Effects of fecal microbiota transplantation on behavioral abnormality in attention deficit hyperactivity disorder-like model rats

（注意欠如多動症様モデルラットの行動異常に対する腸内細菌叢移植の効果）

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ABSTRACT

Attention deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by inattention, hyperactivity, and impulsivity. ADHD symptoms not only impact patients and their families but also impose societal costs. Current treatments for ADHD, including environmental adjustments and medication, are symptomatic and require long-term management. Recently, the link between gut microbiota dysbiosis and various psychiatric and neurological disorders has become evident. The effectiveness of fecal microbiota transplantation (FMT) from healthy individuals in treating autism spectrum disorder, a neurodevelopmental disorder related to ADHD, has been demonstrated. However, despite suggestions of a relationship between ADHD and gut microbiota, few studies have explored the efficacy of FMT for ADHD. In the current study, we used 16S rDNA analysis to show that ADHD-like model rats possess a gut microbiota that is distinct from that of healthy rats, and we demonstrated that FMT from healthy rats improved hyperactivity in ADHD-like model rats. Our findings suggest that differences in gut microbiota underlie ADHD-like behaviors and that FMT may be an effective treatment for ADHD.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by developmentally excessive and life-disrupting levels of inattention, hyperactivity, and impulsivity. In children, the worldwide average prevalence of ADHD is approximately 5%.[1] For symptomatic adult ADHD, accounting for the global demographic structure in 2020, the estimated prevalence is 6.76%.[2] ADHD imposes costs on both individuals with ADHD and their families, as well as on society, because of the associated higher crime rates, healthcare expenses, and specialist education.[1] Although the heritability of ADHD is estimated to be as high as 60%–80%, there is no consistent evidence for a causative gene.[3] The interaction of genetic risk with the small effects of multiple variants and environmental risk factors, such as perinatal risks (maternal drinking and smoking, stress, low birth weight, prematurity, exposure to toxins, and traumatic brain injury) and psychosocial adversity (parent-child relationships, family adversity, low income, early deprivation) may collectively contribute to the development of ADHD.[3] Because of the complex and unresolved nature of its underlying causes, there is currently no definitive treatment that can cure ADHD. The main treatments for ADHD currently consist of pharmacotherapy and behavioral intervention.

Medication for the treatment of ADHD includes methylphenidate, which directly inhibits dopamine transporters and increases dopamine levels in the brain,[4] and atomoxetine, which indirectly increases dopamine levels.[5] Since 1999, hundreds of rigorous trials have consistently confirmed the efficacy and safety of these drugs when taken at therapeutic doses in children from 4 years old to adults.[6] Nevertheless, debate continues regarding the risks and benefits of long-term use, particularly of the former, because of concerns about the possibility of abuse related to its central stimulant effects.[1] Additionally, behavioral interventions, including applied behavior analysis, social skills training, and parent

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training, have been demonstrated to be effective.[7] However, these interventions must be based on detailed behavioral assessments, and the supporters who conduct the assessments must have skills and expertise, making quality assurance a challenge. For these reasons, behavioral therapy does not always provide stable therapeutic effects for all ADHD patients.[8]

Gut bacteria and the brain communicate bidirectionally via chemical transmitters, neuronal pathways, and the immune system (gut-brain axis). Thus, microbiota and molecules derived from them influence host behavior and nervous system function.[9] Differences in gut microbiota composition between healthy individuals and patients have been reported in various psychiatric disorders.[10] Moreover, fecal microbiota transplantation (FMT) from healthy donors has ameliorated depression, anxiety-like symptoms, and behavior, while transplantation from patients exacerbates these behaviors.[11] There is an increasing body of evidence linking the pathogenesis of autism spectrum disorder (ASD), which is classified as a neurodevelopmental disorder like ADHD, to the gut-brain axis.[12] Reports of improvement in ASD symptoms through FMT have been shown in both clinical and animal studies.[13,14] Unlike the growing body of research on FMT in individuals with ASD, investigations into FMT in individuals with ADHD remain limited. In individuals diagnosed with ADHD, several meta-analyses have identified alterations in gut microbiota composition between healthy individuals and patients, although findings have been inconsistent.[15,16] Furthermore, a randomized, double-blind, placebo-controlled experiment reported the amelioration of ADHD symptoms through probiotic supplementation.[17] In a study using an ADHD-like rat model, FMT from ADHD medication-treated model rats exhibiting behavioral improvement resulted in an improvement of ADHD symptoms in naive-ADHD-like model rats.[18] These findings suggest that FMT from a healthy group might contribute to the improvement of ADHD symptoms. However, to the best of our knowledge, the only human study reporting improvement of ADHD symptoms after FMT was a case study of a single patient.[19]

