Reviewer #1:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors:

SPECIFIC COMMENTS TO THE AUTHOR GENERAL COMMENTS:

Authors study the role of microbiota and TLR receptors on an animal model of colonic hypersensitivity. They found that neonatal maternal separation (NMS) in mice induced fecal microbiota dysbiosis characterized by a general decrease in bacterial species richness although in some genera the abundance of bacteria was increased. In addition, NMS induced overexpression of TLR5 in colonocytes whereas the TLR5 agonist flagellin mimic colonic hypersensitivity evoked by NMS. This paper presents interesting data, however I consider that it should be improved at several points shown below.

We would like to thank reviewer #1 for her/his really constructive comments. We have now integrated all manuscript modifications in order to alleviate his concerns.

SPECIFIC COMMENTS:

Materials and Methods: The work is focused on the study of the pathophysiological pathways involved in the development of colonic hypersensitivity. Authors use the colorectal distension test with a pressure transducer catheter coupled to a balloon, an electronic barostat and a system to acquire the signal from the transducer. However, no information is given on the quantification of this signal to show the intracolonic pressure variation (IPV) and to obtain the area under the curve (AUC). In addition, authors should provide information on how these two parameters can reflect an increase in the colonic hypersensitivity.

Information about CRD protocols, and how we proceed to calculate the intracolonic pressure variation (IPV), reflecting the colonic sensitivity, in response to CRD, has been already discussed in previous papers. It was firstly published by Larauche *et al.* (2010) (Reference #18 in the revised manuscript), but also published in our laboratory in British Journal of Pharmacology by Picard *et al.* (2019) (Reference #19 in the revised manuscript). Thus, we have now added in the revised manuscript a brief description on how IPV calculations are made: "After intracolonic pressure recording for each animal along the CRD protocols and signal treatment as previously described [18], intracolonic pressure variation (IPV), reflecting the colonic sensitivity, was calculated as previously described [19] for each distension pressure. Briefly, IPV was calculated by subtracting the integral (area under the curve) of the treated signal corresponding to the 20s preceding the CRD from the integral (area under the curve) of the treated signal during the 20s of CRD stimulation" (lines 158-163 of the revised manuscript).

What numerical criteria have been followed to include NMS animals in each of the following categories: NMS NS and NMS?

We have now added in the Materials & Methods section, a description of the numerical criteria followed to include NMS animals in each category. Those criteria have already been used in our laboratory for another model of CHS induced by bacterial infection (Lashermes *et al.*, 2018 - Reference #20 in the revised manuscript): "Therefore, two groups of NMS mice were defined: NMS non-sensitized (NMS NS) and NMS sensitized (NMS S) mice. The NMS S animals are distinguished according to the area under the curve (AUC) value in response to the distention pressures from 60 to 100 mmHg during CRD procedure [20]. Briefly, if this value is higher than the average AUC of the NH control animals plus twice the SEM value ($AUCN_{MS\ S} \geq AUC_{NH} + 2xSEM_{NH}$), this mouse is considered as

Statistical test used to check if data follow a normal distribution should be given.

We have added in the "statistical analysis" part of the "Materials and methods" section that the kolmogorov-smirnov has been used to check if data follow a normal distribution (lines 231-232 of the revised manuscript).

Results: In general, the Results section is hard to read due to the large amount of numerical data that are written in the text. I suggest to remove all data about means ± SEM in the text of the Results section as well as the statistical significance because they are already shown in the figures.

Sorry for the large amount of written results. We are used to write it but we have now remove all those written data (means ± SEM or statistical significance) on the revised manuscript. We hope it is now more pleasant to read it through.

Why data showing the relative abundance of bacteria belonging to the phylum Bacteroidetes and Firmicutes are not given and only is expressed as "data not shown"? I consider that it is a relevant result and indeed authors used it in the Discussion section.

The data showing the relative abundance of bacteria belonging to the phylum *Bacteroidetes* and *Firmicutes* has been now added in the revised version of the manuscript (lines 265-268 of the revised manuscript and Figure 3C of the revised figures).

