nature portfolio

Peer Review Information

Journal: Nature Microbiology
Manuscript Title: Gut Microbial β-Glucuronidases Regulate Host Luminal Proteases and are depleted in Irritable Bowel Syndrome
Corresponding author name(s): Madhusudan Grover

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Dr Grover,

Thank you for your patience while your manuscript "Gut Microbial β-Glucuronidases Maintain Intestinal Barrier Function by Regulating Host Luminal Proteases" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the end of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, referees #1 and #3 ask that you clarify the limitations of the proteomics, referee #1 asks that you use specific protease inhibitors, and both of these referees also ask that some experiments be repeated with more appropriate methodology e.g. enzyme activity. Referee #3 asks for quantification of bilirubin in vivo and in vitro, and referees #1 and #2 note that improved analysis to identify microbial taxa and proteases associated with high and low protease activity is required.

Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are

available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see:
http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

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-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision, even if a similar study has been accepted for publication at Nature Microbiology or published elsewhere (up to a maximum of 6 months).

In the meantime we hope that you find our referees' comments helpful.

Yours sincerely,

{redacted}

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Reviewer Expertise:

Referee #1: gastroenterology, GI proteases
Referee #2: gut microbiome, intestinal inflammation disorders
Referee #3: gut microbiome, microbial metabolism, omics

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

This paper by Edwison et al. present a very substantial amount of new data that are highly pertinent to the field and that bring new important information and concepts.
Among the key results presented in that paper, the authors observed that microbiota could control proteolytic activity at intestinal mucosa surface, through the production of unconjugated bilirubin. Further, they identified bacterial taxa which loss is associated with high proteolytic activity in the feces of IBS patients. Their results highlight microbial beta-glucuronidases as potential actors in the control of proteolytic activity at intestinal surface.
The significance of the results is very high, but several methodological approaches need attention to ascertain the validity of the conclusions. Often, the authors overstate their results and should be more cautious with their conclusions.

Specifically, several points that would need to be addressed:

- the abstract is poorly informative on the novelty of the findings. It is not clear from the abstract

what is brought by the present study and what was already known. The abstract would have to be re-written to do justice to the quality of the data presented in the rest of the paper.
- A major point missing in the present study is the evaluation of visceral hypersensitivity or pain, which has been shown to be corelated to PA. Either in patients, pain scores, bloating, etc. should have been recorded and compared to all the other parameters studied (in particular taxonomy and the presence of Alistipes taxa), and in mice studies, visceral hypersensitivity should be measured.
- The statement that proteases identified (chymotrypsin-like pancreatic elastases 2A, 3B and PRSS2) in the feces of patients are from pancreatic origin is not supported by any data. The authors should either clearly demonstrate the pancreatic origin, or remove the statement. If their statement is based only on the fact that such proteases are present in the pancreas, this is not sufficient to state that they are from pancreatic origin. Indeed, intestinal epithelial cells for instance, but also other cell types are able to produce chymotrypsin and trypsin-like enzymes.
- The proteomic analysis that has been used is not sensitive enough to document the presence of proteases with a moderate or low expression levels. Only proteases that are present in large quantities could be detected by the approach used in the present paper. This has to be acknowledged and the authors should make clear that their analysis is not exhaustive of all proteases that might be present (indeed, several proteases that are known to be present are not even detected). Considering this, the conclusion that no differences in expression of proteases or protease inhibitors are observed is clearly an overstatement. The authors have to tone down their conclusions.
- Similarly, the authors state that they have assessed specific protease activities (chymotrypsin, elastase, etc). This is not the case. They only have used preferential substrates, but these substrates can be cleaved by several proteases, particularly in the experimental conditions described here. So here again, the authors should be more cautious with their conclusions. They did not measure specific activities.
- For the proteolytic activity measures, they should be complemented by the use of inhibitors, and in particular specific inhibitors (not large spectrum inhibitors).
- Still for the measures of proteolytic activities, the test that is described is not robust (time lapses of measures are not sufficient to fully evaluate proteolytic activity content). How long does the inhibition last? These are important methodology remarks that have to be addressed experimentally.
- For colonic tissue proteomics, no trypsin digestion is noted, then it is not clear how peptides were generated.
- For the trypsin family, there is a strong homology sequence between PRSS1, 2 and 3, which renders proteomic analysis often unconclusive because of not enough specific peptides recovered. How many peptides were recovered to ascertain the specific presence of PRSS2 (and not the other forms of PRSS)?
- Concerning the experiments with monocolonization with GUS expressing coli, it is not clear if the authors have checked or not if after 7 days of colonization, the plasmid is still present in GUS+ bacteria. Is there a selection media? No mention of Antibiotic use is made. All the controls should be described for such experiments.
- Figure 2: Panel C is described as proteomic analysis of colon biopsies for proteases, but some protease inhibitors are present in the list: why? Considering the fact that protease inhibitors are listed in panel 2D.
- The experiments describing the effects of unconjugated bilirubin on trypsin activity are not adequately performed. A lecture for 2 min of enzymatic activity is insufficient. What is the control? No trypsin? Is this inhibition lasting and how long? Is it competitive?

- The authors used bilirubin in GF mice in order to investigate whether this could reduce the proteolytic activity associated with the GF status. But we don't know whether the same proteases are responsible for increased PA in GF or for increased PA associated with post-infectious IBS. The authors would need to determine which proteases are active and responsible for PA in their GF mice.
- The use of UNC10201652 as a specific inhibitor of gut microbial GUS enzymes should be documented in the present study and in the present experimental conditions. Mention of a reference is not sufficient, the authors should demonstrate that treatment with this compound had no effect on hoist proteases.
- The logic of the statement at the end of the first paragraph of the discussion is not clear. The authors mentioned the potential role of A. putredinis in proteolytic activity as a support for the conclusion on the impaired bilirubin deconjugation….. this really does not make sense. It would have to be reformulated.

Reviewer #3 (Remarks to the Author):

The manuscript by Edwinson et al. describes the role of bacterial glucuronidases in maintaining the function of the intestinal epithelium by inhibiting luminal protease activity. The central theme that bacterial glucuronidases are able to inhibit intestinal luminal protease activity by promoting formation of unconjugated bilirubin is intriguing, but difficult to appreciate in this report. It is difficult to interpret the data and "connect the dots". In other words, the relative importance of proteolytic activity, regulation of specific proteases (host or microbial) and specific inhibitors of proteolytic activity in maintaining barrier function is difficult to interpret based on the findings.

