A Comparative Study on Depression and Anxiety of Mice *(Mus musculus)* on a Prebiotic-supplemented Diet versus a Standard Diet during Unpredictable Chronic Mild Stress (UCMS)

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ABSTRACT

Background. Anxiety and depression are becoming increasingly prevalent today and are often aggravated by day-to-day stresses. Because current management strategies are usually accompanied by unpleasant side effects, there is a need to look into alternative treatment regimens - such as prebiotics - that may provide equally effective anxiolytic and antidepressant effects.

Objective. Therefore, the study aims to determine the effect of a combined fructooligosaccharide (FOS) and galactooligosaccharide (GOS) supplemented diet on anxiety and depression levels in mice subjected to Unpredictable Chronic Mild Stress (UCMS).

Methods. Forty male BALB/C mice were subjected to UCMS under a pretest-posttest control group design where the treatment group received prebiotic supplementation throughout the study. Repeated measures ANOVA was run to evaluate between, within, and time interactions of the measured anxiety parameters using the light-dark box test, and depression parameter using the fur coat state assessment.

Results. Results show that (1) the FOS + GOS treatment did not give the treatment group an advantage over the control group during UCMS, (2) both groups grew more anxious and depressed over time, and (3) the treatment group grew more anxious with time in relation to control in terms of the total time spent in the light side.

Conclusion. These imply that the UCMS protocol was successful in inducing stress in mice, but the FOS + GOS regimen failed to provide anxiolytic and antidepressant effects on male BALB/C mice exposed to UCMS.

Key Words: Prebiotics, Unpredictable Chronic Mild Stress, Anxiety, Depression, Light Dark Box, Fur Coat Assessment, Fructooligosaccharide (FOS) and Galactooligosaccharide (GOS)

INTRODUCTION

Depression and anxiety are pervasive and prevalent problems worldwide.[1-3] Global Health Estimates in 2015 report 3.30 million and 3.08 million Filipinos affected by depressive and anxiety disorders, respectively.[2] Chronic stress is an important risk factor for the development of these disorders.[4-5] Stress is a circumstance that disturbs the normal physiological and psychological functioning of a person.[6] While short term responses to stress are adaptive and beneficial, it also has the potential to cause chronic or mood anxiety disorders when neurologic and behavioral

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responses become maladaptive.[4] This causality is important to note, as stress-related disorders have their roots in nuanced interactions between genetic and environmental risk factors, wherein the cumulative physiological effect of stressors causes the dysregulation of multiple systems.[7] The Gut-Brain Axis is a system of highly integrated and regulated complex pathways by which the nervous system and the gastrointestinal system are interconnected.[8] These pathways include the Enteric Nervous System, Autonomic Nervous System, Hypothalamus-Pituitary Axis, and the Central Nervous System. In recent decades, the gut microbiota has shown a bidirectional interaction with the brain, where dysbiosis has been associated with neurological changes.[8] An example of this is the expression of depressive symptoms in patients with Inflammatory Bowel Disease.[9] Given this relationship, influencing the gut microbiota using prebiotics and probiotics has become an interesting strategy to generate new treatments for mental health disorders (e.g., anxiety and depression), cognitive deficits, neurodegenerative disorders, and neuropsychiatric disorders.[7,10-13] A recent meta-analysis of human trials showed that probiotics conferred significant anxiolytic and antidepressant effects, while taking fructooligosachharide (FOS) or galactooligo-saccharide (GOS) did not confer a significant effect when compared to placebo.[14] In contrast, Burokas et al. (2017) found that using a combination of both FOS and GOS on mice models showed significant anxiolytic and antidepressant effects.[12] This contrast then suggests that using a combination of FOS and GOS may provide more potent anxiolytic and antidepressant effects when compared to using these separately. Prebiotics work by stimulating the growth and activity of beneficial bacteria in the gastrointestinal tract, thus using prebiotics in combination may stimulate a broader genera of bacteria causing more profound effects.[15-18] Considering that the Philippines has local and affordable dietary sources of prebiotics such as bananas and onions, the discovery of possible anxiolytic and antidepressant effects of a FOS + GOS based prebiotic may allow Filipino mental health practitioners to integrate diet modification and/or supplementation into their treatment plan for anxiety and depression patients.

Therefore, this present study will clarify previously reported anxiolytic and antidepressant effects of a combined FOS + GOS regimen using a more stress-sensitive mice strain (i.e., BALB/C) and by using a stress protocol that more closely approximates human stresses (i.e., Unpredictable Chronic and Mild Stress).[12] Specifically, the study aims to (1) compare anxiety levels of mice supplemented with prebiotics against mice on standard diet, before and after UCMS, via light dark box test, (2) compare depression levels of mice supplemented with prebiotics against mice on standard diet, before and after UCMS, via fur coat assessment, (3) determine changes in anxiety levels through time, and (4) determine changes in depression levels through time.

MATERIALS AND METHODS

Preparation and Handling of Test Animals

Forty male BALB/C mice (aged 2-3 months old) were obtained from St. Luke's Quezon City Research and Development division where they were fed commercially available Rabbit Pellets by Bantrade Pellet Feeds Manufacturing Company (contains yellow corn, corn by-products, copra meal, distiller's dried grains with solubles, ipil, rice bran, sorghum, wheat pollard, yeast, limestone, tricalcium phosphate, vitamins, and minerals). Sample size for repeated measures ANOVA was calculated by the College of Public Health using the G* power software where power was set at 0.8, and number of groups and measurements were both set to 2. Sample size was adjusted to account for expected mortality. All researchers were certified to be adequately trained in mice handling and feeding by the National Institute of Health in order to minimize performance bias. The population was equally and randomly divided into two groups. The control group was on standard diet while with the treatment group received prebiotic supplementation of 0.5g of FOS and GOS on a daily basis by dissolving the appropriate concentration in their drinking water as prescribed by Burokas et al. (2017).[12] Standard diet refers to the same Rabbit Pellets used by the supplier. Both groups were acclimatized for a total of 1 month, but the treatment group started receiving prebiotic supplementation after 1 week of acclimatization until posttest. After acclimatization, baseline anxiety and depression levels were determined, then both groups were subjected to the UCMS protocol for 4 weeks. After which posttest anxiety and depression levels were measured. Data was then analyzed using repeated measures ANOVA.

