Reports*, 7(1), 2594. <https://doi.org/10.1038/s41598-017-02995-4>
- Cholet, F., Ijaz, U. Z., & Smith, C. J. (2019). Differential ratio amplicons (Ramp) for the evaluation of RNA integrity extracted from complex environmental samples. *Environmental Microbiology*, 21(2), 827–844. <https://doi.org/10.1111/1462-2920.14516>
- Constante, M., Libertucci, J., Galipeau, H. J., Szamosi, J. C., Rueda, G., Miranda, P. M., Pinto-Sanchez, M. I., Southward, C. M., Rossi, L., Fontes, M. E., Chirido, F. G., Surette, M. G., Bercik, P., Caminero, A., & Verdu, E. F. (2022). Biogeographic Variation and Functional Pathways of the Gut Microbiota in Celiac Disease. *Gastroenterology*, 163(5), 1351–1363.e15. <https://doi.org/10.1053/j.gastro.2022.06.088>
- Corrêa, R. O., Castro, P. R., Fachi, J. L., Nirello, V. D., El-Sahhar, S., Imada, S., Pereira, G. V., Pral, L. P., Araújo, N. V. P., Fernandes, M. F., Matheus, V. A., de Souza Felipe, J., dos Santos Pereira Gomes, A. B., de Oliveira, S., de Rezende Rodovalho, V., de Oliveira, S. R. M., de Assis, H. C., Oliveira, S. C., Dos Santos Martins, F., ... Vinolo, M. A. R. (2023). Inulin diet uncovers complex diet-microbiota-immune cell interactions remodeling the gut epithelium. *Microbiome*, 11(1), 90. <https://doi.org/10.1186/s40168-023-01520-2>
- EFSA Panel on Dietetic Products and Allergies (NDA), N. (2010). Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre. *EFSA Journal*, 8(3), 1462. <https://doi.org/10.2903/j.efsa.2010.1462>
- Galipeau, H. J., McCarville, J. L., Huebener, S., Litwin, O., Meisel, M., Jabri, B., Sanz, Y., Murray, J. A., Jordana, M., Alaedini, A., Chirido, F. G., & Verdu, E. F. (2015). Intestinal Microbiota Modulates Gluten-Induced Immunopathology in Humanized Mice. *The American Journal of Pathology*, 185(11), 2969–2982. <https://doi.org/10.1016/j.ajpath.2015.07.018>
- Galipeau, H. J., Rulli, N. E., Jury, J., Huang, X., Araya, R., Murray, J. A., David, C. S., Chirido, F. G., McCoy, K. D., & Verdu, E. F. (2011). Sensitization to gliadin induces moderate enteropathy and insulinitis in nonobese diabetic-DQ8 mice. *Journal of Immunology (Baltimore, Md. : 1950)*, 187(8), 4338–4346. <https://doi.org/10.4049/jimmunol.1100854>
- Goldberg, M. R., Mor, H., Magid Neriya, D., Magzal, F., Muller, E., Appel, M. Y., Nachshon, L., Borenstein, E., Tamir, S., Louzoun, Y., Youngster, I., Elizur, A., & Koren, O. (2020). Microbial signature in IgE-mediated food allergies. *Genome Medicine*, 12(1), 92. <https://doi.org/10.1186/s13073-020-00789-4>
- Itano, A., Maslin, D., Ramani, K., Mehraei, G., Carpenter, N., & Cormack, T. (2023). *Clinical translation of anti-inflammatory effects of Prevotella histicola in Th1, Th2, and Th17 inflammation. Front Med.* 2023.
- Jervis-Bardy, J., Leong, L. E. X., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy, E., Morris, P. S., O'Leary, S., Rogers, G. B., & Marsh, R. L. (2015). Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome*, 3(1), 19. <https://doi.org/10.1186/s40168-015-0083-8>

