PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Protocol: Bugs and Brains, the Gut and Mental Health Study - A mixed-methods study investigating microbiota composition and function in anxiety, depression and irritable bowel syndrome
AUTHORS	Simpson, Carra; Schwartz, Orli; Eliby, Djamila; Butler, Catherine; Huang, Katherine; Simpson-O'Brien, Neil; Callaghan, Bridget; Dashper, Stuart; Gooley, Paul; Whittle, Sarah; Haslam, Nick; Simmons, Julian

VERSION 1 – REVIEW

REVIEWER	Klara Coello CADIC, Psychiatric Center Copenhagen Department O, 6233 Blegdamsvej 9, 2100 Copenhagen, Denmark
REVIEW RETURNED	31-Aug-2020

GENERAL COMMENTS	Peer review report on "Protocol: Bugs and Brains, the Gut and Mental Health study – A mixed-methods study investigating microbiota composition and function in anxiety, depression and irritable bowel syndrome" 1. original paper – clinical study protocol 1.1. Recommendations: minor revision 2. Comments to author: MS.ref.no.: bmjopen-2020-043221 Title: Protocol: Bugs and Brains, the Gut and Mental Health study – A mized-methods study investigating microbiota composition and function in anziety, depression and irritable bowel syndrome Authors: Simpson, Carra; Schwartz, Orli; Eliby, Djamila; Butler, Catherine; Huang, Katherine; Simpson-O'Brien, Neil; Callaghan, Bridget; Dashper, Stuart; Gooley, Paul; Whittle, Sarah; Haslam, Nick; Simmons, Julian Overview and general recommendations: The present study protocol is highly relevant addressing the overlap between depression/anxiety disorders and IBS in participants of female sex and includes a clinical interview, questionnaires and biological samples examining the gut and oral microbiota, urine, saliva and hair metabolites. The design is cross-sectional case-control study of patients with depression and or anxiety disorder (n=40), patients with IBS (n=40), patients with both depression/anxiety and IBS (n=40) and healthy control

	persons (n=40). Additionally, an ancillary component includes only questionnaire data from 1000 female. Participants are aged 18-40 years recruited through advertisements in the Melbourne area and monetarily reimbursed for their time. Participants are excluded if reporting daily smoking, alcohol or receive psychotropic medication the month prior to inclusion. The study started in the end of 2017 and it is not stated when last participant is expected to be included. Power calculations are included, and sample size exceeds the necessary 132 participants to detect an effect size of 0.35. The paper is well written in an easy understandable language with a good study title reflecting the content. It is a strength that authors carefully describe methods of sample collection and analyses and include more participants than needed according to power calculations. Further, the HPA-axis are investigated at at several levels, which is novel and interesting regarding studying gut microbiota and mental health. The methodology in the gut microbiota analyses is sound and as stated by the authors shut gut metagenomic sequencing would be ideal as well as mass spectrometry in the analyses of urine metabolites. Minor points In general, a good informative introduction. However, the last part could be even better. On page 5 in the end of introduction line 28-38: “Several confounding factors have been neglected in the small literature investigating the microbiota in IBS, anxiety and depression…” It is relevant to state that limited is known; however, I think the it would be valuable to state which possible confounders should be addressed in in this study based on prior research. Maybe something like “This research field is still limited; however, it seems reasonable to consider diet, psychosocial factors and so on…”. Further, it would be reasonable to state that smoking (Biedermann et al., 2013; Capurso & Lahner, 2017), alcohol (Capurso & Lahner, 2017). psychotropic medication (Flowers, Evans, Ward, McInnis, & Ellingrod, 2017) and sex seem to influence the gut microbiota and therefore has been eliminated from this study. Concerning Aims and objectives page 5 lines 43-44 you write “…to understand interactions between symptoms and confounding variables”. According to the study design I would suggest changing this to “…to investigate associations between symptoms and possible confounding variables”. Based on one clinical assessment and one biological sample per person it will not be possible to understand possible interactions but rather detect possible associations. You may consider adding your expectations here as well. Methods section, page 7 line 25: FGID must be spelled out first time using this abbreviation Methods section, page 11 line 22: “participants are excluded if they report use of anti-depressant or anti-anxiety medications” – maybe a statement of why you chose to exclude active antidepressant treatment. I understand that treatment could possibly influence your findings, however, excluding those receiving psychotropic treatment also introduces a selection bias. If you change the end

