Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jc6w5w64

# THE IMPACT OF ANTIBIOTICS ON INTESTINAL MICROBIOTA AND COGNITIVE BEHAVIOUR IN RATS

**Noorulain Hyder[1,2*], Ghulam Abbas[3], Farzana Sadaf [1], Ale Zehra[4]**

[1*]Department of Pharmacology, Faculty of Pharmacy, Hamdard University, Karachi, Pakistan
[2]University of Karachi, HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, Karachi, Pakistan
[3]Ziauddin University, Faculty of Pharmacy, Department of Pharmacology, Karachi, Pakistan
[4]Department of Pharmacy Practice, Dow College of Pharmacy, Dow University of Health Sciences

***Corresponding Author:** Noorulain Hyder
*Email: Dr.noorulain@hamdard.edu.pk

**Abstract:**
**Introduction:** The research investigates the alterations and restoration of the gastrointestinal microbiota in sprague dawley rats after broad-spectrum antibiotic administration and its impact on cognitive behaviours.
**Methods:** Rats were randomly assigned to the antibiotic (AT) group and the control group. AT group received an antibiotic regimen of ampicillin, vancomycin, metronidazole, and imipenem for three days, while the control group was given an equivalent volume of normal saline by gavage. Faecal samples were obtained on the fourth day (24 hours after last antibiotic treatment) and at two months for 16S rRNA gene sequencing to assess alterations in gut microbiota. At the two-month mark, cognitive behaviours were evaluated using the operant-based delayed matching to position (DMTP) task and the Y maze.
**Results:** On day 4, the gut microbiota in the AT group exhibited a decline, characterised by a reduction in microbiota diversity ($P<0.0001$), a decrease in Bacteroidetes ($P<0.0001$) and Firmicutes ($P=0.0001$), alongside an increase in Proteobacteria ($P<0.0001$).Following the cessation of prescription antibiotics, the microbiota started to regenerate, showing no significant differences compared to the control group after two months.There was no notable distinction in cognitive behaviours observed between the AT group and the control group.
**Conclusion:** Broad-spectrum antibiotics have the potential to reduce gut microbiota in rats. This reduction is not permanent.The microbiota normalises two months following the cessation of antibiotic treatment.Furthermore, it does not influence the cognitive behaviours of rats.

**Key Word**s: gastrointestinal microbiota, cognition, antibiotic therapy.

## Introduction:

Gut microbiota, also known as gut flora, is comprised of 100 trillion bacteria, which is ten times the total number of human cells. Gut microbiota serves as a source of nutrition for the human digestive tract. There is a possibility that antibiotics, which are one of the primary strategies for intervening in the microbiota of the stomach, might lead to dysbiosis and influence cognitive behaviour. A mixture of medicines, including ampicillin, bacitracin, meropenem, neomycin, and vancomycin, has been shown to have the potential to damage mice's ability to recognise novel objects, according to research

[2]. In mice, the administration of ampicillin results in deficiencies in both their spatial memory and their ability to recognise new objects [3]. However, these studies did not describe whether or not the microbiota in the stomach recovered following antibiotic therapy, nor did they investigate the effects of antibiotics on cognitive behaviour over the long term. Using a combination of four broad-spectrum antibiotics, this research evaluated the alterations, recovery, and influence on cognitive behaviour of the gut microbiota in rats. The study was conducted using rats. In rats, the microbiota in the gut may be reduced by the use of broad-spectrum antibiotics. However, this decline is only going to be slight.After two months have passed after the cessation of antibiotic treatment, the microbiota returns to its usual state.The cognitive behaviours of rats are not impacted in any way by this substance.

