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The effects of repeated antibiotic administration to juvenile BALB/c mice on the microbiota status and animal behavior at the adult age

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Abstract

Recent studies carried on germ −free (GF) animal models suggest that the gut microbiota (GM) may play a role in the regulation of anxiety, mood, and cognitive abilities such as memory and learning processes. Consistently, any treatment disturbing the gut microbiota, including the overuse of antibiotics, may influence the brain functions and impact behavior. In the present study, to address this issue, two wide-spectrum antibiotics (ampicillin and cefoperazone, 1 g/l) were repeatedly applied throughout a 6-week period to initially 21-day-old male BALB/c mice. Antibiotics were administered separately or in a mixed fashion. On the completion of the antibiotic treatment, all mice were subjected to the behavioral tests. The serum levels of corticosterone and brain-derived neurotropic factor (BDNF) were assessed. Gut microbiota profiles were obtained by using denaturing gradient gel electrophoresis system, DGGE, from fecal samples. Ampicillin had a greater impact on both, gut microbiota composition and mice behavior compared to cefoperazone. All antibiotic-treated groups

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manifested a decrease in the locomotor activity and reduced recognition memory. However, the ampicillin-treated groups showed a higher anxiety level as assessed by the open field and the elevated plus maze tests and an increased immobility (behavioral despair) in the forced swim test. Obtained results evidently show that in mice, a repeated antibiotic treatment applied during adolescence, parallel to the changes in GM, affects locomotor activity, affective behavior and cognitive skills in young adults with ampicillin specifically enhancing anxiety- and depressive-like responses. Lower levels of serum BDNF were not associated with cognitive impairment but with changes in affective-like behaviors. Repeated administration of neither ampicillin nor cefoperazone affected basal serum corticosterone levels. This is one of the few studies demonstrating changes in a behavioral phenotype of young-adult subjects who were previously exposed to a repeated antibiotic treatment.

Keywords: Neuroscience, Microbiology

1. Introduction

Although microbiology and neuroscience have developed historically as separate fields, current research pays more and more attention to the role of intestinal microbes in the gut-brain communication. The relationship between brain and gut seems to be bidirectional. Parallel to the well-known 'up to bottom' pathway signaling to the gut emotional states such as anxiety or stress, which may disturb gut functions causing discomfort [1], there seems to be also a 'bottom up' influence of gut microbiota on the brain functions [2, 3, 4, 5, 6, 7]. Up-to-date animal studies seem to prove a relationship between gut microbiota, brain functions and behavior. It has been demonstrated that introduction of pathogen bacteria into intestinal tract may cause changes in the anxiety-like behavior [8, 9] and produce abnormal feeding patterns in infected mice [10]. Hyperreactivity to stress followed by stress-induced memory impairment was also reported after enteric infections in animal models [11, 12]. These behavioral changes were usually observed already at the early stage of infection and could be long-lasting or even permanent. On the other hand, research conducted on germ-free (GF) mice showed that the absence of gut microbiota from birth also affects animals' emotional status and may have impact on memory and social behavior [13, 14, 15, 16]. The differences in the brain chemistry and animal behavior reported between GF and specific pathogen-free (SPF) mice with commensal GM suggests that perturbation in GM may affect brain functions through the impact it has on the turnover and release of neurotransmitters, hormones, and growth factors [17, 18, 19, 20, 21]. In the human contemporary life, apart from diet, one of the most important factors that influence microbial flora of the gut is oral administration of antibiotics which spectrum and use are year by year increasing [22, 23].

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Overuse of antibiotics poses a threat to everyone but especially to infants more susceptible to bacterial infections and more sensitive to the adverse environmental factors. Therefore, in the recent years, studies on the potential harmful effects antibiotics may have on the living organisms gained special clinical importance. At the same time, antibiotic-treatment became another commonly used research method in the studies on gut-brain axis using animal models. Some of the recent mice studies showed that high doses of antibiotics induced changes in gut microbiota associated with hormonal alterations such as changes in BDNF, oxytocin and vasopressin expression, decrease in the adult hippocampal neurogenesis, and changes in anxiety-like responses, exploratory behavior, and cognitive skills [17, 24, 25]. These results show that despite astonishing life-saving properties of antibiotics to combat infectious diseases, their overuse can produce serious side effects. However, the interplay between the long-term exposure to antibiotics and brain disorders has not yet been clearly elucidated in both animal and human studies. Recent studies have focused more on the detrimental effects of antibiotic exposure on various health outcomes in adult subjects and there are still few studies examining the cumulative effect of repeated early-life antibiotic administration on the brain status and behavior at the adult age [24,26]. The aim of the present study was to directly address this issue using a mouse model. Particularly, we tried to determine the correlation between GM perturbed by a repeated antibiotic treatment in juvenile mice and the locomotor activity, affective behaviors and cognitive skills assessed in young-adults. To have an insight into the molecular correlates of these behaviors, we additionally measured the serum levels of BDNF and corticosterone. BDNF is known to be important for both cognitive and affective functions. On the other hand, it is well documented that circulating levels of corticosterone are related to stress and stress-induced behaviors [27, 28].

2. Materials and methods

2.1. Subjects

Experiments were carried out on 21-day old BALB/c mice. This mice strain is a convenient model in antibiotic studies because significant shifts in bacteria composition may occur without symptoms of gut inflammation [17]. To exclude sex as an additional independent variable, only male pups were used in this study. To eliminate litter effect, pups coming from 10 different litters were intermingled between groups. Mice were housed in transparent Plexiglas cages, 5 animals per cage. Throughout the experiments, animals were maintained at a constant temperature (21 °C), under a 12/12 hour light/dark cycle, with free access to water and food (standard commercial mice chow, *Korkutelim* TR). At PD 21, before the antibiotic treatment started, all mice were weighted. In the course of experiments, animals' body weight and their food consumption were weekly recorded.

