Dietary prebiotics promote anxiolytic-like behavior in the open field test and reduce relative adrenal and spleen weight in Fischer 344 rats

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ABSTRACT

It is becoming increasingly clear that the community of microorganisms living in the mammalian gut (the gut microbiota) exerts a significant influence on brain function. Evidence suggests that the microbiota plays a role in anxiety and anxiety-like behavior in animal models. Several studies have demonstrated reduced anxiety-like behavior with administration of probiotics (live bacteria with known health benefits), but less work has been done to investigate the potential anxiolytic effects of prebiotics (dietary substances that promote probiotics already residing in the gut). Prebiotics have several advantages over probiotics as an intervention against anxiety.

We tested the effects of a diet containing prebiotics on anxiety-like symptoms in male Fischer 344 rats, including behavior in the open field test, corticosterone levels, and the weights of the adrenals, spleen, and thymus. One group of animals consumed the prebiotic diet for approximately 4 weeks and underwent behavioral testing during adolescence; another group consumed the diet for 6 weeks and underwent testing during adulthood.

Animals who consumed the prebiotic diet displayed anxiolytic-like behavior in the open field test regardless of age, spending more time in the aversive open center of the field compared to controls. These animals also exhibited reduced relative adrenal and spleen weight. Interestingly, relative adrenal weight was inversely correlated with time spent in the center of the field during the open field test in adult rats. These results suggest an anxiolytic effect of dietary prebiotics. Further investigation is required to determine what changes in the microbiota, metabolome, and brain may have accompanied this anxiolytic effect.

INTRODUCTION

The mammalian gut is home to a diverse community of microorganisms collectively known as the gut microbiota. The gut microbiota exerts an influence on many aspects of host physiology, including metabolism[31], immune function[32], and vascular function[36]. A growing body of evidence indicates that the influence of the microbiota extends even to brain function, including cognition[8,16] and psychological health[1,7,18,21,38]. Deleterious changes in the composition of the microbiota have been implicated in psychiatric disorders such as anxiety and depression[7,18].

The idea that the gut microbiota might play a role in anxiety was first suggested by the observation that germ-free mice (mice delivered and raised in artificial germ-free conditions so as to lack a microbiota) exhibited an exaggerated hypothalamic-pituitary-adrenal (HPA) response to restraint stress compared to mice raised in a conventional environment with exposure to typical microbes[35]. Since then, a series of studies has established that the presence of an intact gut microbiota is important for normal emotional behavior[5,6,11,25,35]. For example, one study showed that germ-free mice displayed more anxiety-like behavior in the open field and marble burying tests than mice who had been conventionalized by exposure to a non-sterile environment[25]. Another study demonstrated that germ-free Fischer 344 rats exhibit more anxiety-like behavior than conventional specific pathogen free rats in the open field and social interaction tests, as well as elevated serum corticosterone levels[6].

Furthermore, studies have shown that ingestion of particular species of bacteria known to produce beneficial effects within the host, known as probiotics[17], reduces anxiety-like behavior. For example, mice treated with the probiotic *Lactobacillus rhamnosus* exhibited reduced anxiety-like behavior in the elevated plus maze as well as reduced stress-induced corticosterone levels[2]. Similarly, administration of a probiotic formulation containing *Lactobacillus helveticus* and *Bifidobacterium longum* decreased anxiety-like behavior in a conditioned defensive burying test in rats and also “alleviated psychological distress” in human subjects based on several self-assessment measures[22].

The successfulness of such attempts to alter behavior through modulation of the microbiota depends on the age of the animal. In general, the microbiota is more malleable in younger animals[26]. For instance, our lab recently demonstrated that exercise was more effective at changing gut microbial composition in juvenile rats than in adults[23]. Furthermore, there seem to be critical periods during early life in which the microbiota has especially powerful and persistent effects on physiology and behavior. Sudo et al. found that colonization of germ-free mice with bacteria from conventional specific pathogen free mice at 14 weeks (adult) had no effect on the exaggerated HPA stress response displayed by these animals; however, colonization at 6 weeks (young adult) returned the response to control levels[35]. In another study, colonization of germ-free mice immediately post-weaning (at 3 weeks of age) reversed the abnormal anxiety-like behavior observed in the light-dark box[5], but the abnormal phenotype of germ-free mice observed by Neufeld et al. persisted following colonization in adulthood[24].