Ethical Considerations

The researchers strictly adhered to the standard of animal care required by the Institutional Animal Care and Use Committee (IACUC)-UP Manila. The study was designated the protocol number: 2018-037. Mice were individually housed in 14 x 7 x 8 plastic cages in the animal room of the Paz Mendoza building, College of Medicine, University of the Philippines-Manila. Housing conditions were maintained at: 25 ± 2°C, humidity at 20%–25% and photoperiod of 12:12 with lights on at 6:00 AM. Mice were fed commercially available mice feeds once a day throughout the study. Distilled water for the control and prebiotic-supplemented water were made available for the mice ad libitum. Fructooligosaccharides and galactooligosaccharides were obtained from BENEO-Orafti and Vision Ingredients Asia, respectively. Mice bedding were replaced 2-3 times a week. The present study subjected the mice to the UCMS protocol with pretest and posttest assessments. Aside from UCMS, no other stressors were deliberately induced by the researchers. Additionally, the pretest and posttest assessments using the light dark box test and fur coat assessment were non-

invasive and merely observational in nature. On the last day of the study, all mice were euthanized by cervical dislocation performed by the laboratory's animal caretaking personnel.

Experimental Study Design

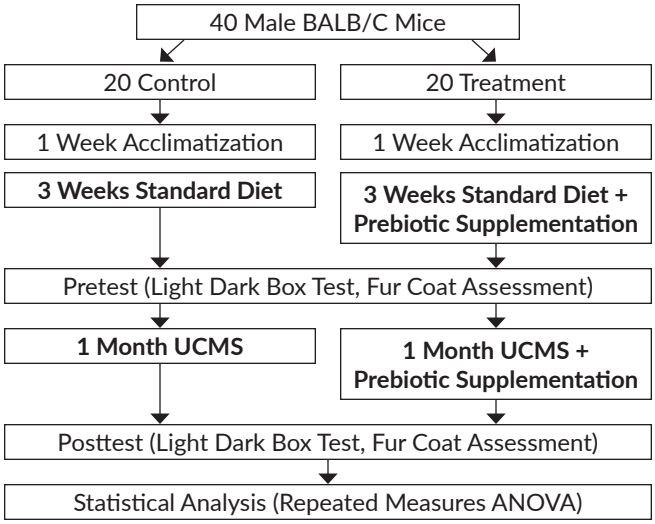

Figure 1. The study utilizes a pretest-posttest control group design. Pretest and posttest levels of anxiety and depression were measured for both groups via the light dark box test for anxiety and fur coat state assessment for depression. Pretest measures were obtained prior to the UCMS protocol, while posttest measures were obtained after. Furthermore, pretest and posttest measures were analyzed for within and between differences, and interaction of treatment with time via repeated measures ANOVA ($\alpha = 0.05$).

Unpredictable Chronic Mild Stress (UCMS)

The UCMS protocol was conducted for 4 weeks. The mice population was stressed via several stressors (Table 1).

Table 1. Stressors with description of their respective methodologies in accordance with the UCMS protocol[19]

Stressor	Description
Cage change	Each mouse was temprarily placed in a cage previously occupied by another mouse and returned to its original cage after 3 hours.
Without sawdust	Sawdust was removed from cages for 1 hour.
Damp sawdust	125 ml of water was poured into each cage to soak the sawdust for 1 hour.
Bath	Sawdust of each cage was removed and replaced with 125 ml of water for 30 minutes.
Cage tilting	Cages were tilted at 45° for 1 hour.
Rat feces	Sawdust in each cage was replaced with ~60 ml of sawdust containing rat feces for 1-2 hours.
Restraint stress	Each mouse was restrained separately in closed and ventilated 50 ml falcon tubes for 15-30 minutes.
Predator sounds	Predator sounds of birds of prey were played for 10 minutes in front of the mice cages.
Cycle disturbances	Change of light/dark cycle (e.g., reversal of light/dark cycle).

Two randomly selected stressors were performed on the population daily. It was ensured that no stressor was conducted two days in a row to prevent habituation.

Light Dark Box Test

The dimension of the box was 46 x 27 x 30 cm, one-third of which was the dark compartment while the remaining two-thirds was for the light compartment. There was a middle partition between compartments with a square opening of not more than 7 x 7 cm to allow crossing[20] (Figure 2). The ceiling of the light compartment box was left uncovered while blue cellophane was placed over the dark compartment. This was done in order to allow observation of mouse behavior while keeping in accordance with the recommended illuminance in the light dark box test protocol. The illuminance, measured in lux, was measured to be 5 or less lux in the dark compartment and from 200-400 lux in the light compartment as prescribed by Serchov et al. (2016).[21]

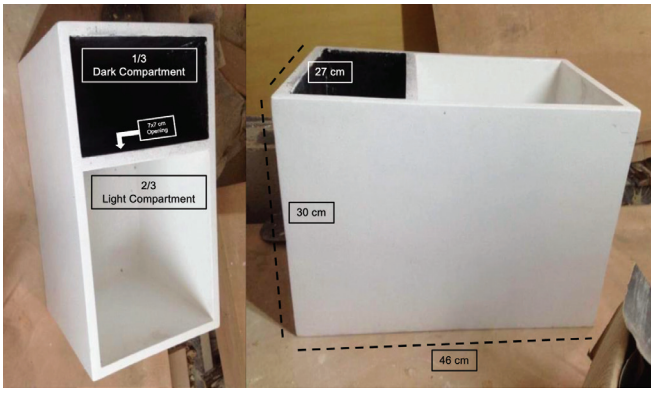

Figure 2. Light Dark Box.

