PEER REVIEW HISTORY

BMJ Open Science publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (http://openscience.bmj.com/pages/wp-content/uploads/sites/62/2018/04/BMJ-Open-Science-Reviewer-Score-Sheet.pdf) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

ARTICLE DETAILS

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<th>TITLE (PROVISIONAL)</th>
<th>Administration of galacto-oligosaccharide prebiotics in the Flinders Sensitive Line animal model of depression</th>
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<td>AUTHORS</td>
<td>Bannach-Brown, A.</td>
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VERSION 1 - REVIEW

| REVIEWER 1             | Anna Paula Ramos Costa |
|                       | University College Cork |
| REVIEW RETURNED       | 14-08-18 |

| GENERAL COMMENTS       | Recommendation Major Revision Comments |
|                       | The preregistered protocol proposal presented in general didn't appeal to me as doesn't propose any novelty in the field. The hypothesis is not clear. It states: little is known about the mechanisms prebiotics can influence behaviour, but does not propose anything to address this gap in the literature. It presents many reserach that supports the fact the prebiotics alter behaviour and the only novelty in the field would be the use of a different Rat strain without explaining this choice. So I would suggest to change the proposal trying to address the real gaps in the field of the Brain-gut axis. |
|                       | In the Methods session there are some points to be clarified: 1) It is not clear the lighting regime proposed. 2) The forced swimming test it's a test to screen antidepressants and not a depressed-like behavior test like the authors state. 3) The power analysis for the forced swimming test is using as a reference a work done in mice, and the power calculation for 2 different species should be different (rats require a pre-test session, which do not occur in mice). 4) the use of tap water to treat the animals with GOS can bring a bias in the sense that the water can be contaminated with unknown virus and bacterias that may influence the effect of the prebiotic, therefore I suggest the use of autoclaved tap water. 5) |
It's not clear if the animals will be kept in the same animal facility room, which could bring another problem, once is well know that in the same environment there is the microbe transfer occurance. Are they going to be put in isolators to prevent this transfer? 6) The method of euthanasia is important for future chemistry analysis. Are the animals going to be perfused before collection of the brain? Which type of analysis you would like to do in future? They are also important to take into account for power calculations and better planning of future outcomes.

Positive points: The authors are concerned about the welfare of the animals and the replacement of oral gavage treatment for syringe feeding is a very good strategy to avoid the stress of gavage treatment. The randomizations and blinding plan is adequate.

| REVIEWER 2 | Natasha Karp  
Astrazeneca |
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I have significant reservations about the proposal. The current documentation gives a thorough description of the experiment and implements latest thinking in good practice of experimental design. However, the manuscript does not convince me that the planned experiments are well thought out in terms of good practice in managing the difficulties of studying the microbiome. I also have reservations about the planned data analysis.

In detail

1. The manuscript fails to discuss the bigger picture limitations of the model or design. I would like a discussion of the translatable risk of the study and limitations of the design.

2. Why are they studying males only when we know disease progression, severity, outcome etc is sex dependent and in fact the disease of interest is more common in females?

3. How are you minimising litter effects? We know that the founder effect in the development of the microbiome is significant and small variation in the initial microbiome will have long term impact of the microbiome.

4. The manuscript correctly identifies that the experimental unit is the cage, one as this is the point of randomisation and two
because of co-housing impact on the microbiome of individuals. However, when it comes to statistical analysis, it isn’t clear that this is being correctly accounted for.

5. The power calculation focuses on data from the open field test. However, the primary outcome is listed as the forced swim test. In reality, for reliable results all variables of interest need to have sufficient power.

6. Moore & Stanley 2016 Clinical translation Immunology discuss the challenges of studies on microbiome. They raise the issue of the high intrinsic variability in microbiota and the high n needed and the inconsistencies in the literature on these topics are likely a result of failing to account appropriately for this. They talk about the need to ensure the starting microbiome is similar and the value of repeat measure designs to unpick the relationship. They highlight the difficulty of insuring the starting microbiome is similar and suggest strategies such as co-housing etc.

7. I note that the housing and husbandry information fails to provide details of the type of facility and the associated health and immune status of the animals.

8. I was also concerned by potential order effects within the experimental studies and how would they minimise these.

9. Exclusion criteria
   o The current wording suggests that unless an animal has a complete complement of data it will be excluded. This seems excessive and wastes data from an animal. I agree if an animal is removed from a study due to an unrelated illness all data from that animal will be excluded. However, technical reasons might lead to a loss of data. For example, a power cut during a screen or a sample dropped. It is appropriate to specify a prior technical reasons and remove on a screen by screen basis.
   o Whilst the authors have specified prior reasons for data exclusion, a later statement “Data cleaning and statistical analysis will be carried...” could be interpreted as free for all on data cleaning. There should be no further data cleaning beyond the rules established. This needs to be clearly clarified.

10. Insufficient information was provided in the force swim test on the outcome variables. It wasn’t clear how the traits of interest were quantified. Eg how does passive behaviour become a quantitative measure – time spent immobile?
Dear Dr Sena and Dear Reviewers, Ana Paula Ramos Costa & Natasha Karp,

Thank you for your time and consideration of the manuscript titled: “Administration of galactooligosaccharide prebiotics in the Flinders Sensitive Line animal model of depression”.

Both reviewers provided thoughtful comments and helpful suggestions. Given external constraints for the timescale of the experiments, the work has already been performed. I understand that the current manuscript will be considered as a Protocol submission rather than a registered report, and so the helpful suggestions for alterations to the experimental design are, unfortunately, longer possible. Where the reviewer comments pertain to issues which can be addressed in a Protocol paper, we have attempted to do so. There have also, in the course of the experiment, been slight deviations from the protocol. These are described and dated in our OSF registration, and our results paper will make it clear where these deviations occurred. However, in the spirit of protocol registration the current manuscript describes our original intentions. We believe the changes we have made in light of your comments has substantially improved the clarity of our manuscript. Changes to the manuscript and reply to each of your comments is detailed below.

Dear Reviewer 1: Ana Paula Ramos Costa

Thank you for your helpful comments. You provided important critiques of a central aspect of the protocol, the hypothesis of the experiment. Further, you highlighted areas of the protocol that were not clear, for this we are grateful.

Comment 1: “The preregistered protocol proposal presented in general didn’t appeal to me as doesn’t propose any novelty in the field. The hypothesis is not clear. It states: little is known about the mechanisms prebiotics can influence behaviour, but does not propose anything to address this gap in the literature. It presents many research that supports the fact the prebiotics alter behaviour and the only novelty in the field would be the use of a different Rat strain without explaining this choice. So I would suggest to change the proposal trying to address the real gaps in the field of the Brain-gut axis.”

- Thank you for this important comment. We understand this comment in 2 parts. Firstly, that the hypothesis is not clear and does not represent the aim of the work. The aim of this work is to contribute to findings of behavioural effects of prebiotics on depressive-like phenotypes in animal models of depression. *We have clarified the aims in text (Introduction > Hypotheses).* Secondly, that the work itself is not novel and that altering our approach would contribute more to the field. We did endeavour to collect evidence regarding the effects prebiotics in animal models of depression using a genetic strain, the flinders sensitive line model. While we can only work with the resources available for this project, we will endeavour to collaborate more widely with experts in microbiome analyses and the broad range of neurobiological processes that are involved in gut-brain axis alterations in
neuropsychiatric research in future to provide a fuller picture of behavioural effects of psychobiotics in animal models of depression.

In the Methods session there are some points to be clarified:

1) It is not clear the lighting regime proposed.

   - Thank you for highlighting this. We understood this to mean that the “time point” information was consuming. We have removed reference to time-points here and all references to time points has been removed throughout to avoid confusion, and instead referred to how many hours after the lights were turned off each procedure was carried out.

2) The forced swimming test it’s a test to screen antidepressants and not a depressed-like behaviour test like the authors state.

   - Thank you for this comment. Yes the FST is a test to screen antidepressants. In doing so, it measures the animals’ activity in the cylinder. We use the measures of behaviours in the test, as indicators of depressive-like behaviour. We therefore have not changed the language about “depressive-like behaviour as measured by the forced swim test” in this protocol.

3) The power analysis for the forced swimming test is using as a reference a work done in mice, and the power calculation for 2 different species should be different (rats require a pre-test session, which do not occur in mice).

   - Thank you for this comment. Published data from similar studies was used to calculate an approximate sample size, one of these studies (Burokas et al., 2017) was conducted in mice and the second (McVey Neufeld et al., 2017) was conducted in rats. We were limited to using the published literature available. We are unaware of any literature which can advise on how a sample size calculation should be altered to take into account differences in variation between species. We have added this limitation to a new section called, “Limitations of the study” at the end of the protocol, also to address Reviewer 2’s comments.

4) the use of tap water to treat the animals with GOS can bring a bias in the sense that the water can be contaminated with unknown virus and bacteria that may influence the effect of the prebiotic, therefore I suggest the use of autoclaved tap water.

