The original reviewer's comments are in *greyed italics* with our responses in normal type face.

To Reviewer #1:

In general this is a well-written paper that provides details of the steps needed to administer probiotics to neonatal mice, various QC steps on the probiotic/synbiotic itself, and how to determine colonisation. The authors correctly suggest that the most interesting aspect of the study is the actual description of the gavage procedure, and I felt that the details of the qPCR etc. were of less general interest. Throughout, the mixture of highly specific instructions (e.g. PCR reactions), with much broader suggestions (e.g. the attempts to provide a generalised framework for assessing CFU for probiotics) can be off-putting, I would prefer the authors just to be absolutely specific about what they did with their synbiotic, other investigators will be able to adapt.

Unfortunately the 'results' are rather poorly presented - comprising a series of assertions that are not supported by details of the numbers of biological/technical replicates, interpretable raw or summarised data, or significance testing. The qPCR plots on their own are not useful, the authors should provide properly annotated and interpreted results or omit the whole section (I feel that a lot of this would be better suited to a standalone paper and is superfluous to a video description of/protocol for neonatal gavage). The authors talk about 'colonization' but provide no information on whether they are talking about in stool, in small intestine etc. From reading the introduction I was hoping for a presentation of longitudinal colonisation data across the length of the small/large intestine, which would have been very interesting, and proper high-quality data on risk of cross colonisation to littermates, but these do not materialise. The comparison of two gavage schedules appears superficial (though hard to judge with inadequate data), and the proposed mechanisms for the effect seen do not make sense to this reader. I think that the minimum 'results' data that are required for this submission are comparisons of safety and colonisation at ages where this is feasible with oropharyngeal gavage, and the issues of colonisation spread might be best dealt with in a submission where they can receive the proper treatment (with much more discussion about how to get around this where littermate controls are crucial and transfer to a foster mother might have impacts on health in and of itself).

We thank the reviewer for their thoughtful and constructive comments on our submission. They were very helpful in re-evaluating the presentation of the manuscript. The suggestions on reorganizing the results were well taken and more information about the technical and biological replicates have been now added to the representative results section and the figures have been annotated to show biological replicates. We hope this will better guide the audience in understanding the results. The reviewer's comments about the results being represented in a stand-alone paper is well taken and we are in the works of further investigating, validating and adding more layers to this data-driven story. However, the results presented in this manuscript are meant to be representative, providing an example of an outcome that can be obtained following the protocol we detail. We understand that some of the information about the qPCR reactions can be adapted by the end-user of the protocol, but we wanted to include it to present a complete picture of our workflow and provide a starting point/ framework for the experimental set up. The unclear representation of colonization commented on by the reviewer has been

addressed and clarified now stating that the whole intestine homogenate was used for the colonization analysis (see introduction (line 117), results (line 440) and discussion (line 567). The reviewer's comments on the gavage schedule were very helpful – our observation of consistent qPCR results between the three biological replicates for both schedules in two technical replicates per sample provided confidence in choosing one schedule over the other. The comments of the reviewer on safety and colonization at different ages were crucial. Specifically, the oropharyngeal gavage though arguably safer, did not provide confidence in the delivery of a calibrated amount of substance. Our protocol can really only provide a starting guide for the setting up the specific colonization experiments, to which the end-user has to then add their specific question of interest, such a colonization over time by potentially using longitudinal stool microbiome tracking.

The most important of the 'specific comments is that the authors provide no detail on how reliably one can administer 30 ul of any solution using a gavage needle and syringe whilst holding a delicate pup, I would have thought this would be the most challenging aspect of the procedure.
I was disappointed not to see a video of the gavage procedure (though perhaps this is provided after review in JoVE?)

