PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Protocol for Gut Bugs in Anorexia Nervosa: An open-label pilot trial of faecal microbiome transfer to restore the gut microbiome in anorexia nervosa
AUTHORS	Wilson, Brooke; Derraik, José; Albert, Benjamin; Leong, Karen; Tweedie-Cullen, Ry; Creagh, Christine; Depczynski, Marysia; Edwards, Taygen; Vatanen, Tommi; Thabrew, Hiran; Cutfield, Wayne; O'Sullivan, Justin

VERSION 1 – REVIEW

REVIEWER	Seitz, Jochen RWTH Aachen University
REVIEW RETURNED	07-Feb-2023

GENERAL COMMENTS	Wilson et al., propose a daring study to use FMT in 20 patients with AN. To date, only two case studies are known, where this has been attempted. While this is a high-risk study, there is evidence for the importance of the gut microbiome in AN. The protocol is well written and easy to follow. The methods of sequencing using both DNA/RNA and conducting shotgun metagenomic as well as metatranscriptomic sequencing are state of the art. I have three major concerns and then some minor issues. First, as this is such a daring, yet important study, why not use a control group and randomize to TAU? Even if they do not receive placebo and/or the same amount of control time points, studying them at primary endpoint and after 12 weeks will at least give some indication of clinical consequences as opposed to mostly academic microbiome-centered outcomes only. Second, as individual stool compositions vary greatly between healthy controls and might represent individual eco-systems, mixing donors has the risk to destroy these individual eco-systems (see e.g. Hata et al., 2019, who used individual donors of AN). Third, as this is such an experimental study with possible side effects, consider only using adults over 18 years of age. Minor issues: Introduction: Less diverse microbiome in AN is disputed, see meta-analysis DiLodovico et al., 2021 Hata et al., 2016: imprecise reporting: not transplanted mice themselves, but their offspring was used in the experiment. Discuss Glenny et al., 2021 here – FMT into gf-mice in another study was without results! Methods: Repeating donor procedures after six months might limit information to be gained as stool is known to vary over time and this complicates

	finding out which stool was indeed suitable. Patients with and without laxative abuse will likely react quite differently. Consider limiting to one of the two subgroups. Consider adding CRP to safety monitoring panel. Consider adding a gastro-intestinal questionnaire, as most patients experience symptoms and change of symptoms could follow FMT (see e.g. Kang et al., 2017) Outcomes: Add Bray-Curtis measure to primary outcome, as mentioned in abstract.

REVIEWER	Prochazkova, Petra Czech Academy of Sciences
REVIEW RETURNED	11-Apr-2023

GENERAL COMMENTS	An open-label pilot trial of faecal microbiome transfer to restore the gut microbiome in anorexia nervosa AUTHORS: Wilson, Brooke; Derraik, José; Albert, Benjamin; Leong, Karen; Tweedie-Cullen, Ry; Creagh, Christine; Depczynski, Marysia; Edwards, Taygen; Vatanen, Tommi; Thabrew, Hiran; Cutfield, Wayne; O'Sullivan, Justin General comments: The submitted manuscript presents a protocol for fecal microbiome transfer to patients with anorexia nervosa. The protocol is nicely written, and it is clear and concise. FMT is mainly used to treat persistent infections with Clostridium difficile, but experimentally is used also to treat various gastrointestinal diseases including colitis, irritable bowel disease, constipation, and others. Since the communication between the gut microbiota and the brain via the so-called “gut-brain axis” was proved, the influence on microbiome composition in various neurological or psychiatric diseases is justified. FMT in patients with anorexia could yield significant findings regarding the role of the microbiota in controlling satiety and hunger centers. I believe that the proposed protocol is suitable for the study, although there are some points where I would suggest some changes or have some questions for clarification. Major comments: 1) Eligibility criteria: Why was the BMI range proposed when the upper value for patients is 19 kg/m2 and the lower value for donors is 18.5 kg/m2. I believe that these values should not overlap for both categories and should be set inversely. 2) Why the authors decided to use a multi-donor FMT approach when a single-donor approach is recommended, at least in experimental studies? 3) FMT capsule administration: The use of laxatives is common in AN patients, but certainly not in all patients. However, I do not see the high prevalence of laxative use as a reason why recipients are not cleansed before receiving the capsule, either with antibiotics or laxatives. Wouldn't it be more effective for accepting foreign microbiota to prepare the recipient in this way? 4) Authors will chose 4 donors and prepare capsules with their stool samples which will be stored at -80C. Authors count on the reserve