   - Thank you for your comment. This factor was considered. The product Bimuno is available to purchase by the general public from health food stores and pharmacies. The general public will consume this product by mixing it with tap water as suggested on the product instructions. Therefore we wanted to preserve the “clinical” setting as much as possible. Further investigation into the impact of different bacteria in tap water on prebiotics could clarify how much of an impact this has.
5) It's not clear if the animals will be kept in the same animal facility room, which could bring another problem, once is well know that in the same environment there is the microbe transfer occurrence. Are they going to be put in isolators to prevent this transfer?

- Thank you for this comment. Although the experiment has already been conducted, this is an interesting consideration. Animals in this study were not housed in isolators or individually ventilated cages as shared housing aimed to mimic the complex microbiota interactions in humans, as this was a disease model study. This would make an interesting experiment to assess the degree of microbe transfer when animals share the same animal facility vs. when housed in isolators and what effects this has on depressive-like and anxiety-like behavioural outcomes. It was however, not the primary aim of this study. Future studies could investigate this in a confirmatory experiment.

6) The method of euthanasia is important for future chemistry analysis. Are the animals going to be perfused before collection of the brain? Which type of analysis you would like to do in future? They are also important to take into account for power calculations and better planning of future outcomes.

- Thank you for this comment. We agree that the method of euthanasia is critical for the validity of various chemical analyses. As this submission has been changed to a protocol, unfortunately, this was not applicable here as brains were not collected in the execution of this experiment.

Positive points: The authors are concerned about the welfare of the animals and the replacement of oral gavage treatment for syringe feeding its a very good strategy to avoid the stress of gavage treatment. The randomizations and blinding plan is adequate.

- Thank you for your feedback.

Dear Reviewer 2: Natasha Karp

Thank you for your comments. You have highlighted keys areas surrounding the experimental design and areas to consider for the external validity and limitations of the study. We have added sections to the protocol to address this. Many thanks.

I have significant reservations about the proposal. The current documentation gives a through description of the experiment and implements latest thinking in good practice of experimental design. However, the manuscript does not convince me that the planned experiments are well thought out in terms of good practice in managing the difficulties of studying the microbiome. I also have reservations about the planned data analysis.
In detail

1. The manuscript fails to discuss the bigger picture limitations of the model or design. I would like a discussion of the translatability risk of the study and limitations of the design.

   - Thank you for highlighting this. We have added limitations of the study design to a new section called “Limitations of the study” at the end of the protocol. In this section, we describe the limitations highlighted in this peer review as well as others. With regards to the translatability risk of the study, we have added a section called “External validity considerations” to the end of the protocol where we discuss the advantages and limitations of translating the possible findings from the study.

2. Why are they studying males only when we know disease progression, severity, outcome etc is sex dependent and in fact the disease of interest is more common in females?

   - Thank you for this comment. We would have liked to investigate the sex effects of prebiotics in FSL and FRL animals. Due to the number of animals used in this experiment, it was our aim to conduct this experiment in males and then expand the investigation by exploring the sex effects. As this is a significantly larger experiment, minimum 2 x 64 animals, we were therefore unable to conduct it with the resources available at this stage in the investigation. This point has been added to the section “External validity considerations”.

3. How are you minimising litter effects? We know that the founder effect in the development of the microbiome is significant and small variation in the initial microbiome will have long term impact of the microbiome.

   - Thank you for this comment. We did not control for litter effects. Animals were co-housed, in standard cages, 1 week prior to the start of the experiment. We hoped that this would allow for the sharing of microbiota between cage mates. We have noted this in the limitations section at the end of the protocol.

4. The manuscript correctly identifies that the experimental unit is the cage, one as this is the point of randomisation and two because of co-housing impact on the microbiome of individuals. However, when it comes to statistical analysis, it isn’t clear that this is being correctly accounted for.

   - Thank you for this comment. The analysis will be carried out using the experimental unit. This has been clarified in text with the addition of “the data from individual animals will be averaged per cage to obtain a value for the experimental unit” to the statistical analysis section.

5. The power calculation focuses on data from the open field test. However, the primary outcome is listed as the forced swim test. In reality, for reliable results all variables of interest need to have sufficient power.
- Thank you for this comment. Published data from similar studies was used to calculate an approximate sample size, one of these studies (McVey Neufeld et al., 2017) was conducted in rats using the open field test. We were limited to using the published literature available. McVey Neufeld and colleagues did not report using the FST in their experiment. However we used data from the open field test as we did not have published FST data from rats, and we were using the open field test as a secondary outcome. As a rough sample size calculation is better than no sample size calculation, we decided to proceed, but we have added this limitation to a new section called, “Limitations of the study” at the end of the protocol.

6. Moore & Stanley 2016 Clinical translation Immunology discuss the challenges of studies on microbiome. They raise the issue of the high intrinsic variability in microbiota and the high n needed and the inconsistencies in the literature on these topics are likely a result of failing to account appropriately for this. They talk about the need to ensure the starting microbiome is similar and the value of repeat measure designs to unpick the relationship. They highlight the difficulty of insuring the starting microbiome is similar and suggest strategies such as co-housing etc.

   - Thank you for this comment. The starting gut microbiota could play a large role in the relationship between prebiotics and behaviour. We aimed to measure the starting microbiome of each animal, and measure the impact of prebiotics on the gut microbiome by taking fecal samples at the beginning and at the end of the experiment. Rather than controlling for similar gut microbiota between the animals at the start of the experiment, which would reduce the variability observed, also does not reflect a naturalistic environment. Humans and rodents have a wide variation in gut microbiota. Therefore, post-hoc grouping of animals into their abundance of bacteria could help predict response to prebiotics. Further, animals in this study were co-housed 1 week prior to the experiment start to ensure that cage-mates shared microbiota and reduce cage variation.

7. I note that the housing and husbandry information fails to provide details of the type of facility and the associated health and immune status of the animals.

   - Thank you for highlighting this lack of reporting. These details have now been added to the manuscript under the heading ‘Methods’ > ‘Animals’.

8. I was also concerned by potential order effects within the experimental studies and how would they minimise these.

   - Thank you for this comment. The order of behavioural tests was not randomised in this experiment. To randomise the order of behavioural tests and be able to statistically test and correct for carry-over effects, we would need to add this as a variable and therefore increase the sample size of groups substantially. All animals were tested on behavioural assessments in the same order, elevated plus maze (EPM), 48 hours rest, pre-swim of the forced swim test (FST), 24 hours rest, open field test of locomotion, and FST test session directly after. We introduced a break of 48 hours between the EPM and FST pre-swim session to minimise effects from the EPM on the FST, and the order was chosen to do from least to most stress-inducing.

9. Exclusion criteria
The current wording suggests that unless an animal has a complete complement of data it will be excluded. This seems excessive and wastes data from an animal. I agree if an animal is removed from a study due to an unrelated illness all data from that animal will be excluded. However, technical reasons might lead to a loss of data. For example, a power cut during a screen or a sample dropped. It is appropriate to specify a prior technical reasons and remove on a screen by screen basis.

- Thank you for highlighting this. We intended that all animals should be included in the analysis of a behavioural test if they completed it. We have changed the wording to hopefully make this clear. “Data for each animal will be included in analysis of the individual tests if they completed the test. If an animal does not complete the open field test they will be removed from the FST analysis as we would be unable to exclude the impact of hyper-locomotion of activity in the FST.”

Whilst the authors have specified prior reasons for data exclusion, a later statement “Data cleaning and statistical analysis will be carried...” could be interpreted as free for all on data cleaning. There should be no further data cleaning beyond the rules established. This needs to be clearly clarified.

- Thank you for highlighting this. We were referring to any data transformations of removal of missing studies. We hope we have clarified this by adding “Potential data transformation and all statistical analysis will be carried out...”

10. Insufficient information was provided in the force swim test on the outcome variables. It wasn’t clear how the traits of interest were quantified. Eg how does passive behaviour become a quantitative measure – time spent immobile?

- Thank you for this comment. We have added more explanation in text as to how the quantitative measures for the FST were calculated.

Further, we identified some minor errors in referencing and a typo in the composition of the control dose which have been corrected.