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2.2. Antibiotic treatment

Prior to the experiments, the animals were randomly assigned to a control (Contr.) and three experimental groups receiving antibiotic treatment (n = 10 each). Antibiotic treatment started on PD 21 (after weaning), lasted for a week and was repeated 3 times one week apart. The timeline of experiments is presented in Fig. 1. The antibiotic treatment consisted of ampicillin (Amp group), cefoperazone (Cef group), and cefoperazone plus ampicillin (CefAmp group). In the CefAmp group, 5 mice (one cage) first received cefoperazone, then ampicillin, and finally again cefoperazone (CefAmpCef subgroup), while the other 5 mice (another cage) received antibiotics in the reversed sequence: first ampcillin, then cefoperazone, and again ampicillin (AmpCefAmp subgroup). To avoid a confounding effect resulting from the stress induced by oral gavage, antibiotics were administered in the drinking water with free access. The weekly dose of antibiotics was administrated in a volume of 400 ml. The concentration of antibiotics in the drinking water was 1 mg/ml. Water and thus antibiotic intake was monitored at the end of each week with antibiotic treatment. Average daily water consumption per mouse was 7 ml which corresponds to 7 mg of antibiotic received per day by a single animal. Dosing of antibiotics was based on previous studies where similar antibiotics were administered to mice in the drinking water [24, 29].

2.3. Behavioral tests

Behavioral tests were applied upon the completion of antibiotic treatment, at 2 months of age. All animals participated in all the tests which were run on the separate days according to the schedule presented in Fig. 1.

2.3.1. Open field (OF) test

The open field test is commonly used to measure general locomotor activity and anxiety in rodents [30]. The test was carried out in a square box with diameters of 60 × 60 cm × 50 cm, made of plain wood painted black and illuminated by a bright light from the ceiling. The animal was placed in the middle of one of the side walls, facing the wall. Its locomotor activity was recorded by the computerized video

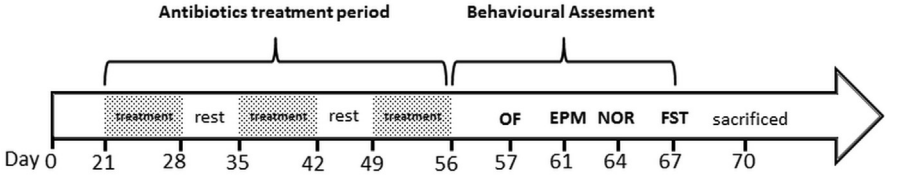

Fig. 1. Experimental timeline of antibiotic administration and behavioral assessment in the open field test (OF), elevated plus maze test (EPM), novel object recognition test (NOR), and the forced swim test (FST).

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tracking system (*EthoVision 3.1 System by Noldus Information Technology*, NL). The open field was divided by virtual lines into 16 equal squares, 12 of which constituted the peripheral zone, and remaining 4, the central zone of the arena. The system recorded time spent and distance moved (ambulation) in each of the zones for 10 min in 5 min intervals [31, 32].

2.3.2. Elevated plus maze (EPM) test

The elevated plus maze is a widely used behavioral test validated to assess the anxiety levels in small rodents [33]. It was constructed of painted black plain wood positioned 80 cm above the room floor and consisted of a central platform (10 × 10 cm), and four crossed arms (50 × 10 cm, each): two open and two closed with darkened Plexiglas walls extending 30 cm above the maze floor. On a single testing session, each animal was placed in the center of the maze facing an open arm and permitted to explore the maze for 5 min. During this time, the number of entries with all four paws to the closed and open arms, the total time spent in the closed and open arms, separately, and the time spent on the central platform were recorded by the computerized video tracking system (*EthoVision 3.1 System by Noldus Information Technology*, NL).

2.3.3. Forced swim test (FST)

FST is considered a valid test for assessing the susceptibility to behavioral despair and depression [34]. Each mouse was challenged by being placed in a cylindrical tank (30 cm height ×20 cm diameter), filled up to 19 cm with a tap water at 24 ± 1 °C temperature. The frequency and duration of periods of immobility were recorded by the experimenter equipped with a video camera. The mouse was considered immobile when it became static in the water, except those motions which were vital to hold its head above the water surface. In the FST, mice were subjected to 6 min swimming session with the last four minutes considered in the data analysis [35].

2.3.4. Novel object recognition (NOR) test

NOR test is commonly applied to evaluate attention, memory retention, and preference for novelty in laboratory animals [36, 37]. Apparatus used for NOR test was an open box made of cardboard, 45 × 30 × 30 cm. A digital video recording device was used to record the animal behavior. Initially, a habituation trial was carried out with the animal freely moving in the box for 10 min. During this time animals' locomotor activity was recorded. Two hours after the habituation trial, two identical objects were placed in two corners of the box (approximately 5 cm from the walls) and mice were allowed to explore the objects for 10 min. All the mice were confirmed to explore each object for at least 10 s. A 10 min testing trial was performed 4 h later.

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For the testing trial, one of the previously used objects was replaced with a novel one. The duration of exploration of each object was scored by trained observers blind to the experimental treatments. Exploration was accepted as valid when the nose of the mice was oriented towards the object at a distance of no more than 2.5 cm or it was touching the object. Objects were made of hard plastics differing in their shape, color and painting patterns. They were mounted to a heavy, flat stone to prevent their displacement by the mice. The ratio b/a+b where "b" is the total exploration time of the novel object and "a" the total exploration time of the familiar object was evaluated as NOR score.

All of the animal experimental procedures were approved by the animal ethics committees of Ankara University and the Middle-East Technical University (METU).

2.4. Biochemical tests

Upon the completion of behavioral tests the animals were sacrificed, their blood samples were collected and stored at −80 °C for the biochemical assays. BDNF, and corticosterone concentrations were measured from mice blood serum using Enzyme-Linked Immune Sorbent Assay (ELISA) (*ELISA Kits*, *Mybiosource*, US).