	of the introduction and argue that you will eliminate possible confounders (se above) you may skip explanation here. Concerning Methods, Fecal Sample page 18 line 21-33. Could you elaborate on why samples should not be collected within a week of menstruation and add a citation? This is new to me and I do not find any citation when I look it up, so possible other readers may also need more information supporting this strategy. Timeframe: when did this study start and when it is suspected to end? At page 26 line 33-34 you write “since the end of 2017, 211 have met screening…”. It would be valuable under methods to introduce the reader to the timeframe. Concerning interviewers, methods page 13 line 53 “Trained interviewers administer a modified version of the SCID-5-RV to assess the inclusion and exclusion psychiatric disorders…” Is it a medical doctor who verify the psychiatric diagnosis and if not – what is the educational background of the “trained interviewer”? and if not, how do you secure accurate diagnosis? Concerning table 2: you have written the inclusion and exclusion criteria in the text and I find this table long and without new information. I suggest it is removed or placed in supplementary. Concerning Ethics and dissemination page 26 line 18-22: “Participants who complete biological samples are monetarily reimbursed for their time….”. I think this information should be moved to the methods section, participants. Biedermann, L., Zeitz, J., Mwinyi, J., Sutter-Minder, E., Rehman, A., Ott, S. J., . . . Rogler, G. (2013). Smoking cessation induces profound changes in the composition of the intestinal microbiota in humans. PLoS One, 8(3), e59260. doi:10.1371/journal.pone.0059260 Capurso, G., & Lahner, E. (2017). The interaction between smoking, alcohol and the gut microbiome. Best Pract Res Clin Gastroenterol, 31(5), 579-588. doi:10.1016/j.bpg.2017.10.006 Flowers, S. A., Evans, S. J., Ward, K. M., McInnis, M. G., & Ellingrod, V. L. (2017). Interaction Between Atypical Antipsychotics and the Gut Microbiome in a Bipolar Disease Cohort. Pharmacotherapy, 37(3), 261-267. doi:10.1002/phar.1890

REVIEWER	Maria Ellionore Jarbrink-Sehgal Baylor College of Medicine, Houston, Texas, USA
REVIEW RETURNED	29-Dec-2020

GENERAL COMMENTS	The authors present a sound study protocol with aims to characterize oral and gut microbial and physiological profiles in anxiety, depression, and IBS relative to controls and also investigate GI and mental health symptoms in a larger sample while accounting for diet, medical history, and psychosocial factors. This study will help fill an existing gap in knowledge on this topic and therefore is clinically relevant. The study is well designed with sound methodology. The questionnaires are adequate and validated and the sampling and sequencing methods described are adequate for study aims.

	While the current study design may inherit limitations by mere study design such as selection bias due to recruitment process and generalizability, the authors' rationale for using this study design is well explained and reasonable given that the disorders of interest such as anxiety, depression, and IBS, are more prevalent in the study population of young females. The novelty of this study includes the in-depth evaluation of each participants' gut and oral microbiome and the use of different comparison groups with well-defined diagnoses. What further strengthens this study are the strict exclusion criteria that will help eliminate known confounders, which many of the existing studies were unable to do. When analyzing the data, I suggest taking into account the quantity and frequency of alcohol use as well as whether participants are undergoing CBT at the time of oral and fecal sampling.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1

Comment 1:
On page 5 in the end of introduction line 28-38: "Several confounding factors have been neglected in the small literature investigating the microbiota in IBS, anxiety and depression…" It is relevant to state that limited is known; however, I think the it would be valuable to state which possible confounders should be addressed in in this study based on prior research. Maybe something like "This research field is still limited; however, it seems reasonable to consider diet, psychosocial factors and so on…". Further, it would be reasonable to state that smoking (Biedermann et al., 2013; Capurso & Lahner, 2017), alcohol (Capurso & Lahner, 2017). psychotropic medication (Flowers, Evans, Ward, McInnis, & Ellingrod, 2017) and sex seem to influence the gut microbiota and therefore has been eliminated from this study.

Response: Thank you for this helpful suggestion. The confounding factors are now discussed on Page 5, Lines 34-51. Instead of referring to the present study in this introductory paragraph, exclusionary status is now specifically discussed in the Aims and objectives Page 6, Lines 12-20.

"Several confounding factors have been neglected in the small literature investigating the microbiota in IBS, anxiety and depression. Due to their effects on microbial composition, future research should consider psychotropic medication use [25, 26], smoking [27], alcohol consumption [27], IBS subtype and severity [15,18], as well as biological sex [28]. Diet, and its interaction with psychosocial factors, is another source of gut microbiota composition variation that has been insufficiently considered in existing research [29,30]. Finally, the extant literature has not often utilised gold-standard diagnostic measures to examine mental health (i.e., clinical interviews), and studies have examined microbiota composition but neglected microbial function [31]."

"Given the associations between biological sex and the microbiota, this study chose to recruit females only, and exclude current smokers or participants with a substance abuse disorder. Possible sources of inter-study variation in previous investigations will be considered (i.e., symptom severity, medication use, diet)."

Comment 2:
Concerning Aims and objectives page 5 lines 43-44 you write "…to understand interactions between symptoms and confounding variables". According to the study design I would suggest changing this to "…to investigate associations between symptoms and possible confounding variables". Based on one clinical assessment and one biological sample per person it will not be possible to understand possible interactions but rather detect possible associations. You may consider adding your expectations here as well.