## Material and Methods

### 1.1 Experimental Animals and Grouping

Three-month-old SPF-grade male SD rats (n=12), weighing 280-300 g, were purchased from the Animal housing facility located at Hamdard University Biotechnology department. [License No. SCXK 2014-0010]. All rats were housed at the Experimental Animal Center of Hamdard University, with 2 rats per cage, an environmental temperature of 20-22°C, humidity of 50%, and a 12-hour light/dark cycle. Food and water are sterilized under high pressure, and all animals have free access to food and water. The experimental rats were randomly divided into two groups, with 6 rats in each group. One group was the antibiotic treatment (AT) group, which was given an antibiotic mixture by gavage for 3 days. Antibiotics The mixed formulation consists of: Ampicillin [180 mg / (kg), Zafa Pharmaceutical Co., Ltd.], Vancomycin [72 mg / (kg), Bosch Pharmaceutical Co., Ltd.], Metronidazole [90 mg / (kg), Sami Pharmaceutical Group Holdings Co., Ltd.], and Imipenem [90 mg / (kg), Merck Sharp & Dohme (MSD) Pharmaceuticals Ltd.], administered twice daily (with a 12-hour interval), 2 ml per dose, for 3 days, followed by routine feeding for 2 months. Another group served as the control group, receiving an equivalent amount of saline via gavage for 3 days, followed by standard feeding for 2 months. On the 4th day (24 hours after the last antibiotic treatment), collect feces for gut microbiota analysis. Conduct tests on gut microbiota and cognitive behavior at 2 months.

### 1.2 Fecal Sample Collection

Fecal samples were collected on Day 4 (24 hours after the last antibiotic treatment) and at 2 months for gut microbiota analysis. Before collecting feces, treat the UV disinfection fume hood for 30 minutes. During collection, wear sterile gloves, collect 0.5 to 10 grams of rat feces in a fume hood, place it in a sterile cryovial, immediately put it on ice and label it, quickly place it in liquid nitrogen, and after collection, immediately store it in a -80°C freezer.

### 1.3 16S rRNA Gene Sequencing

16S rRNA gene sequencing using fecal DNA extraction kits (batch number 154041641, Qiagen, Germany) was used to extract total microbial DNA from each sample. Shanghai Paisei Nuo Biotechnology Co., Ltd. was commissioned to perform high-throughput sequencing on the Illumina MiSeq high-throughput sequencing platform. Using QIIME2 software and VSEARCH software for sequence denoising and operational taxonomic unit (OTU) clustering. Use the R package (v3.1.1) to calculate the number of OTUs and the Shannon diversity index. Using the R package to perform nonmetric multidimensional scaling (NMDS) analysis based on weighted UniFrac distances, showing the differences in OTU composition between samples. Using the RDP Classifier software v. 2.2, classify the OTU representative sequences at the species level with a confidence threshold of 80%. Conduct statistical analysis using R (v3.1.1) software [4].

### 1.4 Delayed Matching to Position (DMT) Task:

Based on Operant Conditioning Reflex Detection in the Operant Conditioning Reflex Testing Room (MED-007-CT, Med Associates, USA, Assessing Rats' Working Memory through the Operant Conditioning Reflex-Based DMT Five days before the training begins, restrict the rats' diet to maintain their body weight at 80% to 85% of their original body weight, but do not restrict their

water intake until the behavioral experiment is completed.

**Training phase procedure:** ① First, conduct the food trough training, each experiment lasts 64 minutes, including 38 food pellets, with an interval of (100 ± 40) seconds; ② Lever press training, each time the left or right lever is randomly extended, the mouse receives a food reward after pressing the lever, each experiment lasts 30 minutes, including 50 lever presses.