Specific issues:

1. Determination of "high PA" seems arbitrary at 90% and it is unclear if there is any basis for this cut-off value. The authors should clarify why these cut-off values were chosen and whether there is any biological significance to these relative values.
2. Differences in alpha/beta diversity are described between high PA and low PA. again it is difficult to understand how these categories were determined. It is not clear why microbial diversity is being considered in the context of proteolytic activity and what that means.
3. Authors should specify how mucosal samples were obtained and how mucosal versus luminal PA was distinguished. The Methods do not describe how luminal versus mucosal enzymatic activities are separated so it is not possible to interpret the significance of these findings. It is important in terms of evaluating the impact on the epithelium or intestinal barrier function.
4. The authors refer to "low PA" and "high PA" microbiota without specifying which proteases they are referring to – host or microbial and that is an important point. The authors refer to protease inhibitory microbial taxa without any clear definition.
5. The authors refer to selection of IBS patients based on several questionnaires and scales but these tools are not each cited with specific references so the validity of the clinical phenotyping is in question without a more complete description in the Methods or Results sections.

Reviewer #4 (Remarks to the Author):

In this study, Edwinson et al. demonstrate that increased intestinal proteases activity in post-infection IBS patients is associated with compositional changes in the gut microbiome and decreased microbial beta-glucuronidase activity. Using metagenomic analysis, the authors identified specific gut bacterial species that are less abundant in post-infection IBS patient and they demonstrate that whole stool transplantation can reverse dysregulated protease activity in ex-germfree mice colonized with stool communities of post-infection IBS patients. Mechanistically, the authors conclude that unconjugated bilirubin, produced by gut microbial beta-glucuronidase, inhibits intestinal proteases to protect the intestinal barrier and contributes to microbiota-host homeostasis in the gut.

I found the addressed question very interesting, elegantly combining clinical observations, omics measurements, and gnotobiotic experiments. The data convincingly support the link between intestinal protease activity, microbiome composition and GUS activity, which is further supported by the performed gnotobiotic experiments to demonstrate that FMT from healthy human donors and GUS overexpression by engineered gut bacteria can reverse dysregulated protease activity. However, the authors' claim that bilirubin is regulating intestinal protease activity seems to lack direct demonstration, not fully supporting the respective conclusion. Overall, the presented study is of high quality and relevance to the field. However, I believe that certain aspects of the manuscript could be improved and clarified:

Major Comments.

1)The authors claim that bilirubin, deconjugated through gut microbial GUS activity, regulates protease activity in the gut lumen. However, the data shown in the manuscript does not seem to fully support such conclusion for several reasons:

i)The performed metabolomics analysis of human samples identified higher levels of urobilinogen, the microbial reduction product of bilirubin, in low-PA compared to high-PA individuals. This raises the question whether bilirubin itself or any of its degradation products impacts protease activity. Also, it remains unexplained why the authors solely focus on bilirubin and consider the contribution of other metabolites that showed an abundance pattern comparable to urobilinogen (Fig. 6B). This needs a more systematic analysis and further explanations.

ii)The authors perform elegantly designed experiments in gnotobiotic mice (i.e., colonization with an E. coli strain overexpressing GUS and administration of GUS inhibitors) to demonstrate that GUS activity regulates intestinal protease activity. To support the claimed protease regulation by bilirubin, free intestinal bilirubin should be quantified in these experiments to demonstrate that GUS and GUS inhibitors indeed lead to increased intestinal bilirubin concentration. Additionally, it would be desirable to also perform the epithelial permeability assays shown in Fig. 3H-J with these animals to directly demonstrate that GUS modulation influences intestinal barrier function, as suggested in the study title.

iii) It is unclear how the performed enzyme assays would allow to assess metabolite inhibition of

protease activity. E.g., 5uL of fecal slurry was diluted in a reaction volume of 200uL to enzymatically assess the protease activity in vitro. As a consequence, fecal metabolites (i.e. bilirubin) are diluted 40 times, which raises the question whether metabolite concentration remains sufficiently high to inhibit proteases. In particular, as the inhibition assays in Fig. 6E were performed in the presence of 200uM bilirubin. To directly support bilirubin inhibition of protease activity, bilirubin should be quantified in these enzyme assays performed with fecal slurries.

Minor Comments.

2) L104-108 and L188-192: The authors used a random forest approach to identify bacterial species that predict diseased state/protease activity. Given that differential analysis was also performed to identify differentially abundant strains, and that linear regression demonstrated high correlation between the abundance of these strains and protease activity, the random forest approach seems redundant and does not provide additional insights. Further, the limited number of humanized mouse data raises the question of overfitting, given the large feature space (gut species, n>1000).

3) L110-134: Metaproteomics analysis of high-PA and low-PA supernatants identified 1,210 and 2,801 unique peptides. This number seems rather low and should be translated into unique proteins detected, as reported for human proteins in mucosa proteomics samples (L129-130). Given that only a few hundred (very abundant) microbial proteins seem to have been detected, differences in microbial protease abundance would likely not be measured, given the detection limit. This and the low proteome coverage in these samples should be critically discussed.

4) Fig. 3D and 3E: It seems that only three of the six high-PA samples show higher protease activity compared to healthy and low-PA samples. This should be discussed in the text and further stratification of the high-PA samples should be considered for downstream analysis.

5) Fig. 3I: 4kDaFITC-Dextran seems to have been undetected in one healthy and one low PA-sample. This is surprising, as creatinine and rhodamine-dextran was measured in the same samples (Fig. 3H and Fig. 3J, respectively). Hence, the authors should make sure that these results are not due to a technical problem that led to wrong conclusions.

6) Reference to Fig 4I is missing in the text.

7) L192-197 and L220-224: The authors perform Kegg pathway analysis using the metagenomics data. Although, a few differentially abundant pathways are identified, this analysis seem quite isolated from the rest of the study and does not seem to contribute to the overall conclusions of the manuscript.

8) I suggest that the authors indicate exact p-values in figures and text, rather than p-value thresholds.

9) I found the discussion quite lengthy and suggest to generally shorten this part of the manuscript.

Author Rebuttal to Initial comments

Reviewer #1:

This paper by Edwison et al. present a very substantial amount of new data that are highly pertinent to the field and that bring new important information and concepts.
Among the key results presented in that paper, the authors observed that microbiota could control proteolytic activity at intestinal mucosa surface, through the production of unconjugated bilirubin. Further, they identified bacterial taxa which loss is associated with high proteolytic activity in the feces of IBS patients. Their results highlight microbial beta-glucuronidases as potential actors in the control of proteolytic activity at intestinal surface.
The significance of the results is very high, but several methodological approaches need attention to ascertain the validity of the conclusions. Often, the authors overstate their results and should be more cautious with their conclusions.

Specifically, several points that would need to be addressed:

1. The abstract is poorly informative on the novelty of the findings. It is not clear from the abstract what is brought by the present study and what was already known. The abstract would have to be re-written to do justice to the quality of the data presented in the rest of the paper.

