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The composition of the gut microbiota shapes the colon mucus barrier

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Nonia Pariente

1st Editorial Decision 30 July 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, all the referees find the topic of interest and in principle suitable for publication here, although all referees bring up a series of minor points that should be addressed before the study can be considered for publication here.

Given that all referees provide constructive suggestions on how to strengthen the work, we would like to invite revision of your study. It would be important to assess whether the animals in room 2 have a mild enteropathy, and providing an analysis of microbiota diversity in wild type mice, as referee 2 suggests, and address all other issues brought up by the referees, many of which are minor controls, statistical analysis or requests for further discussion.

If the referee concerns are adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

In this manuscript, Jakobsson et al. present data that suggest differences in microbiota composition can affect barrier function of the colonic mucus layer. This is an important contribution to the field as it demonstrates clearly that it is not simply the presence of commensal bacteria in the gastrointestinal tract that promotes a well-structured mucus layer; instead, the particular composition of the gut microbiota may have subtle but significant effects on mucus properties. This paper thoroughly describes the observed phenotype, while leaving many questions for further investigation: are specific phylotypes responsible for changes in mucus properties, or are certain functional groups differently represented in these two mouse colonies? Does having a less-structured, more permeable inner mucus layer predispose these mice to inflammatory diseases in the presence of other known risk factors, like a Western diet? This paper helps set the stage to address such questions, and will be of great interest to those studying the interplay between host, microbiota, and IBD.

Figure 1D is missing a legend; 1E is mislabeled as 1D in the legend.

Comment on effect of diet - Food B consistently resulted in more permeable, less-structured mucus in mice from both colonies, as well as changes in microbiota composition. Is this attributable to the food being autoclaved, or a result of the two diets differing in composition? (Food A is 4% fat, Food B is 9% fat, according the supplemental). It would be worth noting in the main text that the two diets are not identical, and if possible, please supply the macromolecular breakdown of both diets in the text (not just % fat).

Candidatus arthromitus is better known to readers as segmented filamentous bacteria (SFB), a phylotype with species specificity, generally not present in humans, with immunomodulatory effects. It is one of the most significant differences between the two mouse colonies but gets only a passing mention in the text, and is never referenced as SFB. It would be worth commenting on this in the text, especially because enumeration data in this paper (higher SFB abundance in Jackson Labs-derived mice) are in direct contrast to previously reported trends (SFB typically only present in Taconic mice; Ivanov et al. Cell 2009). This is also important to note because SFB are associated with an induction of lymphocytes to the lamina propria, a phenotype the authors assess in Fig. 4G

The legend for figure 5C is grouped in with the legend for 5B and not separately labeled. The legend for 5D is mislabeled as 5C.

In the discussion, the authors note that an increase in pathogen-associated phylotypes (Proteobacteria and TM7) is observed in Room 2 mice and may be associated with destruction of mucus and its protective properties. Are there any differences in non-pathogenic bacteria that are capable of mucus consumption (i.e., Bifidobacterium, Akkermansia, Ruminococcus) between mice in the two rooms?

Referee #2:

The gut microbiota composition impairs the colon inner mucus layer barrier
Jakobsson et al.
This is a very interesting and carefully performed paper that compares two independent C57BL/6 colonies in terms of the mucus structure and function. The authors are correct that the results should have an important impact in the assumptions made in strain-combination experiments, where these strains have been maintained separately. It is also important because it shows the highly variable