Anxiety Assessment

Evaluation of the anxious behavior was based on the modified light dark box test used by Hascoët and Bourin (1998).[22] The mice were initially placed in the center of the white portion of the light dark apparatus and allowed to roam for 5 minutes. During this time, the researchers were predominantly absent from the testing site and a video-recording device was placed above each compartment such that the entire compartment was visible. The mice were removed from the apparatus at the end of 5 minutes after which the apparatus was cleaned with 70% unscented ethanol to remove urine and fecal material. There was a waiting period of 5 minutes in between tests to allow evaporation of the alcohol.

Four parameters were measured through video: (1) total time in light, which is the cumulative measured time in seconds spent in the light compartment, (2) the transitions from one compartment to the other, which occurs once all four paws have entered the adjacent compartment, (3) latency time, or the time in seconds that the mouse spends in the light compartment before initially transitioning into the dark

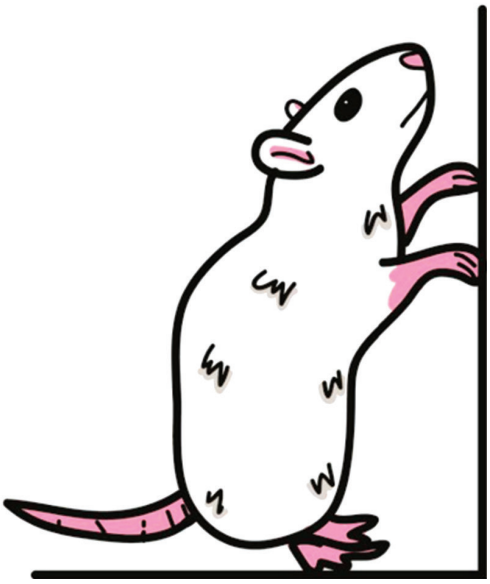

Figure 3. Exploratory rearing.

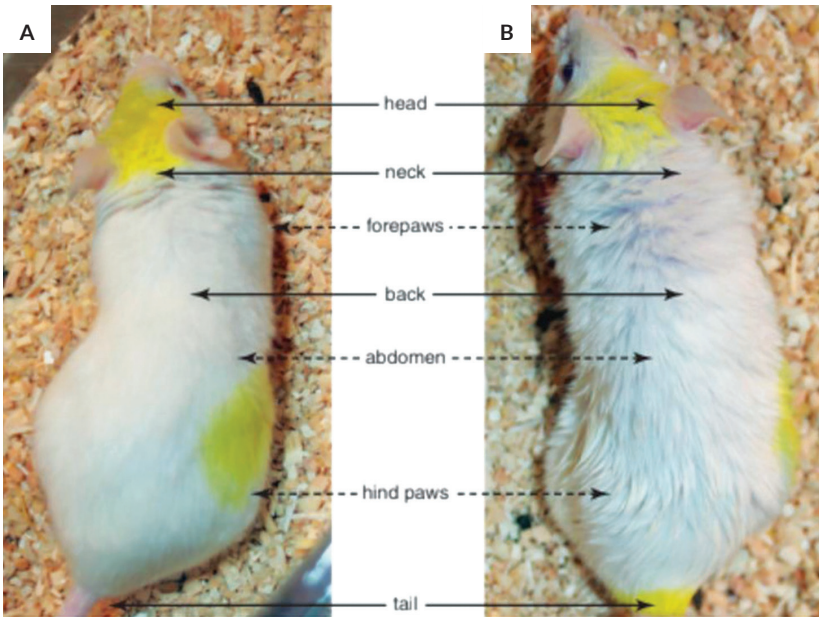

Figure 4. Areas of mouse fur assessed as indicators of depressive behavior.[23]

compartment, and (4) exploratory rears, defined as "directed sniffing with the forepaws directed vertically upon the sides of the chamber"[20] (Figure 3).

Fur Coat Assessment

The fur coat state assessment used was a 0, 0.5 and 1 scoring system to measure depressive behavior. The lowest score possible, 0 (good), is given for smooth and shiny fur, without spiky and tousled patches, while the highest score, 1 (bad), is given to fluffy, spiky, dirty, or stained fur**.** An intermediate value of 0.5 is given when the fur is slightly fluffy with spiky patches.[23] The 7 regions – head, neck, forepaws, dorsal coat, ventral coat, hind paws, and tails – are to be examined by blinded evaluators, then scored using the parameters above. Thus, the maximum score for each mouse is 7. This assessment is correlated to the unhygienic behavior often observed in persons with depression.[23] Fur coat state of each mouse is a mean of independent assessments by two blinded evaluators.

Maneuvers to Minimize Systemic Errors

Each mouse was initially assigned a number from 1 to 40. A numbered list from 1- 40 was then randomly rearranged using an online list randomizer. The mice corresponding to the first 20 items on the randomized list were assigned to the control group while the remaining 20 mice were assigned to the treatment group. Following random allocation, each mice was rearranged in order by number and finally designated a control number. A-1 to A-20 were assigned to mice of the control group while B-1 to B-20 were assigned to mice of the treatment group.

