

The gut microbial metabolites short-chain fatty acids restrain tissue bacterial load, chronic inflammation, and associated cancer in the colon

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Review Timeline:	Submission date:	10-May-2017
	First Editorial decision:	19-Jun-2017
	First revision received:	30-Nov-2017
	Second Editorial decision:	22-Dec-2017
	Second Revision received:	03-Jan-2018
	Third Editorial decision:	01-Feb-2018
	Third Revision received:	28-Feb-2018
	Accepted:	04-Apr-2018

Handling Executive Committee member: Prof. Steffen Jung

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

19-Jun-2017

Dear Dr. Kim,

We are sorry for the delay in the peer review of your manuscript ID eji.201747122 entitled "The gut microbial metabolites short-chain fatty acids support acute but restrain persistent inflammatory response and associated intestinal cancer" which you submitted to the European Journal of Immunology. There was a delay in receiving one of the reports. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be

reconsidered for publication. Our Executive Editor encourages you to clarify the microbiota situation with respect to co-housing of control groups, but also potentially the composition in the animal facility. In addition, literature should be discussed more comprehensively, and it should be more clearly stated what distinguishes your study from earlier studies and how results differ. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

On behalf of
Prof. Steffen Jung

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Reviewer: 1

Comments to the Author

I thank the editors giving me the opportunity to review this manuscript. Kim and colleagues investigated the importance of the metabolite sensing receptors GPR43 and GPR41, that recognize short chain fatty acids (SCFA) for the development of acute and chronic colitis and for the development of inflammation-associated colon cancer. The authors found that GPR43-deficient animals are somehow protected from development of colitis in the acute phase. However, more severe inflammation was observed in the chronic phase. In consequence, GPR43-deficient animals developed more tumours. Further, experiments with bone marrow chimera revealed that non-hematopoietic cells are more important for the observed findings. Supplementation of SCFA or high fibre diets protect from the development of polyps. In part, an impairment of the intestinal barrier in GPR43-deficient animals may explain the observed findings. On the whole, this manuscript adds to the literature that inflammation-associated cancer and not only colitis as previously reported is in part regulated by the SCFA receptor GPR43, but some questions remain.

As pointed out by the authors in the introduction different groups have reported different outcomes in colitis experiments with GPR43-deficient animals in different experimental settings. One possible explanation could be that a different composition of the microbiota may explain these findings. I believe that it is essential to appropriately describe the animal experiments in the method section. In particular, it needs to be stated whether different animals were co-housed before the experiment, how long the animals were co-housed or whether the different groups were kept in different cages.

In case the animals have not been co-housed before the experiment, the author may test whether the observed effects in GPR43-deficient animals for the development of inflammation-associated cancer is transmissible by transferring the gut-microbiota from GPR43-deficient to wt animals and vice versa.

This manuscript does not demonstrate that epithelial cells express GPR43. Expression analysis of GPR43 isolated from the colon could be done.

Whether IL-1 β is cleaved (figure 2) and thereby activated and not only higher expressed has not been investigated.

It remains open, whether SCFA have direct effects on epithelial cells to promote the formation of tight junctions or whether SCFAs supports the integrity of the intestinal barrier by indirect means (.i.e. secretion of cytokines by other cells that support the integrity of the barrier). Either stimulating epithelial cell lines with SCFAs and studying the expression of tight junction proteins and the resistance of the monolayer could test this. Alternatively, intestinal organoids could be stimulated with SCFAs and expression of tight junction proteins determined.

Data was analysed with a student's T test. Whether Gaussian distribution of the data is present has not been examined. A Man Whitney U test may be more appropriate to analyse the data.