Many thanks and kind regards,

Alexandra Bannach-Brown
### REVIEWER 1

**Anna Paula Ramos Costa**  
**University College Cork**

**REVIEW RETURNED** 30-10-18

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<th>GENERAL COMMENTS</th>
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<td><strong>Comments</strong></td>
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<td>I honestly didn't understand why the research group submitted a manuscript as a registered report if there was no time to wait for the reviewers answers about the proposal before starting the work. I was not completely satisfied with the answer presented by the author regarding the protocol suggestions that can no longer be applied as the author stated that &quot;the work has already been performed&quot;. As researchers we do understand funding restrictions, we do understand time restrictions and how hard is to plan animal work, but having this in mind the authors shouldn't submit the manuscript as a registered report. I also don't believe the work fits as a protocol submission because the authors did not state the dates of the experiments and as far I understood the work is finished. I would need much more clarification in this case to make a fair decision.</td>
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### REVIEWER 2

**Natasha Karp**  
**Astrazeneca**

**REVIEW RETURNED** 26-10-18

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studies have limitations and therefore it is important to have a frank and honest discussion on this to allow the results to be interpreted within the context of these issues. As a protocol, the amendments have significantly improved the manuscript and addressed the concerns around the design and validity. There are minor issues below that need to be addressed Issue 1: Litter effects I feel the wording the authors have added around the potential litter effect is down-playing the issue “If animals in a cage were from the same litter they are hypothesised to have more similar microbiota compared to animals in a cage from separate litters.” It isn’t hypothesized, it well established that there can be profound founder effects on the microbiome (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4973323/). I agree the extent the litter effect will be impacting this experiment is unknown, but the current wording is avoiding acknowledging the real risk they have taken. This needs to be reworded. The concept the authors raised in the response to the reviewer that this issue might be minimized by the cohousing prior to the start of the experiment needs to be added to the manuscript where this limitation is discussed. Issue 2: Sex bias The authors in the response to the reviewer letter have argued that the testing of both sexes would have doubled the sample size and thus was not feasible at this point. The authors have reiterated a common misconception. The reality is that when you study both sexes you do not need to double the sample size. Consider when you move from study with just two groups (e.g. control versus treatment) to a factorial design which also includes sex, you only add two terms to the regression analysis and so only lose 2 degrees of freedom. Another way to put it is that in a factorial design, you estimate the treatment effect across groups and use the data from both sexes to estimate the treatment effect and hence the n that is contributing to the sensitivity is split across the two sexes. I do acknowledge there are difficulties to studying both sexes, such as the management of pheromones, however the number of animals is
not an appropriate defence. The wording added to the manuscript “The findings from this experiment will need to be replicated in female animals, with a separate experiment to analyse the sex differences in the effects of prebiotics.” Needs rewording. If you later run a second experiment solely on females you will be unable to test statistically whether the effect depended on sex, any difference could be a batch variation. To answer this question, you would need to run both sexes again. Furthermore, you are not replicating the experiment, as you haven’t assessed the effect of sex at this point. Failure to include females at this stage of the study, means that you will proceed assuming the data is representative which is questionable, or you have to be completed repeat the experiment which is ethically questionable. Please add to the abstract that the study was on male animals. Issue 3: Design taken In the response to the reviewer letter, the authors discussed their design decisions on whether to standardized the gut microbiota. “ We aimed to measure the starting microbiome of each animal, and measure the impact of prebiotics on the gut microbiome by taking fecal samples at the beginning and at the end of the experiment. Rather than controlling for similar gut microbiota between the animals at the start of the experiment, which would reduce the variability observed, also does not reflect a naturalistic environment.” This is an important point and should be added to the discussion on the external validity of the study.

VERSION 2 – AUTHOR RESPONSE

Dear Dr Sena and Dear Reviewers, Dr Ana Paula Ramos Costa & Dr Natasha Karp,

Thank you for your time in reconsidering the manuscript titled: “Administration of galacto-oligosaccharide prebiotics in the Flinders Sensitive Line animal model of depression”.


We have taken into account the comments from your reviewers and added significant sections to the "Background" and "Discussion" relating to the application of the registered reports formats to animal experiments. We are grateful for the comments provided and we believe that they have allowed us to clarify the content of the manuscript.

External Peer Review of Ana Paula Ramos Costa (#1)
Recommendation       Reject
Comments
I honestly didn't understand why the research group submitted a manuscript as a registered report if there was no time to wait for the reviewers answers about the proposal before starting the work. I was not completely satisfied with the answer presented by the author regarding the protocol suggestions that can no longer be applied as the author stated that "the work has already been performed". As researchers we do understand funding restrictions, we do understand time restrictions and how hard is to plan animal work, but having this in mind the authors shouldn't submit the manuscript as a registered report. I also don't believe the work fits as a protocol submission because the authors did not state the dates of the experiments and as far I understood the work is finished. I would need much more clarification in this case to make a fair decision.

- ABB: Thank you for your comment. We can understand your frustration and concern. When we initially submitted the manuscript as a Registered Report we had not yet started the experiment. However, reviewer comments were not received until 5 months later, and constraints of funding and resource (and a time limited PhD project) meant that we had been unable to delay the start of the experiment any further. We acknowledge that Registered Reports have an extremely useful place in scientific publishing; but our experience suggests that the model may need to be adapted for successful application to the field of animal experiments. We hope that you will reconsider this manuscript as a Protocol instead of a Registered Report. We have discussed these issues in the manuscript.

External Peer Review of Natasha Karp (#2)
Recommendation       Minor Revision

It is disappointing that the data has already been collected prior to the process being completed and this has led to the potential benefits of a prior review failing to materialize. There was an opportunity to significantly increase the value of the experiment and reduce the future use of animals that could have been taken (see later discussion around sex). I am, however, pleased that the authors have added a section discussing the limitation of the study. All studies have limitations and therefore it is important to have a frank and honest discussion on this to allow the results to be interpreted within the context of these issues. As a protocol, the amendments have significantly improved the manuscript and addressed the concerns around the design and validity. There are minor issues below that need to be addressed

- ABB: Thank you. We have added to the 'Background' and 'Limitations' sections discussing our experiences of the Registered Reports for animal experiments and the merits and limitations of this publication style.

Issue 1: Litter effects
I feel the wording the authors have added around the potential litter effect is down-playing the issue "If animals in a cage were from the same litter they are hypothesised to have more similar microbiota compared to animals in a cage from separate litters." It isn’t hypothesized, it well established that there can be profound founder effects on the microbiome (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4973323/). I agree the extent the litter effect will be impacting this experiment is unknown, but the current wording is avoiding acknowledging the real risk they have taken. This needs to be reworded. The concept the authors raised in the response to the reviewer that this issue might be minimized by the cohousing prior to the start of the experiment needs to be added to the manuscript where this limitation is discussed.

- Thank you for this comment. We agree had downplayed the risk and that our wording was unclear. We have reworded this section.

Issue 2: Sex bias

The authors in the response to the reviewer letter have argued that the testing of both sexes would have doubled the sample size and thus was not feasible at this point. The authors have reiterated a common misconception. The reality is that when you study both sexes you do not need to double the sample size. Consider when you move from study with just two groups (e.g. control versus treatment) to a factorial design which also includes sex, you only add two terms to the regression analysis and so only lose 2 degrees of freedom. Another way to put it is that in a factorial design, you estimate the treatment effect across groups and use the data from both sexes to estimate the treatment effect and hence the n that is contributing to the sensitivity is split across the two sexes. I do acknowledge there are difficulties to studying both sexes, such as the management of pheromones, however the number of animals is not an appropriate defence. The wording added to the manuscript “The findings from this experiment will need to be replicated in female animals, with a separate experiment to analyse the sex differences in the effects of prebiotics.” Needs rewording.

If you later run a second experiment solely on females you will be unable to test statistically whether the effect depended on sex, any difference could be a batch variation. To answer this question, you would need to run both sexes again. Furthermore, you are not replicating the experiment, as you haven’t assessed the effect of sex at this point. Failure to include females at this stage of the study, means that you will proceed assuming the data is representative which is questionable, or you have to be completed repeat the experiment which is ethically questionable. Please add to the abstract that the study was on male animals.

- ABB: Thank you for bringing this to our attention. We have reworded that section of the ‘Discussion & Limitations’ to reflect this and we have added to the abstract that male experimental animals were used.

Issue 3: Design taken

In the response to the reviewer letter, the authors discussed their design decisions on whether to standardized the gut microbiota. “We aimed to measure the starting microbiome of each animal, and measure the impact of prebiotics on the gut microbiome by taking fecal samples at the beginning and at the end of the experiment. Rather than controlling for similar gut microbiota between the animals at the start of the experiment, which would reduce the variability observed, also does not reflect a naturalistic environment.” This is an important point and should be added to the discussion on the external validity of the study.

- ABB: Thank you for highlighting this. We have added this information to the paper.
Many thanks and kind regards,

Alexandra Bannach-Brown

On behalf of co-authors
BMJ Open Science is committed to open peer review. As part of this commitment we make the peer review history of every article we publish publicly available.

When an article is published we post the peer reviewers’ comments and the authors’ responses online. We also post the versions of the paper that were used during peer review. These are the versions that the peer review comments apply to.

The versions of the paper that follow are the versions that were submitted during the peer review process. They are not the versions of record or the final published versions. They should not be cited or distributed as the published version of this manuscript.