Response: Thank you for your review and the positive and encouraging comments regarding our manuscript. We agree it is critical for the abstract to highlight the breadth and significance of our findings for the field. Consequently, we have restructured the abstract to better convey the known and new findings regarding the regulation of proteolytic activity (PA) in the intestinal tract. The suppression of luminal proteases through microbial β-glucuronidase mediated deconjugation of bilirubin is the key scientific advance made through this manuscript and this is now clearly highlighted in the abstract (page 2).

2. A major point missing in the present study is the evaluation of visceral hypersensitivity or pain, which has been shown to be corelated to PA. Either in patients, pain scores, bloating, etc. should have been recorded and compared to all the other parameters studied (in particular taxonomy and the presence of Alistipes taxa), and in mice studies, visceral hypersensitivity should be measured.

Response: Previous studies have established that luminal proteases mediate visceral hypersensitivity in rodents and humans. The primary focus of this manuscript is to determine the regulation of the luminal proteolytic activity. Our laboratory is not set up to do intestinal sensitivity testing and doing such experiments elsewhere is not currently feasible as these mice are either germ-free or have a very specific microbiota composition making it logistically harder to ship them without risking perturbation of the microbiome. However, we do show in **Table 1** that high PA PI-IBS patients have greater symptom severity than the low PA patients. The IBS symptom severity score is a validated composite score that comprises of pain (severity and frequency), abdominal distension (severity and frequency) and satisfaction with bowel habits. Proteases are established mediators of visceral hypersensitivity and this study provides mechanism of their regulation by commensal microbiota. While having visceral sensitivity data would be very interesting and informative, it is not something we are set up to perform within the time and scope of the current manuscript.

3. The statement that proteases identified (chymotrypsin-like pancreatic elastases 2A, 3B and PRSS2) in the feces of patients are from pancreatic origin is not supported by any data. The authors should either clearly demonstrate the pancreatic origin, or remove the statement. If their statement is based only on the fact that such proteases are present in the pancreas, this is not sufficient to state that they are from pancreatic origin. Indeed, intestinal epithelial cells for instance, but also other cell types are able to produce chymotrypsin and trypsin-like enzymes.

Response: We appreciate the comment and agree that the specific statement of pancreatic origin may be too strong and has been revised (page 5). However, to further investigate and support this conclusion, we examined mucosal proteolytic activity from colonic tissue from mice humanized with high PA and healthy microbiota. Using *in situ* zymography, we determined that there were no differences in the mucosal PA. These new data are presented (page 7, **Supplemental Figure 4A and B**). Furthermore, we have generated additional data demonstrating that distal ileal contents have the highest proteolytic activity which gets promptly suppressed as contents reach the cecum in conventional mice as well as mice humanized with healthy or low PA associated microbiota. In contrast, this suppression in the cecum was not noted in mice humanized with high PA microbiota (**Figure A**). The PA in distal ileal contents was similar among the mice. These findings however do not undermine published findings that epithelium can be an important source of proteases mediating pathophysiology of IBS. It is

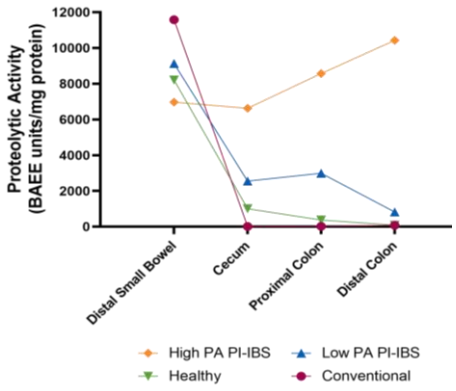

Figure A. Changes in PA across the length of the intestinal tract in conventional and humanized mice

plausible that both excess production by the epithelium and uninhibited luminal pancreatic secretions are important sources and these proteases are uniquely localized to have effects on visceral sensitivity and barrier function. Accordingly, we have acknowledged the possibility in the discussion as well as removed the reference to pancreatic origin (pages 5 and 12).

4. *The proteomic analysis that has been used is not sensitive enough to document the presence of proteases with a moderate or low expression levels. Only proteases that are present in large quantities could be detected by the approach used in the present paper. This has to be acknowledged and the authors should make clear that their analysis is not exhaustive of all proteases that might be present (indeed, several proteases that are known to be present are not even detected). Considering this, the conclusion that no differences in expression of proteases or protease inhibitors are observed is clearly an overstatement. The authors have to tone down their conclusions.*

Response: The reviewer brings up the importance of recognizing the sensitivity of the fecal metaproteomics and tissue proteomics assays. The conclusion pointed by the reviewer "no differences in expression of proteases or protease inhibitors" is for the mucosal expression of proteases. Although low abundance mucosal proteases and protease inhibitors could be missed, these do not seem to explain the large differences in fecal PA observed among the high and low PA states. In fact, several of the previously described mucosal proteases (Trypsin 3, elastase, and thrombin) were detected on our mucosal proteomics but were not significantly different between the two groups. It is also to be noted that several of those previous observations were made in biopsies from inflammatory bowel disease (IBD) patients, which has different pathophysiological basis than irritable bowel syndrome. The overt mucosal inflammation in IBD putatively explains the greater tissue PA observed in those biopsies. However, proteomics continues to be an evolving technology with a lot of further room to increase sensitivity. Hence, as suggested by the reviewer, we have acknowledged the sensitivity as a limitation (page 15).

5. *Similarly, the authors state that they have assessed specific protease activities (chymotrypsin, elastase, etc). This is not the case. They only have used preferential substrates, but these substrates can be cleaved by several proteases, particularly in the experimental conditions described here. So here again, the authors should be more cautious with their conclusions. They did not measure specific activities.*

Response: We realize that even preferential substrates can be cleaved by >1 protease reducing the specificity of activity that is claimed to be tested. This is precisely why we have labeled data in **Supplemental Figure 3** as "trypsin-like activity", "elastase-like activity", etc. These preferred substrates are published: N-p-Tosyl-Gly-Pro-Arg-AMC (trypsin-like; PMID: 2204062), Suc-Ala-Ala-Pro-Phe-AMC (chymotrypsin-like; PMID: 574722), Suc-Ala-Ala-Pro-Val-AMC (neutrophil elastase; PMID: 25458301), Suc-Ala-Ala-Ala-AMC (pancreatic elastase; PMID: 30867416) and Pro-Phe-Arg-AMC (kallikrein; PMID: 591514) and commonly used to reach similar conclusions (PMID: 29911328). There are some novel substrates now available but those are also not entirely specific for proteases they cleave (PMID: 31527638, PMID: 27923620). Additionally, the activity observed complements the expression noted on metaproteomics. However, we have added a statement to limitations that assessment of specific PA is limited by the available tracers (page 15).

6. For the proteolytic activity measures, they should be complemented by the use of inhibitors, and in particular specific inhibitors (not large spectrum inhibitors).

