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Article

The effect of the consumption of the probiotic *B. infantis* on ethanol withdrawal symptoms in planaria (*Dugesia dorotocephala*)

Marissa McCandless and Janine Cupo
Seaford High School, Seaford, New York

SUMMARY

Alcohol use disorder is a chronic, relapsing disease that affects millions of Americans every day. There are limited treatment options for alcohol dependence and alcohol withdrawal symptoms, including depression and anxiety. Previous studies have shown that probiotics can decrease depression in rodents during maternal separation and anxiety in humans. Therefore, we hypothesized that the ethanol-withdrawn planaria who consumed probiotics would have decreased withdrawal symptoms as measured by increased motility compared to the ethanol-withdrawn planaria that were not fed probiotics. The ethanol-withdrawn planaria had a statistically significant decrease in motility compared to the control group, while the planaria that consumed probiotics had no statistically significant change in motility compared to the control group. We determined that there was a statistically significant difference between the ethanol and probiotics-treated group and the control group. Additionally, there was a statistically significant difference between the ethanol and probiotics-treated group and the ethanol-withdrawn group, confirming our hypothesis. Although the results indicated that the consumption of probiotics were unable to significantly counteract the effects of ethanol withdrawal symptoms in planaria, the probiotics significantly increased the ethanol-withdrawn planaria's motility as compared to the ethanol-withdrawn group that was not given probiotics. Given these results, probiotics have the potential to reduce the symptoms of alcohol withdrawal in humans.

INTRODUCTION

Alcohol use disorder (AUD), a chronic, relapsing disease characterized by compulsive alcohol consumption, is an unceasing issue that affects many Americans. About 19.7 million American adults lived with a substance use disorder in 2017, with 74% of these adults living specifically with alcohol use disorder (1). Treatment for AUD is difficult because of the lengthy withdrawal process. Withdrawal is defined as behavior that occurs when ceasing to use an addictive substance. Due to the symptoms that can coincide with alcohol withdrawal, such as depression and anxiety, people often relapse and return to abusing alcohol. Depression and anxiety are disorders that result from an imbalance of neurotransmitters in the brain such as serotonin, dopamine and norepinephrine (2). These neurotransmitters are responsible for initiating nerve impulses through the brain, promoting feelings such as happiness and pleasure. Severe depression and anxiety are often caused by a failure in the neurotransmitter system and are commonly treated with an antidepressant drug. However, if a person is addicted to or withdrawing from alcohol, taking an antidepressant could be dangerous for both their mental and physical state because alcohol can worsen the side effects of an antidepressant, and the antidepressant can exacerbate the effects of alcohol consumption (3). In order to reduce the prevalence of AUD, new treatments must be developed to treat alcohol withdrawal symptoms.

A potential solution is utilizing probiotics as a treatment for the symptoms of depression and anxiety. Probiotics are microorganisms that are introduced into the body to aid in the function of the gut microbiome. The gut microbiome is the community of beneficial microorganisms living in the intestines that help with daily bodily functions (4). The Food and Agriculture Organization and the World Health Organization have defined probiotics as live bacterium that are beneficial for supplementing the body's gut microbiome and its functions (5). Probiotics may serve as a viable treatment for alcohol withdrawal due to the interaction between the gut microbiome and the brain, known as the gut-brain axis (Figure 1). The central nervous system (CNS) and the enteric nervous system (ENS) are communication systems that make up the gut-brain axis (6). The gut-brain axis accounts for how the bacterial communities in the gut microbiome affect mental processes in the brain and vice versa (6). Therefore, the connection between probiotics and ethanol withdrawal symptoms can be made based on previous research that indicated there is a connection between the gut microbiome, where probiotics are found, and the brain, where the symptoms depression and anxiety stem from (6).