**Delayed matching task**: It includes three stages, namely the sample stage, the delay stage, and the choice stage. Sample phase: The room light turns on, the experiment begins, a rod extends (randomly left or right, this rod is the "sample" rod), the mouse presses the rod, the rod retracts, and the delay timer starts. Delay phase: The rat must touch the food trough with its nose. After the delay, the first nose touch will cause both levers to extend simultaneously, entering the choice phase. Choice phase: ① the rat presses the correct lever (the "sample" lever in the sample phase is the correct lever), both levers retract, a food pellet is rewarded, and after a 5 second interval, the next test is conducted; ② the rat presses the incorrect lever, both levers retract, the house light goes out for 5 seconds, which is the "timeout" period. When starting the training, set the delay to 0 seconds. If the accuracy rate of two consecutive experiments exceeds 80%, enter the delay. Delay 1, set the delays to 0s, 1s, 2s, 3s, 4s, 5s, 6s, 7 delays make up a group (block), each delay in a group appears randomly and each delay appears only once, after 7 delays, move to the next group. If the accuracy rate of two consecutive experiments reaches above 80%, proceed to delay 2. Delay 2 is set to 0s, 1s, 2s, 4s, 8s, 12s, 16s. If the accuracy of two consecutive experiments reaches above 80%, start the test experiments. Set the delays to 0s, 2s, 4s, 8s, 12s, 18s, and 24s, conduct five consecutive tests, and record the accuracy for each delay. Conduct test experiments 2 months after antibiotic treatment.

**1.5 Y Maze Detection:** Y Maze has a total of 3 arms, each with an angle of 120°. Each arm measures 60 cm × 10 cm × 35 cm (length × width × height). During the experiment, different geometric shapes were Stick different geometric shapes on each arm as visual markers. Y maze test for assessing spatial memory in rats. The experiment is divided into two stages. The first phase is the training period, using a partition to block one arm. The arm is the novel arm. The rat is placed in the starting arm facing away from the center of the maze, and allowed to freely explore the starting arm and other arms for 10 minutes, then removed. After a 1-hour interval, the second phase begins with the test period. The novel arm is opened, and the rat is placed in the starting arm again, exploring the three arms for 5 minutes. Record the number of times the rat enters each arm and the duration spent in each arm. Each time the experiment is conducted, the novel arm and the starting arm are randomly assigned, and the number of animals in each group with the novel arm on the left and right sides of the starting arm is equal. Conducted 2 months after antibiotic treatment.

**1.6 Statistical Processing**: Data analysis was conducted using SPSS 13.0 software, and the results are expressed as x±s. DMTP comparison used repeated measures two-way ANOVA, while the others used independent samples t-test. $P < 0.05$ indicates statistical significance.

## 2. Results

### 2.1 Antibiotic treatment reduces the number of intestinal floras in rats

The combined treatment with four antibiotics: ampicillin, vancomycin, metronidazole, and imipenem for 3 days can reduce the number of intestinal florae in rats. Compared to the Control group, the number of OTUs in the gut microbiota of rats in the AT group was significantly reduced (see Figure 1A). From the Rarefaction curve, it can be seen that as the sequencing amount continuously increases, the Rarefaction curve flattens. When the sequence depth is sufficient, it can reflect the diversity contained in the current sample (see Figure 1B). Control group rats The Shannon index was (7.93 ± 0.41), and the Shannon index in the AT group rats (3.64 ± 0.57) decreased, with a statistically significant difference ($t = 13.18$, $P < 0.0001$). The NMDS results based on UniFrac distance indicate that the gut microbiota of the AT group rats (AT-1) is different from that of the Control group

(Control-1) (see Figure 1C). At the door level, a total of 15 doors were detected. Among them, the sum of Bacteroidetes and Firmicutes accounts for more than 0.90, making them the main dominant phyla in rat feces. Compared to the Control group, the AT group showed a significant decrease in Bacteroidetes (t = 9.891, $P < 0.0001$, see Figure 1D); a significant decrease in Firmicutes (t = 7.397, P = 0.0001, see Figure 2D); and a significant increase in Proteobacteria (t = 18.37, $P < 0.0001$, see Figure 1D). At the genus level, the abundance of Prevotella, Blautia, Phascolarctobacterium, Sutterella, Bacteroides, Ruminococcus, and Parabacteroides decreased in the AT group of rats (see Figure 1E, F).