Immediately prior to assessment, each mouse was randomly assigned an additional control number in order to avoid measurement bias. The mice were divided into three groups corresponding to the three unblinded chaperones who were tasked with delivering their assigned mice to the assessment areas.

Individual strips of paper labeled from A-1 to A-20 and from B-1 to B-20 were placed together in a bag. A chaperone tasked with delivering a mouse to the assessment area would randomly pick out a strip of paper from the bag. The mouse corresponding to the control number obtained by the chaperone was then assigned its additional control number and brought to the assessment area. The first mouse obtained by the first chaperone was assigned a control number of C1M1, *C1* referring to the first chaperone and *M1* referring to the first mouse obtained by the first chaperone, while control numbers for mice obtained by the second and third chaperones followed the same format. The first chaperone was assigned to 14 mice, the second chaperone was assigned to 13 mice, and the third chaperone was assigned to 13 mice.

During the assessment proper, one mouse at a time was delivered by a chaperone from their cages to an evaluator handling the light dark box. The light dark box test was facilitated by the evaluator, after which the mouse was brought by the chaperone to a second evaluator responsible for the fur coat state assessment. There were a total of three chaperones, three evaluators for the light dark box, and two evaluators for the fur coat state analysis. In order to avoid measurement bias, only the chaperones were aware of the original group classification of each mouse. All evaluators were blinded and were only informed of the chaperone-based control numbers. Only evaluators for their respective assessment tests and mice handled were tasked with measuring their respective parameters. Evaluators for the light dark box test measured the exploratory behavior through video,

while evaluators for the fur coat state assessment scored the depressive state through pictures taken during assessment.

Statistical Analysis

Data was encoded in the Microsoft Excel program during each data collection period. Collected data was composed of pretest and posttest values of four anxiety parameters (e.g., total time in light, time before first transition, number of transitions and number of rears) and one depression parameter (e.g., fur coat state). Stata® software for statistics was used to perform repeated measures ANOVA (between and within means) to identify the effect of treatment between the groups, to determine the effect of treatment across time (i.e., pretest versus posttest measurements), and to determine if there is an interaction between treatment and time. Shapiro-Wilk W test for normality and the Breusch-Pagan/ Cook-Weisberg test for heteroskedasticity was performed to evaluate the adherence of the analyzed parameters to the assumptions of repeated measures ANOVA. Additionally, the epsilon measure for violation of sphericity was obtained to direct the choice of p-values obtained after correction factors have been applied. Parameters that show significant time-treatment interaction was visualized on a contrasts of marginal linear predictions plot to determine the difference of means between groups on pretest and posttest measurements.

RESULTS

Repeated measures ANOVA was run to evaluate (1) the difference between groups through time (i.e., comparison of pretest-posttest difference of both groups), (2) the difference within groups (i.e., comparison of mean pretest with mean posttest), and (3) the interaction of group with time (i.e., correlation of group with time).

In between groups comparison, there was no sufficient evidence to say that the treatment group was significantly different from control through time in the following parameters: total time in light $F(1,3.78)=0.0593$,$p<0.05$; time before first transition $F(1,3.48)=0.0700$, $p<0.05$; total number of transitions $F(1,0.47)=0.4967$, $p<0.05$; and fur coat state $F(1,0.00)=0.9575$, $p<0.05$ (Figures 5.1-5.3). In contrast, there was a significant difference between both groups in terms of total number of rears $F(1,6.20)=0.0173$, $p<0.05$ where the control group significantly exhibited more rears than the treatment through time (Figure 5.4).

Additionally, there is no sufficient evidence to say that there is a significant difference within groups in the following parameters: total time in light $F(1,2.86)=0.0993$, $p<0.05$; and total number of rears $F(1,2.25)=0.1425$, $p<0.05$ (Figures 5.1 and 5.4). This means that there is no change in anxiety level over time in terms of these parameters. In contrast, there is a significant difference within groups in time before first transition $F(1,18.05)=0.001$, $p<0.05$; and fur coat state measures $F(1,34.25)=0.00$, $p<0.05$ (Figures 5.2 and 6). This implies that there is a change in anxiety and depression levels over time in terms of these two parameters.

Furthermore, there is a significant interaction between group and time in the total time in light parameter (Figure 5.1) $F(1,5.43)=0.0254$, $p<0.05$, while no group-time interaction was found in the remaining parameters (Figures 5.2 to 6): time before first transition $F(1,3.17)=0.0831$, $p<0.05$; number of transitions $F(1,0.14)=0.7082$, $p<0.05$; total number of rears $F(1,0.65)=0.4238$, $p<0.05$; and fur coat state $F(1,0.40)=0.5318$, $p<0.05$. Since there is a significant interaction between group and time for the total time in light parameter, a contrast of marginal linear predictions plot shows that during the pretest, the treatment was significantly different from the control ($p=0.004$), but during posttest, the treatment group showed a decrease in total time spent in light closer to the control group's posttest value, with no significant difference ($p=0.826$). This implies that the treatment group significantly grew more anxious with time while the control group's anxiety level remained constant with respect to this parameter.

In summary, it was found that the treatment group did not have significantly different anxiety and depression levels when compared to control (in 4 of 5 parameters) but there was a significant change in anxiety and depression levels in both groups over time (in 2 of 5 parameters) which is in agreement with the observed increased anxiety of the treatment group over time (in 1 of 5 parameters). Interpretation of these findings suggest that (1) the FOS + GOS treatment did not give the treatment group an advantage over the control group in four of five parameters (2) both groups grew more anxious and depressed over time in two of five parameters and (3) the treatment group's anxiety level increased with time while the control group's anxiety level remained constant in context of total time spent in light.

