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The Effects of Prebiotics and Probiotics Following Antibiotic Exposure in a Mouse Model of Autism Spectrum Disorder: A Research Protocol

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Abstract
The human microbiota consists of 10-100 trillion symbiotic microbial cells critical for one's digestive system, immune system and for managing neurological symptoms experienced in neurodevelopmental conditions. With early exposure to antibiotics, an individual's microbiome composition is negatively affected by reducing the diversity of microbial species found in the microbiome and can lead to an imbalance in the Gut-Brain-Axis (GBA). While the direct relationship between the GBA and neurodevelopmental functioning is still unclear, evidence suggests that individuals with a disruptive microbiome and an imbalanced GBA have an increased risk of developing neurodevelopmental disorders, specifically autism spectrum disorder (ASD). To improve microbiome diversity, exposure to a high prebiotic and probiotic diet in the early stages of life can reintroduce beneficial bacteria back into the microbiome and improve microbial diversity. Pre- and probiotics can improve microbiome diversity and restore balance to the GBA. With the introduction of a prebiotic and probiotic diet and a balanced GBA, there is a possibility to reduce the severity of ASD symptoms. By reducing the severity of ASD symptoms, the quality of life of those with severe ASD can potentially be improved allowing them to maintain functional independence. This research protocol intends to utilize an automated video tracking system and three-chambered social approach to evaluate the behavioural symptoms in BTBR strain mice which exhibit symptoms before and after administration of pre- and probiotics following antibiotic exposure.

Keywords: antibiotics; autism spectrum disorder; BTBR mice; gut-brain-axis; institutional animal care and use committee; prebiotics; lactobacillus; microbiome; probiotics

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that impacts how individuals communicate with others and is often characterized by repetitive behaviors, restricted interests, and challenges in learning [1]. According to the Centers for Disease Control and Prevention, about 1 in 36 children have ASD as of 2020, a significant rise from the estimated 1 in 150 children in 2000 [2]. Individuals with ASD often display various comorbidities, including anxiety, seizures, hyperactivity, sensory processing challenges and impaired motor coordination [3]. This is a growing concern because ASD can significantly affect quality of life in these individuals. Recent studies have found a correlation between ASD and gastrointestinal issues such as constipation, diarrhea, and abdominal pain due to an imbalance in the Gut-Brain-Axis (GBA) [2]. The GBA regulates bilateral communication between the nervous system and peripheral intestinal function. The quality of a microbiome is established within the early stages of an individual's life, with early exposure to antibiotics in infancy drastically reducing the quality and diversity of microbes in the microbiome [3]. With a disruptive microbiome, individuals may be at a greater risk of developing neurodevelopmental disorders such as ASD [18]. Emerging evidence suggests the potential role of antibiotics in disturbing microbiota composition in infancy as a possible contributor to the etiology of ASD due to an imbalance of the GBA [2]. These studies illustrate that children with ASD present different intestinal microbe compositions compared to those healthy individuals and have suggested gastrointestinal symptoms in ASD may indicate the underlying inflammatory process [4].

Additionally, there has been an increased interest in prebiotics and probiotics as they may help the behavioral and social functioning of infants with ASD. Among numerous intestinal microbes, probiotics can exert health

benefits to the host through the modulation of the intestinal microbiota. Species belonging to the genera *Lactobacillus* have been reported to be a beneficial probiotic bacterial strain [5]. Prebiotics are plant fibers that work in conjunction with probiotics by serving as fuel for beneficial bacteria [1].

This study uses one-week-old BTBR strain mice because they display typical ASD symptoms [6]. A rodent model was selected because it can be easily monitored and controlled in a laboratory setting. Due to the possible side effects of amoxicillin and the threat of antibiotic resistance, this study was not conducted on infants or children. These symptoms will be monitored in an eight-week-long longitudinal study to compare the severity of ASD symptoms after exposure to a high probiotic diet. This study aims to reduce the severity of ASD symptoms experienced by BTBR strain mice by providing them with a high probiotic diet of $5.0x10^9$ CFU of Lactobacillus twice daily to understand the connections that are interlinked between the microbiome and GBA. If the behavioral symptoms of the mice such as lack of sociability and excessive self-grooming decrease, then a high prebiotic and probiotic diet has a positive effect on the amelioration of ASD symptoms.