functional behaviour of the mucus with relatively small differences in microbiota composition.
The strengths of this paper are i) the care with which the different microbiotas have been characterised at different levels and within different compartments of the intestinal tract; ii) the demonstration that differences in mucus structure are also functionally relevant in terms of bead and bacterial penetration towards the epithelial surface; and iii) that the structural and functional differences are recapitulated after recolonisation of germ free mice with the different microbiotas. The authors discuss the possible effects that microbiota differences may play in generating these structural and functional differences, although identification of responsible species is beyond their current scope (because many suspects such as TM7 or Prevotella are unculturable or extremely fastidious).
I have some comments that the authors may consider.
1. A number of different parameters are shown that suggest that the Room 2 animals may have a mild enteropathy (epithelial proliferation zone, leukocyte content of the lamina propria). Whilst it is certainly reasonable to argue from the histology that there is no colitis, I think that it would be very important to publish the hygiene results from each room to show that there are no major differences. It may also be worth looking at the fecal albumin levels as a hallmark of increased intestinal protein loss in room 2 - also arguing for (or against) an enteropathy. This may then be very interesting, insofar as a microbiota that produces these mild enteropathic changes might be highly susceptible to colitis induction (although I think that this is clearly out of the scope of the present paper).
2. I did not understand the lower segment of Figure 4B from room 2. It appears that there is DAPI staining from eukaryotic cells in the lumen and the red FISH signal is absent?
3. Did the authors look at the diversity of the microbiota in the wild mice? Presumably this would show that extremely different microbiotas are compatible with normal mucus structure and function, also arguing that particular components or compositions of the microbiota are critical for impairment of mucus structure and function.
4. I think that there is an error in the legend to Figure 1 (1D appears to be missing). In Figure 1B I assume that both rooms (with different intestinal segments) are shown at different levels, corresponding to 1A.
5. Figure 4 C-F omits details (which food were the mice fed)?
6. I was intrigued by the differences in shedding of SFB into the ileal lumen. Do the authors think this significant (given that the mucus content is similar)?
7. I think that the legend to Fig. 5D is also missing.
8. What do the authors make of the differences that are generated through an autoclaved diet? (It would be helpful to give the specifics of autoclaving, as this will affect the nutritional content).
9. Which strain of germ free mice was used? I think that the statement that 'It is obvious from this study that the microbiota influences the properties of the
mucus barrier and that this can be observed within the same animal house with
animals of identical genetic background' is almost certainly true. However, perhaps one should be cautious for this interpretation, in that a colitic microbiota that is maintained in a given genetic background can be transmitted to wild type animals (and phenocopy the colitis), and possibly genetic drift between the colonies in rooms 1 and 2 might generate a similar effect with mucus dysfunction or enteropathy.
10. I will gladly share a pdf of suggested typo corrections with the authors, although I cannot attach it to this review.

Referee #3:

General comments: This manuscript by Jakobsson et al. describes the different effects of two gut microbial communities in isolated populations of isogenic C57J/B6 mice, as well as wild caught mice. The authors are interested in determining the effects of the microbiota on mucus layer production and integrity and report that the two different B6 microbiota elicit different effects on the ileal and colonic mucus layer, with one microbiota causing increased penetrability of the inner mucus layer. Causality of these effects due to the microbiota is demonstrated by transplanting the communities into germfree mice and recreating similar effects, although the authors show that diet plays a role as well.

The results of this paper are interesting and leverage a series of novel approaches and technologies that are available to these researchers (in vitro mucus measurements on explants, combined with

microbial ecology and gnotobiotic mice). The report is somewhat descriptive/observational in the current form, although the microbiota transplant experiment is a nice step towards establishing causality. The present results document a new phenotype that will be of interest to many researchers studying molecular mechanisms of host-gut microbiota interaction and provide a basis for future investigations.

Specific comments:
1. Abstract: The unique selling point of the study is certainly the two distinct mucus phenotypes and their correlation to the microbiota, shown by transfer to germ-free mice. The data provided for the abundance of bacteria is inconclusive. Therefore, it might be necessary to keep the focus on the unique selling point, especially for a report format.
2. p. 3. The reference to Qin et al. 2010 is used to support the statement that the microbiota is "typically made up of 500-1,000" species"; although this reference found over 1,000 total species across the subjects surveyed and states "at least 160 species per individual" (abstract). Thus, the stated numbers may be innaccurate (at least with respect to this citation) and depending on whether the authors are referring to the microbiota that could possibly be present across many subjects, or the number per person. Please clarify what is meant.
3. p. 4 presents a very nice summary of the different roles of secreted mucus as a barrier and is a notable asset of the introduction.
4. p. 5. It is argued that the role of diet was "eliminated...by feeding the mice in the two colonies the same chow". The word "eliminated" is too pejorative here since the authors do find a role for diet in impacting the mucus layer readout and also switching the diet fed to the mice in each room cannot restore a diet specific microbiota if the microbiota has been permanently altered by prolonged feeding of one diet. For example, if a continued monotonous diet causes some microbial lineages to go extinct from the microbiota, they cannot be restored by changing diets unless they're in the diet. Perhaps, "but we controlled for the impact of diet, at least in short-term feeding conditions, by feeding..."
5. Introduction (overall and concluding paragraph): A clear rationale behind conducting the present study in relation to the background literature provided in the manuscript is somewhat unclear.
6. p. 6. The strategy for separating the bacteria in "mucus" and "lumen" is somewhat of a concern, although it may not be feasible to perform this separation in a different way unless a microscopic approach like laser-capture microdissection is taken. The reason for concern is the concept, which has largely been developed by the author labs, that the outer mucus layer is the prominent site of microbial colonization, and this would presumably be lost using this separation technique. Addressing this in the discussion as one aspect of design that may have reduced the observed differences in mucus microbiota would be appropriate.
7. p. 10. "A tendency of less secreted MUC2 was observed in room 2 on food B (Fig. S6C)". Even a non statistically significant statement to this effect does not seem warranted based on this data since the means of the two MUC2 box plots in fig. S6C look almost identical, just with less variation in group B.
8. p. 10. The quantitative IHC and microscopy results although statistically significant seem like very minor differences that could be difficult to quantify based on data like that shown in Fig. 4C. Is it really possible to accurately count stained goblet cells so precisely that a difference of 2-3 cells/crypt can be determined with such stat. significance? Some statement in the methods of sample measurement in a blinded fashion (or re-measuring by a blinded evaluator unfamiliar with the project hypothesis) would reinforce the credence that such a small difference can be precisely measured.

