To:
Editor-in-chief
Biomolecules

Re: Response to reviewers with step-by-step description of changes made to the manuscript Rehorova et al.: *Multi-donor Faecal Microbial Transplantation for Critically Ill Patients: Rationale and Standard Operating Procedure*

Dear Editor,
Dear Reviewers,

Thank you for insightful review of our above-named manuscript. In this letter, we describe the changes we have made to our paper and explain them. The revised manuscript in "track changes" format is attached as well as the final revised version.

REVIEWER #1

R1Q1: *English grammar and style: the manuscript has to be revised by an English native speaking person due to relevant concerns and a lot of typos have to be corrected/changed throughout the manuscript*

Answer: The text has been extensively revised as suggested. All corrections can be found in the track-changes version of the manuscript

R1Q2: *line 46 ff: the authors pointed out a variety of disease entities that have been associated with microbiome alterations => since this listing is far from complete: is there any specific reason behind the entities named? If not, could the authors state these associations more in general? Moreover, the listing needs to be re-organized and grammatically adapted*

Answer: There was a missing bracket in the original sentence. We added more general statement and only list those diseases as example.

R1Q3: *line 53ff: this conclusion is over-simplified, incomplete, and finally wrong (by only focussing on SCFA!) => please adapt*

Answer: We changed this part of article as suggested.

R1Q4. *Overall, references used seem out-dated => the authors should add more current literature*

we use more current literature

Answer: We respectfully disagree. Most references are <5 years old and by going step-by-step through older ones, it is difficult to update those without omitting classical landmark paper or using secondary citations (e.g. recent review paper instead of the original paper).

R1Q5: *The relevance of bacteriophages, the virome and mycobiome should be named and discussed in the context of FMT (=> probably relevant effect size for FMT success)*

Answer: We agree and thank for this comment. We have added a section on FMT efficacy to the text, listing all parts of the microbiome. We added this at later section (line 84).

R1Q6: *The specific risk of transmitting multi-resistant organisms/bacteria should be named and discussed, separately (=> see also respective FDA-safety warnings: https://www.fda.gov/safety/medical-product-safety-information/fecal-microbiota-transplantation-safety-alert-risk-serious-adverse-events-likely-due-transmission)*

Answer: This specific risk is now mentioned in the revised text incl. the reference to FDA warning.

R1Q7. *Line 90: although FMT as procedure in the treatment regimen of rCDI is now widely recommend by multiple society guidelines, the statement that "... the method gradually became the golden standard for treatment of recurrent and refractory Clostridium difficile infection (CDI)" should be attenuated since there is still a lack of long term data regarding overall efficacy but especially potential side effects!*

Answer: Agreed. We downtoned the statement from "golden standard" to "well established treatment".

R1Q8. *Line 93 ff: this listing is also very inconclusive (see above) change the text Answer: line 104/105 => a reference is mandatory!*

Answer: We added a reference.

R1Q9. *Line 111 ff: besides the legislative status in the Czech Republic, prospective use of FMT is still very limited (=> study could only be performed under good clinical practice, GCP conditions leading to immens regulatory restrictions and costs of such studies). This topic has to be discussed more in detail and especially within a broader context (out of the Czech perspective)*

Answer: We agree with that and changed this paragraph from very Czech Republic-centered to more general. We added: In collaboration with regulatory authority of one Eeuropean Union country (SUKL) we have developed such an SOPs, which we describe and explain below. Although directly applicable in only one EU country, we believe that it can be useful in its current form or after minor modification in other countries and help to standardize FMT procedure in the trials in the critically ill patients.

R1Q10: *Figure 1: is the admixture of contrast solution part of the SOP => how influences by the contrast solution to the FMT graft have been ruled out and based on which data this has been verified?*

Answer: Thanks for this point. We recognise that we only have anecdotal data and no robust evidence. We have changed Figure 1 saying: Note: Addition of contrast agent is normally not a part of FMT and it should be noticed that there are no robust data on how FMT spreads after rectal administration. We have also down toned the respective part of the methodology (line 189) saying that we still cannot be sure about the spread after administration via rectal tube.

R1Q11: *Line 180 f: effect sizes regarding the FMT application by enema vs. via colonoscopy should be discussed differentially as the latter way of application seems to hold the highest effect size.*

Answer: We have revised the text and mention lower effect size.

R1Q12 *Line 182: the normal small intestine IS NOT STERILE!*

Answer: Words "normally sterile" were deleted from this sentence.

R1Q13: *Line 37: the plural of microbiome ("microbioma") is uncommon => adapt*

Answer: We have changed this.