While the literature on probiotics and anxiety-like behavior is growing, less work has been done to investigate the potential anxiolytic effects of prebiotics. Prebiotics are dietary substances, primarily fibers like those found in many fruits and vegetables, which stimulate the growth and/or activity of beneficial probiotic species already residing in the gut. Prebiotics act as a food source for these probiotics, and when broken down may also produce metabolites that are beneficial to the host[9]. Prebiotics have several advantages over probiotics as interventions by which to modulate host physiology, as described by Liu et al.: First, in order to have an effect, probiotics must be delivered to the gut alive, which represents a significant challenge; this is not true of prebiotics. Second, prebiotics can promote a number of probiotic species at once while probiotics are usually administered one or a few species at a time. Finally, prebiotics may exert direct effects on host physiology in addition to the effects they exert through their influence on the microbiota[17].

Recent data from our lab verify that the prebiotic diet used in the following experiment does encourage the proliferation of probiotics, particularly *Lactobacillus rhamnosus* and other *Lactobacillus* species. These data also indicate that the diet produces a decrease in mRNA levels of c-Fos (a marker of neuronal activation[14]) in the amygdala, suggesting reduced tonic

activation of the amygdala (manuscript in preparation). The amygdala is associated with fear and anxiety[27]. Given that our prebiotic diet promotes probiotics and may reduce activation of a brain region involved in anxiety, we hypothesized that it might also affect anxiety-like behavior.

The following experiment was designed to investigate the effects of a diet containing prebiotics on anxiety-like symptoms in male Fischer 344 rats, including behavior in the open field test, corticosterone levels, and the weights of the adrenals, spleen, and thymus. Furthermore, we aimed to determine how these effects might differ between adolescent and adult animals who consumed the diet beginning immediately post-weaning. We hypothesized that animals who consumed the prebiotic diet would exhibit anxiolytic-like responses compared to controls.

METHODS

Animals

Male Fischer 344 rats (n=32, Harlan Laboratories, Indianapolis, IN) arrived at postnatal day 24 and were pair-housed in Nalgene Plexiglas cages (45 cm x 25.3 cm x 14.7 cm) in a temperature- (22° Celsius) and humidity-controlled animal facility maintained on a 12-hour light/dark cycle (lights on 05:00-17:00). Animals were given ad libitum access to food and water. Each week the animals were weighed, food consumption was calculated, and fecal samples were collected and stored at -80° Celsius. All experimental protocols were approved by the University of Colorado Animal Care and Use Committee.

Experimental design

Half of the animals were assigned to a group fed a prebiotic diet containing galactooligosaccharides, polydextrose, lactoferrin, and milk fat globule membrane 10 (formulated by Mead Johnson Nutrition, Glenview, IL; manufactured by Harlan Laboratories, Indianapolis, IN); the other half were assigned to a group fed a control diet matched for calorie density and macronutrient composition. Within each group, half of the animals consumed their assigned diet for approximately 4 weeks, beginning at postnatal day 24 and

ending at postnatal day 48 (adolescence), when they underwent behavioral testing; the other half consumed their assigned diet for 6 weeks, beginning at postnatal day 24 and ending at postnatal day 66 (adulthood), when they underwent behavioral testing (n=8/group). Animals in both groups were sacrificed via rapid decapitation 6 days following behavioral testing. Adrenal glands, spleens, and thymuses were extracted, weighed, and stored at -80° Celsius; brains and trunk blood were also collected and frozen at -80°.