DISCUSSION

In a meta-analysis of human trials by Liu et al. (2019), the use of either FOS or GOS did not show a significant anxiolytic or antidepressant effect.[14] Meanwhile, Burokas et al. (2017) reported that using FOS and GOS either individually or in combination showed anxiolytic and antidepressant effects in male C57BL/6J mice. But it was observed that using FOS and GOS in combination had superior anxiolytic and antidepressant effect when compared to administering them individually. This is in contradiction with our current findings where we did not observe anxiolytic and antidepressant effect when using a combined FOS + GOS prebiotic solution as a dietary supplement in male BALB/C mice when exposed to unpredictable chronic mild stress (UCMS).

Prebiotics modulate the gut microbiota by influencing the growth of certain microbial taxa over others.[13-14,24-26] The FOS + GOS treatment has been found to increase the abundance of *Bifidobacteria* and *Lactobacilli* over others.[18,26]

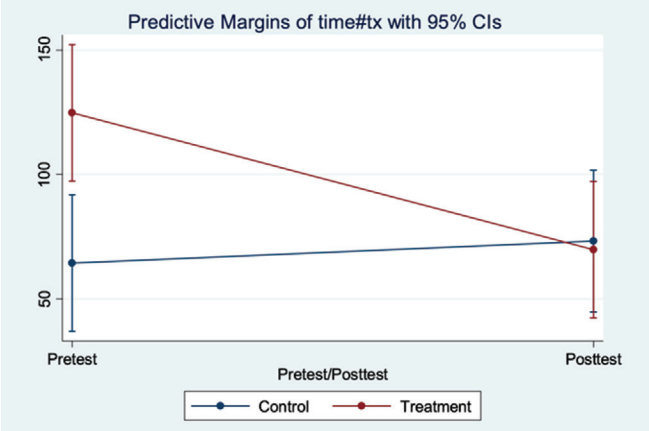

Figure 5.1. Mean total time in light (s) of both groups during pretest and posttest, measured using the light dark box test.

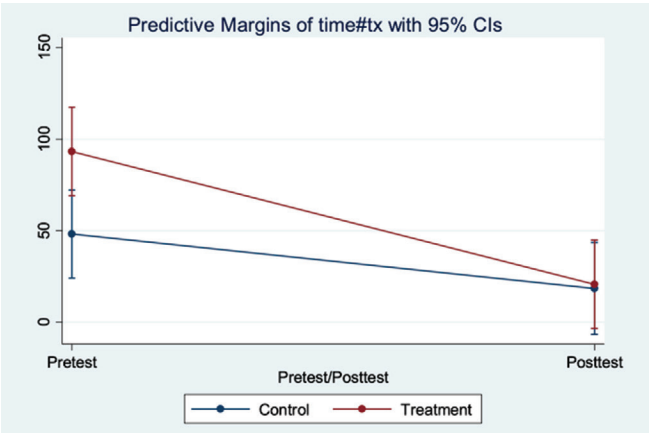

Figure 5.2. Mean time spent in light side before first transition (s) of both groups during pretest and posttest, measured using the light dark box test.

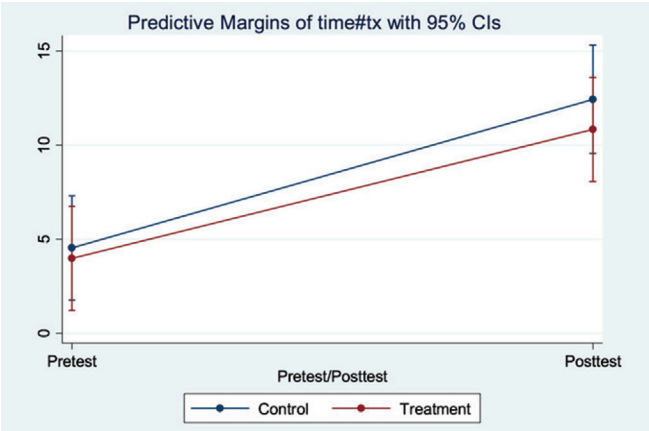

Figure 5.3. Mean number of transitions of both groups during pretest and posttest, measured using the light dark box test.

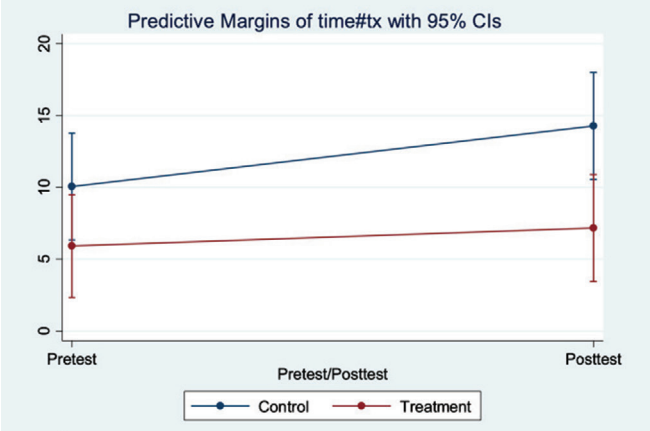

Figure 5.4. Total number of rears of both groups during pretest and posttest, measured using the light dark box test.