2.5. Gut microbiota analysis

For the microflora tracking, at the beginning and at the end of antibiotic treatment, fecal samples were collected from each mouse at an amount of at least four pellets, afterwards they were deep frozen in the liquid nitrogen and stored at −80 °C.

2.5.1. Extraction of DNA and amplification of the DNA target sequence

Total bacterial DNA was extracted from 180−220 mg fecal material using *QIAamp DNA Stool Mini Kit* (*Qiagen*, US) according to manufacturer's instructions. DNA concentrations were determined by BioDrop (*BioDrop*, UK). The 200 bp of the hypervariable V2-V3 regions of the bacterial 16S ribosomal RNA (rRNA) gene were amplified using polymerase chain reaction (PCR) with universal bacterial primers HDA1-GC (5′-**CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG**ACTCCTACGGGAGGCAGCAGT-3′; the GC clamp in boldface) and HDA2 (5′-GTATT CCGCGGCTGCTGGCAC-3′). PCR was performed in 0.2-ml tubes using a high-performance PCR amplification system (*Bio-Rad*, US). For the amplification of the target DNA sequence, the reaction mixture (20 μl) Phusion High-Fidelity PCR Kit (*Thermo*, US) was used. PCR reactions were performed under the following PCR conditions: one cycle of initial denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 15 s and final extension at 72 °C for 10 min [38].

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2.5.2. Denaturing gradient gel electrophoresis (DGGE)

The principle of DGGE is based on running the small quantities of DNA in a gel-based electrophoretic system accommodated with denaturing chemical. During the expansion over the gel, the denaturing chemicals cause melting of DNA along the concentration gradient of the denaturing agent. This leads to separation of DNA based on single-base differences. Profiling of PCR-derived amplicons targeting the 16S rRNA gene (V2 and V3 regions) by DGGE is an adequate and competent approach in order to distinguish the variations between prevalent groups (>1% of total GM) in the GM of animal [39, 40]. DGGE was performed with a DCode universal mutation detection system (*Bio-Rad*, US) utilizing 16-cm by 16-cm by 1-mm gels. Eight percent polyacrylamide gels were prepared and run with 1X TAE buffer diluted from 50X TAE buffer (2 M Tris base, 1 M glacial acetic acid, and 50 mM EDTA). The denaturing gradient was formed with two 8% acrylamide (acrylamide-bis, 37.5:1) stock solutions (*Bio-Rad*, US). The gels contained a 30−50% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7.0 M urea. The electrophoresis was conducted with a constant voltage of 130 V at 60 °C for about 4 h 30 min. The run was stopped when a xylene cyanol dye marker reached the bottom of the gel. Gels were stained with ethidium bromide solution (5 mg/ml; 20 min), washed with deionized water, and viewed by UV transillumination.

2.6. Statistics

Data are presented as means ± standard error of the mean (SEM). Statistical analyses of the weekly water and food consumption per cage were done using a nonparametric Kruskal-Wallis test while the mice' body weight was evaluated by two-way (treatment × time) repeated measure ANOVA. The analyses of behavioral data and BDNF and corticosterone blood levels were done applying Student's t-test, and Mann-Whitney U test. The degree of significance was accepted as less than or equal to 0.05. Statistical analyses were run using SPSS and Prism 6 software (GraphPad Inc.).

3. Results

3.1. Food intake, water intake, and the mouse body weight

Food and water consumption were recorded weekly throughout the whole experiment for each cage separately, with two cages per group and 5 mice per cage. A nonparametric Kruskal-Wallis test used to evaluate these data did not reveal significant between-group differences neither in food nor in water intake.

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The animals' body weight was also recorded weekly for each subject, independently. A two-way (treatment × time) repeated measure ANOVA applied to these data revealed the main effect of time and the time × treatment interaction significant ($F(6:216) = 249{,}021$, $P \leq 0.001$ and $F(18:216) = 2{,}201$, $P \leq 0.004$, respectively), however, the main effect of treatment was yielded insignificant. These results show that, in the course of experiments, the intake of food, intake of antibiotic-containing drinking water, and the mice weight gain were similar in all 4 groups. The mean body weights increased steadily to end at 32 g ± 3 g, 31 ± 3.5 g, 33 ± 4 g, and 32 ± 3 g for Contr., Amp, Cef, and Amp/Cef group, respectively.

3.2. Behavioral tests

Behavioral tests were run on the separate days after the completion of antibiotic treatment in a sequence presented in the Fig. 1.

3.2.1. OF test

Fig. 2 shows locomotor activity of the mice in the peripheral (A and C), and central zone (B and D) of the OF during two consecutive 5-min intervals. To assess the differences between treatment and control groups for each zone and time interval, independently, Student's t-test was applied. In the peripheral zone, during the 1st 5-min interval, Student's t-test confirmed significantly lower locomotor activity in the Cef, Amp, and AmpCefAmp groups compared to control ($p = 0.0263$, $p = 0.0349$, $p = 0.0023$, respectively) and a significantly lower locomotor activity in AmpCefAmp group compared to CefAmpCef group ($p = 0.0186$). As seen from the Fig. 2C, during the 2nd 5-min interval, an overall decrease in the mice locomotor activity was noted in the peripheral zone of the OF and only the difference between AmpCefAmp and control was yielded significant ($p = 0.0198$). In contrast to this, animals' locomotor activity in the central zone during the 1st 5-min interval in the OF was very low with no between-group differences (Fig. 2B). During the 2nd 5-min interval, exploration of the central area apparently increased in the control group, and it appeared to be significantly higher compared to Amp and AmpCefAmp groups ($p = 0.0282$ and $p = 0.0143$, respectively). The distance moved by AmpCefAmp mice in the central zone during the 2nd 5-min interval was also significantly shorter compared to Cef group ($p = 0.0283$) (Fig. 2D).