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If you have any questions on BMJ Open’s open peer review process please email info.bmjos@bmj.com
Administration of galacto-oligosaccharide prebiotics in the Flinders Sensitive Line animal model of depression

Authors: Bannach-Brown, A.,1,2 Tillmann, S.,2 Macleod M.R.,1 Wegener, G.2

Affiliations: 1. CAMARADES, Centre for Clinical Brain Sciences, Chancellor’s Building, Little France, University of Edinburgh. 2. Translational Neuropsychiatry Unit, Department of Clinical Medicine, Risskov Psychiatric Hospital, Aarhus University

Keywords
Animal models of depression, prebiotics,

Abstract
**Introduction:** Major Depressive Disorder is the leading source of disability globally and current pharmacological treatments are less than adequate. Animal models such as the Flinders Sensitive Line (FSL) rats are used to mimic aspects of the phenotype in the human disorder and to characterise candidate antidepressant agents. Communication between the gut microbiome and the brain may play an important role in psychiatric disorders such as depression. Interventions targeting the gut microbiota may serve as potential treatments for depression, and this drives increasing research into the effect of probiotics and prebiotics in neuropsychiatric disorders. Prebiotics, galacto-oligosaccharides and fructo-oligosaccharides that stimulate the activity of gut bacteria, have been reported to have a positive impact, reducing anxiety and depressive-like phenotypes and stress-related physiology in mice and rats, as well as in humans. Bimuno®, the commercially available beta-galactooligosaccharide, has been shown to increase gut microbiota diversity. **Aim:** Here, we aim to investigate the effect of Bimuno® on rat anxiety- and depressive-like behaviour and gut microbiota composition in the FSL model, a genetic model of depression, in comparison to their control, the Flinders Resistant Line (FRL) rats. **Methods:** Sixty-four 5-7 week old rats, 32 FSL and 32 FRL rats, will be randomised to receive Bimuno or control (4g/kg) daily for 4 weeks. Animals will be tested, by an experimenter unaware of group allocation, on the Forced Swim Test to assessed depressive-like behaviour, the Elevated Plus Maze to assess anxiety-like behaviour, and the open field test to assess locomotion. Animals will be weighed and food and water intake, per kg of bodyweight, will be recorded. Faeces will be collected from each animal prior to the start of the experiment and on the final day to assess the bacterial diversity and relative abundance of bacterial genera in the gut. All outcomes and statistical analysis will be carried out blinded to group allocation, group assignments will be revealed after raw data has been uploaded to Figshare. Two-way ANOVA will be carried out to investigate the effect of treatment (control or prebiotic) and strain (FSL or FRL) on depressive-like and anxiety-like behaviour.
Introduction

Major Depressive Disorder (MDD) is the leading source of disability globally (Marcus et al., 2012) and treatment resistance among patients is roughly 50% (Thomas et al., 2013). Therefore, better understanding mechanisms behind MDD and the search for potential effective and novel therapeutic targets are high research and healthcare priorities. Animal models are commonly used to mimic aspects of the phenotype of the human disorder and to characterise candidate antidepressant agents. The Flinders Sensitive Line (FSL) is a well-established and validated genetic model of depression (Overstreet & Wegener, 2013). The FSL rats are bred to display cholinergic sensitivity and later found to display depressive-like behaviour in the forced swim test (FST), compared to their control strain, the Flinders Resistant Line rats (FRL) (Overstreet & Wegener, 2013). FSL rats respond to acute and chronic antidepressant administration and display reduced hippocampal plasticity (Chen et al., 2010) and elevated rapid eye movement (REM) sleep (Benca et al., 1996) in comparison to FRL rats.

Communication between the gut microbiome and the brain may play a role in psychiatric disorders, with research focussing on the bidirectional signalling at the neural, hormonal and immunological levels (Cryan & O’Mahony, 2011). Interventions targeting the gut microbiota may serve as potential treatments for depression, and this drives increasing research into the effect of probiotics and prebiotics in neuropsychiatric disorders. Probiotics have been defined as “live organisms, that when ingested in adequate amounts, exert health benefits.” (Dinan, Stanton & Cryan, 2013). Several probiotic strains have been investigated in psychiatric disorders and have reported effects on behaviour and physiology, in laboratory animals and humans (for a review see Wang and colleagues (2016)). Commercially available probiotic products, “Ecological Barrier” and “Probio’Stick”, have been tested in FSL rats (Abildgaard et al., 2017; Tillmann, et al., 2018). Prebiotics, defined as galacto-oligosaccharides and fructo-oligosaccharides that stimulate the activity of Bifidobacteria and Lactobacilli in the gut, have also been reported to have a positive impact, reducing anxiety and depressive-like phenotypes and stress-related physiology in mice and rats (Burokas et al., 2017; Mika et al., 2017; McVey Neufeld et al., 2017; Thompson et al., 2017; Savignac et al., 2013) and in humans (Perez-Cornago et al., 2016; Schmidt et al., 2015; Tillisch et al., 2013). Further, prebiotics have been shown to increase the diversity of gut microbial composition, with evidence from mice (Burokas et al., 2017) and rats (Mika et al., 2017). Thompson and colleagues (2017), however, showed no difference in gut microbiota composition in F344 rats receiving prebiotics. One prebiotic that is commercially available is Bimuno®. Bimuno® contains beta-galactooligosaccharide (B-GOS) produced from lactose in cow’s milk (Bimuno, 2018).
Based on previous studies showing promising effects of other prebiotics to reduce depressive- and anxiety-like behaviour, we will investigate the effect of Bimuno® on rat behaviour and gut microbiota composition in the FSL model, a genetic model of depression, in comparison to their control FRL rats.

**Hypotheses:** We hypothesise that FSL animals receiving Bimuno® prebiotics will display reduced depressive-like behaviour in the FST and reduced anxiety-like behaviour in the Elevated Plus Maze (EPM) in comparison to control (substance without active ingredients, Bimuno® Free Sugars). As our secondary outcome, we hypothesise that FSL animals receiving prebiotics will display increased diversity in the gut microbiome, in comparison to FSL animals receiving control, as measured on true beta diversity. We want to analyse gut microbiome diversity because we hypothesise that this is the mechanism through which prebiotics influence behaviour. Little is known about these mechanisms and we therefore aim to shed light on the commensal influence of prebiotics (Sherwin et al., 2016). We hypothesise that animals receiving Bimuno® prebiotics will have altered weight and food intake in comparison to animals receiving control.

**Methods**

**Animals:**

Ethics has been approved by Aarhus University animal ethics committee (permission ID 2012-15-2934-00254). 5–7 week-old male FSL and FRL rats bred in-house at TNU, Aarhus University, will be used.

Animals will be housed in pairs, in standard cages with a plastic bottom and metal rack top half, purchased from Techniplast (Cage 1291H Eurostandard Type III H, 425 × 266 × 185 mm, Tecniplast, Italy). The bedding material in each cage will be made out of wooden chips (aspen wood from Tapvei®, Finland) along with access to a tunnel shelter, nesting material, and a wooden stick. Animals will be maintained in a 12hr light/dark cycle with lights off at 1300hrs (time point 0). Seven days prior to the experiment start, the animals will be moved to the experimental facility and the new lightning regime will start immediately. Animals will be under the care of FELASA-accredited in-house animal technicians. Animals will have tap water and standard chow (purchased from Brogaarden®, Altomen 1324).

**Power calculation to determine the number of animals:**

Our sample size calculations are based on published behavioural findings from Burokas and colleagues (2017) and McVey Neufeld and colleagues (2017).
Data were extracted from Burokas et al., (2017) who investigated effects of prebiotics in the FST using male C57L/6J mice (Fig. 6D in the publication). These data (mean, SEM, and group numbers) were used to run a one-way ANOVA and determine an eta squared (= SSbetween/SStotal) of 0.579. This eta squared value was used to compute effect size $f = \sqrt{\frac{\text{eta}^2}{1 - \text{eta}^2}}$ which is 1.1747. This effect size was used in the power calculation carried out in R (v. 3.4.3) using the function “pwr.anova.test”. A significance level of 0.01 and a power of 0.9 were chosen. This gave the result of 6 experimental units per group. An experimental unit is the entity subjected to an intervention independently of all other units where it’s possible to assign two experimental units to different treatments groups (NC3Rs, 2018).

Data were extracted from McVey Neufeld et al., (2017) who used prebiotics and probiotics in a maternal separation model of depression in the open field using male Sprague-Dawley rats. Data is from the amount of time spent in the centre of the open field (Fig 1.B in the publication) for the model group. These data (mean, SD or SEM, and group numbers) were used to run a one-way ANOVA and determine an eta squared (= SSbetween/SStotal) of 0.522. This eta squared value was used to compute effect size $f = \sqrt{\frac{\text{eta}^2}{1 - \text{eta}^2}}$ which is 1.046. This effect size was used in the power calculation carried out in R (v. 3.4.3) using the function “pwr.anova.test”. A significance level of 0.01 and a power of 0.9 were chosen. This gave the result of 6 experimental units per group.

Based on the a priori sample size calculations above and experience from previous in-house experiments, a conservative estimate of sample size for this study of 8 experimental units per group was selected. This number is two per group larger than the power calculation and was selected to account for possible attrition or possible exclusions throughout the experiment (see criteria below). With 8 experimental units per group, power of 90%, and a significance level of 0.01, we are powered to detect an effect of $f = 0.86$. This effect size we consider biologically relevant, in order to see a relevant reduction in immobility behaviour in the FST. The full R code for these calculations is provided in the appendix.

