Reducing the amount of *Clostridium difficile* in the gut microbiome reduces the behavioral projection of cognitive activity in rats

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Abstract. It is already known that the gut microbiome bacteria colony of *Clostridium difficile* produces 10-1,000-fold more para-cresol (p-cresol) than other known p-cresol-producing bacteria in the gut. A notable link between a high concentration of p-cresol, a phenolic compound produced by *Clostridium difficile*, and its penetration into the brain and various neurological disorders has been demonstrated over the past decades. The present study demonstrates a possible link between the amounts of *Clostridium difficile* in the gut microbiome and p-cresol levels in the brain with cognitive activity. Herein, the amount of *Clostridium difficile* in the gut microbiome of rats was decreased through administration of vancomycin, a well-known antibiotic used for eliminating the effects of gut infections caused by the growth of the colony of *Clostridium difficile*. In these rats in the experimental group, the behavioral projection of cognitive activity was significantly decreased, compared to the rats in the control group. These data may thus indicate a potential link between *Clostridium difficile* colonies in the gut and cognitive functions of the brain. It is suggested that this interaction is carried out through the dopaminergic activity of the brain.

Introduction

A diverse microbial community of the human gut microbiome has been shown to metabolize aromatic amino acids (1,2). The secondary metabolites produced by these reactions can affect multiple aspects of human well-being. Among others, the intestinal bacterium, *Clostridium difficile* (*C. difficile*), produces para-cresol (p-cresol) by metabolizing tyrosine via p-hydroxyphenylacetate. For a number of years, the final phenolic product, p-cresol, was known as a uremic toxin, since, at high concentrations, this compound can induce chronic renal disease symptoms (3). Other p-cresol-producing bacteria also are present in the human gut, between them are different strains of and *Clostridiaceae, Bifidobacteriaceae, Enterobacteriaceae, Coriobacteriaceae, Bacteroidaceae, Fusobacteriaceae* and *Lactobacillaceae* families (4). Still, compared with these, *C. difficile* produces 10-1,000-fold more p-cresol and can be regarded as a potent source of this metabolite (1,4). Due to its hydrophobicity, this compound easily penetrates the intestinal mucosa and reaches the bloodstream and all organs, including the brain (5). The p-cresol level is often significantly higher in the urine of patients with autism spectrum disorder (ASD) and epilepsy. It can be used as a possible biomarker of disease severity (3,6,7). In previous studies, the inhibitory effects of p-cresol on the enzyme, dopamine beta-hydroxylase (DBH), were shown to result in an increase in dopamine levels and the diminished production of norepinephrine (8,9). Thus, the inhibition of DBH possibly dysregulates the activities of dopaminergic neurons and can cause the aggravation of certain diseases, including major depressive disorder and bipolar disorder (10). DBH gene mutations and decreased levels of DHB in maternal serum have been shown to be associated with autism in children (11,12), suggesting the involvement of alterations in the dopaminergic signaling in C. difficile-induced precipitation and aggravation of autism spectrum disorder.

The majority of clinical laboratories assume that all *C. difficile* isolates are sensitive to vancomycin, the medication most frequently used in the treatment of *C. difficile*-related illnesses (13). Recurrent *C. difficile* infections are often treated with vancomycin taper regimens (14). Previous studies have demonstrated that oral vancomycin treatment causes a considerable improvement in the symptoms of patients with ASD (15,16). In a recent study, the authors found that a low concentration of p-cresol, compared with a high concentration, potentiates nerve growth factor-induced differentiation via the secretion of brain-derived neurotrophic factor (BDNF) in cultured PC-12 cells, demonstrating that low doses of p-cresol, in contrast to a high toxic dose, can affect neuronal cell structural remodeling (17). The present study examined the effects of vancomycin on the behavioral responses of rats in open-field tests and

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novel object recognition tasks. The results presented herein demonstrate that the treatment of rats with vancomycin affects the behavioral activity of the animals. These changes occur in parallel with the decreases in p-cresol levels in the brain. These data may indicate a direct effect of p-cresol on the behavioral responses of rats.