3.2.2. EPM test

Statistical analysis of the EPM data with the Student's t-test revealed that the Amp group spent significantly more time in the maze closed arms compared to control and the Cef group ($p = 0.0099$ and $p = 0.0173$, respectively) and significantly less time in the maze open arms compared to the Cef group ($p = 0.0371$) with

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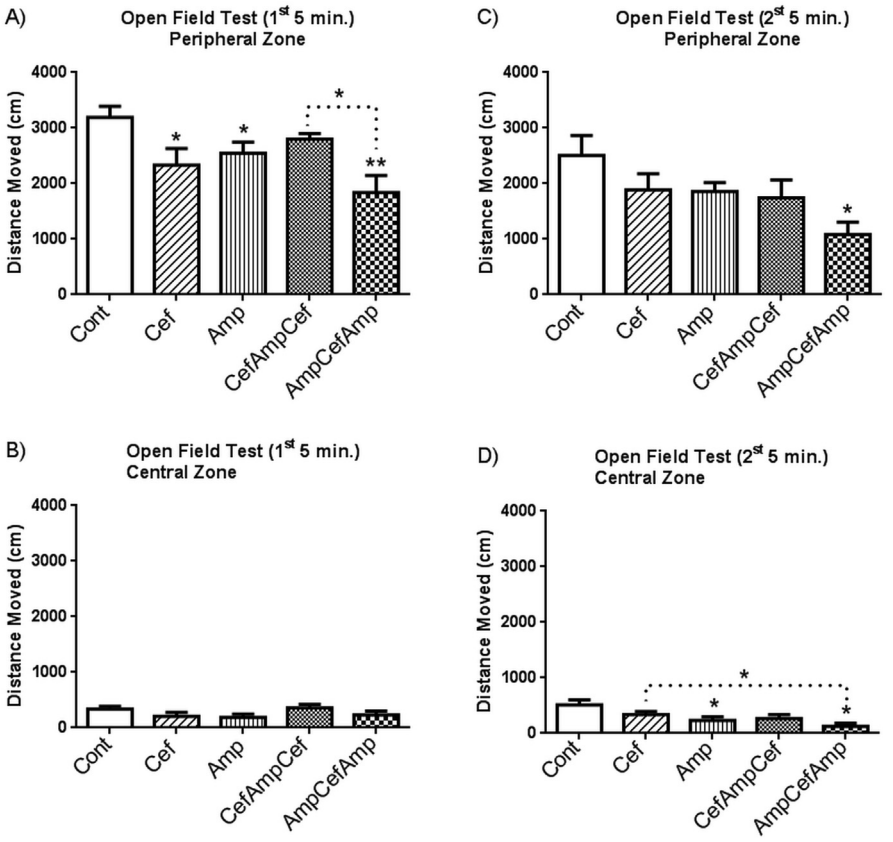

Fig. 2. The mean distance moved in the peripheral zone (A & C) and the central zone (B & D) of the Open Field during the 1st (upper row) and the 2nd (bottom row) 5-min intervals in control and antibiotic groups. Error bars denote SEM and asterisks the level of significance: *$p \leq 0.05$; **$p \leq 0.01$.

no significant difference between the control and the Cef group (Fig. 3). Additionally, Amp group spent significantly less time in the central arena of the maze compared to control ($p = 0.0089$). AmpCefAmp group also spent less time in the open arms and more time in the closed arms of the EPM compared to both control and the Cef group but these differences did not reach the required $p \leq 0.05$ significance level.

3.2.3. FST

Fig. 4 presents mean total immobility time for control and treatment groups. Student's t-test applied to these data confirmed significantly longer total time of immobility (passive floating) in the Amp and AmpCefAmp groups compared to control ($p = 0.0477$ and $p = 0.0181$, respectively). Amp group also showed significantly longer floating time compared to Cef group, as assessed by the Mann-Whitney U test ($p = 0.0368$) with no difference between control and Cef group.

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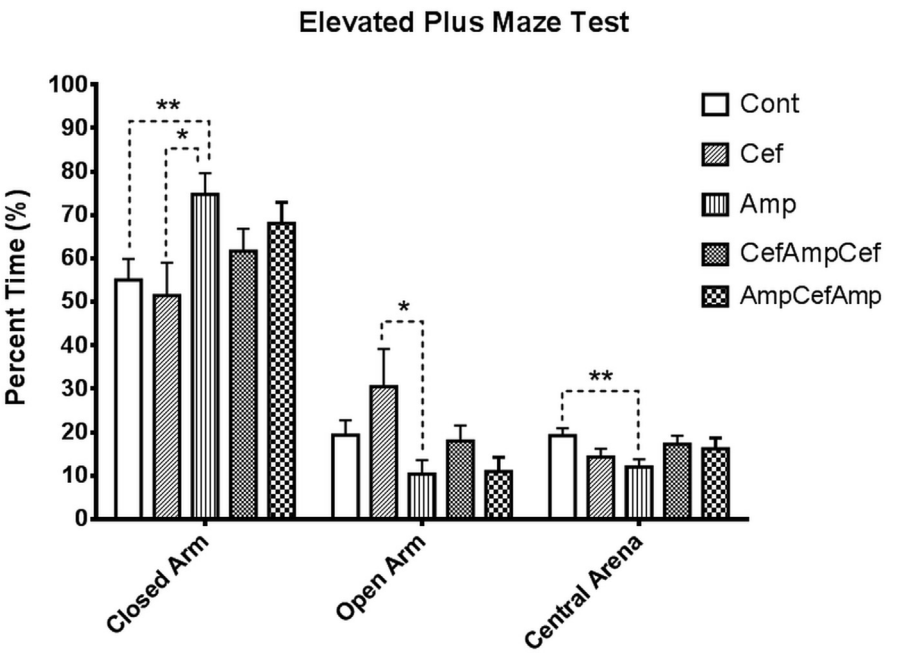

Fig. 3. The mean percent time spent in closed arms, open arms, and the central zone of the Elevated Plus Maze in control and antibiotic groups. Error bars denote SEM and asterisks the level of significance: *p $\leq$ 0.05; **p $\leq$ 0.01.