Typographical errors and minor points:

1. p. 2. Abstract: spell out "SPF"
2. p. 3. "from establish host infections" should be changed to "establishing"
3. p. 4. "inner mucus layer separate bacteria" should be changed to "separates"
4. p. 5. "normal wild-type" in first line. This is redundant. Maybe just retain "wild-type" or define normal if it means something other than genetic background.
5. p. 5. 16S sequencing should be changed to 16S rRNA gene sequencing (also in the results section)
6. p. 7. It might be useful for readers if the term "segmented filamentous bacteria" is inserted next to the mention of Candidatus arthromitus, since the common name has been used for a much longer period and likely has more immediate reader recognition. Also, it has been proposed that animal gut

SFB now be referred to as Candidatus Savagella (Environ Microbiol. 2012 Jun;14(6):1454-65).
7. P. 8. "microbiota that were maternal bacterial transmitted" should read "maternally and the word bacteria may be superfluous since its implied in microbiota.
8. p. 9-10. The discussion here about the integrity of the mucus in various conditions is somewhat vague since it relies on terms like "less structured", "well-stratified" and then "thinner and less organized". Its understood that this layer is dynamic and heterogeneous as well as technically difficult to image and quantify, but this section reads as highly qualitative, especially in contrast to the previous section in which bead penetration depth was quantified nicely. Perhaps more clearly indicating in the representative micrographs *where* the inner and outer mucus layers are defined (i.e., with mark ups on the images) based on the MUC2 staining would assist. These delineations are mentioned in the description but not indicated on the micrographs (for example, in fig. 4A where it looks like the room 2 mice still have a discrete layer that is not colonized.
9. P. 10. "...cells in lamina propria was also estimated". The word estimated here should be exchanged with something like "measured" since the former implies imprecision and the values being discriminated appear quite precise given the samll difference and significance.
10. P. 12. "production of mucus is far from". "Is" should be replaced with "are".
11. P. 13. "This would then talks against". Should be "talk" or a different term like "contraindicate a mucus promoting...".
12. . 15. The final clause on the page "also studies that might not directly involve large intestine" isn't clearly attached to the previous half of the sentence. Consider revising.
13. P. 18. Mention of alpha and beta diversity: the Greek symbols are missing for these abbreviations.

Figure and tables:

1. Fig. 5. Panels A-D are shown, but the legend only lists A-C. It looks like "A" in the legend was split into A/B in the figure. Also, no red FISH staining is at all apparent in the final panels, just blue DAPI staining, which could also be the bacteria.
2. Table 1. Its not clear from the legend what groups the statistical tests were made between. Presumably, the most important test for this study (and that which is shown) is between rooms, but the first mention of table 1 is in reference to the mucus/lumen comparison and this is also shown in this table. Perhaps separate tables would clarify; or, please state clearly what groups are compared. Also, in this table many taxa are listed as "<1". Is this correct? It seems like a limit of detection for the number of sequences surveyed, but in the same samples, sometimes fractional values (e.g., 0.01) are given.

1st Revision - authors' response 22 October 2014

Resopnse to reviewer's comments:

Referee #1: *In this manuscript, Jakobsson et al. present data that suggest differences in microbiota composition can affect barrier function of the colonic mucus layer. This is an important contribution to the field as it demonstrates clearly that it is not simply the presence of commensal bacteria in the gastrointestinal tract that promotes a well-structured mucus layer; instead, the particular composition of the gut microbiota may have subtle but significant effects on mucus properties. This paper thoroughly describes the observed phenotype, while leaving many questions for further investigation: are specific phylotypes responsible for changes in mucus properties, or are certain functional groups differently represented in these two mouse colonies? Does having a less-structured, more permeable inner mucus layer predispose these mice to inflammatory diseases in the presence of other known risk factors, like a Western diet? This paper helps set the stage to address such questions, and will be of great interest to those studying the interplay between host, microbiota, and IBD.*

Figure 1D is missing a legend; 1E is mislabeled as 1D in the legend

Answer: Figure legend 1D is added.

Comment on effect of diet - Food B consistently resulted in more permeable, less-structured mucus in mice from both colonies, as well as changes in microbiota composition. Is this attributable to the food being autoclaved, or a result of the two diets differing in composition? (Food A is 4% fat, Food B is 9% fat, according the supplemental). It would be worth noting in the main text that the two diets are not identical, and if possible, please supply the macromolecular breakdown of both diets in the text (not just % fat).