Materials and methods

Animals. Animal care during the experimental procedures was carried out by the recommendation of the Ilia State University Research Projects Ethics Commission (Decision no. R/266-23) and by the Council of Europe Directive 2010/63/EU for animal experiments. Young adult (weight, 250-300 g) male Wistar rats obtained from breeding colony of the vivarium of I. Beritashvili Center of Experimental Biomedicine (Tbilisi, Georgia) were housed in cages (6 rats per cage) and provided with food and water available *ad libitum* and maintained under conditions at a temperature of 20-22˚C and 40-55% humidity on a 12-h light/dark cycle (lights on at 7:00 a.m.). The rats were subjected to an enforced swimming test in order to exclude endogenic depression. Prior to commencing treatment, all rats were marked and separated for 24 h. A total of 2 g of fecal specimens from a 12-h period were randomly collected from each animal and stored at -80˚C. Following these procedures, a total of 12 male rats (10 weeks old) were randomly assigned into the following subgroups: i) The control group; and ii) the vancomycin-treated group. Behavioral procedures were conducted between 9:00 a.m. and 11: 00 a.m.

Following behavioral testing, all animals were separated again for 24 h. A total of 2 g of fecal specimens from a 12-h period were randomly collected from each animal and stored at -80˚C. Before and after placebo/vancomycin treatment, the fecal specimens were subjected to the qualitative detection of *C. difficile*. After performing behavioral tests, the rats were euthanized by rapid decapitation using a hand-operated guillotine. Decapitation was selected for euthanasia to avoid chemical contamination for further analysis (https://rsawa.research.ucla.edu/arc/euthanasia-decapitation/). After each use, the decapitation equipment was cleaned of any biological fluids with ethanol and water and then wiped. Rat brains were isolated on ice and homogenized rapidly in a 5x volume ice-cold PBS (pH 7.4) using a hand Dounce homogenizer (Kontes Glass Company), then centrifugated for 20 min at 1,000 x g at 4˚C.

Vancomycin treatment. Treatment was commenced 5 days following a 24-h isolation to exclude the effects of social isolation. The rats in the vancomycin-treated group received vancomycin once a day (light cycle) (5 mg/kg body weight, average 1 mg per animal) with bread for 3 weeks. The dose was selected based on published data to minimize the effects of vancomycin on other bacterial species (18). Vancomycin inhibits cell wall assembly by binding to the D-Ala-D-Ala termini of lipid II and has been shown to exhibit a high activity against *C. difficile* (19). The rats in the control group received the same portion of bread at the same time of the day. Behavioral tests were commenced on the second day following the completion of vancomycin treatment.

Open field test. The potential effects of vancomycin on locomotion and anxiety-like behaviors were assessed using the open field test. The device was an opaque Plexiglas arena with a size of 100x100x50 (H) cm. To ensure that the rats spent time in either the center or the outside of the arena, it was divided into 25 small squares. The rats were positioned in the center of the arena, and their behavior was monitored and examined for 10 min. For each rat, the total distance traveled, the total time spent and distance traveled in the center segment, and the number of grooming and raising sessions were recorded (20,21).

Novel object recognition task. Each animal received 10-min habituation sessions in a plastic cage (50x50x40 cm^3) that was equally lit and devoid of objects. The animals were permitted to investigate two identical objects that were always placed in the same location within the box for 5 min (training session) at 24 h following the habituation session. At 60 min after the 5-min training session, the test session was started to assess memory retention. Both well-known and unfamiliar objects were displayed simultaneously during the training phase throughout the 10-min test period. However, during the testing phase, the placement of new objects was changed fictitiously and randomly to avoid the animals' innate preference for one site over another. All objects (which were provided in duplicate) had the same material and size, but various shapes. Boxes and other materials were cleaned with 70% alcohol and allowed to air dry between each change of the animals. The camera recorded the time spent exploring, defined as smelling or touching the object with the nose. The time spent examining each object or novel object divided by the total time spent exploring both objects throughout the training and testing stages multiplied by 100 in the training and test phases, respectively, was finally defined as the discrimination ratio (also known as the recognition index) (20-22). The time spent examining known and novel objects was separately recorded to calculate the discrimination ratio for new object recognition. This ratio was defined as the exploration time differential for the novel object divided by the total exploration time. The more time spent investigating the novel object, the more successfully the memory could distinguish it from the familiar object (23). Therefore, an increase in the discrimination ratio during the test phase indicated an improvement in recognition memory, whereas a decrease indicated the opposite. To reduce any potential item preference that may skew the results, diverse objects were choose in order to be easily distinguished by the rats, while having a similar complexity level (texture, form, color patterning, brightness, etc.) (24). It was ensured that the rats could climb on the objects to prevent induced preference. By contrast, the capacity to climb on an object may boost interest in exploration (time spent simply sitting on an object is not counted toward the exploration time). As the loss of an object due to damage during experimentation could interfere with the continuation of testing and possibly result in harm or injury to the animal, objects made of non-breakable material were used. In the experiment, two similar objects were placed horizontally in the box (i.e., one in the north-west corner and one in the north-east corner) during the training phase; one unfamiliar object was substituted for one of the familiar objects during the testing phase (23,25).