Reviewer: 2

Comments to the Author

Major comments

The effects of GPR43-deficiency on DSS-induced inflammation are described as “apparently contradictory, warranting additional studies...”. It would be helpful if the authors were able to briefly describe how the additional studies - this manuscript, for instance - should avoid the confounding factors that have prevented a clear understanding of the effects of GPR43-deficiency to date. Without an improved approach, “additional studies” are not likely to provide additional insights. Without a more thorough analysis of the literature to date, this work would appear to offer only a small incremental advance for this field. Similarly, the effects of SCFA on colon cancer have been previously reported. The authors do not explain why their data provide a significant advance for this field.

The authors report that lack of GPR43 causes an increase in acute inflammation, while suppressing more chronic inflammation. The mechanisms underlying these paradoxical responses are not sufficiently explored or explained. The authors do not quantify bacterial load, or look at antigen-specific immune responses, at either their ‘acute’ or ‘chronic’ time points. Without these data, their results are very

difficult to interpret. It is therefore unreasonable for the authors to claim, for instance, that "This indicates that SCFA receptors function to promote the acute immune response to prevent chronic inflammatory responses."

Minor comments

In describing Figure 5, the authors claim that their data show "a relatively more important role of non-hematopoietic cells...". However, there appears to be no significant difference between the WT → Gpr43^{-/-} and the Gpr43^{-/-} → WT mice. Thus, this statement is not supported by the data.

In both Figure 5 and Figure 7, spleen weights should be compared to WT untreated spleen weights. This will help to generate context and meaning for the authors' data. Which of these spleens are bigger than the normal spleens in the authors' mouse colonies?

First Revision – authors' response

30-Nov-2017

Reviewer: 1

Thanks for your constructive comments.

As pointed out by the authors in the introduction different groups have reported different outcomes in colitis experiments with GPR43-deficient animals in different experimental settings. One possible explanation could be that a different composition of the microbiota may explain these findings. I believe that it is essential to appropriately describe the animal experiments in the method section. In particular, it needs to be stated whether different animals were co-housed before the experiment, how long the animals were co-housed or whether the different groups were kept in different cages. In case the animals have not been co-housed before the experiment, the author may test whether the observed effects in GPR43-deficient animals for the development of inflammation-associated cancer is transmissible by transferring the gut-microbiota from GPR43-deficient to wt animals and vice versa.

Response: We took this comment seriously and performed co-housing or used bedding-sharing experiments to equalize microbiota. The data indicate that the beneficial effect of GPR43 in suppressing colon cancer is not mediated by any changes in the microbiota due to GPR43 deficiency (Figure 4).

This manuscript does not demonstrate that epithelial cells express GPR43. Expression analysis of GPR43 isolated from the colon could be done. It remains open, whether SCFAs have direct effects on epithelial cells to promote the formation of tight junctions or whether SCFAs support the integrity of the intestinal barrier by indirect means i.e. secretion of cytokines by other cells that support the integrity of the barrier).

Response: This point raised by the reviewer has been established already. Epithelial cells highly express GPR43 and activation of GPR43 is important for mounting acute inflammatory responses (ref 13). Moreover, impact of SCFAs or GPR43 on epithelial tight junction has been reported (ref 14). We mentioned this in the text (page 12).

Whether IL-1 β is cleaved (figure 2) and thereby activated and not only higher expressed has not been investigated.

Response: Inflammasome activation for production of active IL-1b in response to GPR43 activation has been reported (ref 32). Moreover, we found an issue with the PCR and deleted this data from the manuscript.

Data was analysed with a student's T test. Whether Gaussian distribution of the data is present has not been examined. A Man Whitney U test may be more appropriate to analyse the data.

Response: Since the sample number is rather small, we performed both T-test and Man Whitney U test (all figures).

Reviewer: 2

Thanks for your constructive comments.

Major comments

The effects of GPR43-deficiency on DSS-induced inflammation are described as "apparently contradictory, warranting additional studies...". It would be helpful if the authors were able to briefly describe how the additional studies - this manuscript, for instance - should avoid the confounding factors that have prevented a clear understanding of the effects of GPR43-deficiency to date. Without an improved approach, "additional studies" are not likely to provide additional insights. Without a more thorough analysis of the literature to date, this work would appear to offer only a small incremental advance for this field. Similarly, the effects of SCFA on colon cancer have been previously reported. The authors do not explain why their data provide a significant advance for this field.