**Prebiotics Administration:**

The prebiotic and control treatment will be administered for 28 consecutive days (4 weeks). The treatments will be administered within the first hour after lights off, the first hour of the animals’ active phase.

We will use the commercially available prebiotic product “Bimuno®” Powder (Bimuno, United Kingdom), which contains Galactooligosaccharides (B-GOS). A dose of 4 g/kg dissolved in tap water will be used per animal per day, administered by syringe feeding. The dose will be adjusted each week according to the
weight of the animals. This prebiotic was chosen due to its superior effect on behaviour over FOS (Savignac et al., 2013). This dose was given to recreate the findings in previous literature (Savignac et al., 2013; Williams et al., 2016).

Control Administration:

The control for the prebiotics will be the Free Sugars (BFS; consisting of 27% lactose, 23% glucose, and 50% galactose). The control will be administered at a dose of 4 g/kg/day. This follows the dosing regimen of previous literature (Williams et al., 2016; Savignac et al., 2013). This control will be administered simultaneously to the prebiotics administration via syringe feeding in a volume of 2 ml. The dose will be adjusted each week according to the weight of the animals.

Syringe-feeding Details:

Treatment will be administered via syringe-feeding, the prebiotic, within a sweetened vehicle of glucose, is mixed with tap water, and added to a syringe. This is a newly established method for the accurate individual dosing of probiotics in rats (Tillmann & Wegener, 2017). With a training phase of roughly 3–4 days, to allow the rats to become accustomed to the administration and the taste, the rats willingly consume the mixture and approach the edge of the cage when the syringe is presented. This new method has been used for volumes of probiotic + vehicle solution up to 3 ml. This method of administration has been chosen to reduce the stress associated with oral gavage, and to increase the accuracy of dosing with administration of prebiotics in drinking water. In this experiment, the prebiotic Bimuno will be added to tap water to give a total volume of 2 ml, as the smaller the volume, the sweeter the solution, which is thought to be more desirable for the rats to consume. Animals will be fed at the start of the active cycle, time point +0 hours.

Measures to Reduce the Risk of Bias

Randomisation to Treatment and Control & Allocation Concealment

On the first day of the experiment, animals will be moved from the breeding facility into the experimental facility. Animals are pair-housed; the two animals in each cage will be the same strain and will receive the same treatment. Cages will be randomly assigned to a group, Treatment or Control, to ensure allocation concealment during the handling and administration of treatment throughout the experiment.
Randomisation will be carried out using block randomisation with the online tool, The Sealed Envelope (https://www.sealedenvelope.com/simple-randomiser/v1/lists), by a colleague not involved in the day-to-day running of the experiment. Cages will be labelled with a unique randomisation code (e.g. GU9, LI3, etc) and a list of which treatments are given to which cage will be read off each day. This is to minimise potential unconscious bias by ‘remembering’ which cages get which treatment. Treatments will be identified as A or B. The cages (the experimental unit) will be assigned randomly to treatment and the observational unit is the individual animal where the outcome of interest is measured. The observational unit (the animal) is nested within the experimental unit. The order of the cages will be randomised in the racks at the beginning of the experiment so as to reduce possible effects from air-conditioning vents and/or being closer to the door. The placement of the cage will not be taken into consideration as a variable during analysis of the outcome data. Animals will have 7 days to acclimatise to new housing facilities. When the experiment and treatment administration begins, the experimenter will be blinded to which solution (prebiotic or vehicle control) each rat receives. The prebiotics and free sugars are delivered in unmarked sachets (only with company logo and batch number) of 3.56g. Sachets will be removed from their identifying boxes and moved into plastic boxes marked e.g. A+B, to signify which groups will receive the sachets, by a colleague not involved in the day-to-day running of the experiment. They will put enough sachets for the duration of the experiment. This allows the primary experimenter to prepare and administer the treatments each day for 28 days in a blinded manner.

**Blinded Assessment of Primary Outcome**

The primary outcome is the FST. This outcome is recorded on video and scored manually. The videos will be assessed blinded, before the group identity of the animals is revealed. The same procedure will be carried out for the open field test. All videos will be analysed after all behavioural outcomes have been carried out. The primary experimenter will be formally unblinded to the true group identity after data analysis files have been uploaded to Open Science Framework (OSF).
Outcome Assessment:
Behavioural assessment will occur during the rats’ active phase, starting approximately 1 hour after administration of prebiotics, time point +1 hour, and lasting approximately 3 hours, until time point +4 hours.

Forced Swim Test
On day 26 of the experiment, at time point +1 hr, at the start of the animals’ active phase, the FST will be performed. Clear glass cylinders (60 cm h × 24cm Ø) filled with water up to 40 cm will be used. The temperature of the water will be kept at 25 °C ± 1°C. On the first day, the pre-swim session, the animals will be placed in the tanks for 15 minutes. On the second day of testing, animals will be placed into the tanks for 5 minutes. Both sessions will be recorded by video camera. Both testing sessions will be conducted in red light conditions. Three behavioural parameters will be assessed from the video footage, passive behaviour, immobility, and 2 active behaviours, swimming and climbing behaviour. Passive behaviour is defined as “the rat making no further movements beyond those needed to keep its head above the water” (Abildgaard et al., 2017). For each 5s period, the predominant behaviour will be recorded (immobility,
swimming, or climbing). All swimming sessions will be scored by an experimenter blinded to the group assignment of the animals.

Open Field Test

Locomotor activity will be assessed day 27, immediately prior to the second FST session. Locomotor activity will be assessed in a 100 cm × 100 cm (x 20 cm H) black open field arena. Each animal will be placed in the arena in the same starting location. Animals will be assessed for 15 minutes in red light because the animals will be tested in their active phase at time point +1hr. All sessions will be video recorded and analysed using Noldus Ethovision XT9. Locomotor activity will be measured from the video recording, assessed as the distance each animal moved in centimetres. The arena will be cleaned with ethanol between each animal. All video recordings will be scored by an experimenter blinded to the group assignment of the animals.

Elevated Plus Maze

Anxiety behaviour will be assessed on day 24 in the elevated plus maze. The plus-shaped maze has two open arms and two closed arms (length: 50 cm x width: 10cm) and the centre zone measures 10 cm x 10 cm. Each animal will be placed in the arena in the centre, facing the same open arm. Animals will be assessed for 5 minutes and will be tested during their active phase at time point +1hr. The light intensity in the open arms will be 80–100 lx and 20 lx in the closed arms. Animals will be kept in an adjacent dark experimental room and moved individually into the bright experimental room for testing. All sessions will be video recorded and analysed using Noldus Ethovision XT9. Anxiety behaviour will be measured by calculating the time spent in the open arms in proportion to the time spent in the open arms and closed arms and number of entries into open arms, and number of entries into open arms (defined as entire body of rat in the open arm). The arena will be cleaned with ethanol between each animal. All video recordings will be scored by an experimenter blinded to the group assignment of the animals.

Body Weight & Food Consumption

Animals will be weighed every week throughout the experiment, to assess if prebiotics administration influences weight gain, and also to adjust the dose of the prebiotics or control administered (4g/kg). Weekly food and water intake (per kg bodyweight) in the home cage will also be recorded.
Microbiota Analysis

Fecal boli will be collected at the start of the experiment (day 1) and on day of euthanisation (day 28). Fecal boli collected at the start and the end of the experiment will be collected from directly each animal into sterile tubes and frozen and stored at −80°C. Fecal boli will be used to analyse the composition of gut microbiota. DNA will be isolated from the fecal boli using the isopropanol DNA extraction method (Hart et al., 2015). 16S rRNA amplicon sequencing on V4-5 will be carried out on fecal boli from individual animals. Broad sequencing will be carried out by an external biotech company, DNA Sense, in Aalborg, Denmark. The alpha diversity, the beta diversity, and the abundance of genera, more specifically, the relative abundance of *Bifidobacterium* and *Lactobacillus* genera will be analysed as GOS prebiotics have previously been shown to enhance diversity and these specific genera (Monteagudo-Mera et al., 2016; Liu et al., 2017; Burokas et al., 2017).

Dissection

Animals will be euthanized on day 28 and the whole brain will be removed. Brains will be stored in formaldehyde at −20°C. Brain chemistry analyses to assess neurotransmitter receptor mRNA expression and growth factors such as BNDF, will be carried out, funding permitting. A protocol annex for these additional analyses will be submitted to the Open Science Framework prior to commencement.

Exclusion Criteria:

1. Animals will be excluded from the study if they at any point during the study display illness as assessed by trained, in-house, veterinarians on a daily basis. Animals will be euthanized immediately to end suffering.
2. No animals will be excluded from the statistical analysis if they successfully complete all aspects of the study.
3. Potential reasons why an animal might not complete all aspects of the study include technical difficulties during the video recording, or other circumstances where the raw data or data collection process has been compromised and statistical analysis is not possible.
4. There are no exclusion criteria based on performance on behavioural tests.
5. Potential reasons why an animal might be excluded from microbial analysis include failure to produce fecal boli on two separate occasions during the day of collection, or technical difficulties during the sequencing.