	donors if any of the selected donors won't be able to come. Wouldn't it be better to prepare more capsules from the 4 selected ones, which would be frozen and ready for the next use? The authors would have avoided further variation from other microbiomes. 5) There is no mention of whether the preparation of stool capsules would use an anaerobic environment to preserve anaerobic bacteria. In addition, and I consider this to be the biggest weakness of the protocol, donor stools will not be sequenced first to determine microbial composition. It is known that even healthy people can experience large differences in microbiome composition. I would recommend selecting, for example, 10 healthy people, sequencing their microbiomes, and selecting the 4 with the greatest diversity and similarity to each other based on microbiome diversity and similarity analysis. 6) treatment: To my opinion, 2 days are too little for FMT. The composition of the microbiome tends to return, and it is desirable to give the new environment sufficient impulses to accept the new microbiota. I think 4 days for FMT is the minimum, I would recommend more like 2 weeks. 7) From studies on the microbiome in patients with AN is known, that these patients have affected the metabolism of thyroid hormones and also cholinesterase. I would strongly recommend assessing also cholinesterase levels in the blood of patients with AN. 8) Statistical analysis: The analysis of alpha diversity is reliable as well. Diversity changes during the treatment should be analyzed (PERMDISPER etc). 9) According to the Power analysis, 18 participants are necessary for the study. The authors plan to recruit 20 participants. As the study lasts several weeks, it is likely that some patients will withdraw from the study or fall ill or fail to meet the inclusion criteria, so I think the number of 20 is too low and it could be that there will be fewer patients at the end of the study and the study will not have statistically significant data. Minor comments: - section Introduction does not include the newest study on the microbiome in patients with AN – Prochazkova et al., 2021, Gut Microbes. - timeline of the study would be clearer

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Dr. Jochen Seitz, RWTH Aachen University
We would like to thank Dr Jochen Seitz for his helpful feedback on our study protocol. Please see our responses below.

Major concerns
1) First, as this is such a daring, yet important study, why not use a control group and randomize to

TAU? Even if they do not receive placebo and/or the same amount of control time points, studying them at primary endpoint and after 12 weeks will at least give some indication of clinical consequences as opposed to mostly academic microbiome-centered outcomes only.
> As FMT has not been studied as a treatment for those with anorexia nervosa (AN), it is essential to establish feasibility and engraftment to determine whether it is possible or sensible to conduct a large RCT. This includes two critical points; (i) that the treatment is acceptable to those with AN. Given the food aversion seen in anorexia nervosa, will ingesting a large number (n=20) of treatment capsules be acceptable to participants. (ii) Will the donor microbiome become engrafted in participants following treatment? If not, then there would be no point in undertaking a large RCT .

2) Second, as individual stool compositions vary greatly between healthy controls and might represent individual eco-systems, mixing donors has the risk to destroy these individual eco-systems (see e.g. Hata et al., 2019, who used individual donors of AN).
> We have used the same 4-donor design in our previous FMT trial (Leong et al., 2019; PMID: 31005929) and observed that one donor in both the male and female pool dominated strain engraftment (Wilson et al., 2021; PMID: 33985595). The dominant donors were characterised by higher microbial diversity (relative to the other donors), and a high Prevotella to Bacteroides ratio. Despite this, all donors contributed to strain engraftment and microbiome alterations were maintained even out to 6 months following FMT. This is in contrast to a previous FMT study that used a single donor approach and found recipient microbiomes reverted back to their baseline composition by 18-weeks post-FMT (Kootte et al, 2017; PMID: 28978426). This suggests to us that providing recipients with a range of healthy ecosystems does not impair, and may in fact enhance, their ability to sustainably engraft.

3) Third, as this is such an experimental study with possible side effects, consider only using adults over 18 years of age.
> In discussion with Eating Disorders Association New Zealand (EDANZ), the largest anorexia support group in New Zealand, there was a collective view that 16 year old women, who are legally able to consent, should not be excluded from participating in the study. We encourage the attendance of a support person at all treatment and assessment visits and have set up a rigorous safety monitoring plan to ensure all participants receive wrap around care throughout the study period. We have previously trialled FMT in adolescents as young as 14 with no serious adverse events (Leong et al., 2020; PMID: 33346848).