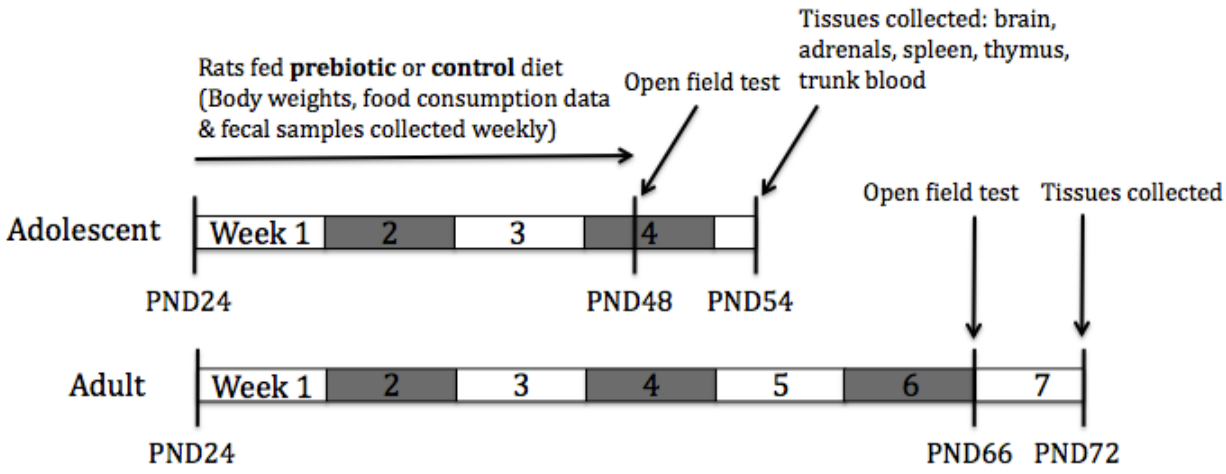

Figure 1. Experimental timeline.

Open field test

Behavior in an open field is a well-established measure of anxiety-like behavior in rodent models[12,15,28]. At postnatal day 48 (for adolescents) or postnatal day 66 (for adults) at approximately 09:00 (during the light cycle), rats were subjected to open field testing. The open field apparatus was a 60 cm x 60 cm square chamber with 50 cm-tall walls, constructed out of non-reflective black Plexiglas. The apparatus was located in a small, quiet room and dimly illuminated from overhead by red and white light (approximately 30-40 lux) to simulate rats' preferred environment (i.e., darkness) as much as possible. A video camera mounted approximately 1.5 m above the apparatus tracked the animal's movement within the field for the duration of the 10-minute test (ANY-maze video tracking software, Stoelting Co., Wood Dale, IL). An approximately 25 cm x 25 cm square area in the center of the

apparatus was designated as the center of the field, and the time the animal spent in this area was quantified.

Corticosterone assay

Trunk blood was collected immediately following decapitation and centrifuged to isolate plasma, which was frozen for future analyses. Plasma corticosterone levels were measured using ELISA (corticosterone ELISA kit, Enzo Life Sciences, Farmingdale, NY) as previously described[3]. Briefly, 10 μL of plasma was diluted 1:50 with assay buffer to a final volume of 100 μL and then processed according to kit instructions. Processed samples along with corticosterone standards were incubated in a 96-well plate coated with donkey anti-sheep antibody, together with a sheep polyclonal antibody specific for corticosterone, and corticosterone that was covalently attached to an alkaline phosphatase molecule, on a plate shaker at 500 rpm for 2 hours. The plate was then washed to clear unbound reagent and substrate was added. After another 1 hour of incubation the reaction was stopped and the intensity of the yellow color that was produced was read with a microplate reader (EMax precision microplate reader, Molecular Devices, Sunnyvale, CA) at 405nm. Concentrations were compared against the standard curve and expressed as micrograms per deciliter (μg/dL) of plasma.