In P12 lines 255-266: This paragraph is confusing: 1) This statement is not correct: "NMS S mice were characterized by a decreased abundance of bacteria of the genera Bacteroides …, Barnesiella … and Allobaculum … compared to control NH mice". In fact, as can be seen in Fig. 3C, Bacteroides are less abundant in NMS S mice when compared with NMS NS. However, it is similar to NH mice. Thus, significance of NMS S observed in figure is "$$$" and not "***". 2) In the same paragraph authors stated: "whereas the relative abundances of Clostridium … and Lachnoclostridium … were increased in these NMS animals with CHS". This is not true because Fig 3C shows that the relative abundance of Clostridium in NMS S animals is similar to NH animals. Only NMS S animals shows fewer abundance compared to NMS NS animals ($).

We are sorry for our mistake on the original manuscript. We have now made corrections in the revised version of the manuscript to make it clearer (lines 269-275 of the revised manuscript).

This point is very important because this result is also misunderstood in the Discussion section (P15 lines 342-343) Thus, I suggest rewriting this paragraph in Results and Discussion sections with more precise comparisons.

We have now corrected the results section and also made some modifications in the Discussion section after removing comparison to control NH mice and to keep only the NMS NS animals, which are mice without CHS (lines 344-346 of the revised manuscript).

Abstract: The Results section of the abstract is too short. Author should explain more in deep their main results, particularly those of fecal microbiota diversity. If it is necessary, Material and methods section can be shortened.

We have added more details in the Abstract about our results on microbiota analysis: "… characterized by a significant decrease of species richness, an alteration of the core fecal microbiota and a specific increased relative abundance of flagellated bacteria" (lines 59-60 of the revised manuscript).

Figures: The text of the figure legends is too large. I suggest deleting the statistical test used in the experiments. In Fig. 1B, the term "20" (minutes) that appear just to the right of "Intrarectal

instillation" I think it should be replaced with "30" Legend of Fig. 2: 1) I suggest changing the statement: "* or $ p.

On the figure 1B, it is the term "20" which should be there. In fact, the CRD protocol after FliC instillation last 20 min so we defined the reference point for the "30 min" post FliC instillation as in the middle of the distension ramp, which is 10 min. Thus, we used a 20 min resting period before to start the "30 min" ramp CRD. About the text of the figure legends being too large, we have removed all information about statistical test used. We hope that figure legends are now easier to read.

<u>Reviewer #2:</u>

Scientific Quality: Grade B (Very good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Accept (General priority)

Specific Comments to Authors:

It is well written paper with accepted abstract ,introduction ,method and discussion results section need simplified tables to present the results it would be a good advantage.

As suggested by the reviewer #1, we have removed all data about means ± SEM in the text of the Results section as well as the statistical significance because they are already shown in the figures as mentioned by the reviewer #1. We hope it is now more pleasant to read it through.

Science editor:

It is an interesting study, relevant results have impact. The methodological approach is well-designed and the discussion well-grounded.

Language Quality: Grade B (Minor language polishing)

Scientific Quality: Grade B (Very good)

Company editor-in-chief:

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors.

Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...".

All figure legends have been changed accordingly to the guidance.

Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file.

A new file named "Revised figures" has been added.

Please authors are required to provide standard three-line tables, that is, only the top line, bottom line, and column line are displayed, while other table lines are hidden. The contents of each cell in the table should conform to the editing specifications, and the lines of each row or column of the table should be aligned. Do not use carriage returns or spaces to replace lines or vertical lines and do not segment cell content.

The table 1 has been changed accordingly to the guidance.

Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022.

The Copyright ©The Author(s) 2022 has been added to the PPT file.

Before final acceptance, when revising the manuscript, the author must supplement and improve the highlights of the latest cutting-edge research results, thereby further improving the content of the manuscript. To this end, authors are advised to apply a new tool, the Reference Citation Analysis (RCA). RCA is an artificial intelligence technology-based open multidisciplinary citation analysis database. In it, upon obtaining search results from the keywords entered by the author, "Impact Index Per Article" under "Ranked by" should be selected to find the latest highlight articles, which can then be used to further improve an article under preparation/peer-review/revision. Please visit our RCA database for more information at: https://www.referencecitationanalysis.com/.

