



**SDI Review Form 1.6**

**PART 1:**

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| Journal Name:            | <a href="#">British Microbiology Research Journal</a>                                    |
| Manuscript Number:       | <b>MS: 2013_BMRJ_3068</b>                                                                |
| Title of the Manuscript: | <b>Lactobacillus rhamnosusGG (LGG) protects cells from Clostridium difficile toxins.</b> |

**General guideline for Peer Review process is available in this link:**

**(<http://www.sciencedomain.org/page.php?id=sdi-general-editorial-policy#Peer-Review-Guideline>)**

- This form has total 9 parts. Kindly note that you should use all the parts of this review form.



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**PART 2: Review Comments**

|  | <b>Reviewer's comment</b> | <b>Author's comment</b> <i>(if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)</i> |
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| <p><b>Compulsory</b> REVISION comments</p> | <p><b>L72:</b> why the no-toxigenic strain <i>C. difficile</i> ATCC700057 was used if no results upon the cellular model are presented in this manuscript? The authors only show a decrease in the log units due to the presence of the lactobacilli, but there is not discussion about the rationale to include this no-toxigenic strain in the study. I advise to remove this strain / results.</p> <p><b>L113:</b> it has not been clearly indicated which is the control (used to refer the results) in the Vero cell experiments. If I have understood correctly, the control must be the cellular line grown in MEM alone (without addition of bioactives); this is correct? In addition, why to use the VERO cell line (monkey kidney) when there are available many cell lines from human colon adenocarcinome (e.g. HT29 and Caco2)? Colon is the ecological niche of <i>C. difficile</i> and the place of injury when an overgrowth, due to different causes of this bacterium occurs. Thus, it seems that the best model to study the antagonic effect of a probiotic will be colonic cells.</p> <p><b>L173:</b> it could be interesting to include the pH of the co-cultures ATCC9689 vs. different dilutions of GG and also the counts of GG (as a control to ascertain that the dilution ratio was accurate). The pH results will give an idea about the metabolic activity of GG that could explain the 1-log unit decrease of the pathogen growth. The comparison of pH values between the toxigenic and no-toxigenic strains (sentence L171-173) does not give indication about the performance of the probiotic.</p> <p><b>L177:</b> the incubation period included in this sentence (48 h) is not coincident with that indicated in figure 2 legend (24 h). Please verify. Since the results of the no-toxigenic strain were not included (to check if other clostridial extracellular factors could have effect on VERO cells) it could be highly recommended to include results of the amount of extracellular toxins present in the co-cultures</p> | <p><b>Thank you very much for your comments. We have addressed each point accordingly.</b></p> <p><b>L72.</b> We decided to use the toxigenic ATCC strain after assessing the degree of cytotoxicity induced on Vero cells. The ATCC strain caused the highest level of toxicity. We used the negative control (non-toxigenic strain) in our cytotoxicity assay as well but data were not shown as it did not cause cytotoxicity. We have included this in the discussion part of the revised version</p> <p><b>L113.</b> That is correct. On L113 we were explaining about positive and negative control wells. The positive control wells were incubated with the CFS or Cell lysate of the toxigenic strain. The negative control wells were incubated with that of the non-toxigenic strain. We have made this clear in the revised version. Vero cells have been used for similar studies by Trejo et al., 2010; Tasteyre et al., 2002 ...etc. In addition, they are well established cell lines in our lab.</p> <p><b>L173.</b> We have included the pH results in the revised manuscript.</p> <p><b>L177.</b> The 48h incubation is referring to the duration of the co-cultures incubation. On the legend for Fig 2, we are referring to the duration the Vero cells were incubated with the CFS/cell lysate. We have cleared that on the revised manuscript. We included the non-toxigenic strain in all our cytotoxicity studies and found out no cytopathic effect.</p> <p><b>L1194, L200.</b> In the revised manuscript, we have modified the discussions regarding Fig 4 and Fig 5 differently to confirm the suppression of the growth of the toxigenic strain.</p> <p><b>L143.</b> We have removed the table.</p> |
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|  | <p>(ATCC9689 with GG) to compare with the single ATCC9689 culture. This will be the direct, and no debatable, way to probe the effect of GG in the production of extra-cellular toxin by <i>C. difficile</i>.</p> <p><b>L194:</b> the results presented in figure 4 do not give valuable information since only corroborates that strain ATCC9689 harbors the <i>TcdA</i> and <i>TcbB</i> gens and the ATCC700057 (no-toxigenic) do not. Besides, it cannot be expected that co-cultivation with probiotic GG “eliminate” the genes encoding for toxins; thus, this sentence is not correct. The expression of these genes (which was analyzed by q-RT-PCR in this study) or the quantification of the intracellular toxins (as indicate above) by using, for example, specific antibodies and ELISA test (which was not done) will demonstrate the effect of the probiotic on intracellular toxin production.</p> <p><b>L200:</b> results presented in figure 5 need clarifications. The relative expression of the <i>tcdA</i> and <i>tcdB</i> genes should be corrected by the expression of a housekeeping or reporter gene (as example 16 rDNA) in order to avoid differences due to variation in the growth rate of clostridia in each co-culture condition. Afterward, data of expression in co-cultures could be referred to the expression level in the single culture.</p> |  |
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| <b><u>Minor</u></b> REVISION comments   | <p><b>L143:</b> please, remove this table since collects information already published in literature.</p> <p><b>L191:</b> it seems that there is a mismatch between the percentages of “cell viability increase” reported in this sentence and that showed in figure 3. Accordingly to this figure, the correlation between percentages and GG ratio should be 20 – 30 – 15%. Please verify.</p> <p><b>L260:</b> remove the citation number 9; it should be Dupuy et al. 2008? In other case, this sentence must be removed since was not included in the main text.</p> <p><b>L338:</b> the reference Segarra-Newham was not included in the main text.</p> | <p><b>L191.</b> We have corrected the error according to the reviewrs comment.</p> <p><b>L260.</b> We have made the correction.</p> <p><b>L338.</b> We have removed the reference from the list.</p> |
| <b><u>Optional/General</u></b> comments | <p>This article analyses the capability of <i>Lacbobacillus rhamnosus</i> GG to counteract the bioactivity of <i>Clostridium difficile</i> toxins upon the cellular line Vero. The experimental design is adequate, although clarifications are required, as well as for the results section.</p>                                                                                                                                                                                                                                                                                                                                                            | <p><b>Thank you very much for your comments.</b></p>                                                                                                                                                 |