Data analysis

Weekly body weights and food consumption were analyzed separately for adolescent and adult groups, using repeated measures ANOVA to compare diets (n=8/group). Open field test data (i.e., percent time spent in the center of the field and distance traveled) were analyzed using a 2 x 2 ANOVA with diet and age as factors (n=8/group); organ weights and plasma corticosterone levels were also analyzed in this manner. The correlation between relative adrenal weight and percent time spent in the center of the field during the open field test in adult rats was assessed via simple regression (n=16). All data were subjected to Grubbs' test for outliers; one outlier in the adolescent prebiotic group was excluded from the plasma corticosterone analysis. Data are presented as mean ± standard error of the mean (StatView statistical analysis software, SAS Institute Inc., Cary, NC).

RESULTS

Body weights and food consumption did not differ between diets

Weekly body weights and food consumption were examined separately for adolescents (who consumed their assigned diet for approximately 4 weeks) and adults (who consumed their assigned diet for 6 weeks). Both adolescents and adults increased their food consumption and gained weight throughout the experiment ($p<0.0001$). No differences were observed between diets.

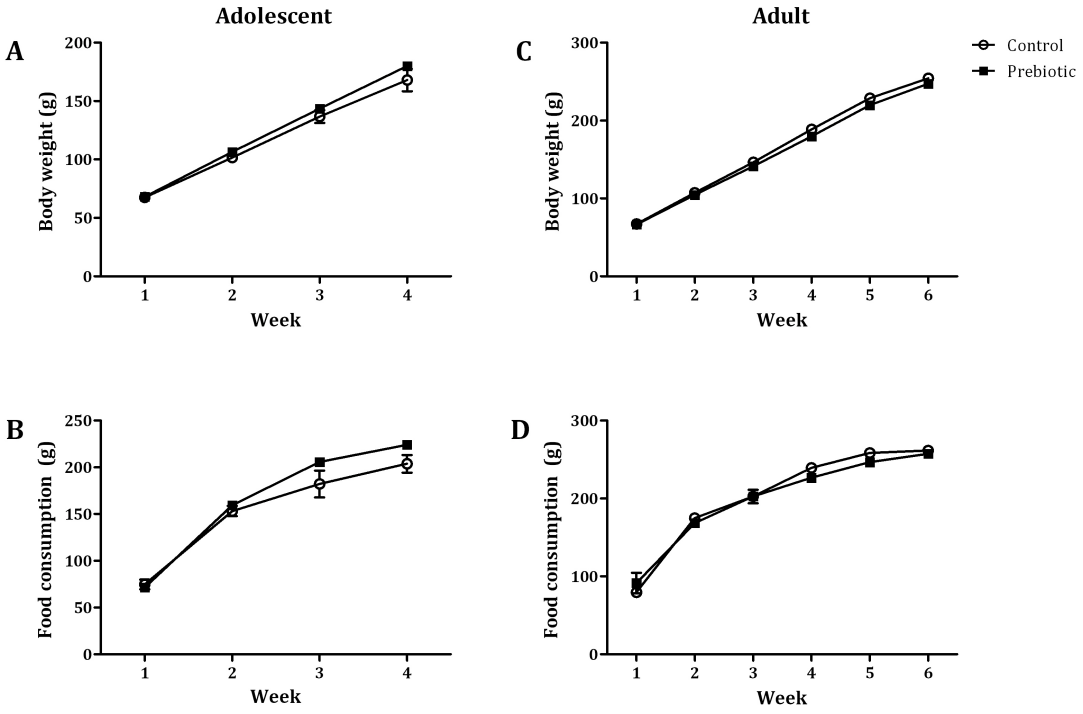

Figure 2. Weekly body weights and food consumption throughout experiment. (A) Average weekly body weights for adolescents on prebiotic diet compared to control diet. Animals gained weight throughout the experiment but no differences were observed between diets. (B) Average weekly food consumption for adolescents on prebiotic diet compared to control diet. Animals increased their food consumption throughout the experiment but no differences were observed between diets. (C) Average weekly body weights for adults on prebiotic diet compared to control diet. Animals gained weight throughout the experiment but no differences were observed between diets. (D) Average weekly food consumption for adults on prebiotic diet compared to control diet. Animals increased their food consumption throughout the experiment but no differences were observed between diets.