Experimental Procedure:

Step 1: Animals are bred in house – At 5-7 weeks of age, rats will be moved into experimental housing and have a 7-day acclimatisation/habitation period to the animal housing facility prior to the start of the experiment. The FSL rats will be randomised into 2 groups; Treatment & Control. The FRL rats will be randomised into 2 groups; Treatment & Control.

Step 2: On day 1 of the experiment, each rat will be weighed and fecal boli will be collected. Then the first administration of prebiotics or control will be given.

Step 3: Animals will remain continuously on this treatment regimen for 4 weeks (28 days). Rats will be weighed every week. The animals’ daily food and water consumption will be recorded.

Step 4: On day 24 animals will be subjected to the elevated plus maze.

Step 5: On day 26 animals will be subjected to the pre-swim of the FST.

Step 6: On day 27 the open field test and the FST will be carried out.

Step 7: On day 28 animals will be euthanized and brains will be harvested from all animals.

The timing of outcome assessments is displayed in table 1.

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment</th>
<th>Length of Admin</th>
<th>Elevated Plus Maze</th>
<th>FST &amp; Open Field</th>
<th>Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSL</td>
<td>Prebiotics</td>
<td>4 weeks</td>
<td>Day 24</td>
<td>Day 27</td>
<td>Day 28</td>
</tr>
<tr>
<td>FSL</td>
<td>Control</td>
<td>4 weeks</td>
<td>Day 24</td>
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</tr>
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</tr>
</tbody>
</table>

Table 1. Timing of outcome measure administration to each group.
Data Analysis Pipeline:
Data from the open field and FST will be recorded in Ethovison. Videos will be stored on a network drive. Video from each animal will be scored blinded to the animal’s group assignment. Data cleaning and statistical analysis will be carried out in R Studio using the latest version of R.

Statistical Analysis:
Firstly, a student’s t-test will be performed to compare performance in the FST between the FSL and FRL rats, to confirm that the FSL rats do indeed display increased depressive-like behaviour. To test the hypothesis that probiotics improve depressive-like behaviour on the FST and anxiety-like behaviour in the EPM, the primary outcome being immobility time, a two-way ANOVA will be conducted with 2 independent variables, treatment (control or prebiotic) and strain (FSL or FRL). Group differences will be investigated with a Tukey post-hoc test. If data are not normally distributed, a base-10 log transformation will be carried out. If data do not meet assumptions for homogeneity of variances in the ANOVA or t-test, a Welch correction will be used. For the ANOVA, a Kruskal-Wallis test will be conducted if residuals are not normally distributed. A tukey post-hoc test will be carried out to investigate group differences; this will be carried out also if data are corrected/transformed data. All raw data, transformed (if applicable), and data analysis code will be uploaded to Figshare.

Principal Coordinate Analysis will be conducted based on 16s rRNA sequencing data to quantify the beta diversity, clustering based on Operational Taxonomic Unit (OTU), and to measure the relative abundance of genera. The relative abundance of *Bifidobacterium* and *Lactobacillus* genera, as well as total bacteria, will be compared between groups.
References:


Administration of galacto-oligosaccharide prebiotics in the Flinders Sensitive Line animal model of depression

Authors: Bannach-Brown, A.,1,2 Tillmann, S.,2 Macleod M.R.,1 Wegener, G.2

Affiliations: 1. CAMARADES, Centre for Clinical Brain Sciences, Chancellor’s Building, Little France, University of Edinburgh. 2. Translational Neuropsychiatry Unit, Department of Clinical Medicine, Risskov Psychiatric Hospital, Aarhus University

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Abstract

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Introduction

Major Depressive Disorder (MDD) is the leading source of disability globally (Marcus et al., 2012) and treatment resistance among patients is roughly 50% (Thomas et al., 2013). Therefore, better understanding mechanisms behind MDD and the search for potential effective and novel therapeutic targets are high research and healthcare priorities. Animal models are commonly used to mimic aspects of the phenotype of the human disorder and to characterise candidate antidepressant agents. The Flinders Sensitive Line (FSL) is a well-established and validated genetic model of depression (Overstreet & Wegener, 2013). The FSL rats are bred to display cholinergic sensitivity and later found to display depressive-like behaviour in the forced swim test (FST), compared to their control strain, the Flinders Resistant Line rats (FRL) (Overstreet & Wegener, 2013). FSL rats respond to acute and chronic antidepressant administration and display reduced hippocampal plasticity (Chen et al., 2010) and elevated rapid eye movement (REM) sleep (Benca et al., 1996) in comparison to FRL rats.

Communication between the gut microbiome and the brain may play a role in psychiatric disorders, with research focusing on the bidirectional signalling at the neural, hormonal and immunological levels (Cryan & O’Mahony, 2011). Interventions targeting the gut microbiota may serve as potential treatments for depression, and this drives increasing research into the effect of probiotics and prebiotics in neuropsychiatric disorders. Probiotics have been defined as “live organisms, that when ingested in adequate amounts, exert health benefits.” (Dinan, Stanton & Cryan, 2013). Several probiotic strains have been investigated in psychiatric disorders and have reported effects on behaviour and physiology, in laboratory animals and humans (for a review see Wang and colleagues (2016)). Commercially available probiotic products, “Ecological Barrier” and “Probio’Stick”, have been tested in FSL rats (Abildgaard et al., 2017; Tillmann, et al., 2018). Prebiotics, defined as substrates that are selectively utilised by a host organism providing a health benefit (Gibson et al., 2017), have also been reported to have a positive impact, reducing anxiety and depressive-like phenotypes and stress-related physiology in mice and rats (Burokas et al., 2017; Mika et al., 2017; McVey Neufeld et al., 2017; Thompson et al., 2017; Savignac et al., 2013; Savignac et al., 2016) and in humans (Schmidt et al., 2015; Kazemi et al., 2018). Further, prebiotics have been shown to increase the diversity of gut microbial composition, with evidence from mice (Burokas et al., 2017) and rats (Mika et al., 2017). Thompson and colleagues (2017), however, showed no difference in gut microbiota composition in F344 rats receiving prebiotics. One prebiotic that is commercially available is Bimuno®. Bimuno® contains beta-galactooligosaccharide (B-GOS) produced from lactose in cow’s milk (Bimuno, 2018).

These previous studies show promising effects of other prebiotics to reduce the depressive-like and anxiety-like phenotypes in stress models of depression (Burokas et al., 2017; McVey Neufeld et al., 2017), and
resilience to a stressful exposure (Mika et al., 2017; Thompson et al. 2017). Bimuno® shows an effect on anxiety-like behaviour in response to a single LPS insult (Savignac et al., 2016) and GOS prebiotics have an effect on BDNF levels (Savignac et al., 2013). Based on previous studies showing promising effects of other prebiotics to reduce depressive- and anxiety-like behaviour, we will investigate the effect of Bimuno® on rat behaviour and gut microbiota composition in the FSL model, a genetic model of depression, in comparison to their control FRL rats.

**Hypotheses:** We hypothesise that FSL animals receiving Bimuno® prebiotics will display reduced depressive-like behaviour in the FST and reduced anxiety-like behaviour in the Elevated Plus Maze (EPM) in comparison to control (substance without active ingredients, Bimuno® Free Sugars). We aim to contribute to the literature describing the behavioural effects of prebiotics in animal models of depression. As our secondary outcome, we hypothesise that FSL animals receiving prebiotics will display increased diversity in the gut microbiome, in comparison to FSL animals receiving control, as measured on true beta diversity. We want to analyse gut microbiome diversity because we hypothesise that this is the mechanism through which prebiotics influence behaviour, we therefore aim to shed light on the commensal influence of prebiotics (Sherwin et al., 2016). We hypothesise that animals receiving Bimuno® prebiotics will have altered weight and food intake in comparison to animals receiving control.

**Methods**

**Animals:**

Ethics has been approved by Aarhus University animal ethics committee (permission ID 2012-15-2934-00254). 5–7 week-old male FSL and FRL rats bred in-house at TNU, Aarhus University, will be used. Animals are bred in a closed breeding colony. Animals will be transferred from the closed breeding colony to a conventional animal facility and housed here for the duration of the experiment. All animals bred in house are checked every 3 months for infectious agents in accordance with FELSA recommendations (Mähler et al., 2014, http://journals.sagepub.com/doi/pdf/10.1177/0023677213516312 ). Animals did not have any known infections at the start of the experiment and were healthy, as assessed by FELSA-accredited in-house animal technicians.

Animals will be housed in pairs, in standard cages with a plastic bottom and metal rack top half, purchased from Techniplast (Cage 1291H Eurostandard Type III H, 425 × 266 × 185 mm, Tecniplast, Italy). The bedding material in each cage will be made out of wooden chips (aspen wood from Tapvei®, Finland) along with access to a tunnel shelter, nesting material, and a wooden stick. Animals will be maintained in a 12hr light/dark cycle
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Our sample size calculations are based on published behavioural findings from Burokas and colleagues (2017) and McVey Neufeld and colleagues (2017).