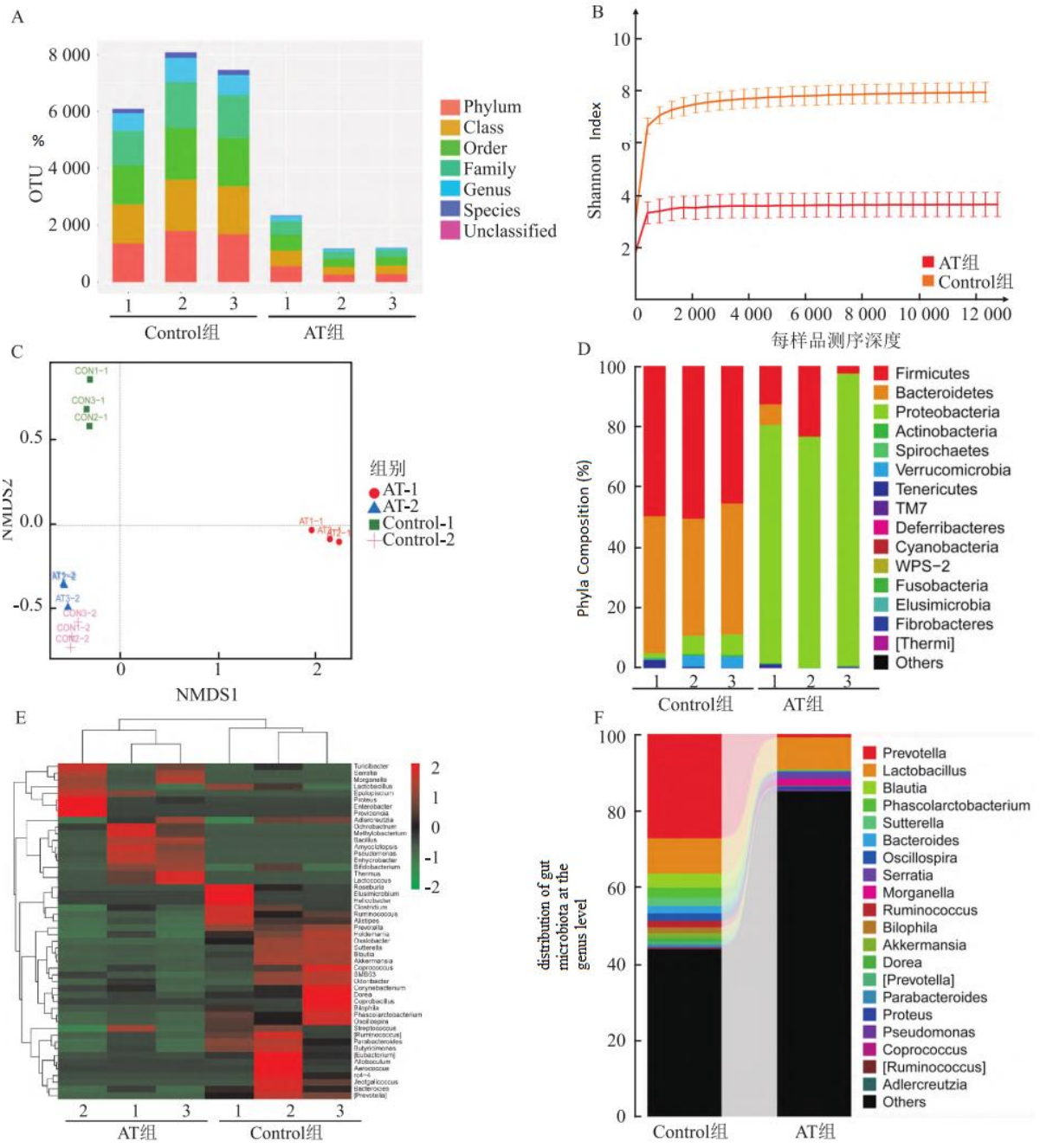


**Figure 1 Differences in gut microbiota between AT and Control groups of rats on Day 4**

**A**: OTU numbers at various taxonomic levels; **B**: Rarefaction curves; C: Sample two-dimensional ordination plot from Weighted UniFrac NMDS analysis, AT-1: Gut microbiota of rats in the AT group on Day 4, Control-1: Gut microbiota of rats in the Control group on Day 4, AT-2: Gut microbiota of rats in the AT group 2 months post-antibiotic treatment, Control-2: Gut microbiota of rats in the Control group 2 months post-antibiotic treatment; D: Composition and abundance

distribution of gut microbiota at the phylum level; E: Heatmap of genus-level community structure combined with clustering analysis; F: Composition and abundance distribution of gut microbiota at the genus level