Response: We revised the introduction part (page 3-4) to clearly state the goal of this study. With the new data with the co-housed mice, our manuscript delineates the function of SCFAs and their receptors in decreasing tissue bacterial load, inflammation and colon cancer. Moreover, the results demonstrate that the beneficial effect of GPR43 is not mediated by microbiota differences. We have revised the text to make the points clear (page 6-7).

The authors report that lack of GPR43 causes an increase in acute inflammation, while suppressing more chronic inflammation. The mechanisms underlying these paradoxical responses are not sufficiently explored or explained. The authors do not quantify bacterial load, or look at antigen-specific immune responses, at either their 'acute' or 'chronic' time points. Without these data, their results are very difficult to interpret. It is therefore unreasonable for the authors to claim, for instance, that "This indicates that

SCFA receptors function to promote the acute immune response to prevent chronic inflammatory responses."

Response: We have added the bacterial load data. Tissue bacterial load remains increased in Gpr43^{-/-} mice but not in WT mice during AOM/DSS-induced colon cancer development. This abnormal increase in tissue bacterial load explains the high inflammatory activity and increased colon cancer development in GPR43^{-/-} mice (Figure 4C and 9D). These results were obtained from co-housed mice to rule out any differences in microbiota between WT and GPR43^{-/-} mice.

Minor comments

In describing Figure 5, the authors claim that their data show "a relatively more important role of non-hematopoietic cells...". However, there appears to be no significant difference between the WT → Gpr43^{-/-} and the Gpr43^{-/-} → WT mice. Thus, this statement is not supported by the data.

Response: It depends on panels (i.e. readouts). We revised the text accordingly to reflect lack of statistical significance between the two groups in some panels (page 7 and 8).

In both Figure 5 and Figure 7, spleen weights should be compared to WT untreated spleen weights. This will help to generate context and meaning for the authors' data. Which of these spleens are bigger than the normal spleens in the authors' mouse colonies?

Response: We have added lines (showing average weight of the spleen and MLN in sex/age-matched control WT animals) to the graphs (Figure 3D, 4D, 4E, 5C, 6C, and 8E).

Second Editorial Decision

22-Dec-2017

Dear Dr. Kim,

Thank you for submitting your revised manuscript ID eji.201747122.R1 entitled "The gut microbial metabolites short-chain fatty acids restrain tissue bacterial load, chronic inflammation, and associated cancer in the colon" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

Unfortunately, Referee #2 was not satisfied with the revisions made and further major revision is requested. The journal does not encourage multiple rounds of revision and you should fully address the concerns of the referee in this final round of revision. Should you disagree with any of the referees'

concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. ****In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. For the flow cytometry results, please ensure to show the full gating strategy (detailed instructions below). Failure to do this will result in delays in the re-review process.****

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referee before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,

Marta Vuerich

On behalf of

Prof. Steffen Jung

Dr. Marta Vuerich

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Reviewer: 1

Comments to the Author

I do not have any questions anymore

Reviewer: 2

Comments to the Author

Comments on the authors responses

On reviewing the previous version of the manuscript, I asked for a more thorough review of the literature in the introduction, to explain how this manuscript might resolve some of the contradictions found in the previous literature. Rather than adding this additional information, the authors seem to have simply removed any reference to these contradictions. This is unsatisfactory. The authors have still not explained how their data contribute to this field.

The authors have now quantified the bacterial load in their AOM/DSS mice, in addition to their previous data on total eubacteria in the GPR43^{-/-} mice after chronic DSS treatment. This increase in chronic bacterial load correlates nicely with their increased tumour burden and increase in chronic inflammation. There are still no data describing the tissue bacterial load in the acute model, where 'inflammatory' responses are reduced. The paradoxical effects of GPR43 on the acute and chronic responses are therefore still difficult for the authors to explain. They have also presented no data relating to antigen specificity in these experiments, or any comments relating to this, as I originally requested.