Response: We have performed additional experiments suggested by the reviewer. A pilot set of fecal supernatants *in vitro* were treated with a range of protease inhibitors (Serine protease inhibitor: *AEBSF*, *Nafamostat*, *UAMC-00050*; Cystine protease inhibitor: *E64*; Elastase inhibitor: *Elafin*; Thrombin inhibitor: *Dabigatran*). Only the serine protease and specific elastase inhibitors suppressed the PA, further strengthening our conclusion that the high PA is driven by these proteases. Similar to the preferential substrates, the inhibitors are also limited by the lack of specificity as they target broader protease families instead of unique proteases. However, now multiple lines of evidence are pointing towards serine protease and elastase activity as drivers of high fecal PA. These new data are presented (page 6 and **Figure 2E**).

7. Still for the measures of proteolytic activities, the test that is described is not robust (time lapses of measures are not sufficient to fully evaluate proteolytic activity content). How long does the inhibition last? These are important methodology remarks that have to be addressed experimentally.

Response: The proteolytic activity measurement assays based on the rate of substrate (FITC-casein or preferential) turnover and changes in fluorescence intensity over time are established and extensively used (PMID: **2204062**, PMID: **574722,** PMID: **25458301,** PMID: 22167196, PMID: 591514 PMID: 29911328. PMID: 31056700, PMID: 29777136 PMID: 30923071). To measure the enzyme kinetics, and thereby protease activity, we measured the rate of turnover of

a range of substrates, preferential or otherwise, kinetically (5min, 30 sec intervals) at 37°C (nmoles/min). The rate of substrate turnover was determined using a standard curve generated for free 7-amido-methylcoumarin (Excitation/Emission = 380/46) after which was converted to specific, or preferential enzymatic, activity (nmoles/min/µg) using the protein concentration obtained for each sample by the Bradford method. A more specific description of the assay can be found in the **Supplemental materials** section of the manuscript. We believe that the reviewer is referring to inhibition caused by unconjugated bilirubin. In this revision, we have examined other metabolites in the bilirubin metabolism pathway for inhibition of PA (**Supplemental Figure 9A-B)**. Unconjugated bilirubin successfully inhibited proteolytic activity across all concentrations tested. Critically, we found despite the shorter time course for the experiment (5min) the presence of 10µM unconjugated bilirubin caused a robust and sustained inhibition of protease activity. This would suggest at high enough concentrations, the presence of unconjugated bilirubin alone can cause long lasting protease inhibition both *in vitro* and *in vivo*. This claim is supported by our **Figure 6E** where mice treated orally with 200µM unconjugated bilirubin have significantly lower fecal trypsin activity compared to control treated animals. Additionally, we have since assessed luminal PA along the GI tract and found that a majority of luminal PA inhibition occurs in the cecum, and luminal PA remains low along the GI tract in low PA humanized animals (**Figure A**) indicating sustained inhibition occurs and is long lasting.

8. For colonic tissue proteomics, no trypsin digestion is noted, then it is not clear how peptides were generated.

Response: Trypsin digestion was not used for this analysis. A Slow Off-rate Modified Aptamer (SOMAmer)–based capture array was used to capture and measure the relative abundances of proteins from colonic tissue. Protein extracted from colonic biopsies and were subsequently bound to chemically modified DNA aptamers to form a complex that was then can be transformed from a protein signal to a nucleotide signal which is quantified using relative florescence using a microarray. These DNA aptamers are modified with functional hydrophobic groups which allow for a greater degree of specificity and allows for identification of nearly identical proteins. This assay has broadly been used to characterize the human plasma proteome (PMID: 29875488), highlight potential biomarkers of Alzheimer's disease (PMID: 24768341) and even describe the proteome of IBD patients (PMID: 28129359). A total of 1305 human proteins can be identified with this approach, where tissue derived serine proteases such as PRSS1,2,3 amongst others are part of the panel. The SOMAmer–based capture assay has a

reported median intra- and inter-run coefficient of variation of ~5% with median lower and upper limits of quantification in buffer at between ~1 pM and ~1.5 nM, and in serum samples between ~2.95 pM and ~1.5 nM (PMID: 21165148).

9. For the trypsin family, there is a strong homology sequence between PRSS1, 2 and 3, which renders proteomic analysis often unconclusive because of not enough specific peptides recovered. How many peptides were recovered to ascertain the specific presence of PRSS2 (and not the other forms of PRSS)?

Response: The reviewer makes an important point regarding sequence homology with the family of trypsin-based proteases. To avoid misidentification with other proteases, we identified and assigned a total of four unique peptide sequences that could be mapped, and were distinct, to PRSS2. The peptide sequences that were identified as distinct for the identification of PRSS2 were LSSPAVINSR, TLDNDILLIK, TLDNDILLIKLSSPAVINSR and VYNYVDWIKDTIAANS. These peptide sequences had partial homology with PRSS1/3; however, each sequence had complete overlap and 100% identity with PRSS2 giving us confidence in that we properly identified PRSS2 in our samples.

10. Concerning the experiments with monocolonization with GUS expressing coli, it is not clear if the authors have checked or not if after 7 days of colonization, the plasmid is still present in GUS+ bacteria. Is there a selection media? No mention of Antibiotic use is made. All the controls should be described for such experiments.

Response: GUS$^+$ *E. coli* contains a plasmid that allows the bacteria to constitutively express β-glucuronidase, but also encodes for antibiotic resistance towards kanamycin. Additionally, these transformed bacteria have resistance to tetracycline. To ensure mice were colonized, we collected pellets from GF, control *E. coli* or GUS$^+$ *E. coli* colonized mice 7 days post colonization and cultured these pellets in both standard and selective media in a shaking incubator at 37°C. Overnight bacterial growth in selective media containing both kanamycin and tetracycline confirmed colonization of mice by GUS$^+$ *E. coli* as the bacterium has plasmid DNA that encodes for both kanamycin and tetracycline resistance. We did not see any growth from pellets collected from mice monocolonized with control *E. coli* when grown under the kanamycin and tetracycline selective media conditions; however, we did see growth in standard media indicating the control *E. coli* did colonize the mouse. Growth was not observed from

pellets collected from germ-free mice in either selective, or standard media giving us confidence that not only were these mice not contaminated during the experiment, but also that the technical approach was sound, and animals colonized with either GUS$^+$ *E. coli* or control *E. coli* were not contaminated. An in-depth description of the experimental method and controls for this experiment is provided in the **supplemental materials** section (page 12).

11. Figure 2: Panel C is described as proteomic analysis of colon biopsies for proteases, but some protease inhibitors are present in the list: why? Considering the fact that protease inhibitors are listed in panel 2D.

Response: Thank you for pointing out this error. SLPI and PI3 were incorrectly included in **Figure 2C**. We have removed these and have included them in **Figure 2D** with the other protease inhibitors.

