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The Enduring Effects of Early Life Stress on the Microbiota-Gut-Brain Axis are Buffered by Dietary Supplementation with Milk Fat Globule Membrane and a Prebiotic Blend

O' Mahony, Siobhain (contact); McVey Neufeld, Karen-Anne; Waworuntu, Rosaline; Pusceddu, Matteo; Manurung, Sarmauli; Murphy, Kiera; Strain, Conall; Laguna, Mamen; Peterson, Veronica; Stanton, Catherine; Berg, Brian; Dinan, Timothy; Cryan, John

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Submission date: 09-Nov-2018
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Editorial Decision: Minor Revision
Revision Received: 12-Jun-2019
Accepted: 25-Jun-2019

Editor: Patricia Gaspar
Reviewer 1: Anne Teissier
Reviewer 2: Monika Fleshner

1st Editorial Decision 10-Jan-2019

Dear Dr. O' Mahony,

First of all, apologies for the time it has taken to deal with your manuscript, this was mainly due to the holiday period. It has now been reviewed by two external reviewers as well as by the Section Editor, Dr. Patricia Gaspar, and ourselves.

Both reviewers thought your work to be of great importance and warrants publication in EJN. However, as you will see from their remarks, they have both suggested a number of changes in the presentation of the results and the discussion in order to clarify the main conclusions that can be derived from the present study. In particular, reviewer 2 would like to see more methodological caution on the effects of cage grouping and stressful behavioral tests on the results of the microbiota. Reviewer 1 made useful suggestions to clarify the main effects of the MFGM relative to the dietary supplements in the observed effects and to be more specific on the effects that can or cannot be related to changes in the microbiota. Please carefully address each of the points that they have raise in a revised version of your manuscript.

Pleas also address the following points in your revised version.
- In accordance with EJN policy, please replace bar charts with more informative scatter plots, hybrid plots or similar.
- Include the figure legends at the end of the main text.
- Please supply a graphic abstract and text (see below).
- The abstract contains too many abbreviations and one is even not defined (PND).
- Higher resolution figures will be needed for publication.
- Fig 7: we probably need a better explanation of the axes.

When revising the manuscript, please embolden or underline major changes to the text so they are easily identifiable and please don't leave 'track change' formatting marks in your paper. Please ensure that you provide a text and a figure file for the Graphical Abstract (as detailed in the instructions below). When

carrying out your revisions please refer to the checklist below and visit the EJN author guidelines at www.ejneuroscience.org

When finalized, please upload your complete revised manuscript onto the website, as a Word file (.doc, or .docx). Please also ensure that a complete set of tables and figures is included as separate files, even if these have not changed from the originals. At this stage it is necessary to provide high resolution figures. Please see important instructions below.

Please go into https://mc.manuscriptcentral.com/ejn - Author Centre - manuscripts with decisions where you will find a 'create a revision' link under 'actions'. We ask that you please indicate the way in which you have responded to the points raised by the Editors and Reviewers in a letter. Please upload this response letter as a separate Word (.doc or PDF) file using the file designation "Authors' Response to Reviewers" when uploading your manuscript files. Please DO NOT submit your revised manuscript as a new one. Also, please note that only the Author who submitted the original version of the manuscript should submit a revised version.

If you are able to respond fully to the points raised, we would be pleased to receive a revision of your paper within 12 weeks.

Thank you for submitting your work to EJN.

Best wishes,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 1

Comments to the Author
The paper nicely describe an effect of milk fat globule membrane (MFGM) treatment for preventing some aspects of maternal separation (MS) phenotype in rats. The interest of the study is high since several clinical studies have started on microbiot and early life stress and these results enlight potential treatments and a better understanding of the pathophysiology of early life stress. They also confirm effects of MFGM treatment on the microbiota. The experiments assessing behaviore and visceral sensitivity are robust and well interpreted. Nevertheless, several important points need to be clarified and strengthen before publication:

- The effects of MFGM on the microbiota are not clearly described which blunt the message of the paper. The figures are too small and effects are unvisible by the reader then one need to trust the text. Moreover, the results are very diluted among changes in family, genus and phylum. I would recommand to pick one representation and describe it clearly, by splitting the bar graphs for each family for exemple. A brief explanation of alpha and beta diversity is also missing. Finally, other studies reported defects in microbiota after MS which are not the same (fusibacteria, lachnospiraceae, muscispirillum), and this should be discussed more in details, maybe mentionning differences in mouse lines (germ free versus normal

breeding). Importantly, the only change the author observe after MS are in paenibacillacea and it does not seem to be reversed after MFGM treatment, with our without probiotic, which is also not clearly mentionned. However, the message of MFGM effect on the microbiota which was already described by others is discussed.