Comments on new data

When referring to the new figures 4D and E, the authors calling to have demonstrated increased 'numbers' of T cells. But no data on numbers are shown in these figures.

The authors do not show the new PCR data that demonstrate that their transgenic mice and controls have “shared” microbiota profiles. These data should be shown, perhaps in the supplementary figures. Similarly, the authors do not show the data which, they claim, show an absence of changes in microbiota during colon cancer development. Without access to these data, it is not possible to evaluate the authors’ claims.

Inn Figure 2, for instance, the authors state that Students t-test, Mann-Whitney and ANOVA were performed. And that ANOVA was used where there were reported measures. This statement is confusing. All the panels contain repeated measures, so only ANOVAs should have been performed, with appropriate post-tests. From the authors’ statements, it is not at all clear whether the appropriate statistical test have been used, both in Figure 2 and elsewhere.

The experiments using co-housing or shared bedding are a welcome addition, but do not address whether the effect of GPR43 is via an effect on the microbiota, because the authors have not shown that their GPR43^{-/-} and control mice have the same microbiota profile - this would need to be checked, at the very least, but 16S sequencing of stool samples. Alternatively, the discussion relating to this aspect of the manuscript could be improved to make this aspect more clear.

Overall

The authors have improved the part of their manuscript that relates to chronic DSS treatment, inflammation, and tumour development, but have not really addressed the questions relating to the acute aspects of their data, or provided convincing data to address the role of the microbiota in their experiments. Nor have they adequately described the statistical test they have used in many of their figures.

Second Revision – authors’ response

03-Jan-2018

Reviewer 2 : Thanks for the your review again. It seems that you missed the data on bacterial load at acute phase in Fig 9D. While we appreciate your comments, we disagree on a couple of points such as the need to study antigen specificity. We hope that you would understand our position on the points. Otherwise, we believe that all other requests have been fulfilled by the second revision.

1. On reviewing the previous version of the manuscript, I asked for a more thorough review of the

literature in the introduction, to explain how this manuscript might resolve some of the contradictions found in the previous literature. Rather than adding this additional information, the authors seem to have simply removed any reference to these contradictions. This is unsatisfactory. The authors have still not explained how their data contribute to this field.

Response: We have added a fairly extensive review of literature to identify issues that our study has addressed. Please understand that we had to rephrase the introduction by replacing the original text to incorporate new results obtained. We reinforced the introduction and discussion sections to provide sufficient background information and interpretation. We also made the contribution of this work clear (page 3-4, 12-13).

2. The authors have now quantified the bacterial load in their AOM/DSS mice, in addition to their previous data on total eubacteria in the GPR43^{-/-} mice after chronic DSS treatment. This increase in chronic bacterial load correlates nicely with their increased tumour burden and increase in chronic inflammation. There are still no data describing the tissue bacterial load in the acute model, where ‘inflammatory’ responses are reduced. The paradoxical effects of GPR43 on the acute and chronic responses are therefore still difficult for the authors to explain. They have also presented no data relating to antigen specificity in these experiments, or any comments relating to this, as I originally requested.

Response: It seems that the reviewer missed the data (tissue bacterial load at acute and chronic phases following AOM/DSS treatment) that are already shown in Figure 9D. Tissue bacterial load during acute inflammatory responses has been previously reported and this reference is cited (ref 13), and we tried not to perform the repetitive work. Regarding the antigen-specificity issue, there is no evidence currently available for the idea that SCFAs and their receptor regulate antigen specific inflammatory and oncogenic processes. Therefore, it is deemed too early to study the aspect at this time point.

3. Comments on new data

When referring to the new figures 4D and E, the authors calling to have demonstrated increased ‘numbers’ of T cells. But no data on numbers are shown in these figures.