Table I. Effects of vancomycin on anxiety-like behaviors in the open field and novel object recognition tests.

Test	Parameter	Control	Vancomycin	P-value
Open field	Line cross (number)	337	206	0.00888[a]
Open field	Center time (sec)	17	0	0.02757[b]
Open field	Center cross (number)	8	2	0.05528[c]
Open field	Rearing (number)	196	104	0.00711[a]
Novel object	Preference of 2nd object (day 2), %	59	36	0.0024[a]

[a]The result was significant at P<0.01; [b]the result was significant at P<0.05; [c]the result was significant at P<0.1 (not significant at P<0.05).

Qualitative detection of C. difficile. The glutamate dehydrogenase (GDH)-based detection of *C. difficile* in the rat fecal specimens from both groups was made before and after placebo/vancomycin treatment using the Abnova™ Clostridium difficile GDH ELISA kit (KA3381, Abnova). Briefly, 2 g fecal specimens from a 12-h period obtained were randomly collected from each animal and stored at -80˚C. Before and after treatment, the fecal specimens were analyzed simultaneously to avoid the technical error of the results. The fecal specimens of each animal were mixed and homogenized using a wooden applicator stick to ensure adequate sampling, and 0.2 g of the obtained sample were transferred to the test tube with 400 μl Sample Diluent for vortex and decantation (15 min). All ensuing procedures were performed according to the manufacturer's protocol. The fecal specimens of each animal were analyzed in triplicate. Respective positive and negative controls were used to confirm the validity of the experiment. The absorbance was determined at a wavelength of 450, and 630 nm was used as a reference wavelength.

Assessment of the P-cresol amount. The concentration of p-cresol in the brain homogenate was performed using an ELISA-based kit (EK711090; AFG Bioscience) according to the manufacturer's protocol. The rat brains were homogenized rapidly in a 5x volume PBS (pH 7.4) using a hand Dounce homogenizer, then centrifuged for 20 min at 1,000 x g at 4˚C. The supernatant was removed and assayed immediately.

Statistical analysis. One-way analysis of variance (ANOVA) was applied when analyzing all the data obtained from the unique objective and open field tests. ANY-maze Video Tracking Software 60000 (Stoelting Co.) was used for this purpose. Tukey's post hoc multiple comparison test was used to identify the statistically significant differences between the groups. Statistics were used to express the data as the mean ± SEM, and a value of P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of vancomycin on novel object recognition. The analysis of the effect of vancomycin on the novel objective recognition test revealed that both groups spent approximately the same amount of time investigating the two familiar objects in the first trial (training phase). There was no statistically significant difference between the groups. Object recognition memory was tested by swapping out the object of the first trial for copies of the original (familiar object) and unfamiliar objects in the second trial (test phase). Performance for examining the novel object reflected memory for novel object identification. The vancomycin-treated group exhibited a substantial decrease in the new objective recognition rate compared to the control group (P<0.05; Table I).

Effects of vancomycin on grooming and rearing. No significant differences were observed among the groups in the grooming number (data not shown); however, the rearing number was significantly lower in the vancomycin-treated group (Fig. 1A). Rearing is a vertical activity; however, unlike locomotion, it is carried out while the animal stands on its rear legs and rests against the cage walls. The animal performs the movement in an aim to become familiar with the environment and, generally, to look for a food source. The velocity of movement in the center and the periphery of the box (Fig. 1D) was assessed by the corresponding line crossing quantity and was significantly lower in the vancomycin-treated group compared to the similar parameters of the control group (Fig. 1B and C). The vancomycin-treated group did not spend time in the center (Fig. 2).