12. The experiments describing the effects of unconjugated bilirubin on trypsin activity are not adequately performed. A lecture for 2 min of enzymatic activity is insufficient. What is the control? No trypsin? Is this inhibition lasting and how long? Is it competitive?

Response: Irreversible covalent binding, adduct formation followed by degradation is the most common mechanism of serine protease inhibitors (PMID: 12475205). For this experiment, the control was trypsin alone, in the absence of any potential metabolite inhibitor, to confirm that indeed the enzyme was catalytically active. This was confirmed as we saw an effective conversion of substrate to product based on increased fluorescence over time in the trypsin alone control condition. A negative, no trypsin control well was also included and served as a background for analysis. No activity or increase in fluorescence was detected in the negative control during the time course of the experiment. To further strengthen our observation, we have now examined additional metabolites in the bilirubin deconjugation pathway and demonstrate that only unconjugated bilirubin suppresses PA and not biliverdin, conjugated bilirubin, mesobilirubin or urobilinogen. These new data are presented two ways, one, normalized to the trypsin control, and secondly as change in fluorescence over time in the presence of the specific bilirubin degradation metabolite to begin addressing the duration of protease inhibition **(Supplemental Figure 9A and B)**.

To better understand the inhibitory capacity of bilirubin metabolites we performed a series of pseudo-first-order kinetics analyses on the enzymatic activity of trypsin in the presence of these

potential bilirubin-based inhibitors. We found that the observed rate of inactivation constant (k_{obs}) to be greatest in the presence of unconjugated bilirubin (k_{obs}= 0.006642s^{-1}) which is consistent with our previous findings that unconjugated bilirubin had the greatest inhibitory capacity of the bilirubin metabolites assessed in this manuscript (**Table 2**). Additionally, we carried out a series of experiments that examined how different concentrations of the prospective bilirubin metabolite inhibitors affected enzymatic activity in order to determine both the dissociation constant (K_i) and the maximum inhibition rate (k_2). The ratio of k_2/K_i is known as a second-order inhibition rate which is commonly used as a parameter to define and report inhibition of enzymatic activity. This was done by plotting the 1/kobs versus 1/[I], where I is the tested inhibitor. We found the greatest inactivation of trypsin to be found in the presence of unconjugated bilirubin (666.6667$M^{-1}s^{-1}$, **Table 2**). We also determined the necessary metabolite concentration required to cause a 50% inhibition of enzymatic activity (IC_{50}). The IC_{50} values for all potential bilirubin derived inhibitors are also reported in **Table 2**, and we report the lowest IC_{50} value for all the tested metabolites to be unconjugated bilirubin (3.478µM).

13. The authors used bilirubin in GF mice in order to investigate whether this could reduce the proteolytic activity associated with the GF status. But we don't know whether the same proteases are responsible for increased PA in GF or for increased PA associated with post-infectious IBS. The authors would need to determine which proteases are active and responsible for PA in their GF mice.

Response: As shown in **Figure 6E**, the control treated GF mice had higher trypsin-like activity which is suppressed upon treatment with unconjugated bilirubin. To address this comment, we have examined the specific activity of FSNs generated from GF mice using the preferential substrates that we described in the manuscript. We found that GF mice had a proteolytic profile that was consistent with what was observed in the FSNs from high PA PI-IBS humanized animals, identifying higher chymotrypsin, pancreatic and neutrophil elastase activity in GF mice compared to low PA animals. The similar profile seen in GF mice compared to high PA humanized mice would suggest similar proteases are active in these two groups and are what are responsible for the observed high fecal PA. We have added the newly generated specific activity data collected from GF to the existing **Supplemental Figure 3B** where the specific activity for humanized mice is presented.

14. The use of UNC10201652 as a specific inhibitor of gut microbial GUS enzymes should be documented in the present study and in the present experimental conditions. Mention of a

reference is not sufficient, the authors should demonstrate that treatment with this compound had no effect on hoist proteases.

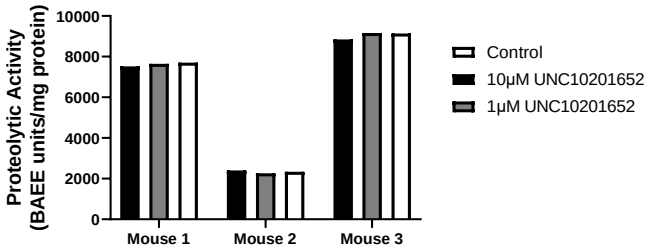

Figure B. Treatment of high PA mouse FSNs *in vitro* with UNC10201652

Response: GUS enzymes are glycosidases that specifically target and hydrolyze the glycosidic R-groups on bilirubin with no known or reported proteolytic/protease activity. For clarification, we are not proposing that GUS enzymes inhibit proteases, but it is the unconjugated bilirubin generated by these enzymes inhibits the host proteases. To test this hypothesis we used UNC10201652 which is a specific inhibitor of gut microbial GUS enzymes and therefore we would not expect it to have any effect on host proteases directly. To address the reviewer's concern that UNC10201652 may be inhibiting host proteases directly, we completed an experiment where we exposed high PA FSNs to UNC10201652 *in vitro*. We measured the PA of the FSNs in the presence and absence of UNC10201652 and found that when tested at different concentrations, UNC10201652 did not affect the PA of the FSNs tested (**Figure B**).

15. The logic of the statement at the end of the first paragraph of the discussion is not clear. The authors mentioned the potential role of A. putredinis in proteolytic activity as a support for the conclusion on the impaired bilirubin deconjugation….. this really does not make sense. It would have to be reformulated.

Response: We have revised the statement in question. The aim of the sentence was to highlight that the dysbiosis and loss of critical microbiota like *A. putredinis* following intestinal infection can lead to impaired bilirubin deconjugation and as a result, impaired inhibition of host proteases. The rewritten sentence should clarify this conclusion (page 12).

Reviewer #3:

The manuscript by Edwinson et al. describes the role of bacterial glucuronidases in maintaining the function of the intestinal epithelium by inhibiting luminal protease activity. The central theme that bacterial glucuronidases are able to inhibit intestinal luminal protease activity by promoting formation of unconjugated bilirubin is intriguing, but difficult to appreciate in this report. It is difficult to interpret the data and "connect the dots". In other words, the relative importance of proteolytic activity, regulation of specific proteases (host or microbial) and specific inhibitors of proteolytic activity in maintaining barrier function is difficult to interpret based on the findings.

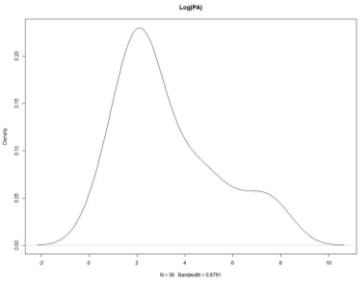

Figure C. Distribution of Log-transformed PA values in healthy volunteers

Specific issues:
1. Determination of "high PA" seems arbitrary at 90% and it is unclear if there is any basis for this cut-off value. The authors should clarify why these cut-off values were chosen and whether there is any biological significance to these relative values.