Minor issues
Introduction:
4) Less diverse microbiome in AN is disputed, see meta-analysis DiLodovico et al., 2021
> We have updated our introduction as specified below:

Lines 65-71 “Multiple studies have shown that the gut microbiome is perturbed in individuals with AN [14–25]. Early reports suggested AN microbiomes were typically less diverse when compared against healthy age-matched controls [19, 21]. However, more recent observations do not support a simple reduction in microbial diversity being linked to AN [16, 17, 24], but rather, a difference in the relative abundances of specific taxa. In particular, a recent systematic review determined that AN individuals harboured proportionally less fiber-utilising taxa (e.g. Roseburia sp.) and more mucin-degrading taxa (e.g. Akkermansia sp. and Methanobrevibacter smithii) [26].”

5) Hata et al., 2016: imprecise reporting: not transplanted mice themselves, but their offspring was used in the experiment.
> We have amended our reporting of this study to clarify this (see updated paragraph below point 6)

6) Discuss Glenny et al., 2021 here – FMT into gf-mice in another study was without results!

> We have updated our introduction to include the study by Glenny et al., 2021 as well as the most recent study by Fan et al., 2023, as specified below:

Lines 82-94 “A contributory role of the gut microbiome in AN symptomatology was demonstrated when germ-free mice were inoculated with the gut microbiome derived from either healthy human donors or donors with AN [35]. The offspring of mice who received the ‘AN microbiome’ showed reduced body weight and a concomitant reduction in food intake. Interestingly, when the ‘AN microbiome’ mice ate the same amount of food as the ‘healthy microbiome’ mice, they gained less weight suggesting they were less efficient at converting food into body mass [35]. Furthermore, the ‘AN microbiome’ mice had reduced serotonin levels and displayed anxiety-related and compulsive behaviours [35]. Similar weight gain differences were observed in another germ-free mice experiment that utilised both AN and healthy control donors, with differences being linked to altered expression of appetite-suppression and thermogenesis genes [17]. By contrast, another study found no difference in body weight or daily food intake between mice receiving transplantations of AN- or healthy-donor microbiota [36]. Further research is therefore required to determine whether the gut microbiome acts as a potential mediator of disease in AN and is thus a suitable target for therapeutic intervention.”

Methods:
7) Repeating donor procedures after six months might limit information to be gained as stool is known to vary over time and this complicates finding out which stool was indeed suitable.
> In this study, we will endeavour to use the same set of donors for all recipients. We have selected a maximum storage time of 6 months to ensure encapsulated bacteria remain viable, based on long-term storage (-80°C) experiments. If we are unable to recruit all 20 participants within a 6 month period, donors will be invited back, rescreened, and a fresh batch of capsules prepared. If a donor fails re-screening we will replace them with an eligible backup donor. This approach prioritises treatment safety and viability, while limiting unnecessary increases in microbial heterogeneity.

Notably, in a previous study which used a similar strategy (i.e. multiple batches of capsules from the same set of donors), we found the same donor microbiomes dominated strain engraftment (Wilson et al., 2021; PMID: 33985595). Furthermore, the variance we observed within donor microbiomes throughout the donation period was minimal relative to the differences between donors.

8) Patients with and without laxative abuse will likely react quite differently. Consider limiting to one of the two subgroups.
> We agree that laxative abuse may impact FMT outcomes and as such we have added an exclusion criterion, “regular laxative use”.

9) Consider adding CRP to safety monitoring panel.
> We have added CRP to our blood monitoring panel.

10) Consider adding a gastro-intestinal questionnaire, as most patients experience symptoms and change of symptoms could follow FMT (see e.g. Kang et al., 2017)
> We were unable to obtain a validated GI questionnaire for young women with AN. However, we will capture this information as part of our pre-screening interview and subsequent adverse event recording.

11) Outcomes: Add Bray-Curtis measure to primary outcome, as mentioned in abstract.
> We have amended our primary outcome statement to include Bray-Curtis dissimilarity, as specified below:

Line 318 “Primary outcome

• A shift in gut microbiome composition at 3 weeks post-FMT (Bray-Curtis dissimilarity). The shift should exceed the drift in gut microbiome composition measured over the 3 weeks between enrolment and baseline."