Qualitative detection of C. difficile. The qualitative detection of *C. difficile* GDH in the animal fecal specimens was made using relevant ELISA tests before and after placebo/vancomycin treatment. The analysis of the obtained data with positive and negative controls (4.29 and 0.081, respectively) revealed positive tests only in the fecal specimens of the vancomycin-treated rats (Fig. 3). Since oral vancomycin is the first-line treatment against the *Clostridium* species, and *C. difficile* is a major source of p-cresol, the p-cresol content was determined in the brains of the control and vancomycin-treated rats. It was found that the content of p-cresol in the vancomycin-treated animals was significantly lower compared to that of the control group rats (Fig. 4). These data indicate that changes in the amount of p-cresol may occur due to the effects of vancomycin on the intestinal microbiome, apparently through a decrease in the *Clostridium* species.

Discussion

The enteropathogen, *C. difficile*, an opportunistic anaerobic bacterium, can be carried by animals and humans and distributed globally (26). *C. difficile* has also been found in rodents,

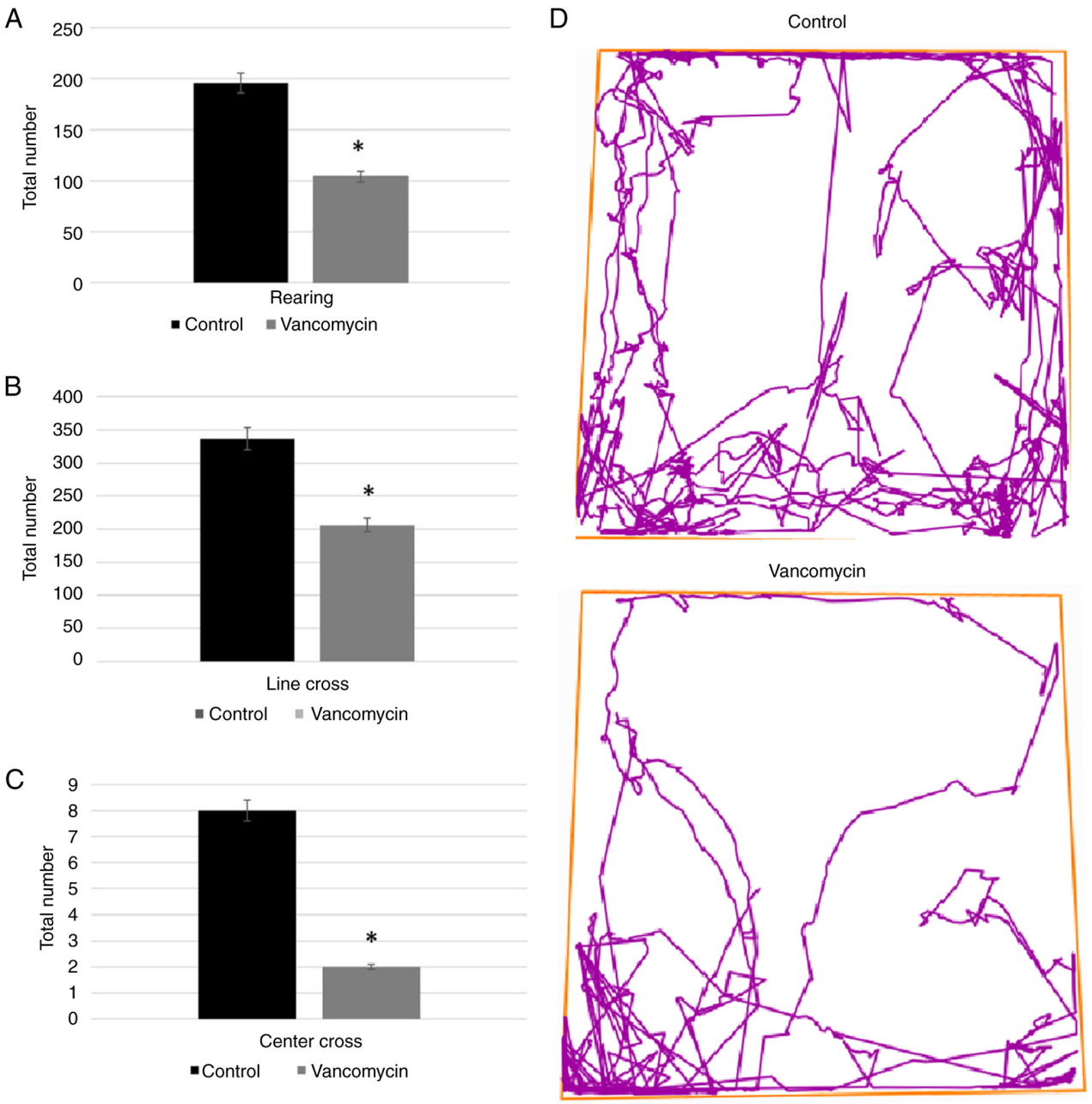

Figure 1. Effects of vancomycin treatment on the open field test parameters. (A) Rearing, (B) line cross, (C) center cross. Values are the mean ± SEM. (D) Representative track plot reports recorded during the 10-min test sessions (using ANY-maze Video Tracking Software). *P<0.05, compared to the control group.

including rats and mice (27). *C. difficile* produces high concentrations of p-cresol through the fermentation of tyrosine, which, apart from perturbations in the beneficial microbiome through gut-brain axes, changes the dopamine metabolism of the brain (4).