Answer: The diets are, as pointed out in the comment by the referee 1, different in composition which could contribute to the phenotype. The effect of autoclaving is not well described. The vendor states "It is fortified with extra nutrients to compensate for nutrient losses during autoclaving and ensure nutritional adequacy". No information of what the losses are can be obtained. In addition the autoclavable food is processed with silicon dioxide to minimize clumping. The text is altered on page 5 for clarity to**: "The two colonies were maintained on different diets with different composition, but…"**
A more detailed composition of each diet is included in materials and methods of the Expanded view.

Candidatus arthromitus is better known to readers as segmented filamentous bacteria (SFB), a phylotype with species specificity, generally not present in humans, with immunomodulatory effects. It is one of the most significant differences between the two mouse colonies but gets only a passing mention in the text, and is never referenced as SFB. It would be worth commenting on this in the text, especially because enumeration data in this paper (higher SFB abundance in Jackson Labs-derived mice) are in direct contrast to previously reported trends (SFB typically only present in Taconic mice; Ivanov et al. Cell 2009). This is also important to note because SFB are associated with an induction of lymphocytes to the lamina propria, a phenotype the authors assess in Fig. 4G
Answer: Thank you for this comment. The name segmented filamentous bacteria has been added in the tables and the name clarified in the text with (segmented filamentous bacteria, SFB) and the significant difference stated in the text on page 7 as "**SFB also showed a small but significant difference between the rooms in the distal colon with higher levels in Room 2."** It is also discussed on page 15-16 **"SFB is prominent in the ileum mucus as a result of its ability to attach to the epithelium. This bacteria that is able to stimulate the immune system is more common in Room 2 and might influence the mucus properties but, it is mostly considered to foster the intestinal homeostasis in healthy mice something that is in contrast with a less developed mucus (Schnupf *et al.*, 2013)."**

The legend for figure 5C is grouped in with the legend for 5B and not separately labeled. The legend for 5D is mislabeled as 5C.

Answer: Figure legends for figure 5 have been corrected.

In the discussion, the authors note that an increase in pathogen-associated phylotypes (Proteobacteria and TM7) is observed in Room 2 mice and may be associated with destruction of mucus and its protective properties. Are there any differences in non-pathogenic bacteria that are capable of mucus consumption (i.e., Bifidobacterium, Akkermansia, Ruminococcus) between mice in the two rooms?

Answer: This is a very relevant comment but no such evidence has been found. Akkermansia is not detected at all in the analysis and there is no difference in *Bifidobacterium* between the rooms. There is a higher abundance of *Ruminococcus* in room 2, but a significant difference is only detected in ileum lumen and cecum and is then less likely to explain the mucus alterations observed in distal colon.

Referee #2:*The gut microbiota composition impairs the colon inner mucus layer barrier Jakobsson et al. This is a very interesting and carefully performed paper that compares two independent C57BL/6 colonies in terms of the mucus structure and function. The authors are correct that the results should have an important impact in the assumptions made in strain-combination experiments, where these strains have been maintained separately. It is also important because it shows the highly variable functional behaviour of the mucus with relatively small differences in microbiota composition. The strengths of this paper are i) the care with which the different*

microbiotas have been characterised at different levels and within different compartments of the intestinal tract; ii) the demonstration that differences in mucus structure are also functionally relevant in terms of bead and bacterial penetration towards the epithelial surface; and iii) that the structural and functional differences are recapitulated after recolonisation of germ free mice with the different microbiotas. The authors discuss the possible effects that microbiota differences may play in generating these structural and functional differences, although identification of responsible species is beyond their current scope (because many suspects such as TM7 or Prevotella are unculturable or extremely fastidious). I have some comments that the authors may consider.

1. A number of different parameters are shown that suggest that the Room 2 animals may have a mild enteropathy (epithelial proliferation zone, leukocyte content of the lamina propria). Whilst it is certainly reasonable to argue from the histology that there is no colitis, I think that it would be very important to publish the hygiene results from each room to show that there are no major differences.

Answer: The hygiene reports show similar results and the information is presented in the Expanded view to give the reader a better control of the status of the rooms. Both rooms are located within the same SPF barrier unit and all routines are the same and the personnel works at both locations.
The Experimental procedures in the animals section of the Expanded view now includes:
"Health reports from sentinel animals screened from the two rooms using FELASA annual serology, FELASA EU annual microbiology, Pinworm and fur Mite panel and Protozoal PCR panel (IDEXX Bioresearch, Ludwigsberg, Germany) were negative for all results except positive for Murine Norovirus and sentinels from Room 2 were positivefor *Helicobacter spp./Helicobacter hepaticus* (not identified in the 16SrDNA sequencing results of any animals in the study). Pooled fecal material from several rooms including either room 1 or 2 was positive for Entamoeba muris (cysts were however not detected in fecal histology samples of analyzed animals)."