Response: We have changed the word accordingly (page 7).

4. The authors do not show the new PCR data that demonstrate that their transgenic mice and controls have “shared” microbiota profiles. These data should be shown, perhaps in the supplementary figures. Similarly, the authors do not show the data which, they claim, show an absence of changes in microbiota during colon cancer development. Without access to these data, it is not possible to evaluate the authors’ claims. The experiments using co-housing or shared bedding are a welcome addition, but do not address whether the effect of GPR43 is via an effect on the microbiota, because the authors have not shown that their GPR43^{-/-} and control mice have the same microbiota profile - this would need to be checked, at the very least, but 16S sequencing of stool samples. Alternatively, the discussion relating to this aspect of the

manuscript could be improved to make this aspect more clear.

Response: Now we have added the confirmatory data (Supp Fig. 2). The 16S rRNA gene PCR data showing the two strains are indistinguishable in selected bacterial groups and therefore the GPR43 effect is not likely to be mediated by the microbiota. We did not perform the requested deep sequencing study of the microbiota to simply confirm the effect of the cohousing. However, we did add more to the discussion on this aspect (page 13).

5. In Figure 2, for instance, the authors state that Students t-test, Mann-Whitney and ANOVA were performed. And that ANOVA was used where there were reported measures. This statement is confusing. All the panels contain repeated measures, so only ANOVAs should have been performed, with appropriate post-tests. From the authors' statements, it is not at all clear whether the appropriate statistical test have been used, both in Figure 2 and elsewhere.

Response: Unlike the reviewer pointed out ("All the panels contain repeated measures") only panel A is repeatedly measured in this figure. Anyway, we used two-way ANOVA followed by Bonferroni's multiple comparison for repeated measure data (Figure 1A, 2A, 3A, 6A, 7B) and one-way ANOVA followed by Tukey's Multiple Comparison Test for differences among multiple groups (Figure 1B-E, 2B-E, 6B-C, 7C, 8C, 8E, 9A-C, E). Additionally, we used Mann-Whitney U-test (2-tailed) in response to reviewer's previous question for single data set comparisons.

Third Editorial Decision

01-Feb-2018

Dear Dr. Kim,

Thank you for submitting your revised manuscript ID eji.201747122.R2 entitled "The gut microbial metabolites short-chain fatty acids restrain tissue bacterial load, chronic inflammation, and associated cancer in the colon" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

Unfortunately, Referee 2 was not satisfied with the revisions made, since he still had concerns about statistical analysis used. Therefore we invited a third referee to evaluate the statistics and based issues raised further major revision is requested. Referee 3 was instructive in providing guidelines how statistical analysis should be performed, and you are strongly encouraged to perform analysis as suggested. The journal does not encourage multiple rounds of revision and you should fully address the concerns of the

referee in this final round of revision. Should you disagree with any of the refereesTM concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Please specify the number of mice/samples per experiment. Please provide detailed gating strategy for all flow cytometry data as detailed below. Failure to do this will result in delays in the re-review process.**

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Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

On behalf of
Prof. Steffen Jung

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Reviewer: 2

Comments to the Author

1) I thank the authors for amending the introduction to highlight the contributions that their work makes to continuing controversies in the field.

2) The legend describing Figure 9D states that the figure shows "(D) Tissue bacteria load in co-housed mice following AOM/DSS-induced colon cancer. Mice were treated with AOM and 3 cycles of 1% DSS in drinking water and sacrificed at day 62-63 post AOM injection." Perhaps the legend could be modified to indicate that some of the animals were sacrificed at day 16-17, so that other readers do not make the same mistake as me?

I am sympathetic to the authors comment that analysis of the antigen-specific immune response is beyond the scope of this work. I have asked them twice to comment on this issue in the paper. Such a sentence would still be appropriate to add where they show data relating to T cell responses but do not discuss whether these changes, in their opinion, might be in antigen-specific populations of cells, or the consequence of systemic inflammatory changes. Their data might indicate the latter. Either way, I would still like to see such a comment.