Like humans, planaria have a gut microbiome that affects many of their bodily functions. Planaria are free-living flatworms that can be found in either spring or saltwater ponds and rivers. Planaria are ideal organisms to experiment with because they present little ethical concerns and are known to experience the effects of a drug similar to the experience of humans (7, 8). Multiple studies have been conducted showing that planaria who consume drugs such as ethanol, nicotine and sucrose develop withdrawal symptoms (7, 8, 9). Additionally, planaria have simple nervous and digestive

systems that are relatively easy to analyze. The planaria nervous system is organized with axons and dendrites and contains multipolar nerve cells, which are similar to the organization of a human's nervous system (10). Not only do they have a similar structure, but planaria also have many of the same neurotransmitters as humans, such as serotonin and norepinephrine (10, 11). These neurotransmitters impact human depression and anxiety and may affect planaria in a similar way. Furthermore, planaria have a digestive system that is analogous to the human digestive system; the planaria's gut microbiome is comprised of multiple bacteria populations that are also found in a human's gut microbiome (12). These similarities make the drug effects that planaria experience comparable to the effects that humans experience.

The goal of this study was to asses a connection between relief from alcohol withdrawal symptoms and the consumption of probiotics. If probiotics were found to be effective in reducing anxiety and depression in planaria, they could be further studied as an aid for people experiencing the ethanol withdrawal process. Therefore, we asked whether the consumption of *Bifidobacterium infantis* will reduce the symptoms of ethanol withdrawal in planaria (*Dugesia dorotocephala*). The specific bacterial strain, *Bifidobacterium infantis*, was utilized since previous studies have shown its effectiveness in reducing depression and anxiety (6, 13). Ethanol, the alcohol found in alcoholic beverages, is frequently used for experiments involving planaria addiction and withdrawal. Therefore, we placed planaria in ethanol for 24 hours and then removed them and placed them immediately in spring water (7, 14). To study symptoms of depression and anxiety in planaria, we used a line crossing

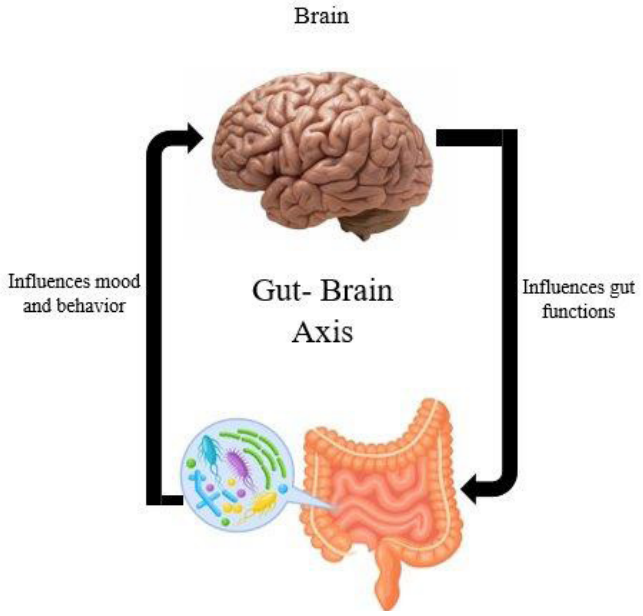

Figure 1: Gut-Brain Axis. The interaction between the gut microbiome and the brain demonstrating the influence of the gut microbiome on mood and behavior.