It is unfortunate that the reviewer did not get a chance to view the video attached to the manuscript and we hope that the video is received without fail in this iteration of the review submission. The video addresses the reviewer's concern about the details of gavaging a young pup. More elaborate information has now also been added to the manuscript to better portray the action of gavaging in words (please refer to protocol step 4). This video also best pictures the usefulness of the blue dye used in the gavage, as requested by the reviewer.

Please provide details of the contributions of each of the 13 authors

Freddy Francis designed and conducted the experiments. Natallia Varankovich assisted with the probiotic quantification, gavage work and filiming of the video. Byron Brook, Nelly Amenyogbe, Rym Ben-Othman Bing cai, Danny Harbeson and Aaron Liu all assisted with monitoring the mice pre and post gavage. They also assisted in proof reading and editing the protocol document and video as it was undergoing development. Ben Dai assisted with establishing breeding schedules. Shelly McErlane and Kris Andrews are animal care facility staff who trained and assisted us with the development of this novel protocol. Tobias R. Kollmann and Pinaki Panigrahi are guides who have assisted us with ideation, development and implementation of the developed protocol and video production.

Response to the specific comments:
-*82 - not certain 'immunomodulatory effects' is the right way to describe, there may be other important mechanisms, the authors immediately go on to state that the mechanisms are not fully understood*

- Regarding the use of the term immunomodulatory, it was used in the introduction to present information from the literature. The connection of probiotics to the immune system has already been made in the published literature(Amenyogbe, Kollmann, &

Ben-Othman, 2017). The statement about the transfer of compounds has been re-written to provide a better explanation.

-98 - probiotics are live bacteria, not 'compounds', and in reference 11 the mothers microbiota is modulated by antibiotic exposure but I'm not sure milk-transfer of antibiotic is a major mechanistic issue

-102 - remove 'furthermore', 106 remove 'thus'

- Grammatical errors pointed by the reviewer have been addressed.

-110 - spell out LP first time it's used in the main body of the manuscript

- LP is spelled out in the body of the manuscript.

-111 - description of the synbiotic would be better suited to the methods

- The explanation of synbiotic is now addressed in procedure step 2.

-110 - I would suggest that LP colonisation was your main readout of the efficacy of the strategy, and colonisation spread was a secondary outcome? If so be clear about this

- Regarding the readout of this procedure – our primary aim was to safely administer the liquid compound to a newborn mice with confidence in the amount delivered. The secondary readouts of degree of colonization and spread were to give some examples for the end-user to acquire data using our procedure.

-120 - 'carried out pertaining to the guidelines established by the support staff at the Animal Care Facility at the University of British Columbia' I'm not clear what is meant by this, I think you could remove and just say this investigative work was approved by ACC

- The protocol was contributed to and approved by two different animal care centers at the university and at the research institution and thus both names are provided to give appropriate credit.

-Quantification - you are referring to 'saturation', but make no reference to actually determining whether the solution is saturated. It seems that you are just taking a commercial dried (lyophilized?) synbiotic, making a serial dilution and plating out to determine CFU count. How does this compare to the stated CFU count for this preparation? Presumably you spread the dilutions on the plate though no mention is made of this. Also, are you counting up to 250 colonies on a quadrant of a plate? That seems incredibly ambitious - I actually think it would be more practical to spread on the whole plate and measure?
-Preparation - you now seem to be adding the probiotic and prebiotic separately is that the case? In which case the preceding quantification step was superfluous since you could surely administer more-or-less as much as you want if you were just resuspending bacteria in some solute? Did you compare lyophilized bacteria to provision of growing bacteria e.g. at log phase from broth culture? 'FOS and malodextric reach saturation at 0.3 g/mL' - has this been determined empirically? Is a second probiotic CFU quantification essential every time?