Methods

One-week-old male BTBR strain mice will be selected and placed in standard conditions (12-hour light-dark cycle, 22℃). This strain of mice displays traits characteristic of ASD, such as abnormalities in social learning, strict adherence to routines, and enhanced stress responses making it a suitable ASD model [8]. Twenty-four mice will be randomly assigned into two groups for the two levels of the independent variable. The experimental group will consume a diet high prebiotic and probiotic and the control group will consume a regular mice diet. Using specifically lactobacillus rhamnoses GG due to it being a common strain found in probiotic supplements and having a powerful adhesive capacity. This probiotic has been frequently used due to its ability to aid the immune system to prevent and treat gastro-intestinal infections and diarrhea[9]. We will be obtaining the product Lactobacillus Rhamnosus with FOS, 5 billion CFU [10], from the supplier Swanson because t mimics the effects of taking a dietary supplement. For the prebiotics we will be administering Fructooligosaccharides due to this already being in the probiotic capsules in a small quantity. This capsule will amplify the amount to 5g of FOS. This product NutraFlora FOS, pure powder is bought from a supplier called NOW FOODS [11]. The gut microbiota of the mice in both groups will be analyzed before the procedure using fecal samples, and data will be recorded for future comparisons. Both groups will receive doses of 0.02 mL twice daily for four weeks of amoxicillin (“Fish Mox”) [12]. This antibiotic was selected because it is commonly used to treat children with ear infections or chest infections such as pneumonia [16]. The mouse model for human gut microbiota will assess gut health pre- and post-antibiotic exposure, to better understand how antibiotics affect gut microbial composition [17]. This model uses the fecal bacteria collected from the feces samples [13] and then performs the 16S rRNA gene sequencing to analyze the gut microbiome found in mice [14]. The microbiota will be analyzed after antibiotic exposure, and it is hypothesized that healthy bacteria will be reduced. To ensure standardization, an independent-samples t-test will be used to compare the means of gut bacteria for both groups before and after long-term antibiotic exposure.

The automated video tracking system with a 3-chambered social approach will be used to collect qualitative data regarding the symptoms of ASD for both groups [7]. In this study, the lack of reciprocal social interaction and the presence of repetitive behaviours are the qualitative measures for ASD. In rodents, social interactions can include frontal approach, following one another, nose-to-nose sniffing, crawling over or under each other, pushing, chasing, and wrestling [3]. The number of interactions and the duration will be recorded using video tracking, and the means of the two groups will be compared. The apparatus will contain two chambers and a central area where the mouse will be placed. One chamber will contain another mouse, and the other will contain a novel object [3]. The control group mice should prefer to engage in social interactions with the other mice. In contrast, the BTBR strain mice that exhibit ASD symptoms should prefer non-social interaction and will explore the novel object. Repetitive behaviour in rodents can be characterized by excessive self-grooming. The number of times and duration the mice engage in self-grooming will be recorded for both groups. The test will be repeated 2 times with the automated video tracking system for 72 hours. Therefore, the mice at 3 weeks old will have to do a behavioural test after consuming amoxicillin and the mice will complete the test again at 11 weeks old for 72 hours after the treatment ends. We will allow the mice to stay with their mother during the amoxicillin treatment until they are 3 weeks old this is to ensure that they are not placed in a stressful environment. The prebiotics and probiotics will be combined, and gavage fed into their stomach. This is to ensure that the proper amounts of the prebiotics and probiotics enter the stomachs so they can have the maximum effect.

The second part of the study involves the use of pre- and probiotics to determine if there is a strong relationship between gut health and the severity of ASD symptoms. The first group will continue consuming a regular mice diet consisting of various plant materials and grains [15]. The second group will consume a prebiotic and probiotic-enhanced diet twice daily with $5x10^9$ CFU of *Lactobacillus*. The mice will adhere to their specialized diets for eight weeks [16] before their microbiota is analyzed for any changes. The automated video tracking system will be used

to collect data from both groups regarding changes in sociability and repetitive behaviours. A repeated-measures analysis of variance (ANOVA) test will be utilized to evaluate if there is an association between gut microbiota and ASD symptoms. The within-subjects factor is time (pre and post the eight-week procedure), and the between-subjects factor is the experimental and control groups. The expected outcome is that there will be a reduction in ASD symptoms in the group that consumed a high pre- and probiotic diet. This procedure will work according to the animal care expectations and obtain consent from the Institutional Animal Care and Use Committee (IACUC) [18].