A recent study by the authors using rat pheochromocytoma cells (PC-12 cells) demonstrated the effects of p-cresol on the secretion of BDNF and neurofilament subunit expression (17). Low doses of p-cresol enhanced BDNF secretion and nerve growth factor induced the differentiation in PC-12 cells in culture. It was hypothesized that low doses of p-cresol may cause mild oxidative stress that stimulates the release of BDNF by turning on redox-sensitive genes (17). Given that the intestinal microbiome is the main source of p-cresol, the equilibrium between gut microbiome strains, particularly *Clostridium* species, and various endogenous neuroactive substances, such as opioids, may directly affect neuroplasticity.

Vancomycin is increasingly used as the first-line drug in the treatment of *Clostridium* infections (28). Notably, the selected dose of vancomycin and the route of administration (oral) used in the present study significantly decrease its bactericidal effects on other target bacteria (e.g., *Staphylococcus aureus*). It was recently shown that oral vancomycin administration selectively affected indole and p-cresol-producing gut bacterial taxa from *Clostridium* species (29). The specificity of vancomycin against *Clostridium* lies in its mechanism of action. Vancomycin is a glycopeptide antibiotic that inhibits cell wall synthesis in susceptible bacteria. This antibiotic binds to D-alanyl D-alanine

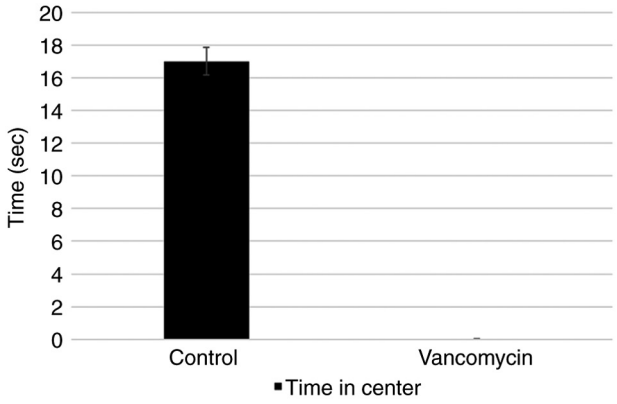

Figure 2. The mean value of time spent in the center for the control and vancomycin-treated groups of rats. Values are the mean ± SEM. P<0.05, compared to the control group.

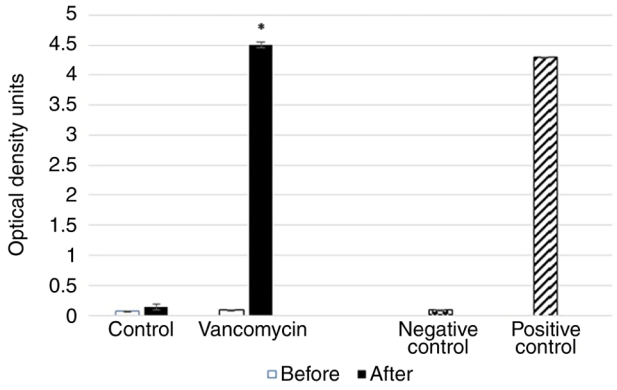

Figure 3. Qualitative glutamate dehydrogenase-based detection of *Clostridium difficile* in animal fecal specimens. The results are expressed in optical density units as the mean ± SEM (n=3). Statistical analysis was performed using one-way ANOVA. *P<0.05, compared to the control group.