Prebiotic diet increased time spent in center of field during open field test

Rats who consumed the prebiotic diet spent significantly more time in the center of the field during the open field test ($F(1,28)=10.463$, $p=0.0031$). Adult rats spent more time in the center of the field than adolescents ($F(1,28)=18.698$, $p=0.0002$). Movement during the test (i.e., distance traveled within the testing apparatus) did not differ between diets or between adolescents and adults.

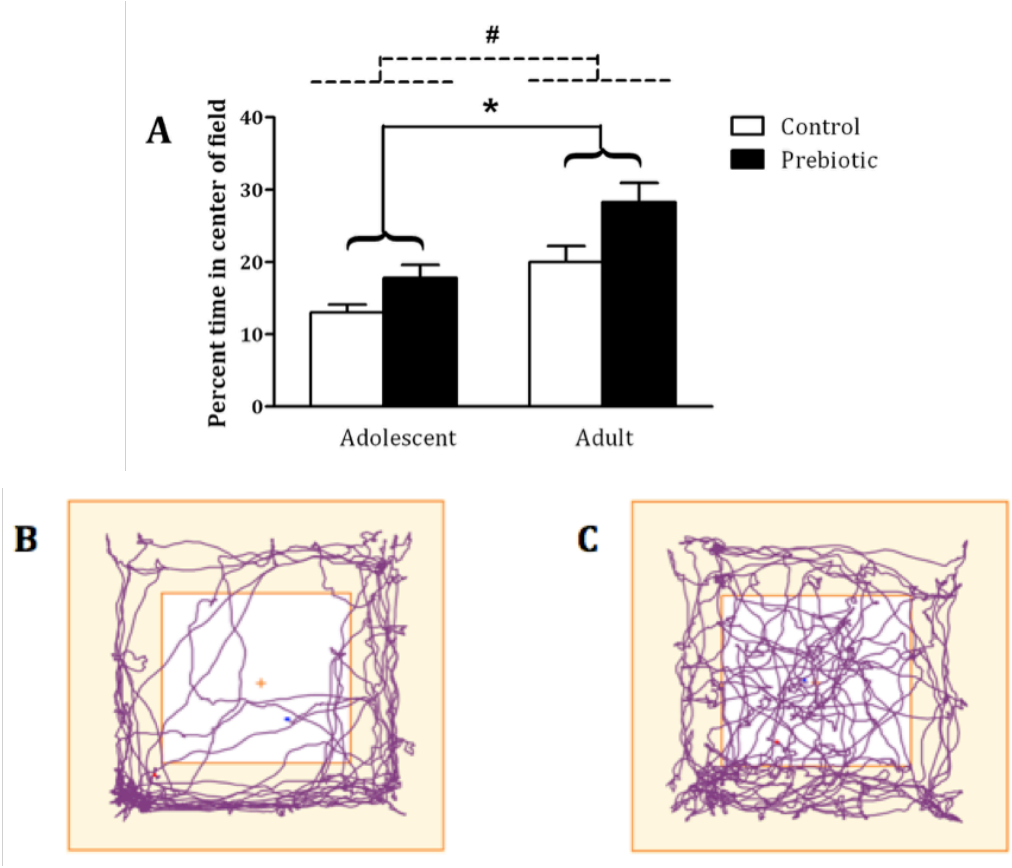

Figure 3. (A) Average percent time spent in the center of the field during the open field test for adolescent and adult rats on prebiotic or control diet. Rats on the prebiotic diet spent more time in the center of the field than rats on the control diet, and adult rats spent more time in the center of the field than adolescents. * denotes a significant main effect of diet at $p<0.05$; # denotes a significant main effect of age at $p<0.05$. (B) Example track plot showing the movement of a rat on the control diet within the open field apparatus throughout the 10-minute test. The animal tends to move along the periphery of the apparatus, avoiding the center of the field. (C) Example track plot showing the movement of a rat on the prebiotic diet within the open field apparatus throughout the 10-minute test. The animal crosses into the center of the field more often compared to the rat on the control diet.