3) I thank the authors for this response.

4) The addition of these data and discussion are very helpful, and are sufficient.

5) The following panels still contain multiple comparisons, but, according to the legend, have been analysed using statistical tests that do not correct for multiple comparisons: 3B, 4B (left panel), 5A, 7C, 8E, 9D.

In some cases, the data are described in the legend as being analysed using both ANOVA, and Mann-Whitney tests. This is also inappropriate.

This issue still needs some attention. I am concerned that if the correct tests were used, some of the significant differences would no longer appear significant.

Reviewer: 3

Comments to the Author

Since this is an already ongoing review process and I was invited by the Editorial office to join in to have a look at statistical methods, I will only comment on the statistical methods applied in figures 1 to 9

according to the figure annotations.

My recommendations are as follows:

- If you apply two-way ANOVA please report whether the interaction of time AND group is returning a significant result including effect size (i.e. partial eta squared). Otherwise the reader does not know which effect you are referring to when reporting significances - merely time or the interaction of time and group. The latter usually is of interest when applying two-way ANOVA. If you are calculating multiple two-way ANOVAs in order to test a hypothesis, multiple-testing adjustments like Bonferroni or Bonferroni-Holm are recommended in order to control type 1 family-wise error inflation. Report the corresponding thresholds of significance after correction, i.e. the new significance levels.
- Applying one-way ANOVA in small samples such as this is usually not covered by the implications of the central limit theorem. Accordingly, the assumption of normally distributed sample characteristics will have to be tested for each group in each ANOVA. If the assumption is violated within at least one group included into ANOVA, a non-parametric test (Kruskal-Wallis) is indicated. Dunn's test should be used to do pairwise group comparisons in case of sig. Kruskal-Wallis test. Depending on the statistical software that is used adjusted p-values will be presented by default or will have to be calculated manually. A Mann-Whitney U-test with multiple test adjustment might be considered as alternative if Dunn's test is not provided by the statistical software package. In case the assumption of normal distribution is met for all groups and one-way ANOVA is significant, Tukey post-hoc test can be applied in cases of equal sample size of groups and similar population variances - it also provides control of type I error inflation. If there is any doubt about similar population variances of groups, Games-Howell post-hoc is a good alternative
- Calculating multiple Mann-Whitney U-tests in order to test a hypothesis requires multiple test adjustments as already outlined above

In order to address the issues raised by the referees, the aforementioned points should be taken into account.

Third Revision – authors' response

28-Feb-2018

Point-by-point response

Reviewer: 2 : We appreciate your specific comments on statistical analyses.

The legend describing Figure 9D states that the figure shows "(D) Tissue bacteria load in co-housed mice following AOM/DSS-induced colon cancer. Mice were treated with AOM and 3 cycles of 1% DSS in drinking water and sacrificed at day 62-63 post AOM injection." Perhaps the legend could be modified to indicate that some of the animals were sacrificed at day 16-17, so that other readers do not make the same mistake as me?

Response: This has been revised as suggested (page 29).

The changes, in their opinion, might be in antigen-specific populations of cells, or the consequence of systemic inflammatory changes. Their data might indicate the latter. Either way, I would still like to see such a comment.

Response: We have included this issue in the discussion (page 12). The increased Th17 cell response in GPR43 deficiency appears to be a bystander response as the consequence of inflammatory changes but we cannot rule out the possibility that it may involve antigen-specific responses.

5) The following panels still contain multiple comparisons, but, according to the legend, have been analysed using statistical tests that do not correct for multiple comparisons: 3B, 4B (left panel), 5A, 7C, 8E, 9D. In some cases, the data are described in the legend as being analysed using both ANOVA, and Mann-Whitney tests. This is also inappropriate. This issue still needs some attention. I am concerned that if the correct tests were used, some of the significant differences would no longer appear significant.