Data were extracted from Burokas et al., (2017) who investigated effects of prebiotics in the FST using male C57L/6J mice (Fig. 6D in the publication). These data (mean, SEM, and group numbers) were used to run a one-way ANOVA and determine an eta squared (= SSbetween/SStotal) of 0.579. This eta squared value was used to compute effect size \( f = \sqrt{\left(\frac{\eta^2}{1 - \eta^2}\right)} \) which is 1.1747. This effect size was used in the power calculation carried out in R (v. 3.4.3) using the function “pwr.anova.test”. A significance level of 0.01 and a power of 0.9 were chosen. This gave the result of 6 experimental units per group. An experimental unit is the entity subjected to an intervention independently of all other units where it’s possible to assign two experimental units to different treatments groups (NC3Rs, 2018).

Data were extracted from McVey Neufeld et al., (2017) who used prebiotics and probiotics in a maternal separation model of depression in the open field using male Sprague-Dawley rats. Data is from the amount of time spent in the centre of the open field (Fig 1.B in the publication) for the model group. These data (mean, SD or SEM, and group numbers) were used to run a one-way ANOVA and determine an eta squared (= SSbetween/SStotal) of 0.522. This eta squared value was used to compute effect size \( f = \sqrt{\left(\frac{\eta^2}{1 - \eta^2}\right)} \) which is 1.046. This effect size was used in the power calculation carried out in R (v. 3.4.3) using the function “pwr.anova.test”. A significance level of 0.01 and a power of 0.9 were chosen. This gave the result of 6 experimental units per group.

Based on the a priori sample size calculations above and experience from previous in-house experiments, a conservative estimate of sample size for this study of 8 experimental units per group was selected. This number is two per group larger than the power calculation and was selected to account for possible attrition or possible exclusions throughout the experiment (see criteria below). With 8 experimental units per group, power of 90%, and a significance level of 0.01, we are powered to detect an effect of \( f = 0.86 \). This effect size
we consider biologically relevant, in order to see a relevant reduction in immobility behaviour in the FST. The full R code for these calculations is provided in the appendix.

**Prebiotics Administration:**

The prebiotic and control treatment will be administered for 28 consecutive days (4 weeks). The treatments will be administered within the first hour after lights off, the first hour of the animals’ active phase.

We will use the commercially available prebiotic product “Bimuno®” Powder (Bimuno, United Kingdom), which contains beta-galactooligosaccharides (B-GOS). A dose of 4 g/kg dissolved in tap water will be used per animal per day, administered by syringe feeding. The dose will be adjusted each week according to the weight of the animals. This prebiotic was chosen due to its superior effect over FOS (Savignac et al., 2013). This dose was given to recreate the findings in previous literature (Savignac et al., 2013; Williams et al., 2016).

**Control Administration:**

The control for the prebiotics will be the Bimuno free sugars (BFS; consisting of 50% lactose, 27% glucose, and 23% galactose). The control will be administered at a dose of 4 g/kg/day. This follows the dosing regimen of previous literature (Williams et al., 2016; Savignac et al., 2013). This control will be administered simultaneously to the prebiotics administration via syringe feeding in a volume of 2 ml. The dose will be adjusted each week according to the weight of the animals.

**Syringe-feeding Details:**

Treatment will be administered via syringe-feeding, the prebiotic, within a sweetened vehicle of glucose, is mixed with tap water, and added to a syringe. This is a newly established method for the accurate individual dosing of probiotics in rats (Tillmann & Wegener, 2017). With a training phase of roughly 3–4 days, to allow the rats to become accustomed to the administration and the taste, the rats willingly consume the mixture and approach the edge of the cage when the syringe is presented. This new method has been used for volumes of probiotic + vehicle solution up to 3 ml. This method of administration has been chosen to reduce the stress associated with oral gavage, and to increase the accuracy of dosing with administration of prebiotics in drinking water. In this experiment, the prebiotic Bimuno will be added to tap water to give a total volume of 2 ml, as the smaller the volume, the sweeter the solution, which is thought to be more
desirable for the rats to consume. Animals will be fed at the start of the active cycle, within the first hour after lights off.

Measures to Reduce the Risk of Bias

*Randomisation to Treatment and Control & Allocation Concealment*

On the first day of the experiment, animals will be moved from the breeding facility into the experimental facility. Animals are pair-housed; the two animals in each cage will be the same strain and will receive the same treatment. Cages will be randomly assigned to a group, Treatment or Control, to ensure allocation concealment during the handling and administration of treatment throughout the experiment. Randomisation will be carried out using block randomisation with the online tool, The Sealed Envelope ([https://www.sealedenvelope.com/simple-randomiser/v1/lists](https://www.sealedenvelope.com/simple-randomiser/v1/lists)), by a colleague not involved in the day-to-day running of the experiment. Cages will be labelled with a unique randomisation code (e.g. GU9, LI3, etc) and a list of which treatments are given to which cage will be read off each day. This is to minimise potential unconscious bias by ‘remembering’ which cages get which treatment. Treatments will be identified as A or B. The cages (the experimental unit) will be assigned randomly to treatment and the observational unit is the individual animal where the outcome of interest is measured. The observational unit (the animal) is nested within the experimental unit. The order of the cages will be randomised in the racks at the beginning of the experiment so as to reduce possible effects from air-conditioning vents and/or being closer to the door. The placement of the cage will not be taken into consideration as a variable during analysis of the outcome data. Animals will have 7 days to acclimatise to new housing facilities. When the experiment and treatment administration begins, the experimenter will be blinded to which solution (prebiotic or vehicle control) each rat receives. The prebiotics and free sugars are delivered in unmarked sachets (only with company logo and batch number) of 3.56g. Sachets will be removed from their identifying boxes and moved into plastic boxes marked e.g. A+B, to signify which groups will receive the sachets, by a colleague not involved in the day-to-day running of the experiment. They will put enough sachets for the duration of the experiment. This allows the primary experimenter to prepare and administer the treatments each day for 28 days in a blinded manner.

*Blinded Assessment of Primary Outcome*

The primary outcome is the FST. This outcome is recorded on video and scored manually. The videos will be assessed blinded, before the group identity of the animals is revealed. The same procedure will be carried
out for the open field test. All videos will be analysed after all behavioural outcomes have been carried out. The primary experimenter will be formally unblinded to the true group identity after data analysis files have been uploaded to Open Science Framework (OSF).

![Experimental Design Setup](image)

**Figure 1. Experimental Design Setup**

**Outcome Assessment:**

Behavioural assessment will occur during the rats’ active phase, starting approximately 1 hour after administration of prebiotics, 1 hour after lights off, and lasting approximately 3 hours, until 4 hours after light off.

**Forced Swim Test**

On day 26 of the experiment, 1 hour after lights off, at the start of the animals’ active phase, the FST will be performed. Clear glass cylinders (60 cm h × 24cm Ø) filled with water up to 40 cm will be used. The temperature of the water will be kept at 25 °C ± 1°C. On the first day, the pre-swim session, the animals will be placed in the tanks for 15 minutes. On the second day of testing, animals will be placed into the tanks for 5 minutes. Both sessions will be recorded by video camera. Both testing sessions will be conducted in red light conditions. Three behavioural parameters will be assessed from the video footage, passive behaviour,
immobility, and 2 active behaviours, swimming and climbing behaviour. Passive behaviour is defined as “the rat making no further movements beyond those needed to keep its head above the water” (Abildgaard et al., 2017). For each 5s period, the predominant behaviour will be recorded (immobility, swimming, or climbing). Counts of behaviour on the three behaviours will be summed and time spent across the swim session (5mins) will be calculated, e.g. 12 x 5 second periods of immobility = 60 seconds out of 5 minutes, 27 x 5 second periods of swimming = 135 seconds out of 5 minutes, 21 x 5 second periods of climbing = 105 seconds out of 5 minutes. All swimming sessions will be scored by an experimenter blinded to the group assignment of the animals.

Open Field Test

Locomotor activity will be assessed day 27, immediately prior to the second FST session. Locomotor activity will be assessed in a 100 cm x 100 cm (x 20 cm H) black open field arena. Each animal will be placed in the arena in the same starting location. Animals will be assessed for 15 minutes in red light because the animals will be tested in their active phase at 1 hour after lights off. All sessions will be video recorded and analysed using Noldus Ethovision XT9. Locomotor activity will be measured from the video recording, assessed as the distance each animal moved in centimetres. The arena will be cleaned with ethanol between each animal. All video recordings will be scored by an experimenter blinded to the group assignment of the animals.