### 2.2 Recovery of Rat Gut Microbiota Two Months Post-Antibiotic Treatment

Two months after antibiotic treatment, the OTU counts of the gut microbiota in the AT group of rats recovered, showing no statistically significant difference compared to the Control group (see Figure 2A). The diversity of gut microbiota also recovered, with the Shannon index of the Control group being (8.83 ± 0.24) and the Shannon index of the AT group being (9.13 ± 0.34). There was no statistically significant difference between the two groups (see Figure 2B). The NMDS results based on UniFrac distance indicate that the gut microbiota of the AT group (AT-2) can recover two months after antibiotic treatment, showing no statistically significant difference compared to the Control group (Control-2) (see Figure 1C). At the phylum level, after 2 months, the numbers of Bacteroidetes and Firmicutes returned to baseline, with no statistically significant difference compared to the Control group (see Figure 2C). At the genus level, after 2 months, the quantities of Prevotella, Ruminococcus, Bacteroidetes, Sutterella, and Phascolarctobacterium recovered, showing no statistically significant difference compared to the Control group (see Figure 2D).

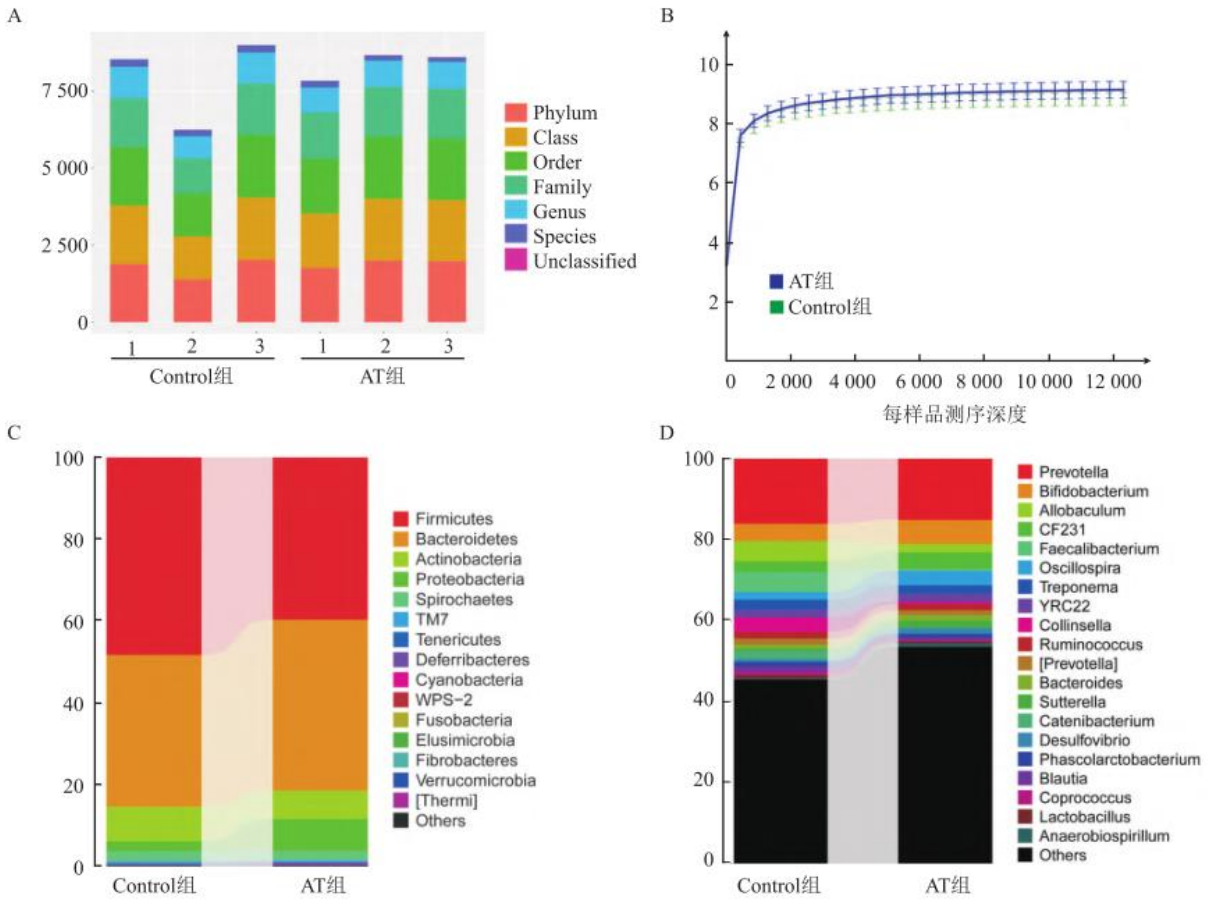


**Figure 2: Gut microbiota of AT group and Control group rats after 2 months of antibiotic treatment A: OTU numbers at various taxonomic levels; B: Rarefaction curve; C: Phylum-level microbiota composition and abundance distribution; D: Genus-level microbiota composition and abundance distribution**

**2.3 The Impact of Antibiotic Treatment on Working Memory**: Cognitive Behavior of Control and AT Group Rats Assessed by DMT-P Based on Operant Conditioning The results showed that with the increase in delay, the correct lever-pressing rate of rats in both the Control group and the AT group decreased. Control group rats. The correct lever pressing rates for the control group rats at 0s,

2s, 4s, 8s, 12s, 18s, and 24s delays were 100.0%, 99.5%, 98.8%, 93.0%, 88.3%, 81.5%, and 80.8%, respectively; the correct lever pressing rates for the AT group rats at 0s, 2s, 4s, 8s, 12s, 18s, and 24s delays were 98.8%, 99.0%, 96.0%, 91.2%, 83.7%, 83.0%, and 77.3%, respectively. At each delay, the difference in the correct lever-press rate between the two groups of rats was not statistically significant ($F$ = 3.029, $P$ = 0.112, see Figure 3).