Prebiotic diet reduced relative adrenal and spleen weight

Adrenal glands

Rats who consumed the prebiotic diet had significantly reduced adrenal gland weight to body weight ratio at time of sacrifice compared to rats on the control diet ($F(1,28)=13.519$, $p=0.0010$). Adult rats had significantly smaller adrenal glands relative to body weight than adolescents ($F(1,28)=43.498$, $p<0.0001$).

Spleen

Rats on the prebiotic diet exhibited reduced relative spleen weight compared to rats on the control diet ($F(1,28)=5.071$, $p=0.0324$). Adult animals had relatively smaller spleens than adolescents ($F(1,28)=98.283$, $p<0.0001$).

Thymus

Adult rats had significantly smaller thymuses relative to body weight compared to adolescents ($F(1,28)=17.728$, $p=0.0002$).

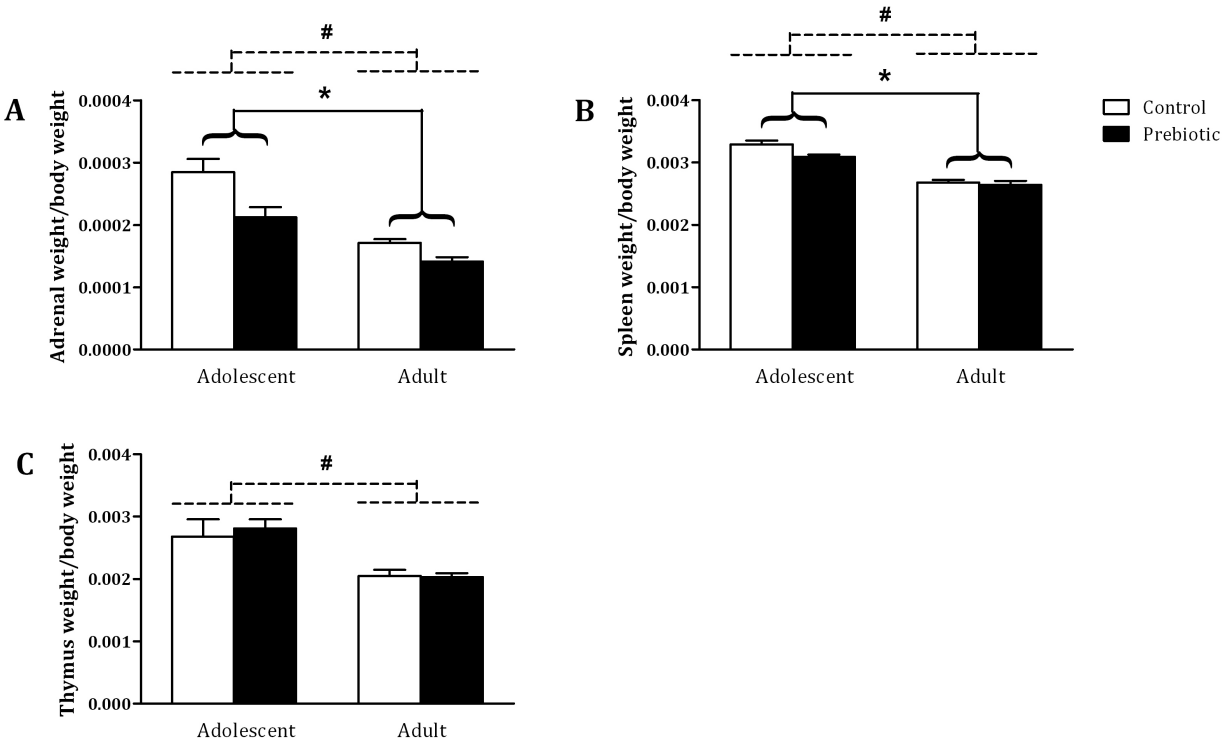

Figure 4. Organ weights. (A) Average adrenal weight to body weight ratio for adolescent and adult rats on prebiotic or control diet. Animals on the prebiotic diet exhibited reduced relative adrenal weight, and adults had relatively smaller adrenals compared to adolescents. (B) Average spleen weight to body weight ratio for adolescent and adult rats on prebiotic or control diet. Rats on the prebiotic diet exhibited reduced relative spleen weight, and adults had relatively smaller spleens compared to adolescents. (C) Average thymus weight to body weight ratio for adolescent and adult rats on prebiotic or control diet. Adults had relatively smaller thymuses compared to adolescents. * denotes a significant main effect of diet at $p<0.05$; # denotes a significant main effect of age at $p<0.05$.