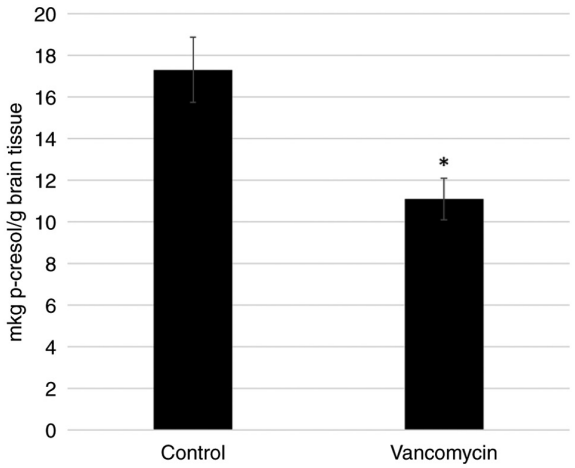

Figure 4. Effects of vancomycin administration on the content of p-cresol in the rat brains. The results are expressed in mkg p-cresol/g brain tissue as the mean ± SEM (n=4). Statistical analysis was performed using one-way ANOVA. *P<0.05, compared to the control group. p-cresol, para-cresol.

and inhibits glucosyltransferase and the P-phospholipid carrier, thereby preventing the synthesis and polymerization of N-acetylmuramic acid and N-acetylglucosamine within the peptidoglycan layer of bacteria (30). In the case of *C. difficile*, the binding of vancomycin disrupts the cell wall synthesis process, weakening or killing the bacteria. One of the reasons vancomycin is effective against *C. difficile* infections is that this bacterium is susceptible to the antibiotic due to the unique composition of its cell wall. Unlike certain other Gram-positive bacteria (e.g., *Bacillus, Campylobacter, Lactobacillus*), the cell walls of *C. difficile* contain a unique peptidoglycan structure that allows vancomycin to bind effectively and disrupt cell wall synthesis (31,32). This inhibition weakens bacterial cell walls and ultimately causes the leakage of intracellular components, resulting in cell death. Thus, lysed cell fragments, including GDH, can be liberated into the stool. The present study revealed an increase in the release of such fragments under the effects of vancomycin.

The present study is the continuation of previous research (7). The results of the present study regarding the novel object recognition test revealed that vancomycin administration in the rats significantly decreased the new object recognition processes compared to the control rats. Additionally, the results from the open field test revealed that the vancomycin-treated rats traveled a substantially shorter distance in the periphery of the box than the control group. Furthermore, compared to the control group, this one's movement in the box's middle was noticeably farther. Moreover, the number of rearings in the vancomycin-treated group was significantly lower than that in the control group rats. These data suggest that vancomycin decreases rats' behavioral projection of cognitive ability. This may indicate a potential link between *C. difficile* colonies in the gut and the cognitive functions of the brain. Furthermore, the administration of vancomycin in rats, in parallel with disruptions in behavioral responses, decreases the content of p-cresol in the brain. It is thus suggested that this interaction is carried out through the dopaminergic activity of the brain.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GT proposed the main hypothesis for the study, designed the main experiments and participated in the writing of the manuscript. NK carried out the main experiments with animal behavior. EZ designed the main stages of the research protocol, and organized the study and participated in data analysis. NN carried out the animal euthanasia, decapitation and brain homogenization. TB performed the biochemical analysis

of p-cresol, and participated in behavioral recording and analysis. LS performed the statistical analysis of the obtained data and prepared the figures and graphs for the study. DM performed the interpretation of the obtained data, prepared the introduction of obtained data, and prepared the introduction and discussion sections of the manuscript. EZ and TB confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Animal care during experimental procedures was carried out by the recommendation of the Ilia State University Research Projects Ethics Commission (Decision no. R/266-23) and by Council of Europe Directive 2010/63/EU for animal experiments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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