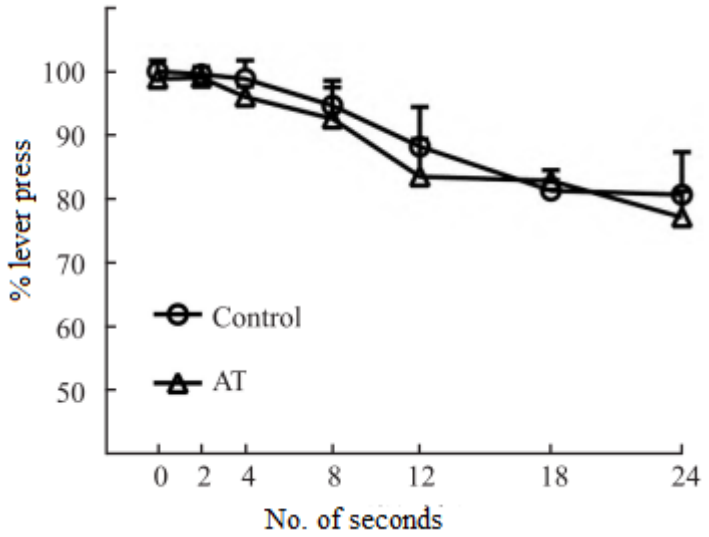


**Figure 3: Detection of working memory in Control and AT group rats using DMTP**

**2.4 The Impact of Antibiotic Treatment on Spatial Memory:** The number of times and the time rats explore the novel arms in the Y-maze reflect their spatial memory function. The Y-maze test results showed that there was no statistically significant difference in the number of times and the duration the two groups of rats explored the novel arms. The ratio of the number of exploratory movements using the novel arm to the total number of exploratory movements in the Control group was (0.43 ± 0.01), and in the AT group, it was (0.46 ± 0.02). There was no statistically significant difference between the two groups (t = 1.131, $P$ = 0.284, see Figure 4A). Similarly, the ratio of time spent by rats in the Control group exploring the novel arm to the total exploration time was (0.54 ± 0.03), and for the AT group, it was (0.56 ± 0.03). The difference between the two groups was not statistically significant (t = 0.381, $P$ = 0.711, see Figure 4B).

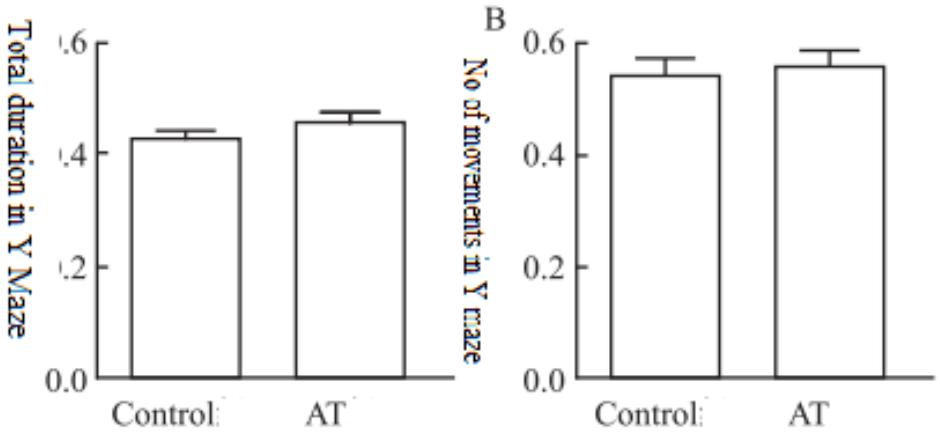


**Figure 4**: **Detection of spatial memory in Control and AT group rats using the Y-maze**
**A: Ratio of the number of entries into the novel arm; B: Ratio of the time spent in the novel arm**

## 2 Discussion

The gut microbiota of healthy adults is primarily composed of two phyla: Bacteroidetes and

Firmicutes, which together account for over 0.90. Other phyla include *Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia* [5]. The gut microbiome is the "second genome" of humans, colonizing the human gut from birth [6]. Microorganisms and hosts engage in mutualistic symbiosis, playing important roles in nutrient metabolism, maintaining intestinal mucosal barrier function, and immune regulation [7-8]. The gut microbiota plays an important role in regulating brain function and behavior, thus the concept of the "microbiome-gut-brain axis" has been proposed [9-10]. Antibiotic treatment affects the quantity and structure of the normal gut microbiota, causing dysbiosis. Research [3] indicates that the number of *Firmicutes* in ampicillin-treated mice decreases, leading to deficits in spatial memory in the Morris water maze and new object recognition memory in the mice. Vancomycin and ampicillin treatment of mice with transient forebrain ischemia increases the abundance of *Proteobacteria*, exacerbating cognitive impairment