Time spent in center of field during open field test was inversely correlated with relative adrenal weight in adults

We found a significant correlation between relative adrenal weight and percent time spent in the center of the field during the open field test in adult rats, such that percent time spent in the center of the field increased as relative adrenal weight decreased (p=0.0311, R-squared=0.291).

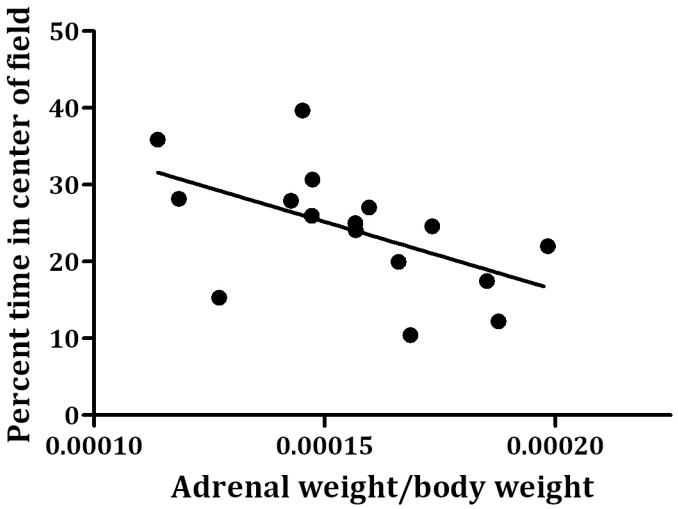

Figure 5. Correlation between adrenal weight to body weight ratio and percent time spent in the center of the open field during the open field test in adult rats.

Prebiotic diet had no effect on plasma corticosterone levels

Plasma corticosterone levels did not differ between diets or between adolescents and adults.

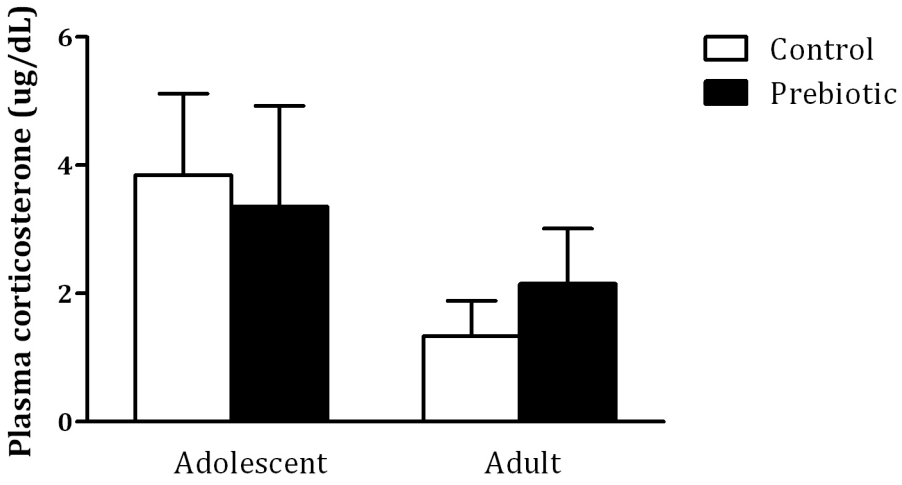

Figure 6. Average plasma corticosterone levels in adolescent and adult rats on prebiotic or control diet. There were no differences in plasma corticosterone between diets or between adolescents and adults.