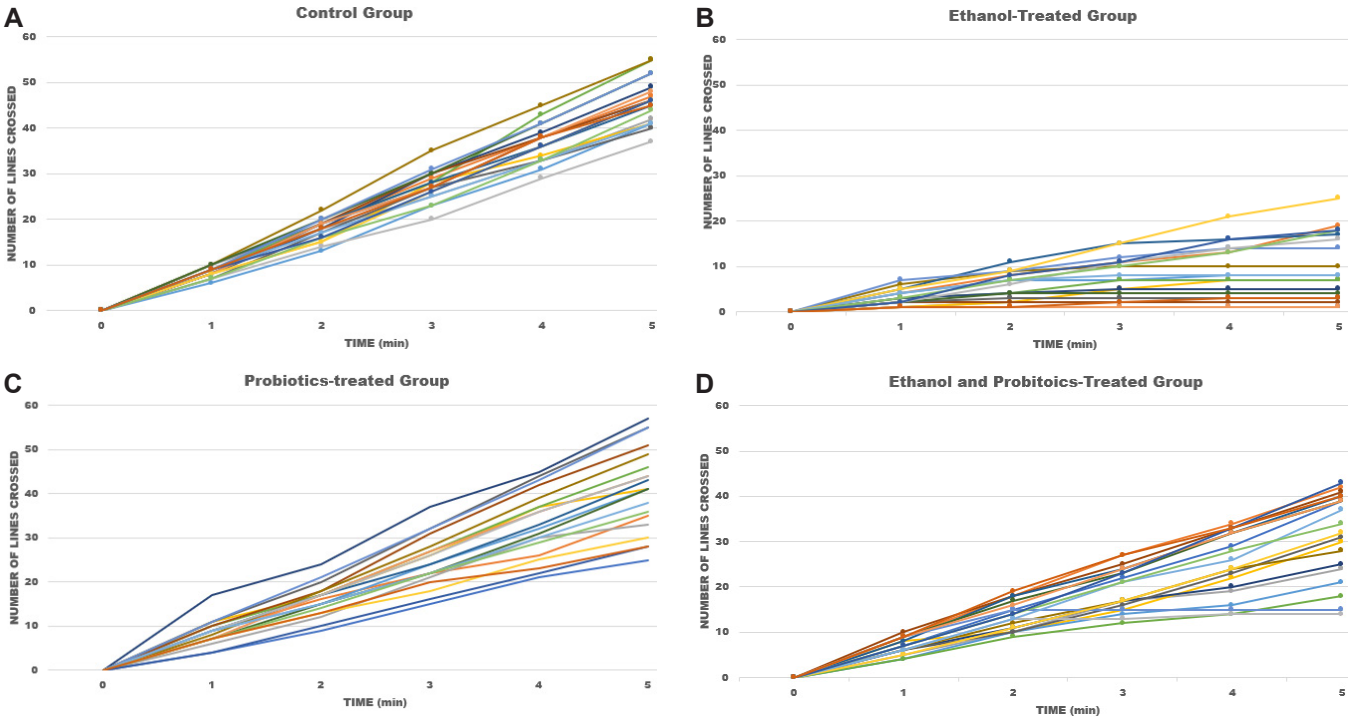

Figure 2: Cumulative number of lines crossed for each individual trial under four different conditions. A: Number of lines crossed per planaria across a five minute period for control group. B: Number of lines crossed per planaria across a five minute period for ethanol-treated group, C: Number of lines crossed per planaria across a five minute period for probiotics-treated group, D: Number of lines crossed per planaria across a five minute period for ethanol and probiotics-treated group (each line of the graph represent a planaria)

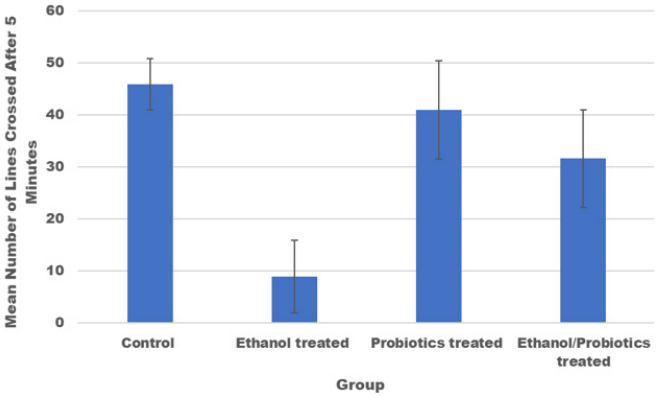

Figure 3. Mean number of lines crossed in five minutes during line crossing assay to determine motility. Error bars represent the standard deviation.

assay, that measures motility in planaria and has been validated by previous research (7, 14). We hypothesized that the ethanol-withdrawn planaria who consumed probiotics would have decreased withdrawal symptoms as measured by increased motility compared to the ethanol-withdrawn planaria that were not fed probiotics. We found that probiotic consumption was effective in diminishing ethanol withdrawal symptoms in the planaria. However, probiotics did not restore the planaria to their normal behavior.