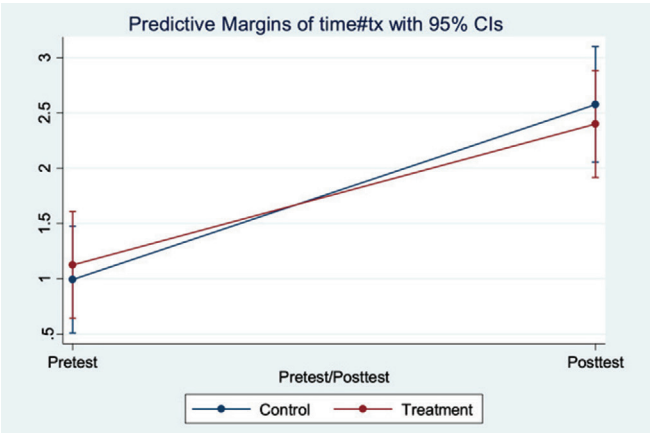

Figure 6. Mean fur coat state assessment scores of both groups during pretest and posttest.

Thus, for prebiotics to take effect, there must be an initial population of its target microbes. Xiao et al. (2015) created a comprehensive gut bacterial gene catalog created from mice of diverse genetic backgrounds, housing locations and diet.[27] The catalog ranks the 20 most abundant bacterial genera across all samples. *Lactobacilli* were among the top 20 core genera in mice, while the omnipresence of *Bifidobacteria* cannot be established. Therefore, the expected anxiolytic and antidepressant effects of the FOS + GOS treatment may have been negatively affected with only one of its two targets most likely present in the mice of the current study. Aside from this, Collins & Reid (2016) showed that the relative abundance of gut microbes also plays as a factor in the prebiotic effect.[24] Thus, a baseline assessment of gut microbiota composition may have accurately determined the initial gut community composition and microbe relative abundance in the present study, however, this was beyond the scope of the present study.

In addition, recent studies state that gut microbial composition is dependent on supplier, housing, diet, feeding pattern, gender, and strain.[27-29] This dependence on strain may explain the contradiction between the present findings to that of Burokas et al. (2017), while the single mice source, uniform housing and diet in the current study contributes to the accuracy of present findings.[12]

Various mice strains exist for varying purposes. It is worth noting that this is the first study to evaluate the use of FOS + GOS on male BALB/C mice exposed to UCMS. Male BALB/C mice were found to be significantly more sensitive to the UCMS protocol when compared to C57BL/6J and DBA/2J mice.[30] This apparent hyperreactivity of BALB/C mice to UCMS was the present study's primary reason for choosing the said strain. This move is supported in Savignac et al. (2015) which states that the BALB/C mice strain is the ideal model in investigating stress-related disorders due to their innate sensitivity to stress.[10] It is therefore suggested that the hypersensitivity of BALB/C to UCMS would elucidate and clarify the anxiolytic and antidepressant effects of the FOS + GOS treatment demonstrated by Burokas et al. (2017) who used C57BL/6J mice.[12] Present findings showed that both groups grew more anxious and depressive by the end of UCMS, and the treatment did not show any marked difference from the control. Aside from the previous discussion on how initial gut microbiota is dependent on mice vendor and strain which may eventually translate into a differential effect of prebiotics, the apparent hyperreactivity of BALB/C mice may have played a confounding role in establishing the therapeutic effects of the FOS + GOS treatment.

The exact composition and administration of FOS + GOS may also be responsible for the study's contradicting findings with that of previous works. The study by Burokas et al. (2017) only provided the supplier (Healy Group, Ireland), but did not provide specific technical product details for either the FOS or the GOS used. While it might be surmised that there are similar characteristics across the different products marketed as FOS and GOS (as a result of their synthesis and degrees of polymerization) evidenced by the GRAS studies by ChromaDex Spherix Consulting (2017) and Heimbach (2011), the exact composition of the actual product might differ (for example, the FOS in our study is composed of 93.2-95.8% oligofructose).[31-32] Additionally, Burokas et al., (2017) utilized a combination of FOS and GOS dissolved in drinking water for 0.3-0.4 g/mouse/day without specifying the exact ratio. It may only be speculated that each prebiotic contributed half (0.15-0.2 g/mouse/day). In contrast, our study utilized 0.5 g/mouse/day in a fixed 1:1 ratio of 0.25g FOS and 0.25g GOS. Given this difference in the administered amount and coupled with uncertainty about the technical specifics of the FOS and GOS used by Burokas et al. (2017), the absence of a significant effect may be attributed to these differences.

It is unlikely that the observed absence of a neuroprotective effect of the prebiotics may be attributed to its handling by the researchers. According to Charalampopoulos and Rastall (2012), GOS is extremely stable, much so that heating a solution to 100˚C at pH = 2 degrades only 5%; and that GOS is stable for several months when stored at 37˚C at pH = 2. FOS on the other hand was reported to be less stable. Heating a FOS solution to 145˚C at pH = 3.5 for only 10 seconds resulted in the hydrolyzation of approximately 10%, while exposure to 85˚C for 30 minutes causes a significant reduction in its prebiotic activity score.[33] Despite the lack of certain environmental controls such as measurement of room temperature, it is unlikely that the storage and handling of the prebiotics have approximated the aforementioned conditions mostly related to the pasteurization process.[30] Given that the prebiotics and the prebiotic solution were stored in an air-conditioned room, it is unlikely that these have degraded so much as to nullify its efficacy and render them without effect.

The UCMS protocol published by Mineur (2003) was utilized by this study. However, although the protocol enumerates a list of stressors from which the researchers can choose, Willner (2005) stated that proximal stressors, which are stressors performed within the cage (cage change, sawdust change, without sawdust, damp sawdust, "bath," cage tilting, rat feces, and restraint stress) were more potent in eliciting behavioral changes than distal stressors (predator sounds and cycle disturbances).[19,34] In our study, more proximal stressors were used, with predator sounds done only five times and cycle disturbance done only once during the whole 4-week stressing period. While this is demonstrably effective in stressing the mice and inducing anxiety and depression over time, it might be that the extensive use of proximal stressors on a highly susceptible strain might have further contributed to the masking effect on the probable efficacy of FOS + GOS.[10]

The physical setup of the laboratory might have also contributed to the results of the study. According to Porsolt and Papp (1998), it is important to keep stressed animals and controls in different holding rooms and minimize excessive ventilation in these rooms.[35] Alario et al. (1986) also report that chronic noise stimulus is moderate stress to mice, such that it was able to increase corticosterone levels but not affect their adrenal morphology.[36] Given that the mice used in this study were housed in a room exposed to construction noise, shared by other mice and rats of other research groups, and both the control and treatment groups had to be placed next to each other, these factors may have contributed additional stress to the mice, which may explain both the increased initial levels of stress, as well as masking in the effect of FOS + GOS.

