

May 27<sup>th</sup>, 2020

## LETTER IN RESPONSE TO THE REVIEWER'S COMMENTS

Dear Dr. Subrata Ghosh and Andrzej S Tarnawski, MD, PhD  
Editor-in-Chief of the *World Journal of Gastroenterology*

We are resubmitting our Manuscript No.: 56606 entitled: “Lactobacillus bulgaricus inhibits colitis-associated cancer via a negative regulation of intestinal inflammation” to the *World Journal of Gastroenterology*.

We sincerely appreciate your valuable feedback and would like to thank you for all suggestions and comments. All comments are addressed and summarized below with changes indicated in **bold** font in the main text. All co-authors have agreed to the resubmission with these revisions.

We would like to justify the inclusion of an author who is a Pathologist (MD, Ph.D.) who carried out the histological analysis for tumor sections as well as interpreted the data in her lab (as per suggested for Reviewer 2). Additionally, this author have contributed critically revised and approved this final manuscript version.

Thank you again for the privilege of having our work reviewed and improved by this peer review process. We are truly grateful.

### ▪ Comments from the Editorial Office:

We are pleased to inform you that, after preview by the Editorial Office and peer review as well as CrossCheck and Google plagiarism detection, we believe that the academic quality, language quality, and ethics of your Manuscript No.: 56606 basically meet the publishing requirements of the *World Journal of Gastroenterology*. As such, we have made the preliminary decision that it is acceptable for publication after your appropriate revision. Upon our receipt of your revised manuscript, we will send it for re-review. We will then make a final decision on whether to accept the manuscript or not based on the reviewers' comments, the quality of the revised manuscript, and the relevant documents.

***Response: Thank you for the privilege of having our work reviewed and improved by this peer review process. We are truly grateful.***

## Step 1: Please select revise this manuscript or not

Please login to the F6Publishing system at <https://www.f6publishing.com> by entering your registered E-mail and password. After clicking on the “Author Login” button, please click on the “Manuscripts Needing Revision” under the “Revisions” heading to find your manuscript that needs revision. Clicking on the “Handle” button allows you to choose to revise this manuscript or not. If you choose not to revise your manuscript, please click on the “Decline” button, and the manuscript will be WITHDRAWN.

**Response:** *Ok. Done.*

## Step 2: Key points of revising the manuscript

(1) **Scientific quality:** Please resolve all issues in the manuscript based on the peer review report and make a point-to-point response to the issues raised in the peer review report.

**Response:** *Ok. Done.*

(2) **Language quality:** Please resolve all language issues in the manuscript based on the peer review report. Please be sure to have a native-English speaker edit the manuscript for grammar, sentence structure, word usage, spelling, capitalization, punctuation, format, and general readability, so that the manuscript’s language will meet our direct publishing needs.

**Response:** *Ok. Done.*

(3) **Special requirements for figures:** Figures must be presented in the order that they appear in the main text of the manuscript (numbered as 1, 2, 3, *etc.*). The requirements for the figures and figure legends include: (A) All submitted figures, including the text contained within the figures, must be editable. Please provide the text in your figure(s) in text boxes; (B) For line drawings that were automatically generated with software, please provide the labels/values of the ordinate and abscissa in text boxes; (C) Please prepare and arrange the figures using PowerPoint to ensure that all graphs or text portions can be reprocessed by the editor; and (D) In consideration of color-blind readers, please avoid using red and green for contrast in vector graphics or images.