Response: We agree that 'association' better reflects the study design, thank you for this suggestion. This has been changed Page 6, Line 6. Given the scale of this study, and the large number of outcomes that will be examined in independent journal articles, we chose not to include expectations in this section.

Comment 3: Methods section, page 7 line 25: FGID must be spelled out first time using this abbreviation

Response: Thank you for your keen eye. This has been elaborated upon on Page 4, Line 31.

Comment 4: Methods section, page 11 line 22: "participants are excluded if they report use of anti-depressant or antianxiety medications" – maybe a statement of why you chose to exclude active antidepressant treatment. I understand that treatment could possibly influence your findings, however, excluding those receiving psychotropic treatment also introduces a selection bias. If you change the end of the introduction and argue that you will eliminate possible confounders (see above) you may skip explanation here.

Response: As per your helpful suggestion, discussion of psychotropics as a confounder is now discussed in detail in the introduction.

Comment 5: Could you elaborate on why samples should not be collected within a week of menstruation and add a citation? This is new to me and I do not find any citation when I look it up, so possible other readers may also need more information supporting this strategy

Response: This has been further clarified Page 12, Lines 45 to 50:

"Participants are asked to wait at least three days after they cease menses to begin collection, and to not collect samples within one week of menstruation, due to the effect of menstrual cycling on endocrine outcomes [39]. A preliminary literature also describes an oestrogen-gut microbiota axis [40], therefore we sought to avoid peaks in oestrogen secretion that may confound microbial analyses."

Comment 6: Timeframe: when did this study start and when it is suspected to end? At page 26 line 33-34 you write "since the end of 2017, 211 have met screening…". It would be valuable under methods to introduce the reader to the timeframe.

Response: This is an important point, which is now discussed Page 6, Line 36:

"Recruitment for the study began in October 2017 and all phases are expected to be completed by

December 2021”

Comment 7: Concerning interviewers, methods page 13 line 53 “Trained interviewers administer a modified version of the SCID-5-RV to assess the inclusion and exclusion psychiatric disorders…” Is it a medical doctor who verify the psychiatric diagnosis and if not – what is the educational background of the “trained interviewer”? and if not, how do you secure accurate diagnosis?

Response: The training of the interviewers and process for validating diagnoses is now more clearly described Page 12, Lines 9-21:

“All interviewers hold a tertiary degree in psychological sciences or related fields, are trained in diagnostic psychological interviewing, and were required to complete Management of Clinical Aggression training (MOCA) [38] and Applied Suicide Intervention Skills Training (ASIST, LivingWorks). Fortnightly clinical meetings are conducted with the research clinical psychologist (OS) to maintain interviewer consistency and discuss any risks presented by participants. Interrater reliability will be conducted for at least 20% of interviews from eligible participants to assess diagnostic-level consistency.”

Comment 8: Concerning table 2: you have written the inclusion and exclusion criteria in the text and I find this table long and without new information. I suggest it is removed or placed in supplementary
Response: Table 2 has now been placed in Supplementary Materials to avoid repetition. It describes some additional information not described in detail in text, such as the precise time periods of diagnoses, as measured by the SCID-5-RV.

Comment 9: Concerning Ethics and dissemination page 26 line 18-22: “Participants who complete biological samples are monetarily reimbursed for their time….”. I think this information should be moved to the methods section, participants.

Response: This paragraph has been moved to the Methods section (Page 8, Line 36).

Reviewer 2

Comment 1: When analyzing the data, I suggest taking into account the quantity and frequency of alcohol use.

Response: Thank you this helpful suggestion. Bugs and Brains Study participants report the frequency of their alcohol use during the semi-structured clinical interview, as well using self-report questionnaires. Participants who meet criteria for a substance use disorder are excluded from all study groups. As you have suggested, we do expect variation in alcohol intake within the sample and this will be considered upon analysis. We have now edited the sentence Page 19, Lines 21-24 to more clearly describe this:

“General linear models will first investigate the relationships between mental health, dietary patterns, exercise, GI health, oral health, early life adversity, substance use (including alcohol), and medical history ...”

Comment 2: When analyzing the data, I suggest taking into account whether participants are undergoing CBT at the time of oral and fecal sampling.

Response: Thank you for this suggestion. Lifetime psychotherapeutic treatment history is indeed collected during the semi-structural clinical interview. This has now been described on Page 12, Line 3-5. We agree that consideration of psychotherapy is an interesting direction, although it is unclear whether we will have sufficient power to compare CBT specifically. Recognising its importance, we intend to explore these associations if possible:

“Trained interviewers administer a modified version of the SCID-5-RV to assess the inclusion and exclusion psychiatric disorders, described in detail in Supplementary Materials. Researchers also collect a hospitalisation and mental health treatment history (e.g., psychotherapeutic and pharmacological)...”