RESULTS

To determine whether consumption of probiotics would be successful in reducing the effects of ethanol withdrawal, planaria were treated with ethanol for 24 hours after being fed probiotics in their food. Then, line crossing assays were conducted to quantify planaria motility as measured by the number of lines crossed by the planaria in five consecutive minutes (7, 9). Reduced planaria motility is reflective of depression and anxiety induced by ethanol withdrawal in planaria and has been demonstrated as an effective way to measure planaria withdrawal from a substance (7, 14, 15).
During the five minutes of each line crossing assay, the cumulative number of lines crossed were recorded for each individual trial (Figure 2). Then, the means and standard deviations were determined (Figure 3). The average number of lines crossed by the control group was 45.9, by the ethanol-treated group was 8.9, by the probiotics-treated group was 41 and by the ethanol and probiotics-treated group was 31.6 (Figure 3). The R^2-value (correlation coefficient), which represents the linear relationship amongst the data, was found for each group (Table 1).

We performed a one-way ANOVA (Table 2) {F(3,76)=85.95, p=2.33E-24}. These ANOVA results suggest that there was a statistically significant difference among the data from each treatment group. We conducted a Tukey HSD post-hoc test following the ANOVA to see where the differences within the data lie. The p-values for comparing the control and ethanol-treated group (p=0.001), the control group and the experimental group (p=0.001), and the

	R^2-Values			
Planaria Number	Control	Ethanol-treated	Probiotics-treated	Ethanol/Probiotics-treated
1	0.999	0.429	0.996	0.997
2	0.999	0.984	0.988	0.998
3	0.998	0.690	0.987	0.987
4	0.990	0.946	0.989	0.975
5	0.993	0.783	0.998	0.985
6	0.991	0.864	0.998	0.985
7	0.998	0.757	0.989	0.992
8	0.996	0.428	0.997	0.998
9	0.993	0.631	0.998	0.989
10	0.998	0.713	0.998	0.997
11	0.998	0.911	0.997	0.997
12	0.999	0.690	0.996	0.998
13	0.999	0.881	0.999	0.659
14	0.998	0.429	0.998	0.999
15	0.997	0.983	0.999	0.760
16	0.995	0.996	0.998	0.994
17	0.999	0.760	0.998	0.991
18	0.994	0.994	0.100	0.999
19	0.997	0.983	0.997	0.993
20	0.998	0.943	0.986	0.992
Mean	0.997	0.790	0.995	0.964

Table 1: R^2 values for Line Crossing Assays.

ethanol-treated group and the experimental group (p=0.001) were all less than 0.01, indicating that there was a statistically significant difference among the data from those groups. The p-value (p=0.213) between the control group and the probiotics-treated group was greater than 0.01, indicating no statistically significant difference among the data in those groups.

DISCUSSION

The R^2-values in Table 1 indicated that there was a linear relationship between the number of lines crossed and time. Planaria have been shown to exhibit exploratory behavior when placed in a novel environment. Since they are mainly nocturnal, once removed from their preferred environment and placed in a new petri dish in a well-lit area, their nearly linear behavior may be due to the planaria's inherent exploratory behavior. The R^2-values indicated that all groups continued to exhibit relatively constant exploratory behavior, thereby allowing motility to be observed and assayed. It is possible that, given more time, the curves would have flattened as the planaria eventually acclimated to their environment.

The results from the ANOVA test {F(3,76)=85.95, p=2.33E-24} indicated that there was a statistically significant difference amongst the data. The Tukey HSD post-hoc test provided p-values and determinations for the differences among the groups. The statistically significant p-value (p=0.001) for the control group and ethanol-withdrawn group signified that the number of lines crossed in the ethanol-

ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	16157.8	3	5385.933	85.95056	2.33E-24	2.724944
Within Groups	4762.4	76	62.66316			
Total	20920.2	79				

Table 2. One-Way ANOVA Results.