**Response:** *Ok. Done! We have prepared as well as arranged all the figures using PowerPoint to ensure that all graphs or text portions can be processed by the editor as per recommendation.*

(4) **Special requirements for tables:** Tables must be presented in the order that they appear in the main text of the manuscript (numbered as 1, 2, 3, *etc.*). Please verify that the tables are referred to in the text by their respective Roman numerals and that the numbering order is correct and format the tables. Please verify that there are no missing or multiple spaces in the text and tables, *e.g.* before or after parentheses, between words, or before or after symbols like +, ×, ±, <, >, ≥, and ≤. Please verify that the special words or letters in the text and tables are correct, *e.g.* *P* (uppercase), *n* (lowercase), *via*, *vs* (lowercase, no punctuation), *in vivo*, *in vitro*, and *et al* (no punctuation) are italicized. **Response:** *Ok. Done.*

**(5) *Special requirements for references:*** Please provide the PubMed numbers and DOI citation numbers to the reference list and list all authors of the references. Please revise throughout. The author should provide the first page of the paper without PMID and DOI numbers. NOTE: The PMID is required, and NOT the PMCID; the PMID number can be found at <https://pubmed.ncbi.nlm.nih.gov>. (Please begin with PMID:) The DOI number can be found at <http://www.crossref.org/SimpleTextQuery/>. (Please begin with DOI: 10.\*\*). Please verify that the references are cited by Arabic numerals in square brackets and superscripted in the text, and that the numbering order is correct. There should be no space between the bracket and the preceding word or the following punctuation. When references in the text and tables are cited with author name(s), it is necessary to manually verify that the name(s) is consistent with the first author's surname in the corresponding reference list.

***Response: Ok. Done. All references were verified again.***

**(6) *Special requirements for article highlights:*** If your manuscript is an original study (basic study or clinical study), meta-analysis, systemic review, the “article highlights” section should be provided. Detailed writing requirements for “article highlights” can be found in the Guidelines and Requirements for Manuscript Revision.

***Response:: Ok. Done. As our manuscript is a basic study we have added the “article highlights” as requirements. So, the “article highlight” section was written at the end of the main text as recommended.***

**(7) *Ethical documents:*** Please double check the accuracy of all ethical documents and verify the completeness of the documents according to the type of manuscript.

***Response:: Ok. Done.***

**(8) *Approved grant application form(s) or funding agency copy of any approval document(s):*** If your manuscript has supportive foundations, the approved grant application form(s) or funding agency copy of any approval document(s) must be provided.

***Response: Ok. Done. We have provided and uploaded the funding agency copy of the approval document from the Conselho Nacional de Desenvolvimento Científico e Tecnológico/ Brazilian National Council for Scientific and Technological Development (CNPq) - as per suggested. Grant Process Number. 140152/2013-0.***

### Step 3: Manuscript revision deadline

The authors must resolve all issues in the manuscript based on peer-review report(s) and make a point-to point response to the issues raised in the peer-review report(s) which listed below:

- **Reviewer #1:**  
**Scientific Quality:** Grade B (Very good)  
**Language Quality:** Grade B (Minor language polishing)  
**Conclusion:** Major revision

#### LIST OF RESPONSES TO THE REVIEWERS' 1 COMMENTS

Reviewer 1 Comments	Authors' Revisions
<ul style="list-style-type: none"><li>• Major points:<ul style="list-style-type: none"><li>- In the manuscript entitled “Lactobacillus bulgaricus inhibits colitis-associated cancer via a negative regulation of intestinal inflammation” the authors present the possibility of prevention of colitis-associated cancer using probiotics (Lactobacillus bulgaricus). I read your document with interest.</li></ul></li></ul>	<b>Response:</b> We appreciate your willingness to revise our manuscript with interest and thank you for all your comments.
1. You showed that the tumor volume and size in Lb treated group were lower. I am interested in these results. Does the tumor develop earlier in the control group? This is a question about the timing of tumor development.	<b>Response:</b> We would like to thank you for this important question. Unfortunately, we did not have colonoscopy or similar equipment available to check tumor development in alive mice. Hence, we were not able to see the exact time that the tumors started to develop. Tumor volumes were only measures at the end of the experiment after euthanasia.
2. In early human CAC, the tumor has usually flat shape. What is the shape of the tumor in this study? Please consider the shape of the tumor.	<b>Response:</b> Although we did not check tumor development in different time points, it is already well established that this AOM/DSS model develops tumors with flat shapes similarly to that ones in humans (De Robertis et al., 2011. doi: 10.4103/1477-3163.78279).
3. Was there a relationship between the size/volume of the tumor and cytokine production?	<b>Response:</b> Thanks for your interesting comment. In agreement with it and as an indicative of some relationship, we have shown that animals treated with Lb exhibited smaller tumors and concomitant decreased levels of proinflammatory cytokines at the end of the experiment when compared to controls. However, although we performed non-parametric bivariate analyzes, we did not include correlation matrix analyzes by Spearman's rank correlation coefficient