Elevated Plus Maze

Anxiety behaviour will be assessed on day 24 in the elevated plus maze. The plus-shaped maze has two open arms and two closed arms (length: 50 cm x width: 10cm) and the centre zone measures 10 cm x 10 cm. Each animal will be placed in the arena in the centre, facing the same open arm. Animals will be assessed for 5 minutes and will be tested during their active phase, one hour after lights off. The light intensity in the open arms will be 80–100 lx and 20 lx in the closed arms. Animals will be kept in an adjacent dark experimental room and moved individually into the bright experimental room for testing. All sessions will be video recorded and analysed using Noldus Ethovision XT9. Anxiety behaviour will be measured by calculating the time spent in the open arms in proportion to the time spent in the open arms and closed arms and number of entries into open arms, and number of entries into open arms (defined as entire body of rat in the open arm). The arena will be cleaned with ethanol between each animal. All video recordings will be scored by an experimenter blinded to the group assignment of the animals.
**Body Weight & Food Consumption**

Animals will be weighed every week throughout the experiment, to assess if prebiotics administration influences weight gain, and also to adjust the dose of the prebiotics or control administered (4g/kg). Weekly food and water intake (per kg bodyweight) in the home cage will also be recorded.

**Microbiota Analysis**

Fecal boli will be collected at the start of the experiment (day 1) and on day of euthanisation (day 28). Fecal boli collected at the start and the end of the experiment will be collected from directly each animal into sterile tubes and frozen and stored at −80°C. Fecal boli will be used to analyse the composition of gut microbiota. DNA will be isolated from the fecal boli using the isopropanol DNA extraction method (Hart et al., 2015). 16S rRNA amplicon sequencing on V4-5 will be carried out on fecal boli from individual animals. Broad sequencing will be carried out by an external biotech company, DNA Sense, in Aalborg, Denmark. The alpha diversity, the beta diversity, and the abundance of genera, more specifically, the relative abundance of *Bifidobacterium* and *Lactobacillus* genera will be analysed as GOS prebiotics have previously been shown to enhance diversity and these specific genera (Monteagudo-Mera et al., 2016; Liu et al., 2017; Burokas et al., 2017).

**Dissection**

Animals will be euthanized on day 28 and the whole brain will be removed. Brains will be stored in formaldehyde at −20°C. Brain chemistry analyses to assess neurotransmitter receptor mRNA expression and growth factors such as BNDF, will be carried out, funding permitting. A protocol annex for these additional analyses will be submitted to the Open Science Framework prior to commencement.

**Exclusion Criteria:**

1. Animals will be excluded from the study if they at any point during the study display illness as assessed by trained, in-house, veterinarians on a daily basis. Animals will be euthanized immediately to end suffering.
2. No animals will be excluded from the statistical analysis if they successfully complete all aspects of the study. Data for each animal will be included in analysis of the individual tests if they completed the test. If an animal does not complete the open field test they will be removed from the FST analysis as we would be unable to exclude the impact of hyper-locomotion of activity in the FST.

3. Potential reasons why an animal might not complete an aspect of the study include technical difficulties during the video recording, or other circumstances where the raw data or data collection process has been compromised and statistical analysis is not possible.

4. There are no exclusion criteria based on performance on behavioural tests.

5. Potential reasons why an animal might be excluded from microbial analysis include failure to produce fecal boli on two separate occasions during the day of collection, or technical difficulties during the sequencing.

Experimental Procedure:

Step 1: Animals are bred in house – At 5-7 weeks of age, rats will be moved into experimental housing and have a 7-day acclimatisation/habitation period to the animal housing facility prior to the start of the experiment. The FSL rats will be randomised into 2 groups; Treatment & Control. The FRL rats will be randomised into 2 groups; Treatment & Control.

Step 2: On day 1 of the experiment, each rat will be weighed and fecal boli will be collected. Then the first administration of prebiotics or control will be given.

Step 3: Animals will remain continuously on this treatment regimen for 4 weeks (28 days). Rats will be weighed every week. The animals’ daily food and water consumption will be recorded.

Step 4: On day 24 animals will be subjected to the elevated plus maze.

Step 5: On day 26 animals will be subjected to the pre-swim of the FST.

Step 6: On day 27 the open field test and the FST will be carried out.

Step 7: On day 28 animals will be euthanized and brains will be harvested from all animals.

The timing of outcome assessments is displayed in table 1.
Table 1. Timing of outcome measure administration to each group.

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment</th>
<th>Length of Admin</th>
<th>Elevated Plus Maze</th>
<th>FST &amp; Open Field</th>
<th>Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSL</td>
<td>Prebiotics</td>
<td>4 weeks</td>
<td>Day 24</td>
<td>Day 27</td>
<td>Day 28</td>
</tr>
<tr>
<td>FSL</td>
<td>Control</td>
<td>4 weeks</td>
<td>Day 24</td>
<td>Day 27</td>
<td>Day 28</td>
</tr>
<tr>
<td>FRL</td>
<td>Prebiotics</td>
<td>4 weeks</td>
<td>Day 24</td>
<td>Day 27</td>
<td>Day 28</td>
</tr>
<tr>
<td>FRL</td>
<td>Control</td>
<td>4 weeks</td>
<td>Day 24</td>
<td>Day 27</td>
<td>Day 28</td>
</tr>
</tbody>
</table>

Data Analysis Pipeline:

Data from the open field and FST will be recorded in Ethovison. Videos will be stored on a network drive. Video from each animal will be scored blinded to the animal’s group assignment. Potential data transformation and all statistical analysis will be carried out in R Studio using the latest version of R.

Statistical Analysis:

Firstly, the data from individual animals will be averaged per cage to obtain a value for the experimental unit. A student’s t-test will be performed to compare performance in the FST between the FSL and FRL rats, to confirm that the FSL rats do indeed display increased depressive-like behaviour. To test the hypothesis that probiotics improve depressive-like behaviour on the FST and anxiety-like behaviour in the EPM, the primary outcome being immobility time, a two-way ANOVA will be conducted with 2 independent variables, treatment (control or prebiotic) and strain (FSL or FRL). Group differences will be investigated with a Tukey post-hoc test. If data are not normally distributed, a base-10 log transformation will be carried out. If data do not meet assumptions for homogeneity of variances in the ANOVA or t-test, a Welch correction will be used. For the ANOVA, a Kruskal-Wallis test will be conducted if residuals are not normally distributed. A tukey post-hoc test will be carried out to investigate group differences; this will be carried out also if data are corrected/transformed data. All raw data, transformed (if applicable), and data analysis code will be uploaded to Figshare.

Principal Coordinate Analysis will be conducted based on 16s rRNA sequencing data to quantify the beta diversity, clustering based on Operational Taxonomic Unit (OTU), and to measure the relative abundance of genera. The relative abundance of *Bifidobacterium* and *Lactobacillus* genera, as well as total bacteria, will be compared between groups.
Limitations of the study and external validity considerations:

The sample size calculation used to estimate the number of animals to be used is based on one study in mice using the FST (Burokas et al., 2017) and one study in rats using the open field test (McVey Neufeld et al., 2017). There are limitations to the use of the outcomes from these papers. Firstly, we do not have published data from the effects of prebiotics in rats for our primary outcome, the forced swim test. Therefore, we combined the use of published FST data from mice and published data from rats in the open field, our secondary outcome. This may yield an inaccurate appropriate sample size calculation however, we are using the available published data to provide a best estimate.

We did not control for littermate effects in this study. Litter mate effects could impact the similarity of microbiota between and within cages. If animals in a cage were from the same litter they are hypothesised to have more similar microbiota compared to animals in a cage from separate litters. Further, if animals in a treatment group are from 2-3 litters compared to animals from more than 3 or 4 litters, there is hypothesised to be higher variability.

The choice of model in this experiment will be the Flinders Sensitive Line rats. This model of depression has a moderate level of face validity (Overstreet et al., 2013) sharing some similarities with the human disorder such as, elevated REM sleep and increased passive behaviour following stress (Overstreet et al., 2013). However, this model does not reflect the full heterogeneity of symptoms observed in depression in humans, and may not reflect the complex interplay between genetics and environment that contribute to the development of depression in humans. Therefore, there may be limited translatability of the findings from this study directly to human populations.

This experiment will be conducted in male animals only. The findings from this experiment will need to be replicated in female animals, with a separate experiment to analyse the sex differences in the effects of prebiotics. Therefore, the generalisability of findings from this study will need to be confirmed with follow up studies.

The choice of behavioural outcome measures that will be used in the experiment are elevated plus maze, open field test, and the forced swim test. The limitations of these behavioural tests have been extensively discussed elsewhere (Commons et al., 2017, Spruijt et al., 2014), we acknowledge the issues with lack of reproducibility across studies. The order of administration of the tests was chosen to be conducted from least to most stress-inducing. All animals will be tested on behavioural assessments in the same order, elevated
plus maze (EPM), 48 hours rest, pre-swim of the forced swim test (FST), 24 hours rest, open field test of locomotion, and FST test session directly after.

The prebiotics will be mixed with tap water. This method of administration is similar to how the prebiotics would be administered in human studies. Although there has not been an investigation into the effects of tap water vs. autoclaved water, we thought this method of administration would maintain ecological validity and mimics the method of administration in human consumption of the prebiotics.
References:


