# **Microbially-produced folate forms support the growth of *Roseburia intestinalis* but not its competitive fitness in fecal batch fermentations**

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We would like to thank both Reviewers for reviewing and providing us feedback on our manuscript. We appreciate the time and effort they have invested in helping us improve our work. Below, you can find our responses to their comments, addressing each point in detail.

Reviewer 1

This is a well-written manuscript with valuable topic in the field of gut microbes. Certainly worth to publish. I have only minor comments to revise.

Comment 1: Lines 61-62 - This sentence is redundant for this paper.

Our response: We appreciate Reviewer’s careful reading of our manuscript. Upon further consideration, we agree that the sentence in question (Line 61-62) may appear redundant in the context of this specific paper. To address this, we have removed the sentence from the revised manuscript.

Comment 2: Line 204 – 2 ul is 0.1 %

Our response: Thank you for your careful review. We have corrected it in the manuscript to accurately reflect that 2 µl is indeed 0.1% of the total volume. The corrected sentence now reads:

*“Each diluted fecal slurry was inoculated at 0.1% (v/v; 2 µl, 10-7 final feces dilution on plate), and the plates were sealed with breathable seal and incubated anaerobically at 37°C in the anaerobic chamber.”*

Comment 3: Line 419 – 6.9 log Roseburia means that there could be less than a single Roseburia cell in the well! (10-7 dilution of feces)

Our response: ?????

Comment 4: Figure 6 – how to explain disappearance of R. intestinalis in D4 and D5?

Our response: Thank you for this important question regarding the disappearance of *R. intestinalis* in donors D4 and D5 as shown in Figure 6. We propose following explanations for this observation:

1. Competitive exclusion: The treatment conditions may have favored the growth of other bacterial species that outcompeted *R. intestinalis*. This could result in *R. intestinalis* being overtaken by other bacterial species that benefited more from the specific nutrient environment created by the treatment.
2. Detection limit: The quantification of *Roseburia* may have fallen below our detection limit in these samples. This does not necessarily mean complete disappearance, but rather a reduction to levels too low for accurate quantification using our current methods.

Comment 5: Conclusion seems to be too straightforward as in two cases of faecal samples (out of five) there was not supporting effect on the growth of Roseburia.

Our response: We appreciate the reviewer’s thoughtful comment regarding conclusion. We agree that our initial conclusion may have oversimplified the complex results observed across the different donors. To address this concern and provide better interpretation of our findings, we have revised our conclusion as follows:

*“In conclusion, this study reveals that R. intestinalis L1-82, a beneficial butyrate-producing member of the gut microbiota, uses different forms of folate produced by folate prototrophs of the human gut. Interestingly, the different folate forms impacted differently its growth kinetics. In the complex community microbiota, we observed that the effects of folate forms and the doses on composition and metabolism were not uniform across all donors tested. While samples from some donors showed no supporting effect on Roseburia growth, others did exhibit changes. This variability highlights the complexity of microbial interactions in the gut ecosystem and suggests that the impact of folate on the gut microbiota may be donor dependent. Our observations suggest that gut microbiota produce folate primarily to fulfil its own requirement, and the effects on specific bacterial groups may vary. Further research with a larger range of fecal donors is necessary to fully determine the possible long-term impact of folate sustained supplementation on the gut microbiota and gut health.”*

Reviewer 2

Summary  
The study investigates the impact of different forms of folate, including natural ones like tetrahydrofolate (THF) and methyl-tetrahydrofolate (M-THF), as well as synthetic folic acid, on the growth and metabolism of gut microbiota, with a special focus on Roseburia intestinalis. Using in vitro fecal batch fermentations, researchers quantified the production of folate by six gut prototrophs and assessed its effect on the auxotrophic R. intestinalis. Results showed that natural folate forms promoted the growth and metabolism of R. intestinalis, but had no observable impact on the overall composition, activity, or abundance of gut microbes during fecal fermentations. This suggests that dietary folate in different forms may have limited effects on the human gut microbiota in vivo. The manuscript is well written and the figures in the manuscript are beautiful. The manuscript can be accepted with minor revisions on the following parts:  
  
  
Comment 1: Line 109, the dot between MgCl2 and 6H2O should be in the center.

Our response: Thank you for your careful attention to detail. We have revised the notation in line 109 to correctly represent the chemical formula as MgCl2‧6H2O

Comment 2: Spacing between paragraphs should be consistent.

Our response: Thank you for noticing this formatting inconsistency. We have carefully reviewed the entire manuscript and corrected the paragraph spacing to ensure consistency throughout the document.

Comment 3: Line 328, R.intestinalis should be italic on the paragraph title.

Our response: Thank you for your attention to detail in formatting. We appreciate you pointing out this oversight. We have corrected the paragraph title to properly italicize *R. intestinalis*, ensuring consistency with scientific nomenclature.

Comment 4: Figure 1, the concentration trends on figure 1D are basically consistent with the figure 1B. How to explain this?

Our response: Thank you for this observation. The consistency in trends between Figures 1B and 1D can be explained by the composition of the inactivated bacterial (IB) extracts from different strains. Specifically, the IB extracts from *Marvinbryantia formatexigens* (MFP), *Blautia hydrogenotrophica* (BHP), and *Blautia producta* (BPP) contain higher concentrations of acetate and lactate compared to the IB extracts of other strains (Supplementary figure XX).

This is significant because *R. intestinalis* L1-82 is known to consume both acetate and lactate for the production of butyrate (REF). This metabolic capability of *R. intestinalis* L1-82 likely contributes to the similar concentration trends observed in Figures 1B and 1D.