	between the independent and dependent variables.
4. CAC is caused by long-term inflammation usually over 10 years. Do you think there is any difference between the acute phase or the chronic phase? Furthermore, based on your research results, what kind of protocol can you consider for clinical application in humans? (probiotics admission period etc.)	<p><b>Response:</b> We really appreciate your comment and suggestion. We have also improved the discussion section related to this topic.</p> <p>Dysregulation of gut microbiota has been associated with increased inflammation and the administration of probiotics have been reported to prevent chronic inflammatory diseases [41]. In recent years there has been growing interest in the possible application of probiotics as a part of combination therapy with conventional treatment of cancer [41,42,43]. However, studies investigating probiotics effects in patients with CRC are still very limited. For clinical application in humans many other studies, mainly randomized controlled trials would be needed to better evaluate the dosage, duration of the intervention and host physiology for confirm these findings [41].</p> <p>In addition, based on literature findings, we believe that a greater amount of probiotic may be necessary for cancer control (<a href="#">Bhagavathi Sundaram Sivamaruthi, et al 2020 doi: 10.1155/2020/3535982</a>; Gianotti L, et al 2010 doi: 10.3748/wjg.v16.i2.167). As prevention we believe that the use of the probiotic 3 times a week would be appropriate, since the bacteria survive for a few days in the intestine. However, as a therapy the daily use for a minimum of 4–6 weeks of the probiotic throughout conventional treatment would be a good possibility (Górska A et al 2019, 10.1007/s00284-019-01679-8).</p>
5. Looking at the title, it feels like a paper targeted human CAC.	<p><b>Response:</b> Thanks for your valuable comment. In order to provide a better understanding of our study we had made some few adjustments in the title including “in AOM/DSS model”. Title: “<i>Lactobacillus bulgaricus</i> inhibits colitis-associated cancer via a negative regulation of intestinal inflammation in AOM/DSS model”.</p>
<ul style="list-style-type: none"> <li>Minor points</li> </ul> <p>6. In this manuscript, you did not show the incidence of tumors in detail. I think you had</p>	<p><b>Response:</b> Thanks for your suggestion. As per suggested and to better describe the range of tumor numbers in each group of mice, we changed the type of graphic to</p>

<p>better describe the range of tumor numbers. Because we would like to know if there are variations in the tumor numbers in each case.</p>	<p>illustrate this variation. In addition, we also changed the sentence “Control mice developed, on average, 9 colorectal tumors, whereas animals treated with L. bulgaricus developed only 3 (Figure 1B).” in the text to “Control mice developed between 4-13 colorectal tumors, whereas animals treated with L. bulgaricus developed only 1-5 (Figure 1B).”</p>
<p>7. I think you wrote the LAB incorrectly in the background of the abstract section.</p>	<p><b>Response:</b> Thanks for the correction. We deleted the repeated words in the sentence of the abstract section as shown: “Previous studies have indicated that lactic acid bacteria (LAB) <u>bacteria (LAB)</u> could be successfully used in managing sporadic CRC, however little is known about their role in CAC.”</p> <p>“Previous studies have indicated that lactic acid bacteria (LAB) could be successfully used in managing sporadic CRC, however little is known about their role in CAC.”</p>