Therefore, in the current study, we investigated the effects of FMT on ADHD-like abnormal behavior in Lister hooded (LH) rats. In previous studies, LH rats exhibited hyperactivity, attention deficit, and impulsivity, and ADHD-like behavior was improved by the ADHD drug atomoxetine; thus, LH rats have been proposed as an ADHD model.[20] Additionally, we previously conducted research focusing on axon initial segment using LH rats as an ADHD model to explore the mechanism of ADHD development.[21]

The current research had three main objectives: (1) to analyze the differences in gut microbiota between ADHD-like rats and healthy controls; (2) to examine whether FMT from healthy rats can ameliorate ADHD-like symptoms in the model; (3) to explore alterations in the brain induced by FMT.

2. Materials and methods

2.1. Animals and experimental design

All animal experiments were performed according to the guidelines of the Ehime University Ethics Committee for Animal Experiments (approved reference number: 05U48-2). Male Wistar rats (Clea Japan, Tokyo) and LH rats (Kyudo, Saga) were maintained in the same facility for at least 7 years after introduction, and were subcultured in the same environment for a sufficient period of time, including the same food (MF: Oriental Yeast, Tokyo), drinking water (tap water), bedding (Ecochip CL-4163: Clea Japan, Tokyo), and bedding changed once a week. The rats were kept in a 12-h light/dark cycle (lights on from 7:00 to 19:00) (4 rats per cage) at a constant temperature of 25 ± 1 °C. Food and water were available ad libitum. The number of doses and duration of FMT administration were determined based on the results of human trials and converted to rats. In other words, FMT was administered to 6-week-old rats every 2 days for a total of 3 times at 11:00. On the 8th day, behavioral tests were performed for 9 consecutive days following a previous study.[20] A day after the behavioral test, fecal samples were collected from each rat and immediately stored at −80 °C. The following day, when conducting tissue collection, the rats were euthanized through inhalation of a 100% concentration of carbon dioxide. After confirming respiratory arrest, the brains and colons of the rats were obtained.

Next, the prelimbic cortex (PrL), orbitofrontal cortex (OFC), and nucleus accumbens (NAcc) were dissected from the brain, and the monoamine content in each of these regions in the right brain was measured using high performance liquid chromatography (HPLC), as previously reported.[20]

2.2. Behavioral tests

Behavioral tests were conducted 7–8-week-old rats. To assess ADHD-like behavior, the open field test (OFT), elevated plus maze (EPM), and Morris water maze (MWM) were administered on consecutive days, with one test conducted per day. The OFT and EPM assessed hyperactivity and anxiety, and the MWM examined short-term memory and memory retention. All behavioral assessments were performed in the evening between 19:00 and 22:00. Tracking was carried out using EthoVision XT 14 (Noldus Information Technology, Wageningen, The Netherlands).

2.2.1. Open field test (OFT)

The OFT, which measures behavioral volume, anxiety, and habituation to a novel environment, was administered in a square box (100 cm long × 100 cm wide × 50 cm high on the wall) for 5 min on five consecutive days. The method followed a previous study.[20]

2.2.2. Elevated plus maze (EPM)

The 5-min EPM was conducted to measure anxiety-like behavior, impulsivity, and hyperactivity of rats. The EPM apparatus, made of black acrylic panels, consisted of four arms, each 50 cm long × 10 cm wide. The closed arms had walls of 50 cm high black acrylic plates. A tracking system determined the frequency with which rats entered the open arms, the duration of their stay, and the total distance traveled in the maze. This method was adapted from a previous study.[20]

2.2.3. Morris water maze (MWM)

The MWM test, conducted using a water-filled pool measuring 150 × 45 cm (diameter × height), and was designed to assess short-term spatial memory and cognitive function. Four spatial landmarks were strategically placed along the pool wall. The MWM test spanned 2 days, designating the first day for learning trials, each consisting of four sessions, and the second day for a probe (transfer) trial. On the first day, rats were allowed to swim in the pool for 90 s, after which they were placed on a transparent platform to consolidate their memories for 15 s, by a series of trials from different starting positions along the pool's periphery. On the second day, the platform was removed, and the rats were released for 90 s. This method was based on previous studies and modified accordingly.[20]

