Author's response to reviews

Title: The protective effect of recombinant Lactococcus lactis oral vaccine on a Clostridium difficile-infected animal model

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Version: 6 Date: 19 June 2013

Author's response to reviews: see over
Dear Dr Christopher Foote,

Please find here our response to Reviewer’s comments regarding the second revision of the manuscript ‘The protective effect of recombinant Lactococcus lactis oral vaccine on a Clostridium difficile-infected animal model’. (Xiao-qiang Yang et al., MS: 4465850277839527).

First of all, we all five authors have adopted unanimously Xiao-qiang Yang as the new corresponding author of this paper after discussion. Besides, we have changed the expression of name belonging to our departments.

We thank the editor and Reviewer #2 for the updated comments. Regarding the comment “Referee 1 was unfortunately unable to review your revised manuscript. However we were satisfied that you had addressed her comments; if you can address the remaining comments of referee 1 we will be happy to publish your manuscript”, we believe the second ‘referee 1’ refers to ‘referee 2’, for that we have made a point-by-point response to ‘referee 1’ in the last revision. Therefore, we have carefully revised the manuscript as requested. A statement of ethical approval for animal experiments, author contributions section, and competing interests section are included in the revised manuscript. The remaining changes are listed below:

**REVIEWER #2:**

**Minor revisions**

**Comment:** The terms ‘secreted plasmid’ and ‘membrane-anchored plasmid’ suggest that the plasmid is secreted and membrane-anchored, whereas this happens to the protein for which the plasmid codes. It would be clearer if the name was changed to ‘secreted protein plasmid’ or the groups were numbered or something like that.

**Response:** The name of the abovementioned plasmid treatment group was changed from ‘secreted plasmid’ to ‘secreted-protein plasmid’ as suggested, in order to avoid
Comment: Method and Materials - Why was cwaM6 chosen as one of the genes in the vaccine with cell wall-anchored proteins?
Response: As mentioned in the Introduction, Page5 line8 “Dieye et al. (7) designed a protein-targeting system for lactic acid bacteria and found that … the expression system constructed with P59, USP45, and the cell wall-anchored sequence of protein M6 (cwaM6) was capable of extracellular secretion of the nuclease as well as anchoring it onto the cell wall of L. lactis and Bacterium lacticum”. As indicated by Dieye et al., the cell-wall-anchored protein expression level is higher than the secreted protein expression level in the Usp45- cwaM6 expression system. Thus, we constructed the L. lactis expression system by gene assembly with the cwaM6 gene and inserted a nontoxic adjuvanted tetanus toxin fragment C to enhance the antigenicity of the expressed exogenous protein.

Comment: Discussion - What could be the advantage of membrane-anchored proteins as vaccine over secreted proteins? Have the authors tried to demonstrate secreted proteins in stool?
Response: The membrane-anchored expression system allows exogenous protein expression on the cell wall. L. lactis, as different from other lactic acid bacteria, has difficulty in colonizing in human and animal intestinal system (Wells, J. M., K. Robinson, L. M. Chamberlain, K. M. Schofield, and R. W. Le Page. 1996. Lactic acid bacteria as vaccine delivery vehicles. Antonie Leeuwenhoek 70:317–330.) and can easily be phagocytosed by M cells in the intestinal system. Thus, protein expressed by the membrane-anchored expression system will be more efficiently delivered to the immune system (Bahey-El-Din M, Gahan CG. Lactococcus lactis-based vaccines Current status and future perspectives. Hum Vaccin. 2011 1;7(1):106-9.), further inducing immune responses. We only detected secreted protein expression in intestinal fluid but not in stool specimens.

The following statement was added/revised in Page23-last few lines: ‘Because membrane-anchored plasmid anchors the recombinant protein onto the cell wall, phagocytosis of L. lactis cells carrying such plasmid can more efficiently deliver the antigen to the immune system than that of secreted-protein plasmid.’

Comment: Discussion - The authors only refer to one article on humoral immunity in CDI in animals, whereas there is a lot of literature on the role of the antibody response in humans. The manuscript would improve if this literature would also be taken into account.
Response: We believe the review refers to the work by Vaerman et al. (1997) regarding ‘the only reference of humoral immunity in CDI in animals’. In fact, searching with the keywords ‘humoral immunity’ + ‘Clostridium difficile’ only yielded 5 hits in PubMed. Yet not all of these hits are available in fulltext. Due to lack of literature resources, we have to retain the abovementioned statement without ambiguity.
further revision.