VERSION 2 – REVIEW

REVIEWER	Klara Coello Copenhagen Affective Disorder Research Center (CADIC), Psychiatric Center Copenhagen, Rigshospitalet, Denmark
REVIEW RETURNED	09-Feb-2021

GENERAL COMMENTS	Dear Authors, As mentioned previously, your study protocol is highly relevant addressing the overlap between depression/anxiety disorders and IBS in participants of female sex and includes a clinical interview, questionnaires and biological samples examining the gut and oral microbiota, urine, saliva and hair metabolites. The paper is well-written and has improved since first revision and I only have a few comments that you may chose to include or not: 1) Limitations: The generalizability of your study is not only restricted to female patients with depression/anxiety age 18-40 years but further restricted to current no smokers, medication free without substance abuse. You may consider adding this to your limitations. 2) Introduction, last paragraph: as the present paper is a study protocol and not a review, I suggest you replace "future reseach should consider" with "it seems reasonable to consider...", Currently it is not clear to me if you mean future studies including the present study or not. I agree, that the added sections in Methods, exclusion criteria makes it clearer. Consider rewriting so the reader understands that in the present study you chose to consider the following confounders as should be done in future studies accordingly. 2) Methods p. 7 line 51: I suggest you write functional gastrointestinal disorder insead of FGID (I know you introduced the abbreviation but as you only use it few times, I think spelling it out is much better) 3) Methods, Depression and anxiety group, page 7 lines 39-50: Maybe you could write a little clearer when patients with current depression can be included and not. What do you define as partial remission? It is confusing to me and maybe you could rewrite it and make it clearer?

	Further, in Exclusion criteria p. 8 line 49-57 you state that no psychotropics are allowed. If a participant is enrolled currently experiencing depression, is it then allowed that psychotropic medication is initiated during the trial? Congratulations with a nice study protocol. I am looking forward to read your results.

VERSION 2 – AUTHOR RESPONSE

Reviewer 1

Comment 1:
Limitations: The generalizability of your study is not only restricted to female patients with depression/anxiety age 18-40 years but further restricted to current no smokers, medication free without substance abuse. You may consider adding this to your limitations.

Response: Thank you for this suggestion. The generalisability of the sample is indeed further limited by these factors. We have incorporated this into the manuscript Page 3: “Due to the possible effects of host sex, age and substance use on microbial outcomes, this study chose to recruit females aged 18 to 40 who were non-smokers, medication free, and without a substance abuse disorder; the generalisability of this study is therefore limited to this specific population.”

Comment 2:
Introduction, last paragraph: as the present paper is a study protocol and not a review, I suggest you replace "future reseach should consider" with "it seems reasonable to consider...", Currently it is not clear to me if you mean future studies including the present study or not. I agree, that the added sections in Methods, exclusion criteria makes it clearer. Consider rewriting so the reader understands that in the present study you chose to consider the following confounders as should be done in future studies accordingly.

Response: We have now incorporated your suggestion:
“Due to their effects on microbial composition, it seems reasonable to consider psychotropic medication use [25, 26], smoking [27], alcohol consumption [27], IBS subtype and severity [15,18], as well as biological sex [28].”

Comment 3:
Methods p. 7 line 51: I suggest you write functional gastrointestinal disorder insead of FGID (I know you introduced the abbreviation but as you only use it few times, I think spelling it out is much better)

Response: We have now written ‘functional gastrointestinal disorder’ in full on Pages 4, 7 and 8.

Comment 4:
Methods, Depression and anxiety group, page 7 lines 39-50:
Maybe you could write a little clearer when patients with current depression can be included and not. What do you define as partial remission? It is confusing to me and maybe you could rewrite it and make it clearer?

Response: This statement was to capture participants with Major Depressive Disorder who experience clinical episodes of brief duration. The wording has been clarified Page 7:

“Given the possibility that participants may experience a Major Depressive Episode of relatively brief duration, participants with Major Depressive Disorder will also be included if an episode is in partial remission (i.e., although full diagnostic criteria are not met, symptoms of the immediately preceding clinical episode are present and have not fully resolved at the time of assessment).”

Comment 5: Further, in Exclusion criteria p. 8 line 49-57 you state that no psychotropics are allowed. If a participant is enrolled currently experiencing depression, is it then allowed that psychotropic medication is initiated during the trial?

Response: Participants are excluded if they begin these medications throughout the study. This has now been clarified Page 8:
“Participants are excluded if they report use of anti-depressants, anxiolytics, other psychotropics, steroids, probiotics, prebiotics or synbiotics in the past four weeks or throughout the duration of their participation....”
“If participants report short-term medication use or illness, they are invited to participate when they meet eligibility criteria.”