It may also be worth looking at the fecal albumin levels as a hallmark of increased intestinal protein loss in room 2 - also arguing for (or against) an enteropathy. This may then be very interesting, insofar as a microbiota that produces these mild enteropathic changes might be highly susceptible to colitis induction (although I think that this is clearly out of the scope of the present paper).

Answer: The question about enteropathy is very difficult as it depends on the methods used and how it is defined. We have taken advantage of the proposed approach to look for protein loss and have used our proteomics data on the mucus that will reflect secreted/loss/leakage of proteins and examined the amount of albumin and alpha 2-microglobulin. We could not detect any difference between the groups from the two rooms. In addition we searched for inflammatory markers as S100A8, S100A9 and Ecp3 in the mucus proteome but such inflammatory markers are not present in any of the samples arguing for a non-inflamed tissue. The observed alterations are thus not leading to enteropathic changes that can be observed. In the text we have added a sentence addressing this **"Inflammatory markers as S100a8, S100a9 and eosinophil cationic protein 3 were not detected and no difference in albumin or alpha 2-macroglobulin was found between the groups. The protein differences were small and not likely to explain the variable mucus properties."**
Additional investigations on susceptibility to develop colitis in these mice is a good idea for continuation of the work but, such experiments we believe are outside the focus of this paper

2. I did not understand the lower segment of Figure 4B from room 2. It appears that there is DAPI staining from eukaryotic cells in the lumen and the red FISH signal is absent?

Answer: The DAPI stain in the lumen is auto-fluorescence sometimes observed in animals on the autoclaved diet and is not detached cells. The stainings have been repeated with less background and the pictures in figure 4B have been exchanged.

3. Did the authors look at the diversity of the microbiota in the wild mice? Presumably this would show that extremely different microbiotas are compatible with normal mucus structure and function, also arguing that particular components or compositions of the microbiota are critical for impairment of mucus structure and function.

Answer: The composition of the flora was not analyzed in these mice. Mice from natural habitats in Europe have been sequenced in another study (Linnenbrink M et al. Mol Ecol. 2013 Apr;22(7):1904-16). The expected large variation in this material would demand a higher number of animals than we have access to. Our experience from these forest mice is that most of them have different types of parasites that have been implicated in having profound effects on the mucus. Parasites contribute to the mucus phenotype by stimulating goblet cell hyperplasia and mucus release. As other than bacteria are not considered when performing 16S sequencing differences in parasites will not be observed. The reason for including the mucus phenotype in this manuscript is to demonstrate that animals in their natural environment develop a dense mucus layer limiting bacterial access to the epithelium. This observation is only used to argue for the Room 1 phenotype that resembles the mucus occurring in mice that live free.

4. I think that there is an error in the legend to Figure 1 (1D appears to be missing). In Figure 1B I assume that both rooms (with different intestinal segments) are shown at different levels, corresponding to 1A

Answer: Figure legend 1D has been added.
Figure 1B contains the same data as in A but separated into the different segments that is responsible for the separation in component 2. The text is clarified by**:**
"A Unweighted UniFrac-based PCoA plot of gut microbial communities from mice in Room 1 (red) and Room 2 (blue) at all intestinal locations.
B Unweighted UniFrac-based PCoA plot of mice gut microbial communities from both rooms at different gut segments…"

5. Figure 4 C-F omits details (which food were the mice fed)?

Answer: Additional information has been added to Figure 4 which now reads:
"C Immunostaining of the Muc2 precursor before it gets *O*- glycosylated using the anti-apoMuc2 antiserum on mice from both room fed Food A. Scale bar is100 µm.
D The crypt length was measured in distal colon on sections from mice in the two rooms on Food A (n=4).
E The number of goblet cells per crypt was counted in distal colon of mice in the two rooms on Food A (n=10).
F The number of cells in lamina propria between two crypts, as shown in Figure S7, was counted in sections from mice in the two rooms on Food A (n=10).
G The number of Ki67 positive cells per crypt was significantly different in distal colon of mice housed in Room 1 and Room 2 on Food A. (n=4, p=0.029).
H Ki67 immunostaining (green) on colonic sections from mice in Room 1 or Room 2 on Food A."

6. I was intrigued by the differences in shedding of SFB into the ileal lumen. Do the authors think this significant (given that the mucus content is similar)?