Comment: Abstract - ‘Is an important pathway’ or, rather, ‘may be an effective strategy’.
Response: Revised as suggested.

Comment: Introduction - Can a reference be given for the statement that CDI incidence is increasing in China?
Response: A reference was added as requested,

Comment: Introduction - Please spell out CLDM the first time it is used.
Response: Page4, line6, clindamycin (CLDM)

Comment: 2.2 ‘Modification’ instead of ‘medication’.
Response: Page7, 2.2-line2, ‘medication’ corrected to ‘modification’

Comment: 2.4 ‘Manufacturer’ instead of ‘manufacture’.
Response: Page11, 2.4-line3, ‘manufacture’ corrected to ‘manufacturer’

Comment: 2.5 How was cytopathic effect scored in the neutralisation assay?
Response: Page12, the methodology for cytotoxicity neutralization assay was referred to the work by Reller et al. (2010) (Reference# 20). The grading criterion adopted form Reller’s work is that, Wells/Samples with 50% or more cell rounding were considered positive if the cytotoxicity was neutralized by specific antitoxin (toxin A or B). We have added the above statement and the corresponding citation to the text and reference, respectively.

Comment: 3.1, last line, One ‘no’ should be removed.
Response: Page15, 3.1-last line, ‘…no animals had no symptoms…’ changed to ‘animals had no symptoms’, as suggested

Comment: 3.2 I do not understand the scores for the membrane-anchored group. Neither do I understand why only the scores for this group are given. The figures are clear enough by themselves. Were any of these differences statistically significant?
Response: Page16 3.2-last line The statement is corrected as follows, ‘Regarding the macropathological and macropathological scores, there are statistically significant differences among the control and three plasmid groups (P<0.005, Fig. 6).’ Fig. 6 is updated by adding the letters indicative of the significance of differences.
Fig. 6 Comparison of macropathological score and histopathological score among all groups of hamsters (a, as compared to control group, \( p = 0.000 \); b, as compared to empty plasmid group, \( p = 0.000 \); c, as compared to secreted-protein plasmid group, \( p = 0.616 \))

**Comment:** 4.2 & 4.3 The authors state in paragraph 4.2 that because their animal model had been treated with clindamycin after vaccination, recombinant *L. lactis* could not have acted as a probiotic. My interpretation is that they assume all *L. lactis* are killed by the clindamycin treatment. Is the MIC for clindamycin of this strain of *L. lactis* known? Furthermore, this assumption could be stated more explicitly. On the other hand, in the next paragraph 4.3, the authors explain the milder clinical course of the animals that were given *L. lactis* with an empty plasmid by its acting as a probiotic. They seem to assume that some *L. lactis* survive clindamycin treatment.

**Response:** Page21-22, 4.2 & 4.3 We did not detect the MIC of clindamycin for *L. lactis*, but conducted *L. lactis* isolation from animals post clindamycin treatment - no *L. lactis* was isolated from clindamycin-treated hamsters. For animals with slight diarrhea, the symptoms were mitigated after administration of *L. lactis* vaccines. These *L. lactis*, as a probiotic, were given by gastric perfusion for enhanced immunization on day 23. The abovementioned questions can be explained by the following points: 1) No *L. lactis* was detected from animals with erythromycin-containing selected medium, biochemical analysis, or PCR assays post clindamycin treatment; 2. Different from other lactic acid bacteria, *L. lactis* is not easy to colonize in human and animal intestinal systems (Wells, J. M., K. Robinson, L. M. Chamberlain, K. M. Schofield, and R. W. Le Page. 1996. Lactic acid bacteria as vaccine delivery vehicles. Antonie Leeuwenhoek 70:317–330.).

**Comment:** The figures still have the names of the hamster groups used in the previous version. Please change.

**Response:** We have corrected the text in Fig. 6.

**Comment:** Could statistically significant differences be indicated in all figures?

**Response:** Letters indicative of the significance of differences were added to Fig. 6 as requested.
Comment: It is difficult to read fig. 9.
Response: Fig. 9 was updated with more details.