Lastly, the observed decreasing trend in total time spent in the light side of the treatment group might imply that the treatment group grew more anxious than the control group. But, if we look at all the other 3 anxiety parameters, this finding may be considered as an outlier. Furthermore, even if there was an apparent decreasing trend, the treatment group was still not significantly different from the control in this parameter.

Furthermore, it must be noted that these present findings are most applicable in the context of the prebiotic, mice strain, and stress protocol used. A FOS + GOS regimen was prebiotic of choice and was administered *ad libitum* following previous studies.[12] Thus, observed depression and anxiety parameters were discussed about this specific prebiotic formulation. The findings of the present study are most applicable to male BALB/C mice since gut microbiota is found to be strain-dependent.[10] The stress protocol employed to elicit behavioral responses in mice was the UCMS protocol since it closely approximates actual human stressors, and does not involve mice starvation and dehydration.[20] Only behavioral responses of mice were assessed in the study to avoid additional stress induced by more invasive methods such as serum analysis. It is also acknowledged that baseline determination and surveillance of gut microbiota composition via qPCR would have provided more insight on the effect of the prebiotic, however, this would be beyond the scope of the present study.

CONCLUSION

The present study has demonstrated that the use of Fructooligosaccharides and Galactooligosaccharides in combination is not significantly effective in lowering the levels of anxiety and depression in mice subjected to unpredictable chronic mild stress. Data from the light-dark box test for anxiety, and fur coat state assessment for depression, revealed no significant difference in the anxiety and depression levels between the control and the treatment group of BALB/C mice. Comparing the posttest and the pretest data, it is evident that the UCMS protocol worked, as the stress levels of both the control and treatment groups significantly increased. Interestingly, the treatment group turned out to be considerably more anxious after the experiment, compared to the control group whose anxiety levels remained constant. Therefore, the use of combined Fructooligosaccharides and Galactooligosaccharides in modulating human behavior remains questionable given that studies using animal models are currently sparse and report contrasting findings as reported in this present work.

Acknowledgments

The authors would like to acknowledge the following people who made this study possible:

To Dr. Darwin Dasig and Dr. Leticia Ibañez, for their constant presence, support, patience, and encouragement throughout our research work, along with their willingness to give insights to help improve our study, our presentation, and our manuscript; to Gracia Fe B. Yu, PhD, for allowing us to house our mice and perform our experiment in the MDL Animal Room; to Dr. Ma. Esterlita Uy and Dr. Herbert Uy, for helping us procure mice from the St. Luke's Medical Center Quezon City and for sponsoring the construction of our light-dark boxes; to the National Institute of Health-UP Manila, for providing us with the necessary skills to handle our mice through their workshop; to the Department of Epidemiology and Biostatistics, College of Public Health, for helping us with the statistical analysis of our data; to Ma'am Yolanda Sanchez, for giving us access to laboratory equipment; to Sir Billy Atendido, for helping us in regularly feeding the mice and cleaning their cages, working with us post-office hours, and overall making sure that our mice, their houses and feeds, and our lab apparatus in the MDL Animal Room was secure and well- taken care of; to our families, for giving us the financial and moral support to accomplish this study; and to each member of the research group who made their sacrifices to make this study possible—thank you.

Statement of Authorship

All authors participated in data collection and analysis, and approved the final version submitted.

Author Disclosure

All authors declared no conflicts of interest.

Funding Source

This paper was funded by the College of Medicine, University of the Philippines Manila

REFERENCES

1. Mohammadi TM, Sabouri A, Sabouri S, Najafipour H. Anxiety, depression, and oral health: A population-based study in Southeast of Iran. Dental research journal. 2019 May;16(3):139.
2. World Health Organization. Depression and other common mental disorders: global health estimates. World Health Organization; 2017.
3. Tiller JW. Depression and anxiety. The Medical Journal of Australia. 2013 Oct 29;199(6):S28-31.
4. McEwen BS, Eiland L, Hunter RG, Miller MM. Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. Neuropharmacology. 2012 Jan 1;62(1):3-12.
5. Hammen C. Stress and depression. Annu. Rev. Clin. Psychol.. 2005 Apr 27;1:293-319.
6. Sadock BJ, Sadock VA. Kaplan and Sadock's synopsis of psychiatry: Behavioral sciences/clinical psychiatry. Lippincott Williams & Wilkins; 2011 Dec 26.
7. Bharwani A, Mian MF, Surette MG, Bienenstock J, Forsythe P. Oral treatment with Lactobacillus rhamnosus attenuates behavioural deficits and immune changes in chronic social stress. BMC medicine. 2017 Dec;15(1):1-4.
8. Mukhtar K, Nawaz H, Abid S. Functional gastrointestinal disorders and gut-brain axis: What does the future hold?. World journal of gastroenterology. 2019 Feb 7;25(5):552.
9. Abautret-Daly Á, Dempsey E, Parra-Blanco A, Medina C, Harkin A. Gut–brain actions underlying comorbid anxiety and depression associated with inflammatory bowel disease. Acta neuropsychiatrica. 2018 Oct;30(5):275-96.
10. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. Bifidobacteria modulate cognitive processes in an anxious mouse strain. Behavioural brain research. 2015 Jul 1;287:59-72.
11. Kim YK, Shin C. The microbiota-gut-brain axis in neuropsychiatric disorders: pathophysiological mechanisms and novel treatments. Current neuropharmacology. 2018 Jun 1;16(5):559-73.
12. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, Stanton C, Dinan TG, Cryan JF. Targeting the microbiota-gut-brain axis: prebiotics have anxiolytic and antidepressant-like effects