R1Q14 *Line 44: the same is true for "pathobiom", that should be pathobiomE? If the authors want to use this term then a specific reference should be given for it*

Answer: The word is not in use in the revised text, only in Ref 31 in the form of "Pathobiome".

REVIEWER #2

This paper try to describe a standardized operating procedure of multi-donor faecal microbial transplantation in ICU patients with critical dysbiosis. FMT indeed attracted much attention recent years on accout for its therapeutical effect in dysbiosis.

R2Q1: *However, the application of FMT in ICU patients needs to be treated with great caution. Since the safety of the FMT procedure is still controversial. In 2020, a case of drug-resistant bacteremia after FMT was reported. The immunity of ICU patients are usually low which may can not endure the FMT operation. There is no evidence that the SOP procedure introduced in this paper is safe for use.*

Answer: We think this comment is important. Although we have never stated in the manuscript that the FMT is safe, publishing a "cook book" for it might give exactly that impression. Consequently, we added a sentence: This SOPs aim to make FMT in critically ill patients as safe as possible, but it should be stressed that good quality data on FMT safety in critically ill patients are still not available.

R2Q2: *The faecal sample from the donor will be frozen before use in this paper, however, most of the FMT cases reported prefer fresh samples. After frozen, bothe benefical and harmful bacteria in the sample may inactivated.*

Answer: We agree although the viability of most microbial species is reduced by freeying-storage-thawing cycle, the FMT still remains highly efficacious. Most trials report a reduction of achieving clinical success of FMT to around 70%. We have chosen frozen samples due to

1. Safety (Sample can be quarantined until the donor is re-tested for transmittable diseases)
2. Logistics (Frozen samples are readily available in ICU whenever a critically ill patients needs them)
3. Standardisation (Use of multi-donor FMT increases the homogeneity of investigational products.

In acute care, searching for and testing the donor is so time consuming, that before the fresh FMT could be delivered it would have been already too late for the patient. Although in theory fresh samples could (and probably will) be more efficient, we see little point to test an intervention, which is not feasible in real life ICU.

R2Q3: *There are many spell mistakes in this paper. For example: in line 82, line 95, line 143…*

Answer: See also R1Q1. We have extensively revised the text to correct typos and spelling errors.

Q2Q4: In line 90 and 94, the bacteria name of the *Clostridium difficile* should be italic. And the name of the genus Clostridium has been updated.

Answer: We have changed to italic. Also in lines 154 and 227.

Answer:

REVIEWER #3

R3Q1: *Řehořová, Cibulková and colleagues present a standard operating procedure for fecal microbiota transplantation (FMT) in critically ill patients. While this is an accepted modality with several controlled trials showing promising results in the treatment of* Clostridioides difficile *infections (CDI), there have been important safety sentinel events that the authors do not adequately cite, including deaths directly related to FMT (e.g. PMID 31665575). In describing the SOP to focus on several key features, there are several aspects of the SOP that require additional justification or clarity*

Answer: Thank you. This is extremely important point – the two deaths resulting from transmition of MDR Enterobacteriaceae from the donor were now added into the revised manuscript, together with reference to FDA warning against this risk (see also answer to R1Q6).

R3Q2: *What are the baseline inclusion/exclusion criteria for the modality in **FMT recipients**? Are all ICU patients to be considered as potential candidates? I would have ethical concerns about including the following sorts of patients:*

1. *Neutropenic patients*
2. *Recent GI surgery*
3. *Known perforated viscus / abdominal free air*
4. *Patients unable to give informed consent*

Answer: We agree and we have added points 1-3 among specific contraindications to line 229. With regards inability of informed consent, this would excluded almost all critically ill patients as most of them are receiving sedation to facilitate endotracheal intubation or have other conditions impairing decision making capacity. Therefore, most trials in ICU patients are rely on a surrogate decision maker, such as the next of kin. Consequently, we do not consider the lack of capacity to give consent a contraindication, rather, this aspect should be reviewed with the respective research ethics board for each trial using FMT in ICU patients.

R3Q3: *In addition to the above, the authors need to comment explicitly on the principal limitation of FMT in the ICU setting: **engraftment**. How is an FMT procedure expected to succeed / engraft in the setting of a patient population where wide-spectrum antibiotic administration is the rule, not the exception? What is the external validity of FMT in a population if antibiotic administration becomes an exclusion criterion? If antibiotics become an exclusion criterion, for how long after discontinuation will FMT be excluded given evidence that some antibiotics remain in the gut for days (i.e. neomycin, enteral vancomycin)?*

Answer: Again, extremely relevant point and it is our fault that we have not made it clearer in the text that we advise to only use FMT at least 48 hours after stopping antibiotics. Although some antibiotics are detectable as long as 6 days after last administration (https://www.tandfonline.com/doi/pdf/10.3109/08910609209141594), we are more pragmatic using the fact, that the profound diarrhoea (the main indication for FMT) is likely to increase the clearance of antibiotics from the gut. It is then the concentration in the blood, which influences the concentration of antibiotics in intestinal lumen. Most common antibiotics used in intensive care, such as broad-spectrum beta-lactams, aminoglycosides, or iv. glycopeptides have relatively short plasma half lives and therefore 48 hours is well above 5 biological half-lifes and it is also consistent with the practice in non-critically ill patients

(https://www.hopkinsmedicine.org/gastroenterology_hepatology/clinical_services/advanced_endoscopy/fecal_transplantation.html).
In the revised manuscript, we have added a paragraph discussing these important aspects.

R3Q4. *Why is a semi-rigid rectal irrigation tube necessary, and why do the authors feel that extensive patient positioning is truly necessary to "[distribute] the transplantate well throughout the length of the colon"? A quick review of successful enema administration of FMT for CDI does not show that this was done in several RCTs, only a simple retention enema. What will the approach be in a patient who cannot participate in these positioning manuevers in the context of critical illness?*

Answer: Retention enema is a technique dependent on voluntary controls of sphincters, and this is not the case for most critically ill patients, who are under influence of sedatives. Consequently, we used
semi-rigid rectal irrigation tube + positioning with inflated balloon of faecal collection system as less invasive alternative to colonoscopy. Of note, patients' positioning does not require active participation by patients themselves and is safe to perform if the patient is haemodynamically stable, which is a prerequisite (See paragraph on *Safety*)

R3Q5: *The ID screening outlined in the supplementary procedure **is not adequate**, particularly given the plan to pool feces from 7 donors in each aliquot. In particular:*

- *HIV screening as outlined does not address the window period problem unless the labs as outlined are collected at baseline and again at the end of the 3 month quarantine period. This is not specified.*
- *Window period infections are also not addressed for the hepatitis virus screening, which does not include NAAT testing or a plan to repeat at any interval.*

Answer: The original document was designed to comply with the recommended local requirements for stool donors (https://www.infekce.cz/DPFMT18.htm), which combine serology testing with questionnaires to detect risk behaviour. Nonetheless, but we agree with the Reviewer #3, that rules should be even stricter for multi-donor FMTs. Consequently, we revise the SOP to mandate full serology testing every 2 months during the period of donation. This also better reflect the established practice of "daily donations" of limited group of long term donors.

R3Q6: *No **donor deferral criteria** are provided, nor is the questionnaire given to the donor for risks of infectious diseases. Would the following donors be deferred:*

1. *History of travel to India 6 months ago?*
2. *History of travel to India 18 months ago but for a total stay duration of 4 months?*
3. *A donor with >1 sexual partner in the last 12 months?*
4. *Donor with history of IBS?*
5. *Patients with an extensive family history of GI malignancies, peptic ulcer disease, or autoimmune disease?*

Answer: We now attach full donor refusal criteria in the Supplementary data file, that are compliant with most of the above.

R3Q7: *Are donors compensated? If so, what measures are in place to prevent conflicts of interest in assessing the donor deferral criteria?*
Answer: Yes, the donors have the inconvenience and lost time compensated a small amount (equivalent EUR 40) for every donation, whilst no compensation is offered for blood test etc. This

makes the process still an act of altruism rather than way of earning money for living. The donor cannot work in healthcare or share a household with anyone who does. The donor also have to adhere to some dietary restrictions (see also R3Q18).

R3Q8: *Insufficient detail is provided about "laboratory exclusion of the presence of ABX-resistant microorganisms." The specific methodology and organisms screened for need to be specified, but at a minimum I would suggest vancomycin-resistant* Enterococcus*, extended spectrum beta lactamase Enterobacterales, carbapenem-resistant Enterobacterales, and methicillin-resistant* Staphylococcus aureus. *The authors should also strongly consider screening/excluding healthy donors colonized with* Clostridioides difficile *OR specifically specify why they feel including these donors in the pool is expected to be low-risk. Donors also need to be screened for* ***norovirus.***

Answer: We thank the reviewer for this comment. In the revised supplementary materials, we provide more details of donor screening, which is fully compliant with recent Czech, European, and British guidelines. In addition to that, we included test for *Enteropathogenic E.coli (EPEC,EAEC,ETEC,EIEC),*Vibrio cholerae, MDRO (VRE, ESBL,CRE), PCR detection of CMV, Rotavirus and Norovirus, and standard microscopic parasitological exam (to detect *Giardinia lamblia, Cryptosporidium parvum, Isospora, Microsporidia, Entamoeba histolytica*). Further, we included virological tests via. Faecal occult blood test will be also performed. A nasopharyngeal swab – PCR will be performed to rule out COVID-19 infection

R3Q9: *The authors, as most in the FMT field, are exclusively focused on bloodborne pathogen and classical enteric infection risk, but I would encourage mention of a plan to screen for the following infections which are routinely shed in stool, saliva, urine, and tears. This can be done either with direct screening of feces (i.e. CMV and HSV1, HSV-2) or screening of the donor (i.e. anal Pap smears for HPV or rectal swabs for HSV) or serological screening of the donor. Donor serological screening to assess risks for donor-derived infection, particularly in immunosuppressed patients, could also be outlined:*

1. *Helicobacter pylori*
2. *Herpes simplex virus 1 and 2*
3. *Cytomegalovirus (CMV)*
4. *Human papillomavirus (HPV)*
5. *JC virus*

Answer: From above listed pathogens, we have added CMV tests. The donor screening is performed in agreement with Czech, European and British guidelines published up to date. See citations at R3Q13.

R3Q10: *An informed consent framework and draft informed consent document should be provided. How many of the above considerations are communicated in writing to prospective patients?*
Answer: The informed consent for the transplant acceptor contains potential FMT risks, including infection transmission, changes in the metabolic properties of the guts, caused by the mikroflóra composition changes are also described (weight changes, insulin resistance changes, mood changes etc.). Other potential risks, described in the consent are bowel perforation or bleeding, connected to the tube insertion.

Answer: We provide an example of informed consent and its English translation as “Supporting material to review process”. Relevant risks – as listed above – are indeed listed.

R3Q11: *In the SOP for "preparation of the transplant," it is not acceptable to call this an SOP and make a statement like "no strict specifications on the mixing process are given." It is also not acceptable to use a kitchen hand-held blender. How could this be used and not result in cross-contamination between samples? How would the blender be disinfected or, preferably, sterilized*

between samples? Overall, I am not satisfied that the methodology outlined here is safe, as there is no detail about ***good manufacturing process*** *details given.*

Answer: Thanks for this relevant comment. We originally used hand-held blender treated with hot water, detergent and afterwards 2% chlorhexidine wipes. Yet, we agree with the reviewer that for formal SOPS a more robust technique is required to standardise the blending and – more importantly – sterilisation to prevent cross-cointamination. In the revised SOPs, we changed the recommended blender to https://www.grainger.com/product/WARING-COMMERCIAL-Lab-Blender-45H280) and recommend mixing time of 2 mis and full sterilization of stainless steel removable jar part.

R3Q12: *The "final preparation of multi-donor transplant" section also sounds unsafe, as there is additional opportunity to contaminate the final product in a 37C water bath. Overall, the authors have not given sufficient detail to describe measures that will be used to prevent contamination of the FMT product during preparation*.

Answer: We agree that graft thawing and mixing process was not described in sufficient details. In the revised SOPs we included more details, incl. the bath sanitation procedure.

R3Q13: *Other donor selection considerations: What is the justification of the exclusion of donors >60yo?*
Answer: This age threshold has been recommended by European consensus conference on FMT (See Cammarota, G. et al. European consensus conference on faecal microbiota transplantation in clinical practice. Gut 66, 569–580, 2017)., as well as by the British joint guidelines (Mullish, B. H. et al. The use of faecal microbiota transplant as treatment for recurrent or refractory Clostridium difficile infection and other potential indications: Joint British Society of Gastroenterology and Healthcare Infection Society guidelines. Gut 67, 1920–1941, 2018) and it based on the fact that microbiome diversity declines with aging, whilst microbiome-associated co-morbidities increase (Mangiola, F., Nicoletti, A., Gasbarrini, A. & Ponziani, F. R. Gut microbiota and aging. Eur. Rev. Med. Pharmacol. Sci. 22, 7404–7413, 2018). Therefore, we also decided to limit donor age to <60 years.

R3Q14. *Are investigators planning to include/exclude donors based on body mass index?*

Answer: Yes. Body weight and height measurement is a part of the donor physical examination. Only persons with BMI between 20 and 30 will be accepted as donors.

R3Q15: *Is diet recorded for donors, and what are the safety provisions for avoiding use of FMT from donors consuming certain donors in recipients with life-threatening food allergies?*

Answer: The description of the dietary limitations is a part of the donor informed consent and adherence to dietary restrictions is regularly checked. Because this is reliant on self-reported data, recipients with severe food allergies will be excluded (See exclusion criterion: „History of severe anaphylactic food allergy").

Dear editor,
We again want to thank all reviewers for extremely helpful and insightful notes and sincerely hope that them the revised manuscript is now acceptable for publication in Biomolecules.

Best regards
František Duška – on behalf of authors