Reviewer: 2
Dr. Petra Prochazkova, Czech Academy of Sciences
We would like to thank Dr Petra Prochazkova for her helpful feedback on our study protocol. Please see our responses below.

Major comments:
1) Eligibility criteria: Why was the BMI range proposed when the upper value for patients is 19 kg/m2 and the lower value for donors is 18.5 kg/m2. I believe that these values should not overlap for both categories and should be set inversely.
> We selected the minimum donor BMI threshold to be 18.5 in accordance with the international consensus for a healthy BMI range between 18.5 - 25. We had originally proposed a narrower BMI range between 13-17 for our AN participants. However, our eating disorder study advisors felt that this BMI range was too restrictive and explained that there would be many individuals with AN whose BMI was above this threshold hence we raised the upper limit to 19. We do not believe that the unlikely situation where the donor BMI is marginally less than the BMI of the recipient with AN has any implications for the potential effectiveness and safety of the microbiome transplant. This is because our donor eligibility criteria carefully selects for donors who have a healthy lifestyle, diet, and body composition, making them quite different to participants with AN.

2) Why the authors decided to use a multi-donor FMT approach when a single-donor approach is recommended, at least in experimental studies?
> A recent meta-analysis of 14 FMT studies across 5 different disease conditions found that the microbial diversity of the donor was a significant factor influencing strain engraftment (Podlesny et al., 2022; PMID: 35931074). Consistent with this, our previous multi-donor FMT study identified specific donor microbiomes that dominated strain engraftment (Wilson et al., 2021; PMID: 33985595); however, the specific characteristics that make one a super-donor are yet to be resolved (Wilson et al., 2019; PMID: 30719428). Our multi-donor design is aimed at minimising the administration of ineffective microbiomes while increasing the overall diversity of treatment. By enabling potential engraftment of strains from multiple donors, we increase the likelihood of identifying clinical benefits arising from these combinations, even if the engrafting organisms are not present within a single donor microbiome.

3) FMT capsule administration: The use of laxatives is common in AN patients, but certainly not in all patients. However, I do not see the high prevalence of laxative use as a reason why recipients are not cleansed before receiving the capsule, either with antibiotics or laxatives. Wouldn't it be more effective for accepting foreign microbiota to prepare the recipient in this way?
> Our study advisors and ethics committee were uncomfortable with the use of laxatives to cleanse the bowel prior to treatment in this vulnerable population. This was due to both direct safety concerns, and the potential risk of introducing or normalizing the use of laxatives in participants with AN who had not used them before. Hence, the pre-FMT bowel cleanse was not included in the protocol. Moreover, the use of pre-FMT antibiotics was also discouraged due to 1) the potential for selecting antibiotic resistant strains, and 2) the retention of antibiotics within the recipient's system – potentially impacting on FMT engraftment.

4) Authors will chose 4 donors and prepare capsules with their stool samples which will be stored at -80C. Authors count on the reserve donors if any of the selected donors won't be able to come. Wouldn't it be better to prepare more capsules from the 4 selected ones, which would be frozen and ready for the next use? The authors would have avoided further variation from other microbiomes.

> As discussed in point 7 to reviewer 1, experiments to measure the impact of long-term storage (-80°C) on bacterial viability within FMT capsules indicated that viability was maintained for up to 6 months, with progressive loss in viability after that time. If we are unable to recruit all 20 participants within a 6 month period, donors will be invited back, rescreened, and a fresh batch of capsules prepared. If a donor fails re-screening we will replace them with an eligible backup donor. This approach prioritises treatment safety and viability, while limiting unnecessary increases in microbial heterogeneity.

5) There is no mention of whether the preparation of stool capsules would use an anaerobic environment to preserve anaerobic bacteria.
> Capsules are prepared under aerobic conditions. Fresh donations are collected on site and immediately processed (taking on average 1-2hrs) to minimise aerobic exposure. We have clarified this now within our protocol:

Line 179 “Donor stools will be processed individually for encapsulation under aerobic conditions.”