- **Reviewer #2:**  
**Scientific Quality:** Grade C (Good)  
**Language Quality:** Grade A (Priority publishing)  
**Conclusion:** Minor revision

## LIST OF RESPONSES TO THE REVIEWERS' 2 COMMENTS

Reviewer 2 Comments	Authors' Revisions
The authors describes in this manuscript following points: 1) Lactobacillus inhibits tumorigenesis in AOM/DSS model 2) Lactobacillus attenuates intestinal inflammation 3) Lactobacillus attenuates inflammatory cytokine production in non-tumorous lesions 4) Lactobacillus attenuates inflammatory cytokine levels in tumor lesions. The study is straightforward in the point of the inhibitory role of Lactobacillus on inflammation in AOM/DSS model. The phenotype observed is interesting and may be important in the IBD field to prevent inflammation-associated tumorigenesis.	<b>Response:</b> We thank you for your comments. The phenotype observed in our study is indeed a relevant aspect that may be important in the IBD field to prevent inflammation-associated tumorigenesis.
The major point which should be determined before further consideration is the molecular mechanisms how Lactobacillus inhibits intestinal inflammation in this model. The authors should provide some evidence about the possible molecular mechanisms how Lactobacillus inhibited intestinal inflammation.	<b>Response:</b> We appreciate your questioning and we added this point in the discussion of the paper. Also, we have added the study limitations. Several researches have indicated that the use of probiotics might improve beneficial microbiota, induce the release of antimicrobials and anticarcinogenic agents that help to remove carcinogens, and modulate immune responses that decrease intestinal inflammation in CRC patients (Bhagavathi Sundaram Sivamaruthi, et al 2020 doi: 10.1155/2020/3535982; Marta Molska, Julita Reguła, 2019 doi: 10.3390/nu11102453; Maya Raman et al, 2012, doi.org/10.4161/gmic.23919). Here, we have shown that <i>L. bulgaricus</i> inhibited colitis-associated cancer via a negative regulation of intestinal inflammation. Although a deeper characterization of the molecular mechanisms underlying <i>L. bulgaricus</i> anti-inflammatory activity, the strength of our findings indicates a relevant and evidenced phenotypic pattern, which may be important in IBD field to prevent inflammation-associated tumorigenesis. To the best of our knowledge this is the first

	study to investigate and provide promising evidences of a preventive effect of the probiotic <i>L. bulgaricus</i> in cancer development in an experimental model of CAC.
<p>Minor points</p> <p>1. Please provide results that <i>Lactobacillus</i> is actually colonized in large intestine in these experiments.</p>	<p><b>Response:</b> In order to answer this question, we would have to do more specific experiments. However, there are indices that <i>Lactobacillus</i> may remain in this region and contribute to protect against CRC. (Marta Molska, Julita Reguła, 2019 doi: 10.3390/nu11102453; Maya Raman et al, 2012, doi.org/10.4161/gmic.23919). In addition, numerous useful compounds produced and metabolized by gut microbiota have been demonstrated to play an essential role in maintaining homeostasis and suppressing carcinogenesis (Bhagavathi Sundaram Sivamaruthi, et al 2020 doi: 10.1155/2020/3535982).</p>
<p>2. Please provide histological findings during the course of the experiments.</p>	<p><b>Response:</b> We have included one paragraph and Supplementary figure (Supplementary File 1) to histologically illustrate the tumors as per suggested. Besides, we also added a paragraph describing the histological analysis into the methods section. “After histopathological evaluation of the tumor sections, we observed that, regardless of treatment, both groups of mice presented morphologically similar neoplastic lesions. In general, colorectal tumors were lesions of the polypoid adenoma type with variation between low and high degrees of dysplasia and mixed inflammation (Supplementary File 1)”. These analyses were conducted by the Pathologist (MD, Ph.D.), who were included as author of our paper.</p>
<p>3. It is better to add some experimental results without AOM treatment.</p>	<p><b>Response:</b> This is a great comment. We are also interested in these findings. So, we have already initiated experiments to investigate the role of <i>L. bulgaricus</i> in DSS colitis (without AOM). However, we still have to finish them. For sure, we look forward to publish these data in future.</p>