Response: The distribution of PA in healthy volunteers follow a log-normal distribution (**Figure C**). We notice a clear bimodality in the distribution (i.e. two populations). The 90% threshold was utilized to define the right-sided distribution, which is clearly different from the rest. This cutoff used provided the biological and statistical rationale for categorizing subjects as high and low PA.

2. Differences in alpha/beta diversity are described between high PA and low PA. again it is difficult to understand how these categories were determined. It is not clear why microbial diversity is being considered in the context of proteolytic activity and what that means.

Response: Microbiota are known to produce both proteases and protease inhibitors. This manuscript is based on our findings that within PI-IBS patients there are differences in the fecal PA. We found there is a population of PI-IBS individuals that group separately from the others based on the fecal PA as demonstrated with a bimodal distribution of the PI-IBS population as a whole which we categorized these two groups as high and low PA. We found the patients with

high PA had greater symptom severity. Additionally, we found a greater abundance of proteases present in the fecal material of high PA individuals compared to low PA through our metaproteomics analysis but did not see any differences in protease inhibitors. This observation that high PA PI-IBS patients were clearly different from the low PA PI-IBS individuals led us towards trying to understand possible mechanisms by which microbiota may be interacting with the luminal proteases. Detecting differences in microbial diversity and composition was an important step in understanding the regulation of luminal proteases. This approach gave us insight into specific bacterial taxa like *A. putredinis* that could then be used to suppress PA. These findings are important in informing potential future therapeutics that could alleviate the symptoms and clinical manifestations of patients diagnosed with high fecal PA.

3. Authors should specify how mucosal samples were obtained and how mucosal versus luminal PA was distinguished. The Methods do not describe how luminal versus mucosal enzymatic activities are separated so it is not possible to interpret the significance of these findings. It is important in terms of evaluating the impact on the epithelium or intestinal barrier function.

Response: Additional details have been added to the methods section about collection of mucosal biopsies in **supplemental materials** (page 6). Additionally, we have now performed mucosal PA using *in situ* zymography. Briefly, tissue sections (fresh-frozen, sectioned 8µm) were then stained with a green-fluorescent SYTOX Green Nuclear Stain (ThermoFisher, S7020) followed by incubation with a low melt agar overlay containing N-p-Tosyl_Gly-Pro-Arg 7-amido-4-methylcoumarin hydrochloride. A single slide for each sample was then incubated at 4°C overnight and served as a background control for that sample while the paired slide incubated at 37°C. The slide held at 37°C with the substrate agar overlay allows for the visualization of localized tissue specific proteolytic activity. Fluorescence intensity was measured under the same settings for both the 4°C and 37°C incubated slides and reported as the difference between the two measurements. Nuclei and PA were pseudocolored blue and green respectively. We did not observe any differences in colonic mucosal PA between the high and low PA tissue samples. These new data have been presented in the **supplemental materials** (**Supplementary Figure 4A-B**). The method for fecal PA measurement as well as fecal proteomics is presented in the **supplemental materials** (page 2 and 5-6 respectively).

4. The authors refer to "low PA" and "high PA" microbiota without specifying which proteases they are referring to – host or microbial and that is an important point. The authors refer to protease inhibitory microbial taxa without any clear definition.

Response: The titles of high PA and low PA microbiota is based on fecal PA assessment using FITC-casein based activity assay. This assay measures the overall PA which can be from host or the bacteria. However, our metaproteomic analysis demonstrates three specific serine proteases of human origin (chymotrypsin like pancreatic elastase 2A, 3B and trypsin 2) were differentially abundant between the high and low PA samples (**Figure 2B**). Regarding the definition of microbial taxa, the differential abundance analysis as well as random forest-based prediction identified microbiota that associates with low PA. We realize that calling them "protease inhibitory microbial taxa" may not be most ideal and have thus changed it to "microbial taxa associated with low PA" (pages 6, 8).

5. The authors refer to selection of IBS patients based on several questionnaires and scales but these tools are not each cited with specific references so the validity of the clinical phenotyping is in question without a more complete description in the Methods or Results sections.

Response: Thank you for the feedback. We have added a paragraph at the start of **supplemental materials** (page 2) that has the details and references for the questionnaires used for clinical phenotyping.

Reviewer #4:

In this study, Edwinson et al. demonstrate that increased intestinal proteases activity in post-infection IBS patients is associated with compositional changes in the gut microbiome and decreased microbial beta-glucuronidase activity. Using metagenomic analysis, the authors identified specific gut bacterial species that are less abundant in post-infection IBS patient and they demonstrate that whole stool transplantation can reverse dysregulated protease activity in ex-germfree mice colonized with stool communities of post-infection IBS patients. Mechanistically, the authors conclude that unconjugated bilirubin, produced by gut microbial beta-glucuronidase, inhibits intestinal proteases to protect the intestinal barrier and contributes to microbiota-host homeostasis in the gut.

I found the addressed question very interesting, elegantly combining clinical observations, omics measurements, and gnotobiotic experiments. The data convincingly support the link between

intestinal protease activity, microbiome composition and GUS activity, which is further supported by the performed gnotobiotic experiments to demonstrate that FMT from healthy human donors and GUS overexpression by engineered gut bacteria can reverse dysregulated protease activity. However, the authors' claim that bilirubin is regulating intestinal protease activity seems to lack direct demonstration, not fully supporting the respective conclusion. Overall, the presented study is of high quality and relevance to the field. However, I believe that certain aspects of the manuscript could be improved and clarified:

Major Comments.
1) The authors claim that bilirubin, deconjugated through gut microbial GUS activity, regulates protease activity in the gut lumen. However, the data shown in the manuscript does not seem to fully support such conclusion for several reasons:

1. The performed metabolomics analysis of human samples identified higher levels of urobilinogen, the microbial reduction product of bilirubin, in low-PA compared to high-PA individuals. This raises the question whether bilirubin itself or any of its degradation products impacts protease activity. Also, it remains unexplained why the authors solely focus on bilirubin and consider the contribution of other metabolites that showed an abundance pattern comparable to urobilinogen (Fig. 6B). This needs a more systematic analysis and further explanations.