- Jovel, J., Patterson, J., Wang, W., Hotte, N., O'Keefe, S., Mitchel, T., Perry, T., Kao, D., Mason, A. L., Madsen, K. L., & Wong, G. K.-S. (2016). Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. *Frontiers in Microbiology, Volume 7-2016*.
<https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2016.00459>
- Könönen, E., & Gursoy, U. K. (2022). Oral Prevotella Species and Their Connection to Events of Clinical Relevance in Gastrointestinal and Respiratory Tracts. *Frontiers in Microbiology, Volume 12*.
<https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2021.798763>
- Kuang, R., & Binion, D. G. (2022). Should high-fiber diets be recommended for patients with inflammatory bowel disease? *Current Opinion in Gastroenterology, 38*(2).
https://journals.lww.com/co-gastroenterology/fulltext/2022/03000/should_high_fiber_diets_be_recommended_for.15.aspx
- Lamas, B., Hernandez-Galan, L., Galipeau, H. J., Constante, M., Clarizio, A., Jury, J., Breyner, N. M., Caminero, A., Rueda, G., Hayes, C. L., McCarville, J. L., Bermudez Brito, M., Planchais, J., Rolhion, N., Murray, J. A., Langella, P., Loonen, L. M. P., Wells, J. M., Bercik, P., ... Verdu, E. F. (2020). Aryl hydrocarbon receptor ligand production by the gut microbiota is decreased in celiac disease leading to intestinal inflammation. *Science Translational Medicine, 12*(566), eaba0624.
<https://doi.org/10.1126/scitranslmed.aba0624>
- Lau, J. T., Whelan, F. J., Herath, I., Lee, C. H., Collins, S. M., Bercik, P., & Surette, M. G. (2016). Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. *Genome Medicine, 8*(1), 72. <https://doi.org/10.1186/s13073-016-0327-7>
- Laursen, M. F., Laursen, R. P., Larnkjær, A., Michaelsen, K. F., Bahl, M. I., & Licht, T. R. (2017). Administration of two probiotic strains during early childhood does not affect the endogenous gut microbiota composition despite probiotic proliferation. *BMC Microbiology, 17*(1), 175.
- Le Leu, R. K., Hu, Y., Brown, I. L., & Young, G. P. (2009). Effect of high amylose maize starches on colonic fermentation and apoptotic response to DNA-damage in the colon of rats. In *Nutrition & metabolism* (Vol. 6, p. 11).
<https://doi.org/10.1186/1743-7075-6-11>
- Marietta, E., Black, K., Camilleri, M., Krause, P., Rogers III, R. S., David, C., Pittelkow, M. R., & Murray, J. A. (2004). A new model for dermatitis herpetiformis that uses HLA-DQ8 transgenic NOD mice. *The Journal of Clinical Investigation, 114*(8), 1090–1097.
<https://doi.org/10.1172/JCI21055>
- Matchado, M. S., Rühlemann, M., Reitmeier, S., Kacprowski, T., Frost, F., Haller, D., Baumbach, J., & List, M. (2024). On the limits of 16S rRNA gene-based metagenome prediction and functional profiling. *Microbial Genomics, 10*(2), 001203.
<https://doi.org/10.1099/mgen.0.001203>

- McCarville, J. L., Dong, J., Caminero, A., Bermudez-Brito, M., Jury, J., Murray, J. A., Duboux, S., Steinmann, M., Delley, M., Tangyu, M., Langella, P., Mercenier, A., Bergonzelli, G., & Verdu, E. F. (2017). A Commensal Bifidobacterium longum Strain Prevents Gluten-Related Immunopathology in Mice through Expression of a Serine Protease Inhibitor. *Applied and Environmental Microbiology*, 83(19), e01323-17. <https://doi.org/10.1128/AEM.01323-17>
- Natividad, J. M. M., Petit, V., Huang, X., de Palma, G., Jury, J., Sanz, Y., Philpott, D., Garcia Rodenas, C. L., McCoy, K. D., & Verdu, E. F. (2012). Commensal and Probiotic Bacteria Influence Intestinal Barrier Function and Susceptibility to Colitis in Nod1^{-/-};Nod2^{-/-} Mice. *Inflammatory Bowel Diseases*, 18(8), 1434–1446. <https://doi.org/10.1002/ibd.22848>
- Nistal, E., Caminero, A., Herrán, A. R., Arias, L., Vivas, S., de Morales, J. M. R., Calleja, S., de Miera, L. E. S., Arroyo, P., & Casqueiro, J. (2012). Differences of small intestinal bacteria populations in adults and children with/without celiac disease: effect of age, gluten diet, and disease. *Inflammatory Bowel Diseases*, 18(4), 649–656. <https://doi.org/10.1002/ibd.21830>
- Niu, B., Li, F., Lv, X., Xiao, Y., Zhu, J., Zhao, J., Lu, W., & Chen, W. (2024). Unveiling the therapeutic potential and mechanism of inulin in DSS-induced colitis mice. *International Journal of Biological Macromolecules*, 280, 135861. <https://doi.org/https://doi.org/10.1016/j.ijbiomac.2024.135861>
- Prasoodanan P. K., V., Sharma, A. K., Mahajan, S., Dhakan, D. B., Maji, A., Scaria, J., & Sharma, V. K. (2021). Western and non-western gut microbiomes reveal new roles of Prevotella in carbohydrate metabolism and mouth–gut axis. *Npj Biofilms and Microbiomes*, 7(1), 77. <https://doi.org/10.1038/s41522-021-00248-x>
- Roque, A., Zanker, J., Brígido, S., Tomaz, M. B., Gonçalves, A., Barbeiro, S., Benítez-Páez, A., & Pereira, S. G. (2024). Dietary patterns drive loss of fiber-foraging species in the celiac disease patients gut microbiota compared to first-degree relatives. *Gut Pathogens*, 16(1), 58. <https://doi.org/10.1186/s13099-024-00643-7>
- Rucker, R. (2022). Allometric Scaling: Comparison of Interspecies Nutritional Relationships and Requirements. In *The Journal of nutrition* (Vol. 152, Issue 11, pp. 2626–2627). <https://doi.org/10.1093/jn/nxac189>
- Santiago, A., Amber, H., Marco, C., Sara, R., Josie, L., Kyle, J., Gaston, R., Laura, R., Rithwik, R., Wolfram, R., Jon, S., Alberto, C., Premysl, B., Heather Jean, G., & Verdu, E. F. (2023). Crohn's disease proteolytic microbiota enhances inflammation through PAR2 pathway in gnotobiotic mice. *Gut Microbes*, 15(1), 2205425. <https://doi.org/10.1080/19490976.2023.2205425>
- Sobh, M., Montroy, J., Daham, Z., Sibbald, S., Lalu, M., Stintzi, A., Mack, D., & Fergusson, D. A. (2022). Tolerability and SCFA production after resistant starch supplementation in humans: a systematic review of randomized controlled studies. *The American Journal of Clinical Nutrition*, 115(3), 608–618. <https://doi.org/https://doi.org/10.1093/ajcn/nqab402>
- Tett, A., Pasolli, E., Masetti, G., Ercolini, D., & Segata, N. (2021). Prevotella diversity, niches and interactions with the human host. *Nature Reviews Microbiology*, 19(9), 585–599. <https://doi.org/10.1038/s41579-021-00559-y>