Results

The study will be completed in a specific time frame. First, permission must be acquired to use twenty-four male BTBR mice from the IACUC. After approval, fecal samples will be obtained from the mice to be analyzed on day one before any treatments. The different bacterial compositions in the fecal samples can be found using 16S rRNA gene sequencing to determine the data and gauge the gut microbiome state, noting which bacteria are prominent and which are lacking. The mice will receive a two-week treatment of amoxicillin dosages containing 0.02 mL twice daily. Next, we will obtain another fecal sample from all the mice looking at the variations in the fecal bacteria found after antibiotic exposure with using 16S rRNA gene sequencing once again to determine the types and concentrations of bacteria in the gut microbiome. With the data obtained, a repeated-measures ANOVA test is performed to analyze the difference in the gut bacteria before and after antibiotic exposure.

After antibiotic exposure, the mice will be randomly sorted into either the control or experimental groups. Half of the mice will receive eight weeks of pre-and probiotic treatment administered in their food, while the other half will continue consuming the regular diet. Behavioural symptoms of ASD will be analyzed using an automated video tracking system before and after dietary changes are applied. After the completion of the treatment which consists of 8 weeks, their microbiota will be analyzed again and compared to the previous samples using a repeated-measures ANOVA to evaluate if the high pre- and probiotic diet had a strong, positive effect on gut microbiome quality. The behavioural symptoms of ASD will be analyzed again using the automated video tracking system and compared to the previous data. As previously mentioned, these symptoms in rodents include sociability and repetitive behaviours measured by the number of times and duration. Lastly, a correlation analysis will be utilized to evaluate if there is an association between gut microbiota quality and ASD symptoms.

The statistical significance is set at $p < 0.05$. Therefore, if our values are below this, we can conclude that there is a significant difference in the gut microbiome levels of the mice before and after antibiotic treatment. Likewise, when this test is conducted a second time then if there is a statistical difference in the gut microbiome before and after the administration of probiotics in the experimental group. This test is completed to ensure standardization and eliminate potential confounding variables for improving behavioural symptoms. We expect the results to yield a Pearson correlation coefficient of 0.7 or greater. This coefficient would indicate the relationship between a high pre- and probiotic diet and the amelioration of ASD behavioural symptoms is strong and positive.

The repeated-measures ANOVA results will allow us to compare multiple groups simultaneously to determine if there is a relationship between them by looking at the p-value ($p < 0.05$) [24]. In addition, when using ANOVA, the F statistic allows for the analysis of multiple groups. It determines if there is variability between the samples. An increase in the F value would indicate a significance between the variables [25].

Discussion

Gut bacteria are able to regulate the central nervous system (CNS) through neural, immune, and endocrine pathways [27]. Gut microbiota can affect these pathways by regulating the hypothalamic-pituitary-adrenal (HPA) axis, which produces neurotransmitters [27]. The neurotransmitters thought to influence ASD are oxytocin and dopamine, which are regulated using the HPA axis. Therefore, understanding that bacteria influences this is crucial since it is an essential link in the GBA, which is still under-researched [28].

One limitation of this study was the use of only male mice. Due to the cyclical hormone release in female rodents, it was expected that behavioural changes would be more challenging to assess. The symptoms of ASD are typically more pronounced in males, as females in general are socialized more quickly and often mask their behavioural characteristics. One study demonstrated that male BTBR rodents demonstrated more frequent ASD-like symptoms, including social approach and self-grooming, than female mice [26]. In the future, this study should be conducted with both male and female rodents. A future extension could be to evaluate sex differences in BTBR rodents after treatment with a high pre- and probiotic treatment.

This study differs from previous literature in that it addresses both how antibiotic exposure at birth could be associated with autism spectrum disorder and how probiotics could ameliorate the symptoms and restore the gut microbiota. Other studies have explored these ideas separately. While there is conflicting evidence regarding whether prenatal antibiotic exposure is associated with an increased risk of ASD, exploring potential correlational factors and how they can be prevented is important [29]. This study adds value to the present literature on ASD because it is longitudinal and simulates the process of

human infant exposure to antibiotics and the restoration of the gut microbiome using probiotics. ASD behavioural symptoms are closely tracked throughout the study to evaluate whether there was a significant improvement. This study is valuable to individuals with ASD because it may illustrate how realistic changes can be implemented to improve symptoms. It is crucial because it demonstrates the importance of the enteric nervous system in regulating ASD symptoms.