Response: Thank you for the review and the encouraging and positive feedback on our manuscript. The specific metabolite produced as a result of β-glucuronidase activity which causes suppression of PA has been of significant interest to us. Initially we only tested conjugated and unconjugated bilirubin inhibition of PA *in vitro*. To address this question, we expanded on this initial experiment to include various products in the bilirubin deconjugation pathway (biliverdin, mesobilirubin, and urobilinogen) for their ability to inhibit the PA. We found that only the unconjugated bilirubin was able to suppress the PA. Mesobilirubin and urobilinogen had some effect, but this was not statistically significant. These results have been provided in the new **Supplementary Figure 9A and B**. Furthermore, we have demonstrated that unconjugated effectively suppresses PA *in vivo* **Figure 6E**. This suggests that unconjugated bilirubin is an effective suppressor of PA. However, it does not exclude the possibility of additional metabolites to play a role in the suppression of proteases. This has been discussed (page 15).

2. The authors perform elegantly designed experiments in gnotobiotic mice (i.e., colonization with an E. coli strain overexpressing GUS and administration of GUS inhibitors) to demonstrate that GUS activity regulates intestinal protease activity. To support the claimed protease regulation by bilirubin, free intestinal bilirubin should be quantified in these experiments to demonstrate that GUS and GUS inhibitors indeed lead to increased intestinal bilirubin concentration. Additionally, it would be desirable to also perform the epithelial permeability assays shown in Fig. 3H-J with these animals to directly demonstrate that GUS modulation influences intestinal barrier function, as suggested in the study title.

Response: We appreciate the comment and to address this question, we performed pilot untargeted metabolomics on two sets of mice: (a) colonized with *E. coli* overexpressing GUS (GUS^+ *E. coli*) or control, (b) D-glucaro-1,4-lactone GUS inhibitor vs control treated. D-urobilin, an end product of bilirubin deconjugation was increased in GUS^+ *E. coli* treated mice (ratio of control/treated= -12.3, *p-value*=0.001). None of the other identified products of bilirubin metabolism pathways were statistically significant. In contrast, mice administered D-glucaro-1, 4-lactone had decreased levels of bilirubin degradation product, C17H20N2O5 (ratio of treated/control=-10, *p-value*=0.03). None of the other metabolites are significantly different. The differences in the metabolic end products are likely due to differences in the baseline states of these mice. Humanized mice given D-glucaro-1,4-lactone have a complex community of diverse microbiota that would be processing all of the downstream metabolites of bilirubin degradation whereas mice monocolonized with GUS^+ *E. coli* lack this complexity which can lead to different metabolomic profiling. We found it encouraging to see in both conditions, metabolites altered support our conclusion that host PA is largely regulated through the activity of GUS enzymes supplied by intestinal microbiota. These new data are presented on page 11 and **Figure 6F**.

Next, regarding your question on intestinal permeability upon GUS modulation. We examined *in vivo* intestinal permeability of mice treated with D-glucaro-1, 4-lactone. Compared to controls, treatment with the GUS inhibitor led to an increased level of serum 4kDa FITC dextran. This observation is indicative of an increase in the leak pathway in mice treated with GUS inhibitors and provides strong evidence that GUS modulation leads to an influence in intestinal barrier function and further supports the work outlined in the manuscript. These results have been added to page 11 and **Supplemental Figure 9C**. We were also interested in the permeability of the mice monocolonized with GUS^+ *E. coli* for 7 days. Here we could not demonstrate a difference in permeability from the controls. We know that intestinal microbiota is required for the maturation of epithelial, immune and barrier function. It is plausible that suppression of protease activity seen is not by itself sufficient for achieving the barrier maturation from the GF state. The

duration of the experiment might play a role as well and it is possible that a longer timeframe may be required to observe alterations in intestinal permeability.

3. It is unclear how the performed enzyme assays would allow to assess metabolite inhibition of protease activity. E.g., 5uL of fecal slurry was diluted in a reaction volume of 200uL to enzymatically assess the protease activity in vitro. As a consequence, fecal metabolites (i.e. bilirubin) are diluted 40 times, which raises the question whether metabolite concentration remains sufficiently high to inhibit proteases. In particular, as the inhibition assays in Fig. 6E were performed in the presence of 200uM bilirubin. To directly support bilirubin inhibition of protease activity, bilirubin should be quantified in these enzyme assays performed with fecal slurries.

Response: Fecal supernatants (FSNs) are generated from both high and low PA individuals as well as humanized mice and assessed for overall proteolytic activity using a FITC-casein based assay, or through the use of specific/preferred substrates to identify the activity of specific enzymes. For each of these experiments, low PA and high PA FSNs are treated the same. Therefore, any potential dilution effects that may be encountered due to using 5µL of FSN in a reaction volume of 200µL should be accounted for experimentally. Low PA FSNs, which have bilirubin metabolites present at greater abundances than high PA FSNs, remain low PA even after dilution. In additional experimentation we have found after assessment of luminal PA along the GI tract that a majority of luminal PA inhibition is occurring in the cecum and remains inhibited along the GI tract whereas luminal PA remains high along the GI tract in high PA humanized animals (**Figure A**). This supports sustained inhibition occurs *in vivo* and that any dilution that occurs in the activity assays is not diluting the potential potency of an inhibitor in high PA feces. Additionally, the data we present in **Figure 2B** indicates that high PA not only have high fecal PA, but also have a greater abundance of fecal proteases compared to low PA individuals. This would suggest unconjugated bilirubin neutralizes PA, but there is also the potential for removal of proteases by an undefined mechanism during, or after neutralization. It is possible that unconjugated bilirubin, bound to luminal serine proteases is followed by targeted degradation; an inhibitory mechanism of serine protease inhibitors (PMID: 12475205). We however did not test this hypothesis regarding the removal of luminal proteases as this was beyond the scope of current manuscript. The experiment conducted in **Figure 6E** involved treating mice orally with 200µM unconjugated bilirubin or a control gavage and was designed to demonstrate whether unconjugated bilirubin could lower fecal PA of germ-free mice *in vivo* as

we have established these mice have high fecal PA. We did not quantify the amount of bilirubin in the *in vitro* enzymatic assays as we used germ free animals and would anticipate these animals to have little unconjugated bilirubin in feces as they are devoid of intestinal microbiota. The same can be said of the 200µM unconjugated bilirubin treated animals as the only unconjugated bilirubin that should be measurable would be that what was administered for the experiment. We think it would be difficult to quantify the bilirubin present in the fecal slurries of these animals and directly ascribe it to inhibition of proteolytic activity as it is possible that some of the unconjugated bilirubin will inherently degrade before reaching the GI tract, and it is possible that the unconjugated bilirubin could be recycled *in vivo* artificially reducing the quantity of unconjugate bilirubin in the feces.

Minor Comments.

1. L104-108 and L188-192: The authors used a random forest approach to identify bacterial species that predict diseased state/protease activity. Given that differential analysis was also performed to identify differentially abundant strains, and that linear regression demonstrated high correlation between the abundance of these strains and protease activity, the random forest approach seems redundant and does not provide additional insights. Further, the limited number of humanized mouse data raises the question of overfitting, given the large feature space (gut species, n>1000).