in the Y-maze, novel object recognition, and Barnes maze tasks. Another study [2] used a combination of five antibiotics—ampicillin, bacitracin, meropenem, neomycin, and vancomycin—to treat mice, resulting in a reduction in gut microbiota and a decrease in bacterial diversity, which impaired the mice's new object recognition memory. These studies suggest that short-term treatment with a single antibiotic or a combination of antibiotics may disrupt the gut microbiota and affect cognitive function. However, it has not been reported whether the gut microbiota will recover after discontinuing antibiotics and whether there will be long-term effects on cognitive behavior. This study applied a combination of four antibiotics: ampicillin, vancomycin, metronidazole, and imipenem, and observed that the antibiotics inhibited the gut microbiota of rats, leading to a reduction in gut microbiota diversity, with a decrease in the numbers of dominant bacteria from the *Bacteroidetes and Firmicutes* phyla. Consistent with previous studies [2], the combination of ampicillin, vancomycin, metronidazole, and imipenem can non-selectively eliminate gut microbiota, leading to a general downregulation of dominant bacteria. Therefore, this study further confirms that short-term antibiotic treatment is an effective model for exploring the causal relationship between the microbiome and its dependent brain function and behavioral changes. In addition to antibiotic treatment, germ-free (GF) animals are also a valuable tool for studying the gut microbiome [12]. Because GF animals do not contain any microbiota, a specific strain can be introduced to observe its effect on host health, or the fecal microbiota of patients (or disease model animals) can be transplanted to observe the impact of gut microbiota on the occurrence and development of diseases. However, GF animals exhibit permanent neurodevelopmental and cognitive behavioral deficits [13], so there are still some areas in the current research on GF animals that need improvement. Compared to GF animal studies, this research confirms that short-term use of broad-spectrum antibiotics does not have long-term effects on cognitive behavior. The results of this study further support that using broad-spectrum antibiotics to inhibit the host's gut microbiota, followed by transplanting fecal microbiota from patients or disease model animals to observe its effects on cognitive function, is a more ideal model.