In terms of clinical-wide applications, this study could encourage medical doctors to advise new mothers to administer probiotics to their newborns if they were exposed to antibiotics during infancy. It might motivate older individuals with ASD to have their microbiome tested to see if probiotics could be beneficial to their gut microbiome and for parents with ASD to discuss how probiotics may help their children. Since the gut microbiome is inherited from the mother, another line of study could investigate how the mother could improve the infant's gut microbiome by taking probiotics during pregnancy [30]. This research protocol bridges the gaps in other studies by tracking changes in behavioural symptoms over time with varying changes in the gut microbiome to give a comprehensive demonstration.

Conclusions

Principally, this study aimed to explore how treatments improving the gut microbiome could be used to improve the behavioural symptoms of ASD, specifically using BTBR mice. Due to this model, the study is easily replicable and can be manipulated to test different pre- and probiotics. This study is critical because it addresses a neurodevelopmental disorder that is becoming more and more prevalent using an alternative approach. The potential of a high prebiotic and probiotic diet minimizing the behavioral symptoms of ASD is unique in the sense that it is a whole-body, holistic, and personalized approach that can be modified to individual needs. With a more recent shift of the Western world towards the whole-body approach, more research will likely be conducted regarding the gut microbiome, nutrition, and lifestyle factors on neurodevelopmental disorders. Another line of investigation could be evaluating how different antibiotics affect the gut microbiome and determining which are the least harmful to beneficial bacteria. Similarly, different strains of probiotics could be tested to see if BTBR mice respond better to specific ones. Lastly, future research could explore if the damage of antibiotics to the gut microbiome could be more easily reversed. Due to the risk-free nature of pre- and probiotics, this treatment could serve as a basis for a preventative medicine regarding ASD.

List of Abbreviations Used

ASD: autism spectrum disorder
BTBR: black and tan brachyury
CFU: colony forming units
CNS: central nervous system
GBA: gut-brain-axis
HPA: hypothalamic-pituitary-adrenal
IACUC: institutional animal care and use committee
rRNA: ribosomal ribonucleic acid

Conflicts of Interest

All author(s) declare that they have no real, potential, or perceived conflicts of interest between the duties or responsibilities that are related to research, and personal institutional, or other interest which may be associated with each author.

Ethics Approval and/or Participant Consent

Our manuscript did not require ethics approval because it was a research proposal. The proposed procedure would require approval from the Institutional Animal Care and Use Committee and the Research Ethics Board. We require approval from the IACUC that the standards we have are accurate, valid and all the animals involved are provided with the best possible treatment and care. Including the proposed research applies with the applicable regulations and guidelines and all staff working with the mice have the proper training and utilize the proper practices. We require approval from the Research Ethics Board to review the ethical implications, the potential risks and benefits, and the relevance of our research.

Authors' Contributions

IK: Drafted the abstract, contributed to drafting the discussion, assisted with research, revised the manuscript, and gave final approval of the version of the manuscript to be published.
JVP: Made contributions to research, drafted the methods and results, revised the manuscript critically and gave final approval of the version of the manuscript to be published.
LK: Made contributions to research, contributed to drafting the discussion and methods, drafted conclusion, completed citations, revised the manuscript critically and gave final approval of the version of the manuscript to be published.
MT: Made contributions to the introduction section, assisted with research, and gave final approval of the version of the manuscript to be published.

Acknowledgements

We would like to thank Scinapse for the opportunity to participate in the undergraduate science case competition (USCC).

Funding

This study was not funded, due to this being a research proposal.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Ricky Chow, Anastasiya Moiseyenko
Article Dates: Received May 18 23; Accepted Sep 13 23; Published Nov 02 23

Citation
Please cite this article as follows:
Kostwinder L, van Paassen J, Keritses I, Tavares M. The effects of prebiotics and probiotics following antibiotic exposure in a mouse model of autism spectrum disorder: A research protocol. URNCST Journal. 2023 Nov 02: 7(11).
https://urncst.com/index.php/urncst/article/view/501
DOI Link: https://doi.org/10.26685/urncst.501