-The part on gene expression changes is also weak. The names of the genes, except for the mineralocorticoid receptor, are only found in the material and methods, when all results should be included in a figure and the two results sections need to be fused.These data are of interest for the rescue of MR expression by MFGM sonce this molecular phenotype is stronglt associated with behavioral response. The choice of GABA receptor as stress response genes should be justified. Moreover, the defects in myelin content should be more carefully discussed, since apparently no changes are visible in several others myelin-related genes and other papers show controvesial results. Nevertheless, the authors discusse the potential role of MS on early oligodendrogenesis which is of high interest.

-The fact the myelin content in the hippocampus parralleles the behavioral deficits induced by MS should be emphasized but also discussed more carefullty with regards to the microbiot since their, changes do not present the same parrallelism.

- Discussion is a little messy and is redundant with the result section. A lot of space is spent on describing benefits of dietetary interventions on bdnf, lipipds content etc, which are not directly related to the topic. On the opposite important discussion points are missing, including the time of intervention, where the authors have previously shown that early alteration of the microbiot leads to permanent behavioral impairement but not permanent microbiota impairement, and an other group has described that germ free animals exposed to MS do not present behavioral impairement but develop the behavioral deficits after colonization in adutlhood (De Palma 2014).The discussion section should be reorganized.

-The author mention a amelioration of MS phenotype with MFGM and to a "greater extand" when combined with PDX/GOS, but no statistical analysis was performed.

- In the method section, in control and test diets, the authors mention an adjustment of corn starch, casein, etc.. which might rather be explicited since the mention earlier that the test diets only differe from control diet by inclusion of GSO/PDX or MFGM-1.

Comments on the form:
- p values need the be explicited and not only mentionned as $p<0.05$, especially for a better avaluation of combinatory effect.
- Some references are missing in the reference part, including Timby 2015 and 2017. Please check carefully this section and throughout the document (ie p 20: (Timby et al)(Mudd et all)).

Reviewer: 2

Comments to the Author
The manuscript entitled, “The enduring effects of early life stress on the microbiota-gut-brain axis are buffered by dietary supplementation with milk fat globule membrane and a prebiotic blend” adds to the emerging evidence that gut microbial modulatory diets can have real and long-lasting impacts on the brain and physiology.

The introduction is well-written and the procedures are clearly described. The most compelling finding was that the prebiotic blend reduced visceral pain sensitivity. This effect was found in both control and early-life stressed rats. This is an important contribution to the field. The work is within the scope of the European Journal of Neuroscience. The quality of experimental tools used to measure the dependent outcomes is high.

There are, however, several major issues associated with experimental design that limit the interpretation of many of the results.

1. The rats were housed 3 per group at PND 21. What impact did this group housing have on the gut microbiota? How is this accounted for in the analyses?
2. Behavioral tests were repeatedly performed on the rats in the following order: open field, novel object recognition, Morris water maze. Although they were separated by 1 week, repeated handling and cognitive testing is disruptive to rats in many ways. The stress of handling, reduced sleep, etc. Do the authors have evidence that rat behavior on each of these tasks was not influenced by previous testing?
3. Measures of visceral pain with 24h fasting, and restraint stress also were done on the same rats after all the behavioral testing. What impact does the pain test have on subsequent restraint? What impacts did the behavioral tests have on pain? How did fasting impact any effect of the gut microbiome measured in these same rats?
4. Finally, rats were sacrificed after all of these behavioral tests, pain tests, fasting, and restraint stress; and brain and cecum were removed. Any changes in the brain or cecum microbiota are uninterpretable using this design.