Treatment Pair	Tukey HSD p-value	Tukey HSD inference
Control vs. Ethanol-treated	0.001	**p<0.01
Control vs. Probiotics-treated	0.213	Not significant
Control vs. Ethanol/ Probiotics-treated	0.001	**p<0.01
Ethanol-treated vs. Ethanol/Probiotics-treated	0.001	**p<0.01

Table 3. Tukey HSD Post-Hoc Results (** indicates significant difference).

withdrawn group was significantly less than the number of lines crossed in the control group. Thus, the planaria experienced the symptoms of withdrawal, depression and anxiety, from the 0.5% ethanol solution, as decreased motility is indicative of these symptoms. These results agreed with previous studies that demonstrated that planaria can experience withdrawal symptoms from this ethanol concentration (7). The Tukey HSD post-hoc test determined that the p-value (p=0.231) from the control and probiotics-treated groups was statistically insignificant, denoting that there was no statistically significant difference between the number of lines crossed in those groups. As a result, we concluded that the $6.67x10^6$ CFU/mL probiotic solution did not have an impact on planaria motility. The statistically significant p-value of 0.001 between the control group and the ethanol and probiotics-treated group demonstrated a significant difference among those groups, indicating that the probiotics were unable to completely reverse the effects of ethanol withdrawal. However, the p-value (p=0.001) between the ethanol-treated group and the ethanol and probiotics-treated group denoted a statistically significant difference between those groups, indicating that the probiotic was effective in increasing the number of lines crossed by the ethanol withdrawn planaria. The probiotic was therefore effective in reducing the symptoms of depression and anxiety in this study. After coming to these conclusions, we failed to reject the hypothesis that the ethanol-withdrawn planaria who consumed probiotics would have decreased withdrawal symptoms as measured by increased motility compared to the ethanol withdrawn planaria that were not fed probiotics.

The results support our hypothesis and offer a new understanding of the benefits of probiotics. Previous studies have shown that probiotics are useful in enhancing the gut microbiome function, in reducing inflammation, which is linked to decreased anxiety, and in decreasing the effects of depression (6, 13). The results of this experiment suggest that probiotics may be a treatment for the ethanol withdrawal symptoms of depression and anxiety as well.

The implications of this study are plentiful. Since probiotics can potentially aid in the alcohol withdrawal process, they may also diminish the occurrence of AUD. Likewise, since the symptoms of withdrawal include depression and anxiety, probiotics could potentially serve as a treatment for these disorders and as a substitute for antidepressants. There are a multitude of possibilities for probiotics in the future, and if research in this field continues to progress, probiotics may be able to solve many health issues that the world is facing today.

MATERIALS AND METHODS

Dugesia dorotocephala (planaria) were obtained from the Carolina Biological Supply Company. The planaria were cared for in a home jar containing Poland Spring water since their natural habitat is spring ponds and rivers (16). This water was changed three times a week and the planaria were fed chicken liver once a week. Many researchers feed planaria chicken liver as their main source of nutrition due to its protein (16). We made ethanol solutions (0.5%) from 70% ethanol obtained from Carolina Biological Supply. Planaria were treated with powder from probiotic pills of Bifidobacterium infantis 35624 ($1x10^9$ colony forming units), from Alforex. Colony forming units (CFU) is the unit used in the scientific community to measure the viable bacteria in a given sample.

First, the planaria were separated into four groups to distinguish between the variables being tested. Each group had twenty planaria and each planaria was considered one trial, equating to twenty trials per group. All planaria were maintained at the same environmental conditions and fed on the same days at the same time, with the only difference being that the probiotics-treated group and the experimental group were fed probiotic soaked chicken liver. The negative control group contained planaria that represented normal planaria behavior, and the results from this group were compared against the rest of the groups' results. The first positive control group was the planaria that were treated with a 0.5% ethanol solution to demonstrate that the ethanol solution caused withdrawal symptoms in the planaria. In the second positive control group, the planaria were fed the probiotic solution to demonstrate that the probiotics did not have adverse effects on the planaria. Finally, the experimental group contained planaria that were fed the probiotics and then exposed to the ethanol solution, to determine whether the probiotics were successful in diminishing the effects of the ethanol withdrawal symptoms of depression and anxiety. The temperature, humidity and light conditions remained the same for all groups.