Response to Second round review

<u>Reviewer #1:</u>

SPECIFIC COMMENTS TO THE AUTHOR

Authors have answered and included in the manuscript satisfactorily all the main questions. However, several minor questions that were included in my referee's comments sent in April have not been answered. They are some minor concerns in the text, and especially in the figures and figure legends. Then, I consider that this paper could be improved at several points shown below before it will be published.

We sincerely apologies about missing responses to the rewiewer's comments after the first revision step. I used the comments received by email and I just realized they were not complete. We have now edited our manuscript in order to answer all rewiewer's minor comments. We hope it is now acceptable for publication.

<u>Figures and Figure legends:</u>

Fig. 3D: Error bars representing SEM are absent, whereas they appear in the Fig 3C.

Done.

Figures 4A and 4C: To avoid confusion, please use the symbol "$" instead "*" in comparisons between NMS S and NMS NS groups, and include this symbol in the figure legend.

Done.

In Figs 2A, 5A and Suppl 1A, in the Y axis, the term "Intracolonic Variation Pressure" should be replaced with "Intracolonic Pressure Variation".

Done.

In Figs 2C and Suppl 1A, in the Y axis, the term "Dextran-FITC" should be replaced with "FITC-Dextran".

Done.

In Figs, 2A, 5A and Suppl 1A, the number of animals used in the experiments ("n=…") can be removed because they are in the Figure legends.

Done. We have also removed this from the figures 3A, 3C and D.

In Fig. Suppl 1A the number of animals used for NMS NS is different in the figure ("n=7") and in the legend of this figure ("n=6").

The right number is n=7 so we have changed the figure legend.

Legend of Fig. 2: I suggest changing the statement: "* or \$ p<0,05; ** or \$\$ p<0,01; \$\$\$=\$ p<0,001, respectively vs. NH or NMS NS groups" by this one: "* p<0,05 and ** p<0,01 vs. NH group; and \$ p<0,05, \$\$ p<0,01 and \$\$\$ p<0,001 vs. NMS NS group"

Done. We have also changed this statement in the figure legends for figure 4A and 4C, following the same rules.

Furthermore, I suggest inserting "and FITC-Dextran" in the last statement: "For AUC and FITC-Dextran, each dot represents one mouse and red lines represent means"

Done.

Legend of Fig. 5: Remove the sentence: "Values are expressed as means and error bars represent SEM", because it is repeated later in the same legend: "dots represent means and error bars represent SEM".

Done.

In addition, I suggest replacing the statement of statistical significance with a shorter one: "*p<0,05, **p<0.01 and ***p<0.001 respect to Baseline".

Done.

Figure legends should be self-explanatory as possible. Thus, some abbreviations should be included in full: "NMS" (in Fig. 1), "OTU" (in Fig. 3) and "FliC" (in Figs. 1, 4 and 5).

Done.

Minor comments in the text:

The scientific name of one species should be in full the first time that it appears in a text, thus replace "E. coli" with "Escherichia coli" in the Materials and Methods section

Done.

"Quantitative Insights into Microbial Ecology pipeline (QIIME)" is twice times abbreviated. In the last paragraph of Materials and Methods you can substituted it by "QIIME".

Done.

The same can be applied by "Flagellin (FliC)", thus it can be replaced with "FliC" the second time that it appears in Materials and Methods.

Done.

The opposite occurs with the term "OTU" that it does not appear in full in the text. Please, replace "OTU" with "Operational Taxonomic Units (OTU)" the first time that it appears in the text

Done.

Replace "LPS" with "lipopolysaccharide (LPS)" the first that it appears in the Introduction section and remove other "lipopolysaccharide (LPS)" that can be found thereafter in the text.

Done.

Replace "Dextran-FITC" with "FITC-Dextran" in the Results section.

Done.

"kolmogorov-smirnov test" should be in capital letters: "Kolmogorov-Smirnov test".

Done.