Below are several minor issues that should be addressed.
1. Was food intake measured? Do the dietary supplements impact weight gain?
2. Why was alpha diversity measured using one metric, i.e., Shannon Diversity?
3. In the discussion and figure 4 caption the authors conclude that prebiotic diet blunts the corticosterone response to restraint. The data don't support that conclusion. Instead, it appears that 30 min after restraint termination, rats ingesting microbial modulatory diets returned to baseline more quickly. This likely reflects a facilitation in feedback, not a constraint in drive. This, one could argue, is far more beneficial to health than blunting a highly adaptive, acute glucocorticoid response.
4. Page 21 in the discussion, the authors describe the corticosterone response as the "stress axis". This is vague language and should be avoided. Finally, there is no evidence to suggest that any impact on circulating corticosterone is due to changes in the brain. Any output or feedback difference can occur at any level of the HPA axis.
5. Page 21, middle of the page. Two sentences in a row beginning with "Hence"
6. Page 12, "data" are plural.
7. Why report only 1 measure of alpha diversity?

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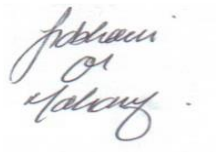
Authors' Response 26-Apr-2019

Dear Professor Foxe,

Thank you and the Reviewers for taking the time to review our manuscript entitled: "The Enduring Effects of Early Life Stress on the Microbiota-Gut-Brain Axis are Buffered by Dietary Supplementation with Milk Fat Globule Membrane and a Prebiotic Blend". We have addressed all of the comments in our manuscript and have written the answers to each of the comments individually below.

We look forward to hearing from you in due course,

Yours Sincerely,



Siobhain O' Mahony PhD

Comments from the Editor

- Changes in the presentation of the results and the discussion in order to clarify the main conclusions that can be derived from the present study.
  - Each of these have been addressed below and amended in the manuscript.
- Reviewer 2 would like to see more methodological caution on the effects of cage grouping and stressful behavioral tests on the results of the microbiota.
  - We have addressed this below.
- Reviewer 1 made useful suggestions to clarify the main effects of the MFGM relative to the dietary supplements in the observed effects and to be more specific on the effects that can or cannot be related to changes in the microbiota.
  - We have done this as described below.
- Please replace bar charts with more informative scatter plots, hybrid plots or similar.
  - We have done so where possible (see Figures 2B, 3A, 3B, 4B)
- Include the figure legends at the end of the main text.
  - We have done this now.
- Please supply a graphic abstract and text (see below).

- We have now included this.
- The abstract contains too many abbreviations and one is even not defined (PND).
- These have been reduced
- Higher resolution figures will be needed for publication.
- These have been provided now
- Fig 7: we probably need a better explanation of the axes.
- We have now added the explanation to the legend and increased the size of the labels.

Reviewer 1

- The effects of MFGM on the microbiota are not clearly described which blunt the message of the paper
- We have now focused on the impact of MFGM more in the discussion.
- The figures are too small and effects are invisible by the reader then one need to trust the text. Moreover, the results are very diluted among changes in family, genus and phylum. I would recommend to pick one representation and describe it clearly, by splitting the bar graphs for each family for example.
- We agree with the Reviewer and we have now included these as supplemental figures. We refer to Table 3 which includes all of the significant changes in microbiota abundance. We had meant to include this in our original version-we apologize for it being missing.
- A brief explanation of alpha and beta diversity is also missing.
- We have now done this in the discussion on page 25 and 26.
- Finally, other studies reported defects in microbiota after MS which are not the same (fusibacteria, lachnospiraceae, muscispirillum), and this should be discussed more in details, maybe mentioning differences in mouse lines (germ free versus normal breeding). Importantly, the only change the author observe after MS are in paenibacillacea and it does not seem to be reversed after MFGM treatment, with our without probiotic, which is also not clearly mentioned. However, the message of MFGM effect on the microbiota which was already described by others is discussed.
- This is now more explicitly discussed on page 23 and 24 of the discussion
- The part on gene expression changes is also weak. The names of the genes, except for the mineralocorticoid receptor, are only found in the material and methods, when all results should be included in a figure and the two results sections need to be fused.
- We have now included a table of the genes analysed in the prefrontal cortex and the hippocampus and more explanation in the discussion has been added.
- The choice of GABA receptor as stress response genes should be justified.
- We have now explained the reasons for the choices on page 11.
- Moreover, the defects in myelin content should be more carefully discussed, since apparently no changes are visible in several others myelin-related genes and other papers show controversial results. Nevertheless, the authors discussed the potential role of MS on early oligodendrogenesis which is of high interest.
- We have now added the Table 2 in with all of the data from our analysis of myelin-related genes. We have also discussed this in more detail in the discussion.