and reverse the impact of chronic stress in mice. Biological psychiatry. 2017 Oct 1;82(7):472-87.
13. Sun J, Wang F, Hu X, Yang C, Xu H, Yao Y, Liu J. Clostridium butyricum attenuates chronic unpredictable mild stress-induced depressive-like behavior in mice via the gut-brain axis. Journal of agricultural and food chemistry. 2018 Jul 24;66(31):8415-21.
14. Liu RT, Walsh RFL, Sheehan AE. Prebiotics and probiotics for depression and anxiety: A systematic review and meta-analysis of controlled clinical trials. Neurosci Biobehav Rev. 2019 07;102:13-23.
15. Markowiak P, Śliżewska K. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients. 2017 Sep;9(9):1021.
16. Toward R, Montandon S, Walton G, Gibson GR. Effect of prebiotics on the human gut microbiota of elderly persons. Gut microbes. 2012 Jan 1;3(1):57-60.
17. Kao AC, Harty S, Burnet PW. The influence of prebiotics on neurobiology and behavior. International review of neurobiology 2016 Jan 1 (Vol. 131, pp. 21-48). Academic Press.
18. Taylor AM, Holscher HD. A review of dietary and microbial connections to depression, anxiety, and stress. Nutritional neuroscience. 2020 Mar 3;23(3):237-50.
19. Mineur YS, Prasol DJ, Belzung C, Crusio WE. Agonistic behavior and unpredictable chronic mild stress in mice. Behavior genetics. 2003 Sep 1;33(5):513-9.
20. Hascoet M, Bourin M. The mouse light–dark box test. InMood and Anxiety Related Phenotypes in Mice 2009 (pp. 197-223). Humana Press, Totowa, NJ.
21. Serchov T, van Calker D, Biber K. Light/dark transition test to assess anxiety-like behavior in mice. Bio Protoc. 2016;6:e1957.
22. HascoëT M, Bourin M. A new approach to the light/dark test procedure in mice. Pharmacology Biochemistry and Behavior. 1998 Jun 1;60(3):645-53.
23. Nollet M, Guisquet AM, Belzung C. Models of depression: unpredictable chronic mild stress in mice. Current protocols in pharmacology. 2013 Jun;61(1):5-65.
24. Tarr AJ, Galley JD, Fisher SE, Chichlowski M, Berg BM, Bailey MT. The prebiotics 3′ Sialyllactose and 6′ Sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: Evidence for effects on the gut–brain axis. Brain, behavior, and immunity. 2015 Nov 1;50:166-77.
25. Collins S, Reid G. Distant site effects of ingested prebiotics. Nutrients. 2016 Sep;8(9):523.
26. Mao B, Gu J, Li D, Cui S, Zhao J, Zhang H, Chen W. Effects of different doses of fructooligosaccharides (FOS) on the composition of mice fecal microbiota, especially the Bifidobacterium composition. Nutrients. 2018 Aug;10(8):1105.
27. Xiao L, Feng Q, Liang S, Sonne SB, Xia Z, Qiu X, Li X, Long H, Zhang J, Zhang D, Liu C. A catalog of the mouse gut metagenome. Nature biotechnology. 2015 Oct;33(10):1103-8.
28. Hugenholtz F, de Vos WM. Mouse models for human intestinal microbiota research: a critical evaluation. Cellular and Molecular Life Sciences. 2018 Jan 1;75(1):149-60.
29. Thaiss CA, Levy M, Korem T, Dohnalová L, Shapiro H, Jaitin DA, David E, Winter DR, Gury-BenAri M, Tatirovsky E, Tuganbaev T. Microbiota diurnal rhythmicity programs host transcriptome oscillations. Cell. 2016 Dec 1;167(6):1495-510.
30. Mineur YS, Belzung C, Crusio WE. Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. Behavioural brain research. 2006 Nov 25;175(1):43-50.
31. ChromaDex Spherix Consulting. GRAS Notice (GRN) No. 721: Generally Recognized As Safe Determination for the Use of VITAGOSTM in Non-Exempt Term Infant Formula and Selected Conventional Foods. [Internet]. 2017 [Cited 10 January 2021]; 6. Available from https://www.fda.gov/media/110690/download.
32. Heimbach J. GRAS Notice (GRN) No. 392: Determination of the GRAS Status of the Addition of Oligofructose to Infant Formula as a Nutritional Supplement. [Internet]. 2011 [Cited 10 January 2021]; 5-6. Available from https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=392&sort=GRN_No&order=DESC&startrow=1&type=basic&search=392
33. Charalampopoulos D, Rastall RA. Prebiotics in foods. Current opinion in biotechnology. 2012 Apr 1;23(2):187-91.
34. Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology. 2005;52(2):90-110.
35. Porsolt R, Papp M. CNS–Psychiatric models of disease: depression. Current Protocols in Pharmacology. 1998;591:598.
36. Alario P, Gamallo A, Beato MJ, Trancho G. Body weight gain, food intake and adrenal development in chronic noise stressed rats. Physiology & behavior. 1987 Jan 1;40(1):29-32.