Answer: We cannot control for the mucus amount to be exactly similar although the sampling is made using the same procedure. The difference in the lumen between the rooms is significant but it is not so clear to draw conclusions of how well the bacteria are attached to the epithelium as there is a considerable variation in the data. We are concerned with the variation and the very small amounts of non-attached SFB in the lumen that could also contain dead bacteria to make such conclusions. There is however a clear indication of more SFB in the mucus of animals from Room 2. We have further clarified the nomenclature in the results section and Table 1 and added a comment in the discussion (see the answer to question 3 from referee #1)

7. I think that the legend to Fig. 5D is also missing.

Answer: Figure legends for figure 5 have been corrected.

8. What do the authors make of the differences that are generated through an autoclaved diet? (It would be helpful to give the specifics of autoclaving, as this will affect the nutritional content).

Answer: The content of the food is explained in more detail in the Experimental procedures of the Expanded view as described above. The autoclaving procedure is 135°C, 35 min and the sterility is controlled by cultures. This information is also included in the Expanded view.

9. Which strain of germ free mice was used? I think that the statement that 'It is obvious from this study that the microbiota influences the properties of the mucus barrier and that this can be observed within the same animal house with animals of identical genetic background' is almost certainly true. However, perhaps one should be cautious for this interpretation, in that a colitic microbiota that is maintained in a given genetic background can be transmitted to wild type animals (and phenocopy the colitis), and possibly genetic drift between the colonies in rooms 1 and 2 might generate a similar effect with mucus dysfunction or enteropathy.

Answer: The C57BL/6 strain of mice was used as germ free for the conventionalization experiments. These mice have been kept separate for an extensive time and are not completely similar to any of the two strains used in the experiment but their origin is from the Jackson background as are the animals from Room2. We agree that genetic variation could play a role but as the two mucus phenotypes are recapitulated in colonized germ-free mice with identical genetic background, microbiota must have a much larger impact than the genetic variation. This is added to the discussion on page 12.

10. I will gladly share a pdf of suggested typo corrections with the authors, although I cannot attach it to this review.

Answer: Thank you for this file and all the suggested corrections. We have corrected the errors accordingly.

Referee #3: *General comments: This manuscript by Jakobsson et al. describes the different effects of two gut microbial communities in isolated populations of isogenic C57J/B6 mice, as well as wild caught mice. The authors are interested in determining the effects of the microbiota on mucus layer production and integrity and report that the two different B6 microbiota elicit different effects on the ileal and colonic mucus layer, with one microbiota causing increased penetrability of the inner mucus layer. Causality of these effects due to the microbiota is demonstrated by transplanting the communities into germfree mice and recreating similar effects, although the authors show that diet plays a role as well. The results of this paper are interesting and leverage a series of novel approaches and technologies that are available to these researchers (in vitro mucus measurements on explants, combined with microbial ecology and gnotobiotic mice). The report is somewhat descriptive/observational in the current form, although the microbiota transplant experiment is a nice step towards establishing causality. The present results document a new phenotype that will be of interest to many researchers studying molecular mechanisms of host-gut microbiota interaction and provide a basis for future investigations. Specific comments:*

1. Abstract: The unique selling point of the study is certainly the two distinct mucus phenotypes and their correlation to the microbiota, shown by transfer to germ-free mice. The data provided for the abundance of bacteria is inconclusive. Therefore, it might be necessary to keep the focus on the unique selling point, especially for a report format.

Answer: We have made alterations in the abstract to focus more on the effect of the microbiota to impact the mucus phenotype.

2. p. 3. The reference to Qin et al. 2010 is used to support the statement that the microbiota is "typically made up of 500-1,000" species"; although this reference found over 1,000 total species across the subjects surveyed and states "at least 160 species per individual" (abstract). Thus, the stated numbers may be innaccurate (at least with respect to this citation) and depending on whether the authors are referring to the microbiota that could possibly be present across many subjects, or the number per person. Please clarify what is meant.

Answer: This sentence has been changed and now reads:
" The microbiota is diverse and typically made up of in total 500-1,000 species with at least 160 species that are shared among individuals and two phyla, the Bacteroidetes and Firmicutes being dominant (Human microbiome project, 2012;Qin *et al.*, 2010). "

3. p. 4 presents a very nice summary of the different roles of secreted mucus as a barrier and is a notable asset of the introduction.

Answer: Thank you for the nice comment.

4. p. 5. It is argued that the role of diet was "eliminated...by feeding the mice in the two colonies the same chow". The word "eliminated" is too pejorative here since the authors do find a role for diet in impacting the mucus layer readout and also switching the diet fed to the mice in each room cannot restore a diet specific microbiota if the microbiota has been permanently altered by prolonged feeding of one diet. For example, if a continued monotonous diet causes some microbial lineages to go extinct from the microbiota, they cannot be restored by changing diets unless they're in the diet. Perhaps, "but we controlled for the impact of diet, at least in short-term feeding conditions, by feeding..."