3.2.4. NOR

Fig. 5 presents mean ratio of the exploration time of a novel object to the total time spent on the exploration of both novel and familiar objects on the test trial, referred to as a NOR score. Score 0.5 means that there was no difference in the exploration time of novel and familiar object. The score higher than 0.5 means that an animal spent more time on the exploration of a novel object, while the score smaller than 0.5 means that an animal spent more time exploring a familiar object. The NOR score was higher than 0.5 only in the control group what means that the control animals spent more time exploring novel object. The between-group comparisons done by the Student's t-test showed significantly lower learning scores in Cef and Amp groups compared to control ($p = 0.0319$, $p = 0.0367$, respectively) with no significant difference between the antibiotic groups.

3.3. Biochemical tests

Fig. 6 presents the mean serum levels of BDNF and corticosterone (A and B, respectively) calculated for each group independently. A nonparametric Man-Whitney U test applied to the BDNF data rendered serum BDNF concentration in the Cef group significantly higher compared to the Amp group ($p = 0.0229$, respectively) with no difference compared to the control. Statistical analysis applied to the blood serum corticosterone data did not revealed any significant between-group differences.

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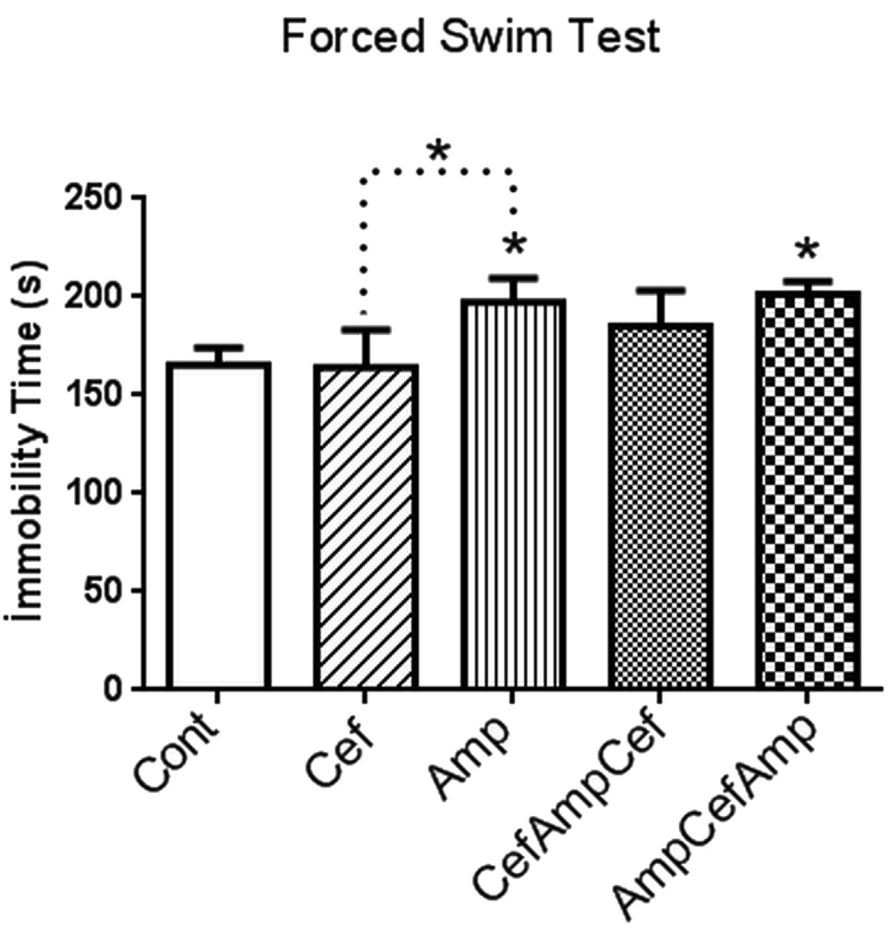

Fig. 4. The mean immobility time in the Forced Swim Test in control and antibiotic groups. Error bars denote SEM and asterisks the level of significance: *p $\leq$ 0.05; **p $\leq$ 0.01.

3.4. Gut microbiota analysis

The gut microbiota profile of control and antibiotic-treated mice groups obtained by DGGE of 16s RNA encoding genes is presented in the Fig. 7.

As seen from the DGGE bands, microbial community profiles found in control mice were altered in the antibiotic-treated groups: in Amp group, 18 bands out of total 33 control bands were missing and 3 new bands were added, while in Cef group, 10 bands were missing with 8 new bands added. Examination of DGGE profile in AmpCefAmp group showed 20 bands missing and 3 bands added. In contrast, in CefAmpCef group only 13 bands were missing and 4 new bands were recorded.