2.3. Microbiota methods and analyses

The nucleotide sequences of the 16S rRNA gene were determined. Initially, genomic DNA from fecal samples was extracted using NucleoSpin Tissue (Macherey-Nagel, Germany). The quality of the extracted DNA was assessed using a Qubit Flex Fluorometer (Invitrogen, Carlsbad, CA, USA). Subsequently, the 16S rRNA sequences in the V3–V4 region were amplified using common primers (341F-805R) by 2X KAPA HiFi HS ReadyMix (Kapa Biosystems, Wilmington, USA). After purifying the polymerase chain reaction amplicons and quantifying them using fluorescence with magnetic beads Sera-Mag Select (Cytiva, Tokyo, Japan), sequencing libraries were prepared using the Nextera XT Index Kit v2 Set A (Illumina, San Diego, CA, USA), and sequencing was performed

using the Illumina MiSeq platform. The sequencing was conducted by G CUBE Co., Ltd.

Data processing and analysis used QIIME2 v2023.2[22] and R version 4.3.2 [23]. The QIIME2 DADA2 plugin[24] was used to denoise, merge, and filter for chimeric reads, and q2-feature-table plugin removed samples with less than 1500 reads. Taxonomic assignments for generating amplicon sequence variants (ASVs) were conducted using a feature-classifier plugin[25] using taxonomic classifiers created from the SILVA database (version 138.1 [26]) with the RESCRIPt plugin.[27] Genetic diversity analysis and visualization were performed using MicrobiotaProcess (version 1.14.1).[28] For generating MicrobiotaProcess input data, phylogeny align-to-tree-mafft-fasttree pipeline[29,30] was used, and the data were handled using phyloseq package (version 1.46.0).[31]

2.4. Fecal microbiota transplantation (FMT)

Fresh fecal pellets from the 6-week-old donor rats were immediately frozen on dry ice. The temperature of the frozen fecal pellets was raised stepwise over 24 h for FMT, and the operation started when the temperature reached 4 °C. The stool was immersed in a solvent that was 2.5 times the weight of the stool for approximately 1 h and filtered three times using five layers of four-fold sterile gauze. NanoGAS® water (WIPO: WO/2019/168034), suggested to increase bacterial colonization, was used as the solvent.[32] NanoGAS® water has previously been used as a solvent for FMT in human clinical trials, and its effectiveness has been confirmed.[33] Each recipient rat was injected with a progressively increasing concentration of the bacterial suspension, adjusted to 0.05 g in the first dose, 0.1 g in the second dose, and 0.15 g in the third dose (per 200 g of rat body weight). Injection was performed under anesthesia with isoflurane by inserting a φ1 × φ2 mm silicone tube through the rectum. The rat's body was held head down for 1 min to prevent loss of the injected material.

2.5. Determining monoamine in brain regions related to ADHD

Noradrenaline (NA), dopamine (DA), and serotonin (5-HT) contents of ADHD-related regions (PrL, OFC, NAcc) in the right brain were measured using HPLC.[20] Aliquots were prepared from brain tissue homogenized using an ultrasonic cell disruption machine in 0.1 M perchloric acid containing 5 mM ethylenediamine tetraacetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 3,4-dihydroxybenzamine (Wako), followed by centrifugation. The filtered supernatant was injected into an HPLC system equipped with a reversed-phase column. The mobile phase consisted of 15% (v/v) methanol with 0.1 M sodium acetate (Wako) and 0.1 M citric acid (Wako), adjusted to pH 3.5, along with 180 mg/L sodium octyl sulfate (Wako) and 10 mM ethylenediamine tetraacetic acid, pumped at a flow rate of 0.25 mL/min. The mean value of the Vehicle group served as the reference, and the percentage change for each individual relative to this reference was calculated to perform statistical comparisons between the Vehicle and FMT groups.