Answer: This is a very valid comment and yes it might very well be so that the food cannot restore the microbiota. However, the differences in the microbiota are the focus of the study and the correction of food is to limit the effect of the food on the measured parameters. The text is altered according to the suggestion and now reads:
"The two colonies were maintained on different diets with different composition, but we controlled for the impact of diet, at least within one generation of offsprings, by feeding the mice in the two colonies the same chow (Food A, standard chow or Food B, autoclaved chow) from weaning."

5. Introduction (overall and concluding paragraph): A clear rationale behind conducting the present study in relation to the background literature provided in the manuscript is somewhat unclear.

Answer: We have rewritten parts of the introduction and specified the aims of the study more clearly to make the rationale more clear.

6. p. 6. The strategy for separating the bacteria in "mucus" and "lumen" is somewhat of a concern, although it may not be feasible to perform this separation in a different way unless a microscopic approach like laser-capture microdissection is taken. The reason for concern is the concept, which has largely been developed by the author labs, that the outer mucus layer is the prominent site of microbial colonization, and this would presumably be lost using this separation technique. Addressing this in the discussion as one aspect of design that may have reduced the observed differences in mucus microbiota would be appropriate.

Answer: Mucus sampling using more refined techniques developed in the lab would not be suitable due to difficulties to keep the materials separated without cross-contamination. The bacteria identified in the ileum represents bacteria located between the villi as the mucus on the tips will be washed away. The mucus sampled in the distal colon is attached to the epithelium and will mainly have bacteria located on the luminal surface of the inner mucus layer or in the inner mucus layer in samples (room 2) that have a penetrable mucus. This represents the bacteria located closest to the epithelium. The outer mucus will *in vivo* be intermixed with the luminal content and a good separation is not very reproducible and depends on fecal material present etc. The ex vivo method used to define the non-attached mucus is as stated above not allowing sampling without risk of contamination. The amounts when using a method like laser capture will render detection limit problems.
This is now discussed in greater detail on page 16 **"The microbiota mucus sample strategy used may have reduced the observed differences in mucus microbiota as the amounts of attached mucus were not controlled for. Unfortunately more elaborate mucus sampling techniques are difficult to perform."**

7. p. 10. "A tendency of less secreted MUC2 was observed in room 2 on food B (Fig. S6C)". Even a non statistically significant statement to this effect does not seem warranted based on this data since the means of the two MUC2 box plots in fig. S6C look almost identical, just with less variation in group B.

Answer: This is absolutely correct and the statement is removed.

8. p. 10. The quantitative IHC and microscopy results although statistically significant seem like very minor differences that could be difficult to quantify based on data like that shown in Fig. 4C. Is it really possible to accurately count stained goblet cells so precisely that a difference of 2-3 cells/crypt can be determined with such stat. significance? Some statement in the methods of sample measurement in a blinded fashion (or re-measuring by a blinded evaluator unfamiliar with the project hypothesis) would reinforce the credence that such a small difference can be precisely measured.

Answer: This is a very valid point and we have performed additional counts both of more samples and by 2 additional evaluators. The new results reveal a variation where a tendency to more goblet cells and more cells in lamina propria of animals from Room 2 is seen but significant differences is not obtained in all the sets and thus the graphs and text have been adjusted accordingly not stating a clear difference in these parameters any more.

Typographical errors and minor points
1. p. 2. Abstract: spell out "SPF"
2. p. 3. "from establish host infections" should be changed to "establishing"
3. p. 4. "inner mucus layer separate bacteria" should be changed to "separates"
4. p. 5. "normal wild-type" in first line. This is redundant. Maybe just retain "wild-type" or define normal if it means something other than genetic background.
5. p. 5. 16S sequencing should be changed to 16S rRNA gene sequencing (also in the results section)

Answer: point 1-5 has been corrected.

6. p. 7. It might be useful for readers if the term "segmented filamentous bacteria" is inserted next to the mention of Candidatus arthromitus, since the common name has been used for a much longer period and likely has more immediate reader recognition. Also, it has been proposed that animal gut SFB now be referred to as Candidatus Savagella (Environ Microbiol. 2012 Jun;14(6):1454-65).

Answer: This has been corrected to also state the name segmented filamentous bacteria in the text and in the tables.

7. P. 8. "microbiota that were maternal bacterial transmitted" should read "maternally and the word bacteria may be superfluous since its implied in microbiota.

Answer: This has been altered in the text.

8. p. 9-10. The discussion here about the integrity of the mucus in various conditions is somewhat vague since it relies on terms like "less structured", "well-stratified" and then "thinner and less organized". Its understood that this layer is dynamic and heterogeneous as well as technically difficult to image and quantify, but this section reads as highly qualitative, especially in contrast to the previous section in which bead penetration depth was quantified nicely. Perhaps more clearly indicating in the representative micrographs *where* the inner and outer mucus layers are defined (i.e., with mark ups on the images) based on the MUC2 staining would assist. These delineations are mentioned in the description but not indicated on the micrographs (for example, in fig. 4A where it looks like the room 2 mice still have a discrete layer that is not colonized.