6) In addition, and I consider this to be the biggest weakness of the protocol, donor stools will not be sequenced first to determine microbial composition. It is known that even healthy people can experience large differences in microbiome composition. I would recommend selecting, for example, 10 healthy people, sequencing their microbiomes, and selecting the 4 with the greatest diversity and similarity to each other based on microbiome diversity and similarity analysis.
> What constitutes an effective donor microbiome is currently unclear. While alpha diversity is a good indicator, sequencing donor microbiomes, prior to administration, would add a 6 week delay before capsules could be used. This would reduce the 6 month capsule storage window and make it less likely that we could complete all treatments with a single batch. Given the extremely stringent donor selection criteria, and resulting small pool of donors, this would negatively impact our ability to complete the trial.

6) treatment: To my opinion, 2 days are too little for FMT. The composition of the microbiome tends to return, and it is desirable to give the new environment sufficient impulses to accept the new microbiota. I think 4 days for FMT is the minimum, I would recommend more like 2 weeks.
> Our previous FMT trial used a two-day dosing schedule and demonstrated sustainable augmentation of the recipients gut microbiome through engraftment of donor stains that were largely retained out to 6 months post-treatment (the most distant time-point assessed) (Wilson et al., 2021; PMID: 33985595).

7) From studies on the microbiome in patients with AN is known, that these patients have affected the metabolism of thyroid hormones and also cholinesterase. I would strongly recommend assessing also cholinesterase levels in the blood of patients with AN.
> We have added cholinesterase to our panel.

8) Statistical analysis: The analysis of alpha diversity is reliable as well. Diversity changes during the treatment should be analyzed (PERMDISPER etc).
> We have updated our study outcomes to make it clear that we will investigate microbiome diversity.

Line 327 “Gut microbiome diversity, composition and functional potential at 6, and 12 weeks post-FMT”

9) According to the Power analysis, 18 participants are necessary for the study. The authors plan to recruit 20 participants. As the study lasts several weeks, it is likely that some patients will withdraw from the study or fall ill or fail to meet the inclusion criteria, so I think the number of 20 is too low and it could be that there will be fewer patients at the end of the study and the study will not have

statistically significant data.
> All participants, even those unable to complete treatment, will still be included in the study, as per our planed ITT analysis. However, to remain powered in case of potential drop outs, we have amended our protocol to read:

Line 345 “To account for a potential dropout rate of 10%, we aim to recruit at least 20 participants who complete treatment and the primary outcome assessment.”

Minor comments:
10) section Introduction does not include the newest study on the microbiome in patients with AN – Prochazkova et al., 2021, Gut Microbes.
> We have updated our introduction to incorporate Prochazkova et al., 2021.

11) timeline of the study would be clearer
> We have revised our study schedule paragraph and amended Table 3 to clarify scheduling with reference to baseline and visit type.

Lines 200-209 “Participants will have an enrolment appointment where we will explain the study in detail, assess their eligibility, and obtain their written informed consent. During this visit, we will perform a whole-body dual-energy X-ray absorptiometry (DXA) scan and administer the Patient Health Questionnaire 9 (PHQ-9) to confirm they meet the BMI criteria and do not have feelings of self-harm/suicidal ideation. We will also collect a stool sample from the participant during this visit and schedule their baseline assessment for 3 weeks time (Table 3). The first treatment dose will be given at baseline after all assessments have been completed. Subsequent treatment doses will be scheduled the following consecutive day/s depending on the participant’s preference for a 2-day or 4-day treatment schedule. Follow-up clinical assessments will be scheduled for 6 and 12 weeks after baseline. Participants will also be asked to collect a stool sample at home 3 weeks after their baseline assessment.”

References
1. Leong et al., 2019; PMID: 31005929
2. Leong et al., 2020; PMID: 33346848
3. Kootte et al, 2017; PMID: 28978426
4. Podlesny et al., 2022; PMID: 35931074
5. Wilson et al., 2021; PMID: 33985595
6. Wilson et al., 2019; PMID: 30719428

VERSION 2 – REVIEW

REVIEWER	Seitz, Jochen RWTH Aachen University
REVIEW RETURNED	29-May-2023

GENERAL COMMENTS	All questions have been addressed.

REVIEWER	Prochazkova, Petra Czech Academy of Sciences
REVIEW RETURNED	29-May-2023

GENERAL COMMENTS	All questions have been addressed.