DISCUSSION

Anxiety disorders are the most common type of mental illness in the United States, affecting an estimated 18% of adults[13] and costing the country over $42 billion per year[10]. Current treatments have significant drawbacks—medications require prescriptions, can be expensive, and may have undesired side effects; therapy can be expensive and inconvenient. By contrast, prebiotics do not require a prescription, are relatively inexpensive, and can be conveniently administered via addition to one's diet. Moreover, rather than having undesired side effects, they are likely to have additional health-promoting effects through their influence on the microbiota.

The current study found that regardless of age, rats who consumed a diet containing prebiotics spent more time in the center of the field during an open field test than controls. The open field test is a well-established method for assessing anxiety-like behavior in

rodents[4,12,28]. It operates on the premise that rodents are generally averse to open spaces, so the more entries into and/or time spent in the open area of the apparatus, the less "anxious" the animal[4,28,29]. This reasoning is supported by evidence that anxiolytic drugs such as benzodiazepines increase entries into the center of the field[4,28]. Thus, our open field test data indicate that the prebiotic diet promoted anxiolytic-like behavior. In contrast to what we expected, given the reported greater plasticity of the adolescent microbiota, this effect was not any more robust in adolescents. This may be because adolescents were on the diet for 2 weeks less than adults, although unpublished data from an earlier experiment supports the conclusion that 4 weeks of a diet containing galactooligosaccharides and polydextrose produced protection against learned helplessness equal to 9 weeks of the same diet (manuscript in preparation). The finding that adults overall spent more time in the center of the field than adolescents is consistent with other studies which have shown that adolescent rats exhibit greater anxiety-like behavior than adults[19,34].

In addition to producing anxiolytic-like behavior, the prebiotic diet also reduced relative adrenal weight compared to controls. The adrenal glands constitute one-third of the hypothalamic-pituitary-adrenal axis, a part of the neuroendocrine system responsible for controlling reactions to stress as well as other body processes. The adrenal cortex produces corticosterone, a major stress hormone. Chronic stress results in adrenal hypertrophy, which is associated with increases in corticosterone levels[30,37]. Given that anxiolytic-like behavior in the open field test increased as relative adrenal weight decreased in adult rats, the reduced relative adrenal weight observed in rats on the prebiotic diet may be an indication of reduced basal stress or "anxiety." The decrease in relative adrenal weight was not accompanied by a decrease in plasma corticosterone, however the reduced relative adrenal weight was replicated in a recent experiment, and the correlation between relative adrenal weight and anxiolytic-like behavior in the open field test was even stronger.

Reduced relative spleen weight was also observed in rats who consumed the prebiotic diet. The spleen acts as a filter for the blood, removing pathogens and senescent red blood cells, and serving other immune functions. It is unclear by what mechanism prebiotics might cause a decrease in relative spleen weight, or what implications this might have. Splanchic

vasoconstriction or a decrease in tonic inflammation could explain both the reduced relative spleen and adrenal weight, however we have no evidence to indicate that either of these conditions accompanied consumption of the prebiotic diet, and the reduction in relative spleen weight in rats on the prebiotic diet was not corroborated by data from a recent experiment. The observation that adults had smaller adrenals, spleens, and thymuses relative to body weight compared to adolescents is consistent with previous studies which found similar decreases in the weight of most internal organs relative to body weight with age[20,33].

Taken together, the results of this experiment suggest that consumption of a diet containing prebiotics has an anxiolytic effect regardless of age. Future work will aim to determine what changes in the microbiota, metabolome, and brain may have accompanied this anxiolytic response. We plan to examine the microbiota and the metabolome through 16S rRNA analysis and targeted short-chain fatty acid analysis of the fecal samples that were collected each week throughout the experiment; we will also attempt to replicate the decreased c-Fos mRNA levels in the amygdala we observed previously, and look for other changes in the brain that may occur with consumption of a prebiotic diet. Ultimately, we hope these endeavors might reveal the mechanisms by which prebiotics may influence anxiety-like behavior.

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