The ethanol solution was prepared by calculating the amount of Poland Spring water and ethanol in milliliters that was necessary to create a 0.5% concentration of ethanol by mass. When planaria are removed from this specific concentration of ethanol, previous studies have indicated that they experience withdrawal symptoms (7, 8). Before each trial for the probiotics-treated group and the ethanol and probiotics-treated group, the probiotic solution was created by breaking apart the pill's protein capsule, pouring the

probiotic powder into 150 milliliters of Poland Spring water, and creating a suspended solution by using a magnetic stirrer. The final probiotic solution consisted of $6.67x10^6$ CFU per milliliter of water. The $6.67x10^6$ CFU/mL concentration was the only solution that was found to have no detrimental effects on planaria motility, after testing the planaria with varying concentrations of probiotics. Additionally, before each trial in these groups, a 0.1-gram piece of chicken liver was soaked in the suspended probiotic solution for five minutes and then fed to the planaria for three hours, which is their normal feeding time (17). In using this method, the planaria were treated with the probiotics through ingestion, similar to how humans take probiotics.

Line Crossing Assay

To conduct a line crossing assay, a planaria is placed in a petri dish containing Poland Spring water which was placed on one-inch grid lined paper. The number of lines crossed by the planaria in five consecutive minutes is recorded (9). Decreased motility is characterized when a planaria crossed fewer lines as compared to the control group which indicates depression and anxiety in the planaria.

Control Group

A planaria was randomly selected from the home jar and placed into a petri dish consisting of Poland spring water. The petri dish was then placed back into a dark cabinet for 24 hours since planaria prefer a dark environment. After 24 hours, the planaria was then moved into a different petri dish and a line crossing assay was performed.

Ethanol-withdrawn Group

A planaria was randomly selected from the home jar and placed into a petri dish consisting of the ethanol solution (0.5%) and the petri dish was then placed back into a dark cabinet for 24 hours. After these 24 hours, when the planaria became addicted to the ethanol, the planaria was removed from the ethanol solution, placed in a new petri dish with Poland spring water and the line crossing assay was conducted.

Probiotics-treated Group

First, the probiotic soaked chicken liver was fed to five planaria, randomly selected from the home jar, in a petri dish filled with Poland Spring water. Five planaria were chosen in order to speed up this process as this group had a lengthy procedure. The chicken liver was removed after the standard amount of time the planaria are given to eat (approximately three hours). The planaria were then placed into separate petri dishes containing Poland Spring water and placed in a cabinet for 24 hours. After the 24 hours, the planaria were moved into new petri dishes containing Poland Spring water and the line crossing assay was performed.

Ethanol and Probiotics-treated Group

Five planaria were randomly selected from the home jar and placed into petri dishes containing the probiotic soaked chicken liver. After being allotted the standard amount of time to feed, the planaria were placed in individual petri dishes containing the 0.5% ethanol solution. The planaria were left in a cabinet in the ethanol solution for 24 hours, and then removed and placed into new petri dishes containing Poland Spring water for the line crossing assay.

Data Analysis

Statistical analyses of the line crossing assay data were conducted using Microsoft Excel to determine means, standard deviations, R2-values and p-values. A one-way ANOVA test was performed to determine the F-value, degrees of freedom between groups and within groups, and a p-value for all the group comparisons. A p-value corresponding to an F-statistic lower than 0.05 would suggest that there is a significant difference among one or more of the treatment groups and a post-hoc test would be performed. A Tukey HSD post-hoc test was performed, since the p-value corresponded to the F-statistic, to determine where the differences lie within the data. P-values were determined from this test and if the values were less than 0.05, there was a statistically significant difference between the groups.

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