- The fact the myelin content in the hippocampus parallels the behavioral deficits induced by MS should be emphasized but also discussed more carefully with regards to the microbiota since their, changes do not present the same parallelism
  - We agree with this Reviewer-this is a very good point and now there is a more detailed explanation on page 22 in the discussion.
- Discussion is a little messy and is redundant with the result section.
  - We have amended this so there isn't so much overlap between the results and the discussion.
- A lot of space is spent on describing benefits of dietary interventions on bdnf, lipipds content etc, which are not directly related to the topic.
  - We agree and have toned them down in the discussion.
- On the opposite important discussion points are missing, including the time of intervention, where the authors have previously shown that early alteration of the microbiota leads to permanent behavioral impairment but not permanent microbiota impairment, and another group has described that germ free animals exposed to MS do not present behavioral impairment but develop the behavioral deficits after colonization in adulthood (De Palma 2014). The discussion section should be reorganized.
  - We agree with the Reviewer that this is a very relevant point and necessary to include here and have expanded on this concept in the discussion (page 22 and 23).
- The author mentions an amelioration of MS phenotype with MFGM and to a "greater extent" when combined with PDX/GOS, but no statistical analysis was performed.
  - We agree that no statistical tests were used to confirm this we were noting that a greater number of changes were noted from the control group in the group treated with the combination of MFGM and prebiotic which is very easy to see in Table 3.
- In the method section, in control and test diets, the authors mention an adjustment of corn starch, casein, etc.. which might rather be explicited since the mention earlier that the test diets only differ from control diet by inclusion of GSO/PDX or MFGM-1.
  - We have now included Supplemental Table 1 that describes all of the diet components.
- - p values need the be explicited and not only mentioned as $p<0.05$, especially for a better evaluation of combinatory effect.
  - These are now described in more detail.
- Some references are missing in the reference part, including Timby 2015 and 2017. Please check carefully this section and throughout the document (ie p 20: (Timby et al)(Mudd et all)).
  - Apologies these were incorrect and have now been amended.

Reviewer 2

- The rats were housed 3 per group at PND 21. What impact did this group housing have on the gut microbiota? How is this accounted for in the analyses?
- This is an interesting point given the coprophagic nature of these animals. Co-housing with 3 rats per cage is acceptable for microbiota analysis and we have shown this on several occasions that this is enough to see differences between groups if they exist.
- Behavioral tests were repeatedly performed on the rats in the following order: open field, novel object recognition, Morris water maze. Although they were separated by 1 week, repeated handling and

cognitive testing is disruptive to rats in many ways. The stress of handling, reduced sleep, etc. Do the authors have evidence that rat behavior on each of these tasks was not influenced by previous testing?

- The Reviewer has a very valid point. In line with the 3Rs and recommendations from our ethical committees both local and national the number of tests and rest time we give the rats is appropriate. We have previously carried out less tests on rats and the results here are comparable in the control groups (e.g. O'Mahony et al., 2009; 2014).

