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.

This manuscript presents a highly significant contribution to the field of microbiota transplantation and antibiotic resistance. Its comprehensive methodology, which combines culture techniques, metagenomic sequencing, and a long-term follow-up period, offers a holistic understanding of the intricate relationship between fecal microbiota transplantation (FMT) and multidrug-resistant (MDR) bacteria. The study's meticulous investigation into MDR bacteria prevalence and abundance before and after FMT provides crucial insights into how FMT can effectively reduce the carriage of MDR bacteria in patients suffering from recurrent Clostridioides difficile infection (rCDI).

One of the most commendable aspects of this research is its extended follow-up period of up to three years post-FMT, allowing for the assessment of long-term impacts on the microbiota composition and antibiotic resistance patterns. This prolonged observation is vital in shedding light on the persistent effects of FMT and provides valuable information for clinicians and researchers alike. Additionally, the paper's use of plasmid predictions and their findings on plasmid-mediated antibiotic resistance add an extra layer of depth to the analysis, offering insights into the transferability of resistance genes.

My overall remarks would be:

1. Long-term follow-up should be better defined than 1-3 years. 1 to 3 years is a significant difference and an important confounding factor in my opinion.
2. Any significant causes of microbiome modulation (antibiotic use, probiotic/prebiotic/postbiotic use, surgery, malignancy, serious illness etc.) should be provided especially for long-term follow-up patients as those would be serious confounding factors for the measured outcomes
3. As stated below, “lost to follow-up” should be discussed in detail as there is a significant difference between long and short-term follow-up patients (22/87)

Other remarks are as follows;

A. “To further explore the effects of FMT in rCDI patients on antibiotic resistance of the gut microbiota, we assess colonisation with MDR bacteria with both culture and metagenomics.” This is a complex sentence and what the authors

aim to deliver with this sentence is unclear. I believe a more comprehensive explanation of the aim of the study would be more appropriate.

B. At the start of the results section; “During the sample collection period the NDFB provided faecal suspensions for 208 FMT treatments of 187 rCDI patients. Eighty-seven pairs of patient stool samples (median age: 73, interquartile range (IQR): 64-81 years, 56 females (64%)) from pre- and short-term post-FMT in 10% glycerol were available for testing for MDR bacteria by culture” The authors mention “availability” for the selection process which was described as “Pre- and short-term post-FMT stool samples of rCDI patients and their corresponding donors were collected between May 2016 and March 2021. Additionally, in February 2021 we contacted 53 patients to obtain clinical information and request a long-term follow-up (LTFU), or long-term post-FMT, stool sample. (~2 years after FMT.)” in the methods section. I believe this “availability” should be better described to rule out selection bias. In addition, in the remark “Eighty-seven pairs of patient stool samples (median age: 73, interquartile range (IQR): 64-81 years, 56 females (64%)”, the patient stool samples can not have a median age or gender percentage, therefore the details of the patients should be provided here.

C. “Sixty-three pairs of raw frozen patient stool samples (median age: 73 years, interquartile range (IQR) 65-81 years; 40 females (63%)) were available for shotgun metagenomic deep sequencing (Table 1)” Same condition applies here as well.

D. “Twenty-two patients provided a long-term post-FMT sample (median age: 73, IQR 64-78 years; 14 females (64%))” The ratio of long-term post-FMT sample size is dramatically low compared to Short-term post-FMT (~3 wks) (22/87= 25%). It is very important to know why this ratio is a quartile of the short term, especially because of the immortal time bias. It is important to provide how many mortalities happened in the patients with MDR (10 in short term with culture) as there are only 2 remaining for the long-term follow-up.

E. “In the long-term, the colonisation rate decreased further to 2/22 (9.1%; $p = 0.0008$ compared to short-term post-FMT).” Although this “p value” points to a significant outcome, there is also a significant drop-out rate (reasons not discussed in the study) which is a confounding factor for this significance. The authors should discuss their statistical analysis approach here and should provide information on this important limitation.

F. The same limitation applies to the “Figure 1. B: Colonisation rates in 22 patients of whom long-term follow-up (~1-3 years after FMT) samples were collected. 5/22 had a MDR bacterium before FMT, 4/22 were colonized 3 weeks after FMT, and 2/22 were still colonized a few years later. In both time intervals, colonisation rates dropped ($p = 0.01$ and $p = 0.005$, respectively” In the same figure’s legend it says “Before FMT, 20/87 patients were colonised by a MDR bacterium, after FMT 10/87 were colonised. The colonisation rate after FMT is significantly lower (McNemar’s chi-square, $p < 0.0001$).” yet, in the LTFU group 5 patients were colonised by a MDR bacterium and after FMT 4 patients are colonized. So there is a significant discrepancy between short-term follow-up and LTFU groups which may be caused by the relatively small sample size. I believe this should also be discussed in the discussion section.

G. In the discussion section; “None of the patients in our cohort reported an infection with an MDR bacterium after FMT and we included no control group without FMT. Therefore, we have no data on risk of infections with MDR bacteria post-FMT.” Does that statement include the LTFU patients? If so, I believe the authors should also provide information/remark about the patients that they couldn’t monitor for long time (87-22: 65 patients)