Answer: Arrows have been added to indicate the inner mucus, when present, in figure 4 and 5 to make it easier to interpret for the reader. The text is also altered to improve the statements in a more stringent way with more precise descriptions of the observations made.

9. P. 10. "...cells in lamina propria was also estimated". The word estimated here should be exchanged with something like "measured" since the former implies imprecision and the values being discriminated appear quite precise given the samll difference and significance.

Answer: This is now changed to counted.

10. P. 12. "production of mucus is far from". "Is" should be replaced with "are

Answer: This is now corrected.

11. P. 13. "This would then talks against". Should be "talk" or a different term like "contraindicate a mucus promoting...".

Answer: This is now reads **"speak against".**

12.p. 15. The final clause on the page "also studies that might not directly involve large intestine" isn't clearly attached to the previous half of the sentence. Consider revising.

Answer: This is now reads **"also studies where this can have an indirect effect".**

13. P. 18. Mention of alpha and beta diversity: the Greek symbols are missing for these abbreviations.

Answer: This has been corrected.

Figure and tables:
1. Fig. 5. Panels A-D are shown, but the legend only lists A-C. It looks like "A" in the legend was split into A/B in the figure. Also, no red FISH staining is at all apparent in the final panels, just blue DAPI staining, which could also be the bacteria.

Answer: Figure legends for figure 5 have been corrected and bacteria are shown by DNA staining with the difference pointed out in the enlargement. FISH-staining was incorrectly added to the figure legend.

2. Table 1. Its not clear from the legend what groups the statistical tests were made between. Presumably, the most important test for this study (and that which is shown) is between rooms, but the first mention of table 1 is in reference to the mucus/lumen comparison and this is also shown in this table. Perhaps separate tables would clarify; or, please state clearly what groups are compared. Also, in this table many taxa are listed as "<1". Is this correct? It seems like a limit of detection for the number of sequences surveyed, but in the same samples, sometimes fractional values (e.g., 0.01) are given.

Answer: The comparison is always between the two rooms with one food at a time and this is now stated in the legend of the table that reads**: "The mean of the relative abundance of dominant phyla (bold), classes (bold) and genera in ileum, distal colon, and caecum samples obtained from mice bred in Room 1 or Room 2. Statistical tests of over or under-representation of bacterial lineages among sample groups (Room 1 versus Room 2) at each sample location were made at the phylum, class and genus levels using Wilcoxon rank-sum test…."**
For values where no significant difference occurs that are less than 1 are described as <1. For values below 1 where there is a significant difference between the rooms the exact values are included in the table. This is to make the table more apprehensible and clear. This is added to the legend of the table: **"For values <1 the exact value is only given if significantly different."**

2nd Editorial Decision 06 November 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, referees 1 and 2, who assessed your revised study, are now positive about its publication in EMBO reports (referee 1 had no further comments to the authors). I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your

manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- The description of the statistical analyses performed, although relatively thorough, is incomplete in the legends to several of the figure panels. Please go through your manuscript carefully once more and ensure that all relevant figures and supplementary figure legends include information on the number of independent experiments measured (which should be at least three if errors are indicateD), the type of error bars used and what the bar represents (mean, median, etc). This should be included in addition to the statistical tests applied and P values obtained, which you already have.

-It is a precondition for publication in EMBO reports that authors agree to make all data that cannot be published in the journal itself freely available, where possible in an appropriate public database. In the case of nucleotide sequence datasets, they should be submitted to an International Nucleotide Sequence Database Collaboration member: GenBank (http://www.ncbi.nlm.nih.gov/genbank/), EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) or DDBJ (http://www.ddbj.nig.ac.jp/).

- We now encourage the publication of original source data -in your case, this would apply to the numerical data behind the graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports. Please contact me if you have any questions or I can help getting the study ready for publication.

REFEREE REPORT:

Referee #2:

A careful revision has been performed for the paper and the concerns of the reviewers are addressed.

2nd Revision - authors' response 01 December 2014

We have now done all the additional minor revisions with the manuscript and expanded view. Enclosed is also the source data file for data behind the graphs. The mass spectrometry proteomics data have been deposited to the ProteomeXchange via the PRIDE partner and the 16S sequencing data is being deposited to ENA at EBI we hope this proceeds well and await the accession number to be added in the proof.

3rd Editorial Decision 02 December 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal. As I mentioned, we will highlight your article with a "Hot off the Press" written by Andrew MacPherson, and our media relations department may also be in touch, as we will likely issue a press release on your study.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