4. Discussion

This study provided one more evidence that repeated administration of antibiotics, here cefoperazone and ampicillin, to mice during adolescence has a serious impact

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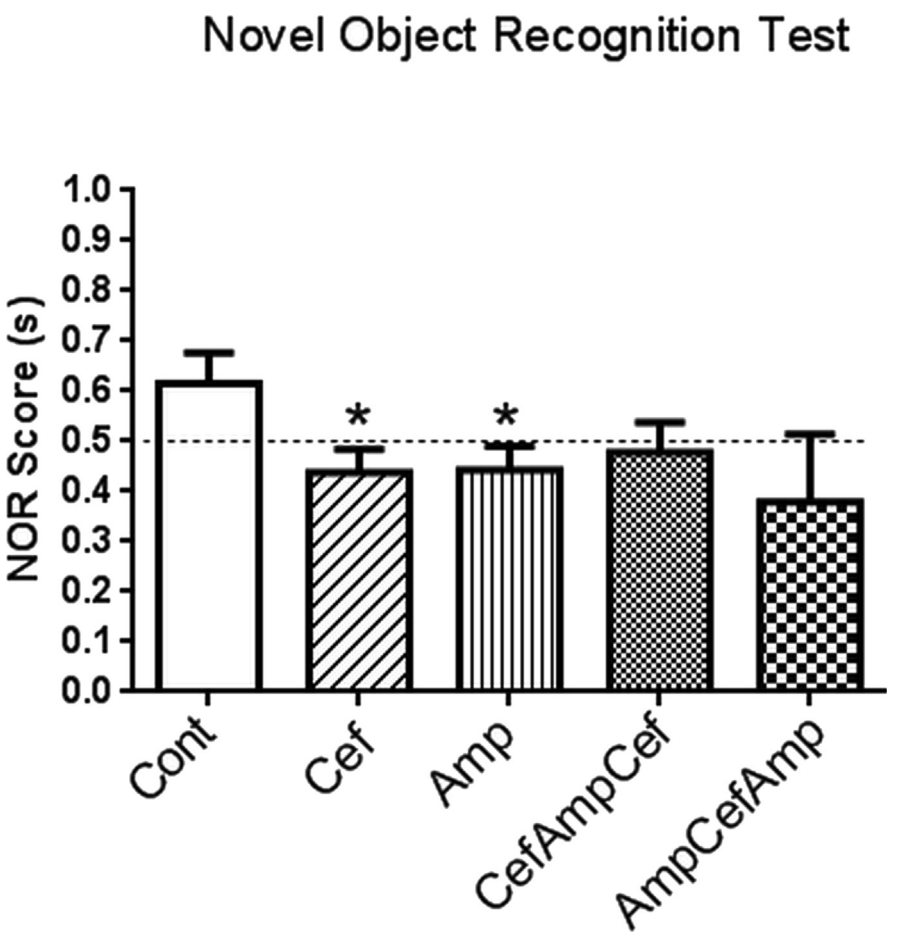

Fig. 5. Mean learning scores in the Novel Object Recognition test for control and antibiotic groups. Error bars denote SEM and asterisks the level of significance: *p $\leq$ 0.05; **p $\leq$ 0.01.

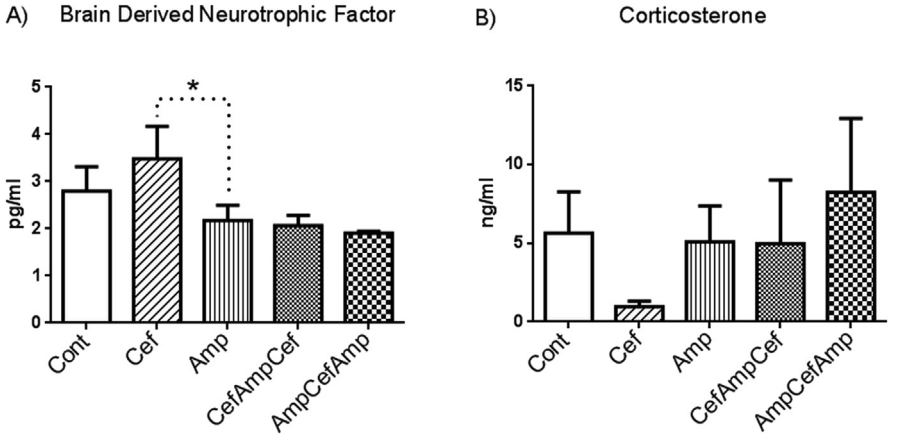

Fig. 6. Serum BDNF (A) and Corticosterone levels (B) in control and antibiotic-treated groups of mice. Error bars denote SEM, and asterisks the level of significance: *p $\leq$ 0.05; **p $\leq$ 0.01.

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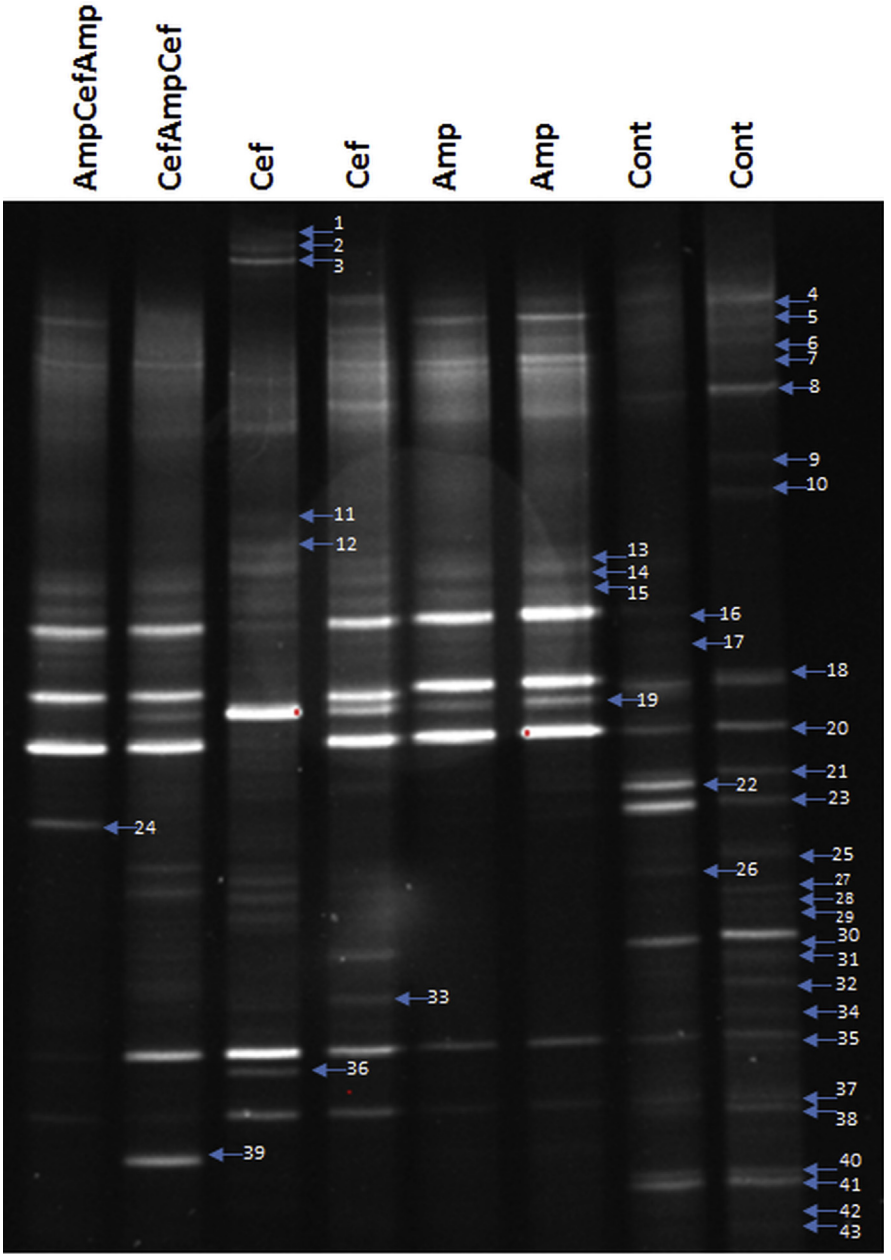