#### Step 4: Editorial Office's comments

The author must revise the manuscript according to the Editorial Office's comments and suggestions, which listed below:

**(1) *Science Editor*:** 1 Scientific quality: The manuscript describes a basic study of the colitis-associated cancer. The topic is within the scope of the WJG. (1) Classification: Grade B and Grade C; (2) Summary of the Peer-Review Report: Reviewer#02527569 thinks this is an interesting study. The phenotype observed is interesting and may be important in the IBD field to prevent inflammation-associated tumorigenesis. The major point which should be determined before further consideration is the molecular mechanisms how *Lactobacillus* inhibits intestinal inflammation in this model. The authors should provide some evidence about the possible molecular mechanisms how *Lactobacillus* inhibited intestinal inflammation. Reviewer#03805206 summarized the authors present the possibility of prevention of colitis-associated cancer using probiotics. However, there are some issues should be addressed. The questions raised by the reviewers should be answered; and (3) Format: There are 4 figures. A total of 40 references are cited, including 5 references published in the last 3 years. There are no self-citations. 2 Language evaluation: Classification: Grade A and Grade B. A language editing certificate issued by AB Traduções was provided. 3 Academic norms and rules: The authors provided the Biostatistics Review Certificate, the Institutional Animal Care and Use Committee Approval Form, the signed Copyright License Agreement, and the ARRIVE form.

**Response: Ok.**

- The authors need to complete the Conflict-of-Interest Disclosure Form.

**Response: Ok. We have completed and uploaded the Conflict-of-Interest Disclosure Form of each author.**

This manuscript does not involve human beings, the Institutional Review Board Approval Form was not applicable. No academic misconduct was found in the CrossCheck detection and Bing search. 4 Supplementary comments: This is an unsolicited manuscript. The study was supported by the Brazilian National Council for Scientific and Technological Development. The topic has not previously been published in the WJG. The corresponding author has not published articles in the BPG.

**Response: Ok.**

- 5 Issues raised:

(1) I found the authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s);

**Response: Ok. Done. We have provided and uploaded the funding agency copy of the approval document from the Conselho Nacional de Desenvolvimento Científico e Tecnológico/ Brazilian National Council for Scientific and Technological Development (CNPq) - as per suggested. Grant Process Number. 140152/2013-0.**

(2) I found the authors did not provide the original figures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor;

**Response:** *Ok. Done! We have prepared as well as arranged all the figures using PowerPoint to ensure that all graphs or text portions can be processed by the editor as per recommendation.*

(3) I found the authors did not write the “article highlight” section. Please write the “article highlights” section at the end of the main text.

**Response::** *Ok. Done. As our manuscript is a basic study we have added the “article highlights” as requirements. So, the “article highlight” section was written at the end of the main text as recommended.*

6 Re-Review: Required.

**Response:** *Ok. Done!*

7 Recommendation: Conditionally accepted.

**Response:** *Ok. Thank you.*

(2) **Editorial Office Director:** I have checked the comments written by the science editor.

**Response:** *Ok. Thank you.*

(3) **Company Editor-in-Chief:** I have reviewed the Peer-Review Report, the full text of the manuscript, the relevant ethics documents, and the English Language Certificate, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted.

**Response:** *Ok. Thank you so much.*

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.

**Response:** *Ok. Thank you.*

All co-authors have agreed to the resubmission with these revisions. The manuscript is consistent with the Guidelines for Authors of the *World Journal of Gastroenterology*.

We are looking forward to hearing from you soon, and we hope to have our manuscript accepted for publication in this renowned journal

Yours Sincerely,  
The authors