Response: Traditional differential abundance analysis methods test association for each taxon, followed by multiple testing correction. Such univariate testing procedure may suffer from power loss if multiple taxa are weakly associated with the phenotype and/or there are interactive effects among the taxa. Random forest, on the other hand, jointly analyzes all the taxa, and could pool individually weak signals as well as exploit the potential interactive effects among taxa. Therefore, random forest coupled by Bortua feature selection offers an alternative way of identifying phenotype-associated taxa, which could be potentially missed by a univariate-based procedure. Due to the incorporation of randomness in the random forest algorithm, random forest could accommodate a large number of features with less overfitting than other model-based approaches (Breiman, L. Random Forests. Machine Learning 45, 5–32 (2001). https://doi.org/10.1023/A:1010933404324). The coupled Boruta feature selection method used resampling method to select the marker taxa and was also less subject to overfitting. Thus, we believe that random forest analysis adds strength to the findings.

2. L110-134: Metaproteomics analysis of high-PA and low-PA supernatants identified 1,210 and 2,801 unique peptides. This number seems rather low and should be translated into unique proteins detected, as reported for human proteins in mucosa proteomics samples (L129-130). Given that only a few hundred (very abundant) microbial proteins seem to have been detected, differences in microbial protease abundance would likely not be measured, given the detection limit. This and the low proteome coverage in these samples should be critically discussed.

Response: The numbers should be corrected as 'Metaproteomics analysis of high-PA and low-PA supernatants identified 1,413 and 2,116 unique peptides. This corresponds to 104 microbial proteins and 143 host proteins (for high-PA) and 124 microbial proteins and 144 host proteins (for low-PA) (page 5). The sensitivity of fecal proteomics has been discussed (page 15).

3. Fig. 3D and 3E: It seems that only three of the six high-PA samples show higher protease activity compared to healthy and low-PA samples. This should be discussed in the text and further stratification of the high-PA samples should be considered for downstream analysis.

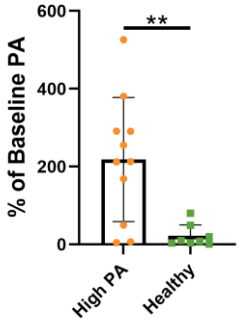

Figure D. Expanded cohort of healthy and high PA PI-IBS humanized mice

Response: This is an important observation and to further assess robustness of this finding, we humanized additional groups of mice with either high PA microbiota (n=5) or healthy microbiota (n=2). Out a total of 11 humanizations with high PA human microbiota (6 from the original cohort and 5 new), 8 had higher PA compared to the healthy and low PA samples (**see Figure D**). In contrast, all 8 humanizations with healthy microbiota resulted in lowering of the PA. This has further strengthened our assertion that high PA microbiota ineffectively suppresses the PA. We chose not to stratify analysis for statistical reasons as an n=3 is a small sample size.

4. Fig. 3I: 4kDaFITC-Dextran seems to have been undetected in one healthy and one low PA-sample. This is surprising, as creatinine and rhodamine-dextran was measured in the same samples (Fig. 3H and Fig. 3J, respectively). Hence, the authors should make sure that these results are not due to a technical problem that led to wrong conclusions.

Response: In the data presented in **Figure 3I**, the serum 4kDaFITC-Dextran was undetected in one of the samples (healthy) but was detected at low levels in the low PA PI-IBS humanized animal (0.039 mg/dL). We have checked the experimental details and find no errors with the presented data. Considering a physiological measurement, factors like stress, circadian rhythm etc. could influence permeability of a tracer in an outlier mouse. This is why we perform these experiments on ≥3 mice and report averages. This has also been observed in subset of mice in experiments by Dr. Jerold Turner, who protocol we have adapted (PMID: 28618266, 34623320)

5. Reference to Fig 4I is missing in the text.

Response: Thank you for pointing this out this omission. This is now provided (page 8).

6. L192-197 and L220-224: The authors perform Kegg pathway analysis using the metagenomics data. Although, a few differentially abundant pathways are identified, this analysis seem quite isolated from the rest of the study and does not seem to contribute to the overall conclusions of the manuscript.

Response: We have removed the KEGG pathway presented in Fig 4 and referenced in L192-197 and included this as a **supplemental figure 6D**. We have retained the KEGG pathway analysis referenced in L220-224 (shown in **Figure 5E**) as we think it importantly highlights a predictive increase in aromatic degradation pathways in mice that did not receive an FMT. FMT resulted in suppression of PA, which implies lower degradation of proteins and amino acids that have an aromatic ring. Interestingly, this pathway was increased in high PA state (**figure 4I**) and gets suppressed after FMT.

7. I suggest that the authors indicate exact p-values in figures and text, rather than p-value thresholds.

Response: The text and the figures have been revised to ensure that exact p-values are reported and not the thresholds.

8. I found the discussion quite lengthy and suggest to generally shorten this part of the manuscript.
Response: We appreciate the feedback and we were able to reduce the discussion by 110 words,

factoring into added text discussing limitations and alternative explanations suggested by the reviewers (pages 11-15).

Decision Letter, first revision:

Dear Dr. Grover,

Thank you for submitting your revised manuscript "Gut Microbial β-Glucuronidases Maintain Intestinal Barrier Function by Regulating Host Luminal Proteases" (NMICROBIOL-21041117A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology Please do not hesitate to contact me if you have any questions.

Sincerely,

{redacted}

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed previous remarks.

Reviewer #4 (Remarks to the Author):

With the additional experiments performed and clarification of the text, the authors have successfully addressed all my previous concerns and I suggest publication of this interesting manuscript.

Given the extensive amount of supplementary tables (120 pages!), I propose to provide the data in a format that is more easy to parse (e.g. csv, xls) than the current word tables.

Decision Letter, final checks:

Dear Dr. Grover,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "Gut Microbial β-Glucuronidases Maintain Intestinal Barrier Function by Regulating Host Luminal Proteases" (NMICROBIOL-21041117A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Gut Microbial β-Glucuronidases Maintain Intestinal Barrier Function by Regulating Host Luminal Proteases". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Microbiology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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If you have any further questions, please feel free to contact me.

{redacted}

Reviewer #1:
Remarks to the Author:
The authors have adequately addressed previous remarks.

Reviewer #3:
None

Reviewer #4:
Remarks to the Author:
With the additional experiments performed and clarification of the text, the authors have successfully addressed all my previous concerns and I suggest publication of this interesting manuscript.

Given the extensive amount of supplementary tables (120 pages!), I propose to provide the data in a format that is more easy to parse (e.g. csv, xls) than the current word tables.

Final Decision Letter:

Dear Madhu,

I am pleased to accept your Article "Gut Microbial β-Glucuronidases Regulate Host Luminal Proteases and are depleted in Irritable Bowel Syndrome" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Please ensure that metabolomics study IDs are live prior to publication.

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