Response: Because statistical analysis has been a major point now, we have recruited a statistician (Dr Hyungjin M. Kim from the Department of Biostatistics, University of Michigan) to re-analyze all data including the ones pointed out by the reviewer. Now, we fully correct for multiple comparisons and used only one method (either ANOVA for multiple group comparisons or U tests for two-group comparisons). Reanalysis did not change the overall conclusions or discussions but somewhat narrowed down the time points of significant differences in repeated measure experiments. Please see page 18 and figure legends.

Reviewer: 3 : We appreciate your general comments on statistical analyses.

- If you apply two-way ANOVA please report whether the interaction of time AND group is returning a significant result including effect size (i.e. partial eta squared). Otherwise the reader does not know which effect you are referring to when reporting significances - merely time or the interaction of time and group. The latter usually is of interest when applying two-way ANOVA.

Response: Both Statistical Analysis and Legends are now clarified to describe group effects, and p-values for the group effect are now provided in legends for each figure. Statistical analysis section describing the analysis involving data over time now reads as the following: "Repeated measures ANOVA was used to determine group effects (Figure 1A, 2A, 3A, 6A and 7B) for indicated time periods, and after finding a significant group effect, pairwise comparisons of interest were made on specific days using multiple two-sample t-tests with Bonferroni's adjustments to account for multiple comparisons."

If you are calculating multiple two-way ANOVAs in order to test a hypothesis, multiple-testing adjustments like Bonferroni or Bonferroni-Holm are recommended in order to control type 1 family-wise error inflation. Report the corresponding thresholds of significance after correction, i.e. the new significance levels.

Response: Legends for each figure now reports the thresholds of significance after Bonferroni adjustments.

- Applying one-way ANOVA in small samples such as this is usually not covered by the implications of the central limit theorem. Accordingly, the assumption of normally distributed sample characteristics will have to be tested for

each group in each ANOVA. If the assumption is violated within at least one group included into ANOVA, a non-parametric test (Kruskal-Wallis) is indicated. Dunn's test should be used to do pairwise group comparisons in case of sig. Kruskal-Wallis test. Depending on the statistical software that is used adjusted p-values will be presented by default or will have to be calculated manually. A Mann-Whitney U-test with multiple test adjustment might be considered as alternative if Dunn's test is not provided by the statistical software package. In case the assumption of normal distribution is met for all groups and one-way ANOVA is significant, Tukey post-hoc test can be applied in cases of equal sample size of groups and similar population variances - it also provides control of type I error inflation. If there is any doubt about similar population variances of groups, Games-Howell post-hoc is a good alternative

- Calculating multiple Mann-Whitney U-tests in order to test a hypothesis requires multiple test adjustments as already outlined above

Response: For any two-group comparisons (e.g., Figure 3B, 3D, 4, 5C, 5D, 8A, 8B), we used a non-parametric Mann-Whitney U-tests. For comparing multiple groups (Figure 1B-E, 2B-E, 6B, 6C and 7C), one-way ANOVA was used, and when significant group effect was found, post-hoc tests were done using Tukey's multiple comparison tests with an overall alpha set at 0.05. We now describe this more consistently in Statistical Analysis section and legends. Based on our prior experience with similar data, we expect our measures not to violate normality assumption once adjusting for primary design factors (e.g., group). We also do not expect tests for normality to show significant violations of the assumption due to small sample size. Nonetheless we inspected the data visually using histograms and performed Shapiro-Wilks test and Shapiro-Francia tests of normality and did not find evidence for violation of normality assumptions.

Fourth Editorial Decision

27-Mar-2018

Dear Dr. Kim,

It is a pleasure to provisionally accept your manuscript entitled "The gut microbial metabolites short-chain fatty acids restrain tissue bacterial load, chronic inflammation, and associated cancer in the colon" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: <https://onlinelibrary.wiley.com/toc/15214141/0/ja>). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European

Journal of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Steffen Jung

Dr. Nadja Bakocevic
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