Review of: "Long-term beneficial effect of faecal microbiota transplantation on colonisation of multidrug-resistant bacteria and resistome abundance in patients with recurrent Clostridioides difficile infection"

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Potential competing interests: No potential competing interests to declare.

- The authors present a paper in which they assess colonization with MDR bacteria and shifts in the resistome upon FMT in rCDI patients. There are only a couple of ongoing trials and a published pilot study on eradication of MDR bacteria by FMT so this study adds important information to an understudied research area. In particular the long-term follow-up and the combination of culture-based and metagenomic approaches are strengths of this study. The comparison of culture and WGS with WMGS nicely shows the added value of combining both approaches to increase sensitivity.

There are however also some limitations inherent to the methodological design that I feel need to be more clearly addressed. Moreover, I have some recommendations for clarification.

The main limitation of this study is the fact that the pre-FMT (baseline) samples of the rCDI patients are collected during or after antibiotic treatment. As antibiotic treatment will most likely result in an expansion of bacteria encoding AMR genes against the antibiotics administered, but also AMR genes that are co-localized on the same MGE's, the shift in the resistome after FMT will be a combination of the FMT and the natural restoration of antibiotic-induced shifts in the resistome. Without a control group the effects of antibiotic exposure and FMT cannot be disentangled and while the authors indeed address this in some way, I feel that this limitation should be more explicitely mentioned.

The fact that the abundance of MDR bacteria (as determined from metagenomic data Fig 1C) were significantly higher in pre-FMT as compared to post-FMT samples further suggests that the antibiotic pre-treatment resulted in a bloom of Enterobacteriales, including ESBL-producing ones, increasing the detection sensitivity of MDR bacteria. The authors also conclude themselves that "As expected in patients pre-treated with antibiotics, we found that MDR bacteria had higher relative abundances in rCDI patients before FMT than after FMT". Moreover, Fig. 2 is a striking example of the antibiotic induced enrichment of Enterobacteriales and consequently a strong argument of the antibiotic-induced enrichment of MDR bacteria and likely also resistome (increased/decreased diversity).

This temporary bloom of Enterobacteriales might have resulted in the rise of MDR Enterobacteriales to levels that are culturable or detectable by WMGS while levels might return to a low abundance that fall under the detection limit – especially given that enrichment was not selective in this study. One way to partially overcome this limitation by study

design and to strengthen the conclusions of the study is to use a more sensitive methods to screen post-FMT samples of patients with pre-FMT MDR bacteria – e.g., targeting the specific MDR genes present in the cultured isolates from preFMT samples using qPCR.

- I suggest to use the new nomenclature for bacterial phyla instead of the old nomenclature (e.g., Bacillota, Bacteroidota and Actinomycetota rather than Firmicutes, Bacteroidetes and Actinobacteria)
- The authors conclude that the ESBL-producing E. coli strains detected in the long-term follow-up samples are likely the result of persisting bacteria as pre-FMT samples also contained ESBL E. coli and similar AST patterns. However, the WGS data for the two LTFU isolates are lacking. Why have these strains not been sequenced and would it be possible to still perform WGS of these isolates as this would provide more definite proof of persistent carriage?
- Lines 142-149 on availability of samples for sequencing is quite difficult to follow. For example, why 56 samples from 8 donors (also good to provide an average and range). From the description in the statistics section and later in the results section it becomes clear that multiple stools were collected per donor, but it would be good to have this information already clarified in the methods section. Also, the overlap between samples used for culture and metagenomics in patients is not entirely clear. I would suggest to include a flow-chart or some other kind of visual representation for further clarification.
- The authors compared the beta diversity between species profiles of donor and patient metagenomes and concluded that the profiles were most different pre-FMT. However, this conclusion should not be based on the differences in p-values alone. I would suggest to present the between-group beta-diversity (e.g., box-plot showing the beta-diversity between each pre-FMT patient sample and each donor sample vs. box-plot of each post-FMT patient sample and each donor sample) and test for differences between the two to substantiate this conclusion.
- For the reasons mentioned earlier, it cannot be included from the increase in richness in patient microbiota and the shift towards the donor microbiome that this is the result of FMT or merely the recovery of antibiotic-induced perturbations. However, the impact of FMT could be substantiated by examining whether the beta-diversity of the patient microbiota post-FMT and the paired donor is smaller than the beta-diversity of patient and unpaired donors. Same for alpha diversity -> do patients receiving FMT from donors with a higher microbial richness/diversity also have a higher microbial richness/diversity upon FMT when compared to patients receiving FMT from donors with a lower microbial richness/diversity?
- Regarding resistome profiles – to what extend are the observations (specifically with respect to pre-FMT and donor profiles) a reflection of the bloom in Enterobacteriales in pre-FMT samples from patients. It would be informative to link resistome to microbial taxonomic profiles (e.g., co-occurrence analysis, Procrustes analysis or else).
- Line 212 “In PCA, donors and patients are compared using PERMANOVA and PERMDISP tests, considering the repeated measure in patients”. How was repeated sampling taken into account, was subject ID used to partial out this effect or was another strategy used?
- Why did the authors not choose to use methods that are specifically designed to test for differential abundance of microbial taxa, such as ZIBR or LinDA, rather than using paired t-tests or equivalent non-parametric tests?