Fig. 7. Representative DGGE gel of fecal microbiota from control and antibiotic-treated mice.

on gut microbiota and animals' behavior after antibiotic cession. In the present study, each antibiotic treatment applied to BALB/c mice pups perturbed the gut microbiota profile differently with all four treatments causing loss of many commensal microbiota components and addition of few new components not recorded in the control group. The between-group comparison of DGGE bands indicates towards greater reduction in the gut commensal microbiota in mice exposed to ampicillin (about 57%) compared to animals exposed to cefoperazone (about 35%). The

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aberrant status of gut microbiota was accompanied by behavioral changes. At the behavioral level, impact of ampicillin was also apparently greater. Both antibiotics decreased animals' general locomotor activity in the OF and impaired mice performance in the NOR task. However, a significant increase in the anxiety-like behavior, as assessed by the distance moved in the central zone of the OF and the time spent in open arms of the plus maze, was noted only in the Amp group. In the Amp group, it was accompanied by a significantly greater degree of behavioral despair manifested in the FST.

In contrast to our results, the absence of the gut microbiota in GF mice models, was previously frequently reported to have an anxiolytic effect, as assessed by the elevated plus maze or light-dark preference test [13, 15, 19]. There are, however, few studies reporting in GF F344 rats but also in GF mice enhanced anxiety-like behavior when exposed to the open field, social interactions, and marble-burring test [41, 42]. These discrepant results paved the way for the hypothesis that gut microbiota is balancing behavioral stress responses decreasing anxiety-like behavior in anxiety-prone strains such as BALB/c mice and F344 rats, and increasing this behavior in anxiety-resistant strains such as Swiss Webster NMRI mice [41, 43]. Indeed, microbiota transfer experiments showed that colonization of BALB/c GF mice with microbiota from Swiss mice decreased, while colonization of Swiss GF mice with microbiota from BALB/c mice increased anxiety in the recipient animals [17]. Further experiments showed that inocculation of anxiety-prone animals with a single bacterial strain such as *Lactobacillus helveticus* r0052 or *Bifidobacterium longum* r0175, may be sufficient to reduce anxiety-like behaviors [44, 45]. Our study shows that, antibiotic-induced perturbation in the composition of commensal GM in anxiety-prone BALB/c mice may increase the anxiety-like behavior and that this behavioral change is antibiotic-specific occurring after ampicillin but not cefaperazone treatment. These results are consistent with the hypothesis that commensal GM may temper the innate neuroendocrine and behavioral responses to stress and thus, in some cases, antibiotic-induced disbiosis may exacerbate anxiety-like behaviors.

Previous studies manifested a significant correlation between the level of anxiety and the degree of behavioral despair [46]. In line with this notion, in the present study, an increased degree of behavioral despair in the FST, and thus inclination towards depression-like symptoms, was observed only in groups with greater exposure to ampicillin and manifesting significantly higher anxiety (Amp and AmpCefAmp groups). Similar result was obtained by Hoban and colleagues [47] who also reported enhanced depressive-like behavior in the FST in young, male Sprague Dawley rats with antibiotic-depleted gut microbiota [47]. On the other hand, probiotic bacteria have been reported to heal this condition. Bravo and colleagues [48] found that mice inoculated with *Lactobacillus. rhamnosus* (JB-1) shows decreased depression-like behaviors in the FST test. Similarly, administration of probiotic

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bacteria- *Bifidobacteria .breve* to BALB/c mice was reported to induce antidepressant-like behavior in the tail suspension test [49].