- Verdu, E. F., Huang, X., Natividad, J., Lu, J., Blennerhassett, P. A., David, C. S., McKay, D. M., & Murray, J. A. (2008). Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 294(1), G217-25.
<https://doi.org/10.1152/ajpgi.00225.2007>
- Vuillermin, P. J., O'Hely, M., Collier, F., Allen, K. J., Tang, M. L. K., Harrison, L. C., Carlin, J. B., Saffery, R., Ranganathan, S., Sly, P. D., Gray, L., Molloy, J., Pezic, A., Conlon, M., Topping, D., Nelson, K., Mackay, C. R., Macia, L., Koplin, J., ... Group, the B. I. S. I. (2020). Maternal carriage of *Prevotella* during pregnancy associates with protection against food allergy in the offspring. *Nature Communications*, 11(1), 1452.
<https://doi.org/10.1038/s41467-020-14552-1>
- Whelan, F. J., Waddell, B., Syed, S. A., Shekarri, S., Rabin, H. R., Parkins, M. D., & Surette, M. G. (2020). Culture-enriched metagenomic sequencing enables in-depth profiling of the cystic fibrosis lung microbiota. *Nature Microbiology*, 5(2), 379–390.
<https://doi.org/10.1038/s41564-019-0643-y>
- Zmora, N., Zilberman-Schapira, G., Suez, J., Mor, U., Dori-Bachash, M., Bashiares, S., Kotler, E., Zur, M., Regev-Lehavi, D., Brik, R. B.-Z., Federici, S., Cohen, Y., Linevsky, R., Rothschild, D., Moor, A. E., Ben-Moshe, S., Harmelin, A., Itzkovitz, S., Maharshak, N., ... Elinav, E. (2018). Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Features. *Cell*, 174(6), 1388-1405.e21. <https://doi.org/10.1016/j.cell.2018.08.041>
- Zou, J., Reddivari, L., Shi, Z., Li, S., Wang, Y., Bretin, A., Ngo, V. L., Flythe, M., Pellizzon, M., Chassaing, B., & Gewirtz, A. T. (2021). Inulin Fermentable Fiber Ameliorates Type I Diabetes via IL22 and Short-Chain Fatty Acids in Experimental Models. *Cellular and Molecular Gastroenterology and Hepatology*, 12(3), 983–1000.
<https://doi.org/10.1016/j.jcmgh.2021.04.014>

Point by Point responses to Reviewer 1's comments:

“Please include sample size consideration in limitations of the discussion.”

Response: Thank you for this suggestion. We have now clarified in the Discussion (limitations paragraph) that this was an exploratory analysis of celiac samples obtained from our biobank and that the sample size was limited in part by the invasive nature of small intestinal aspirate collection. While these aspirates are highly relevant to celiac disease, they are substantially more invasive than fecal sampling; consequently, some patients declined additional procedures. We have explicitly acknowledged this as a limitation in the revised manuscript.

Page 14 (lines 292): “Our cohort was small and exploratory.”
(line 295) “There was limited availability of relevant small intestinal samples for microbial metabolic analysis.”

Suggested changes to results section subheadings:

Line 75 page 4: consider adding “independent of GFD” to subheading.

Line 107 page 5: consider adding “nor gastrointestinal symptoms” to subheading, as this point is notable.

Response: Thank you. We have revised the titles as suggested:

Page 4 (lines 75-76): “Microbial saccharolytic function in the small intestine was lower in CeD than controls independent of GFD”

Page 5 (lines 108-109): “Diet does not fully explain lower microbial saccharolytic function or gastrointestinal symptoms in CeD.”

“Lines 233-235 page 11 are *critical in the premise of the study*** and should be further highlighted (abstract). Now with all the major revisions and new experiments, the title and abstract could align better if they are revisited in the lens of the new findings.”**

Response: The abstract has been amended. The title, however, has been changed following *Nature Com* editorial recommendations.