- What impact does the pain test have on subsequent restraint?
  - Again, this is a very valid point that the Reviewer makes. We do give a washout period of a week for the rats to recover from the colorectal distension before we carry out the stress responsivity test. We include a caveat in the methods to account for the potential of behavioural interference.
- What impacts did the behavioral tests have on pain?
  - We agree that this is also a very valid question and here again we give the rats a week to rest after the Morris water maze before doing the colorectal distension and whilst we cannot say for sure there is no residual effect of the previous tests on the pain behaviours the results we see in the controls groups are comparable to what we have noted before (O'Mahony et al., 2009). Again, all rats are exposed, and we report the schedule of tests.
- How did fasting impact any effect of the gut microbiome measured in these same rats?
  - This is a very valid question. The rats were fasted the night before colorectal distension and we collected the samples for microbiota analysis 2 weeks later and hence we do not expect the fasting to still have an impact at this time.
- Finally, rats were sacrificed after all of these behavioral tests, pain tests, fasting, and restraint stress; and brain and cecum were removed. Any changes in the brain or cecum microbiota are uninterpretable using this design.
  - We thank the Reviewer for noting this and ideally if would be best to analyse the samples from rats not exposed to any behavioural tests but as mentioned above in accordance with the 3Rs and recommendations from our ethical committees the number of rats and tests used is appropriate. Also it is beneficial to be able to correlate behaviour and molecular analysis in the same animals.
- Was food intake measured?
  - This was measured and did not differ between groups.
- Do the dietary supplements impact weight gain?
  - The dietary supplements did not impact on weight gain.
- Why was alpha diversity measured using one metric, i.e., Shannon Diversity?
  - We used several but none showed a significant difference.
- In the discussion and figure 4 caption the authors conclude that prebiotic diet blunts the corticosterone response to restraint. The data don't support that conclusion. Instead, it appears that 30 min after restraint termination, rats ingesting microbial modulatory diets returned to baseline more quickly. This likely reflects a facilitation in feedback, not a constraint in drive. This, one could argue, is far more beneficial to health than blunting a highly adaptive, acute glucocorticoid response.
  - We thank the Reviewer for this comment as the explanation of the effects are clearer now and a better and more accurate description of events. We have made changes to the abstract, results, legend for Figure 4 and the discussion on page 21.
- Page 21 in the discussion, the authors describe the corticosterone response as the "stress axis". This is vague language and should be avoided. Finally, there is no evidence to suggest that any impact

on circulating corticosterone is due to changes in the brain. Any output or feedback difference can occur at any level of the HPA axis.

- We agree and have amended this.
- Page 21, middle of the page. Two sentences in a row beginning with “Hence”
- Thank you-the second “Hence” has been changed to “Furthermore”
- Page 12, “data” are plural.
- This has been amended.
- Why report only 1 measure of alpha diversity?
- We responded to this query above also. We used several measures of alpha diversity but given none were significant we did not report them.

2nd Editorial Decision 28-May-2019

Dear Dr. O' Mahony,

Your revised manuscript has been re-evaluated by one of the external reviewers as well as by the Section Editor, Dr. Patricia Gaspar and ourselves. We are pleased to inform you that we expect that it will be acceptable for publication in EJN after some minor revisions.

As you can see below, although the reviewer indicated that your manuscript has been substantially improved, he/she raises a few issues that need to be addressed more effectively. Please carefully respond to each point raised in a revised version which will not require re-review but will be dealt with by the Editors.

When finalised, please upload your complete revised manuscript onto the website as a Word (.doc or .docx), or .rtf file. Please also ensure that a complete set of tables and figures is included as separate files, even if these have not changed from the originals. At this stage it is necessary to provide high resolution figures. Please see important instructions below.

Please go into https://mc.manuscriptcentral.com/ejn - Author Centre - manuscripts with decisions where you will find a 'create a revision' link under 'actions'. We ask that you please indicate the way in which you have responded to the points raised by the Editors and Reviewers in a letter. Please upload this response letter as a separate Word (.doc) file using the file designation "Authors' Response to Reviewers" when uploading your manuscript files. Please DO NOT submit your revised manuscript as a new one. Also, please note that only the Author who submitted the original version of the manuscript should submit a revised version.

If you are able to respond fully to the points raised, we shall be pleased to receive a revision of your paper within 30 days.

Thank you for submitting your work to EJN.

EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Kind wishes,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 1

Comments to the Author
The authors have addressed the main points asked in the revisions. Nevertheless, several corrections could still improve the paper, mainly in the discussion section which still should be tempered. After these corrections i would accept the paper for publication.
p16: hippocampal genes expression paragraph AND myelin related genes: the name of tested genes should appear in the text.
p19 last paragraph: 2 in iterations in the first sentence
p20 first sentence: Their scores were not different that breast fed controls potentially indicating that the beneficial impact of breast feeding was due to MFGM. This suggestion goes too far from their data, elthough it might have been discussed in the cited papers, and I would remove it.
p20 second paragraph: the authors mention "specific brain regions or neurotransmetters pathways" that should be explicited.
p22: "What is interesting here os that the changes in MAG are related to changes in behavior in our MS model but not in microbiota hence indicating that some of the impact of behavior on stress and dietetary intervention may be due to changes in myelination patterns". This sentence is wrong because (i) parrallel changes in myelin and behavior only concern the time in the quadrant from the MWM, not pain behavior, neither novel object. (ii) no causality has been demonstrated or even could be suggested from myelin. Nevertheless, the correlation remains interesting.
In general, the term behavior should me more specifies throughout the text.
p23: This indicates that possible for the stress associated hypersensitivity of the brain gut axis to remain, despite apprent recovery of the gut microbiota composition. I am surprised no other papers have associated transient microbiot changes with permanent behavioral alterations. This should be cited here.
p26 first paragraph: the definition of alpha and beta diversities belongs to the result section where it is first mentionned. Also "This suggest that the subtle changes in relative abondance were also noted in beta diversity" This sentence should be removed as it is a non significant result.

Authors' Response 11-Jun-2019

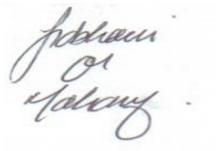
Dear Professors Foxe and Bolam,

Thank you, the Reviewer and Dr. Gaspar for taking the time to review our manuscript entitled: "The Enduring Effects of Early Life Stress on the Microbiota-Gut-Brain Axis are Buffered by Dietary Supplementation with Milk Fat Globule Membrane and a Prebiotic Blend". We have addressed all the

comments in our manuscript and underlined the new changes and have written the answers to each of the comments individually below.

We look forward to hearing from you in due course,

Yours Sincerely,



Siobhain O' Mahony PhD

Reviewer 1

- p16: hippocampal genes expression paragraph AND myelin related genes: the name of tested genes should appear in the text.
  - We have now included these and underlined them-they are in the methods and results section on page 16 and 17.
- p19 last paragraph: 2 in iterations in the first sentence
  - Thank you-we have removed one „in"
- p20 first sentence: Their scores were not different that breast fed controls potentially indicating that the beneficial impact of breast feeding was due to MFGM. This suggestion goes too far from their data, although it might have been discussed in the cited papers, and I would remove it.
  - We agree with the Reviewer and have removed this statement.
- p20 second paragraph: the authors mention "specific brain regions or neurotransmetters pathways" that should be explicited.
  - We have now expanded this section to be more descriptive and included references on page 20.
- p22: "What is interesting here is that the changes in MAG are related to changes in behavior in our MS model but not in microbiota hence indicating that some of the impact of behavior on stress and dietetary intervention may be due to changes in myelination patterns". This sentence is wrong because (i) parrallel changes in myelin and behavior only concern the time in the quadrant from the MWM, not pain behavior, neither novel object. (ii) no causality has been demonstrated or even could be suggested from myelin. Nevertheless, the correlation remains interesting. In general, the term behavior should me more specifies throughout the text.
  - We have now toned this down and have been more specific with regard to the type of behaivour throughout.

- p23: This indicates that possible for the stress associated hypersensitivity of the brain gut axis to remain, despite apprent recovery of the gut microbiota composition. I am surprised no other papers have associated transient microbiot changes with permanent behavioral alterations. This should be cited here.
  - We agree with the reviewer and have now included our own paper which shows just this on page 23.
- p26 first paragraph: the definition of alpha and beta diversities belongs to the result section where it is first mentionned. Also "This suggest that the subtle changes in relative abondance were also noted in beta diversity" This sentence should be removed as it is a non significant result.
  - We agree and have now put the explanations on page 23 and 24. We have removed the suggested line also.