## References

1. Lu ZH, Liu YW, Ji ZH, et al. Alterations in the intestinal microbiome and mental health status of workers in an underground tunnel environment. BMC Microbiol, 2021, 21(1):7.
2. Fröhlich EE, Farzi A, Mayerhofer R, et al. Cognitive impairment by antibiotic-induced gut dysbiosis: analysis of gut microbiota-brain communication[J]. Brain Behav Immun, 2016, 56:140-55.
3. Sarkar SR, Mazumder PM, Chatterjee K, et al. Saccharomyces boulardii ameliorates gut dysbiosis associated cognitive decline [J]. Physiol Behav, 2021, 236:113411.
4. Li Li, Zhang Xin, Zou Lin. Based on genomic and metabolomic analyses, the correlation between changes in gut microbiota and its metabolites and the occurrence of heart failure is discussed. Journal of Anhui Medical University, 2022, 57(3): 407-12.
5. Zhang B, Wang X, Xia R, et al. Gut microbiota in coronary artery disease: a friend or foe? ［J］. Biosci Rep, 2020, 40(5): BSR20200454.
6. Korpela K, Salonen A, Vepsäläinen O, et al. Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in Caesarean-born infants.

Microbiome, 2018, 6(1): 182.
7. Jandhyala S M, Talukdar R, Subramanyam C, et al. Role of the normal gut microbiota[J]. World J Gastroenterol, 2015, 21(29): 8787-803.
8. Li X, He C, Li N, et al. The interplay between the gut microbiota and NLRP3 activation affects the severity of acute pancreatitis in Acta Universitatis Medicinalis Anhui 2022 Sep; 57(9): 1451 mice[J]. Gut Microbes, 2020, 11(6): 1774-89.
9. De La Fuente-Nunez C, Meneguetti B T, Franco O L, et al. Neuro-microbiology: how microbes influence the brain[J]. ACS Chem Neurosci, 2018, 9(2): 141-150.
10. Powell N, Walker MM, Talley NJ. The mucosal immune system: master regulator of bidirectional gut-brain communications[J]. Nat Rev Gastroenterol Hepatol, 2017, 14(3): 143-159.
11. Lee K E, Kim J K, Kim D H. Orally administered antibiotics vancomycin and ampicillin cause cognitive impairment with gut dysbiosis in mice with transient global forebrain ischemia. Front Microbiol, 2020, 11:564271.
12. Ferguson JF, Aden LA, Barbaro NR, et al. High dietary salt-induced dendritic cell activation underlies microbial dysbiosis-associated hypertension[J]. JCI Insight, 2019, 5(13): e126241.
13. Dinan T G, Cryan J F, Stanton C. Gut microbes and brain development have black box connectivity [J]. Biol Psychiatry, 2018, 83(2): 97-9.