Parallel to animal studies, in the recent years, there is growing evidence from clinical trials that there is a link between gut microbiota and depression in human [50]. A differential effect of ampicillin and cefaperazone on anxiety- and depressive-like behaviors is probably related to the differences in the antibiotics' spectrum and their different impact on GM. Further studies on specific changes in the microbiota composition after ampicillin and cefoperazone administration may shed light on this question. In the present work, however, Amp group manifesting increased anxiety-like behavior did not show higher basal level of corticosterone in blood. This result is consistent with findings by some other authors who also reported lack of the difference in the basal corticosterone levels between gut microbiota-depleted GF mice and SPF mice with normal commensal microbiota after the maternal separation stress [51]. It was also reported that consumption of probiotic bacteria (*Bifidobacterium longum* (B.) 1714, *B. breve* 1205) by innately anxious BALB/c mice decreased anxiety- and depressive-like behaviors not affecting the basal corticosterone levels [49]. In other studies by Desbonnet and colleagues [24], after chronic antibiotic treatment in mice, an acute restraint stress induced an increase in the serum corticosterone concentration, however, here too, no difference was found in the baseline corticosterone levels between antibiotic-treated and non-treated mice [24]. These and similar results suggest that lack of gut commensal microbiota or its perturbations do not significantly change the basal activity of HPA axis although response to a stress is often exaggerated under such conditions.

The effects of antibiotics-induced dysbiosis on anxiety- and depressive-like behaviors on the one hand, and locomotor activity on the other hand, are independent of each other and are probably due to different perturbations in the commensal microbiota. In the present study, a significant decrease in the mouse locomotor activity was noted in both Amp and Cef groups while an increase in the anxiety-like behaviors was observed only in groups exposed to ampicillin. Previous studies by other authors also demonstrated changes in both, anxiety-like behavior and locomotor activity in BALB/c GF mice [42] and F344 GF rats [41] compared to commensal fecal microbiota-associated animals. Interestingly, in GF mice, association with *Brautia coccoides* diminished anxiety without affecting locomotor activity while association with *Bifidobacterium infantis* decreased locomotor activity not affecting anxiety levels.

In our study done on BALB/c mice characterized by high emotional activity but low locomotor activity, antibiotic-induced perturbation of GM enhanced innate behavioral traits by increasing emotional responses to novel and stressful conditions and decreasing general locomotor activity. These results are in contradiction to the reports that GF mice entirely deprived gut microbiota demonstrated higher motor

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activity and lesser anxiety with respect to the specific pathogen free (SPF) mice with a normal gut microbiota [42]. These discrepant results draw attention to multiple factors such as animals' strain and sex that, in addition to gut microbiota status, shape animals' behavior [13, 49]. In addition, antibiotic-treatment is not just depleting but it is restructuring gut microbiota, therefore it is not plausible to expect similar effects under these two different conditions.

In contrast to a differential effect of ampicillin and cefoperazone on the affective behavior, both antibiotics showed a detrimental effect on the animals' cognitive performance manifested by a shorter exploration time of a novel object in the NOR test. Similarly to our results, Fröhlich and colleagues [52] reported an impaired performance in the NOR, but not spacial, memory task in adult male C57BL/6N mice subjected to a 5-antibiotic cocktail consisting of ampicillin, bacitracin, meropenem, neomycin, and vancomycin. Cognitive deficits were also found by Desbonnet and colleagues [24] in mice treated with a combination of antibiotics during adolescence. In the NOR test, animal's response to novelty is evaluated basing on the exploratory activity and one may assume that animals with lower general locomotor activity would manifest reduced exploratory activity, just as it was in the present study. It was reported, however, that perturbation of the microbiota in adult BALB/c mice by oral administration of neomycin, bacitracin and antifungal agent pimaricin led to an increase in the exploratory activity with no change in the overall locomotor activity [17]. The latter data suggest a lack of strong correlation between the exploratory and general locomotor activity.

Parallel to the behavioral tests, serum levels of BDNF were evaluated upon cession of antibiotic treatment. The main source of BDNF is the brain tissue, it can cross the blood-brain barrier [53] and a positive correlation between peripheral and central BDNF levels was reported in rodents [54]. Therefore, it is postulated that the serum levels of BDNF may be indicative of its concentration in the central nerves system [55]. In the brain, BDNF, through its impact on neurons' differentiation and survival is important for the development and maintenance of the central nervous system in good conditions. For the last two decades, however, more attention has been paid to the role of BDNF in synaptogenesis and synaptic plasticity, and thus its role in learning and memory [56]. A positive correlation has been found between animals' performance in the NOR test and the BDNF expression in perirhinal cortex and DNA methylation in the hippocampus [57]. On the other hand, serum BDNF levels have been shown to be decreased in depressive and manic episodes associated with cognitive impairment in bipolar patients [58]. In the present study, however, cognitive impairment was registered in all antibiotic groups while serum level of BDNF was significantly lower in Amp group only, as compared do Cef group with no difference between Cef and the control group. This result indicates that serum BDNF in mice with antibiotics−perturbed microbiota did not reflect animals' cognitive status

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but is more associated with anxiety- and depressive-like behaviors. These results are in line with the neurotrophin hypothesis of depression [59, 60].

In summary, in the present study, repeated administration of two common antibiotics, ampicillin and cefoperazone, to juvenile mice produced antibiotic-specific changes in their GM with ampicillin apparently resulting in greater perturbations in the GM profiles. These GM perturbations were associated with behavioral changes which were recorded in young adults upon antibiotic cession and also were antibiotic specific. An overall decrease in general locomotor activity and poorer performance in the cognitive task were registered in all antibiotic groups. Ampicillin treatment additionally significantly enhanced anxiety- and depressive-like behaviors. Lower levels of serum BDNF were associated with changes in affective behaviors but not cognitive impairment. Repeated administration of neither ampicillin nor cefoperazone affected basal serum corticosterone. This work is one of very few studies demonstrating changes in a behavioral phenotype of adult subjects after being exposed to a repeated antibiotic treatment during adolescence. Evidently, different antibiotics through their impact on the GM differently affect animal behavior. Although this and similar studies may have therapeutic implications, at this stage, it is difficult to speculate on the signaling pathways mediating observed behavioral aberrations.

Declarations

Author contribution statement

Taha Ceylani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ewa Jakubowska-Doğru: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Rafig Gurbanov: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Hikmet Taner Teker: Performed the experiments.

Ayse Gul Gozen: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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