- The reviewer's comments regarding the word usage 'saturation' has been addressed and has been elaborated to better explain the empirically determined conditions. The plate count for the CFU was done in different dilutions to find the active concentration of bacteria and plates containing between 25 and 250 colonies counted; there typically are considered ranges when only one dilution would be plated on one plate. This information was poorly and ambiguously worded in the original manuscript and the

colony count ranges have been edited to portray expected colonies per quadrant as the protocol set up for the plate is in the quadrant format. A second plate count every time before gavage is done to have an accurate representation of the CFUs administered and this step also serves as a quality control step for the consistency of CFUs administered.

-203 - measuring from the mouth/incisors? - no, I see later should be the nose, please state.

- The representation of this information was ambiguous and has been now clarified in the manuscript. (Protocol steps 4.1, 4.7 and 4.8; Line 542)

-207 - how do you accurately administer 30 ul? What kind of syringe do you use? How do you deal with dead space?

- The syringe details are now better highlighted in protocol step number 4.1 and the more information on the companies and sizes of the needles and syringes can be found on the addendum (materials and methods document).

-282 - you seem to freeze the whole intestine for later evaluation of colonisation longitudinally through the intestine (after cutting into 'small segments' line 303) - is there a benefit of this compared to just sequencing the intestinal contents? Or intestine with the contents squeezed out? (I can imagine there might be and those data might be provided)
-For the quantification, you provide absolute numbers but what are the units? Per cm intestine?

- We appreciate the reviewer's ideas on getting the whole intestine DNA sequenced. This was however not the focus of our current protocol. The quantification number for the copy counts provided were per 'whole intestine' and can be represented in either per cm intestine (if doing sectional analysis of microbiome) or per gram intestine (if exploring relative abundance). We clarified this in the revised manuscript in the introduction, results and discussion.

-The authors assert that their method is more reliable than oropharyngeal administration but provide no evidence to support this - in fact the fact that more colonisation is achieved with fewer administrations is concerning for the reliability of the technique - is this a real biological effect or stochastic? Has it been replicated by the authors?

- Our primary goal for this procedure is to have no regurgitation of the administered solution, commonly observed with oropharyngeal gavage. The primary success of our result was the elimination of regurgitation of gavage and the consistent copy number representation of the probiotic in our qPCR biological replicates as an added confirmation of the accuracy of this technique. While these two methods complement each other, they were not set up as a validation, i.e. not meant to provide statistical significance. This was now clarified in the manuscript (line 444).

-446 - treatment groups should be separated by cage - but should litter mates still be used? Therefore would the separated neonates need to be 'fostered' by another dam able to provide milk? It depends on how much of an effect/spread is meant by 'a lesser degree' (this is particularly inadequately reported)

- The reviewer's comments on the possibility of using foster mothers to solve cross-colonization of the probiotic is interesting. This was a very important discussion missing form our manuscript and now has been added (line 462).

-508 - 'above the opening of the cardiac sphincter' are the authors trying to gavage into the stomach or the distal oesophagus? I would have thought that they are trying to gavage into the stomach if measuring below the xiphisternum?

- The postulation to describe the higher copy number in pups gavaged every two days has been edited to better portray logic. The gavage needle bulb is not forced past the cardiac sphincter to avoid any damage to the crucial contractile muscle. We have empirically found that the external measurement of needle to the lower end of the xyphoid process has given us the best anticipated results, and clearly state this now in the manuscript.

-536 'observed diminished representation…' - please put all results in the result section and provide data to support assertions

- The results section has been revised to best represent the data presented in accordance with the reviewers concerns.

-549 - avoid 'roadmap', this is a 'protocol'.
-Figures 1-4 are not helpful, data presented needs to be presented properly (keeping a representative PCR plot for example if wished)
-Figure 5 please label all structures. Do you have a picture with the blue food dye?

- The phrasing suggested has been fixed and the graphs are labelled with biological replicates to better present the data.

The results section has been edited to address the overarching concerns of the reviewer. The figures have been revamped to better represent the data. We thank the reviewer again for the thoughtful and detail feedback and hope that we have adequately made edits to address the reviewer's concerns and have improved the quality of our manuscript with their assistance.