2.6. Statistical analyses

The behavioral data are presented as mean ± standard deviation (SD). Behavioral test data analysis was conducted using R version 4.3.0 [23]. Between-group comparisons were executed using unpaired Welch's *t*-test. A significance level of $p < 0.05$ was applied to all tests. Alpha diversity between samples was estimated using the "mp_cal_alpha" function in the MicrobiotaProcess R package.[28] The Wilcoxon rank-sum test was performed using the "wilcox.exact" function in the exactRankTests R package.[34] Beta diversity between samples was estimated using Bray-Curtis distances with the "mp_cal_dist" function from the MicrobiotaProcess R package. PERMANOVA, calculated from the Bray-Curtis distance matrix, was conducted using the "adonis" function in the vegan R package, with 9999 permutations to obtain permutation p-values. For calculating p-values in the cladogram visualization, the Kruskal-Wallis test was performed using the "kruskal.test" function from the coin R package.[35]

3. Results

3.1. Fecal microbiome diversity in ADHD models and Wistar rats

First, we investigated the differences in the gut microbiota between the control group (Wistar rats) and the ADHD-like model (LH rats) at 6 weeks of age, using 16S rDNA analysis (Fig. 1A–C). In the alpha diversity analysis, we found that the Chao1 index, which emphasizes rare species, was significantly reduced in the LH group ($p = 0.0023$, Fig. 1A). In contrast, the Simpson index, which focuses on species evenness, was similar between the two groups ($p = 0.64$, Fig. 1A). Beta diversity analysis revealed significant differences between the two groups. Principal coordinates analysis (PCoA) using Bray-Curtis distances demonstrated significant separation between Wistar and LH rats (Fig. 1B, $p = 1e\text{-}04$). Differential abundance analysis identified bacterial taxa that were more abundant in Wistar rats and those that were more abundant in LH rats (Fig. 1C). At the phylum level, bacterial taxa that were enriched in Wistar rats belonged to Actinobacteriota, Campylobacterota, and Patescibacteria. The phyla Bacteroidota and Verrucomicrobiota were more abundant in LH rats than in Wistar rats. At the class level, bacteria that were more abundant in Wistar rats included Actinobacteria, Campylobacteria, Saccharimonadia, and Alphaproteobacteria, whereas Bacteroidia and Verrucomicrobiae were more prevalent in LH rats. At the order level, Wistar rats exhibited a higher abundance of Bifidobacteriales, Campylobacterales, Erysipelotrichales, Saccharimonadales, and Rhodospirillales, whereas LH rats showed increased levels of Bacteroidales, Monoglobales, and Verrucomicrobiales. At the family level, Wistar rats exhibited increased Bifidobacteriaceae, Atopobiaceae, Barnesiellaceae, Tannerellaceae, Helicobacteraceae, Erysipelotrichaceae, unclassified Oscillospirales, Saccharimonadaceae, and uncultured Rhodospirillales. In contrast, LH rats exhibited a greater abundance of Muribaculaceae, Monoglobaceae, and Akkermansiaceae. At the genus level, Bifidobacterium, Parabacteroides, Desulfovibrio, Turicibacter, and Streptococcus, which are abundant in Wistar rats, have been reported to be associated with mental and neurological disorders. In LH rats, Akkermansia, which is also linked to mental and neurological disorders,[36] was found to be abundant. All genera that showed significant increases in abundance in each group are listed in Table S1.

Next, we compared ADHD-like behavior using the same rats (Fig. 1D–K). In OFT, the total distance traveled tended to be longer in the LH group than in the Wistar group, but the difference was not significant. However, the frequency of entries into the center zone and the time spent there were significantly higher in LH rats. In the EPM, LH rats showed significantly higher activity levels (Fig. 1I). Moreover, the percentage of entries into and time spent in the open arms were significantly increased in LH rats. These findings suggested that LH rats exhibited higher levels of activity and lower levels of anxiety.

Overall, the current findings indicated differences in both behavior and gut microbiota composition and diversity between Wistar and LH rats.

3.2. FMT improved hyperactivity in an ADHD-like rat model, but had no significant effect on indicators of anxiety

It was evident that the composition of the gut microbiota and behavior differed between Wistar and LH rats. However, it remains unclear whether these differences in the gut microbiota contribute to behavioral changes. To address this, we observed the behavior of LH and Wistar rats after FMT derived from Wistar rats (Fig. 2A). Two days after the final FMT, we conducted the OFT for five consecutive days (Fig. 2B–I). The total distance traveled, an index of hyperactivity, was

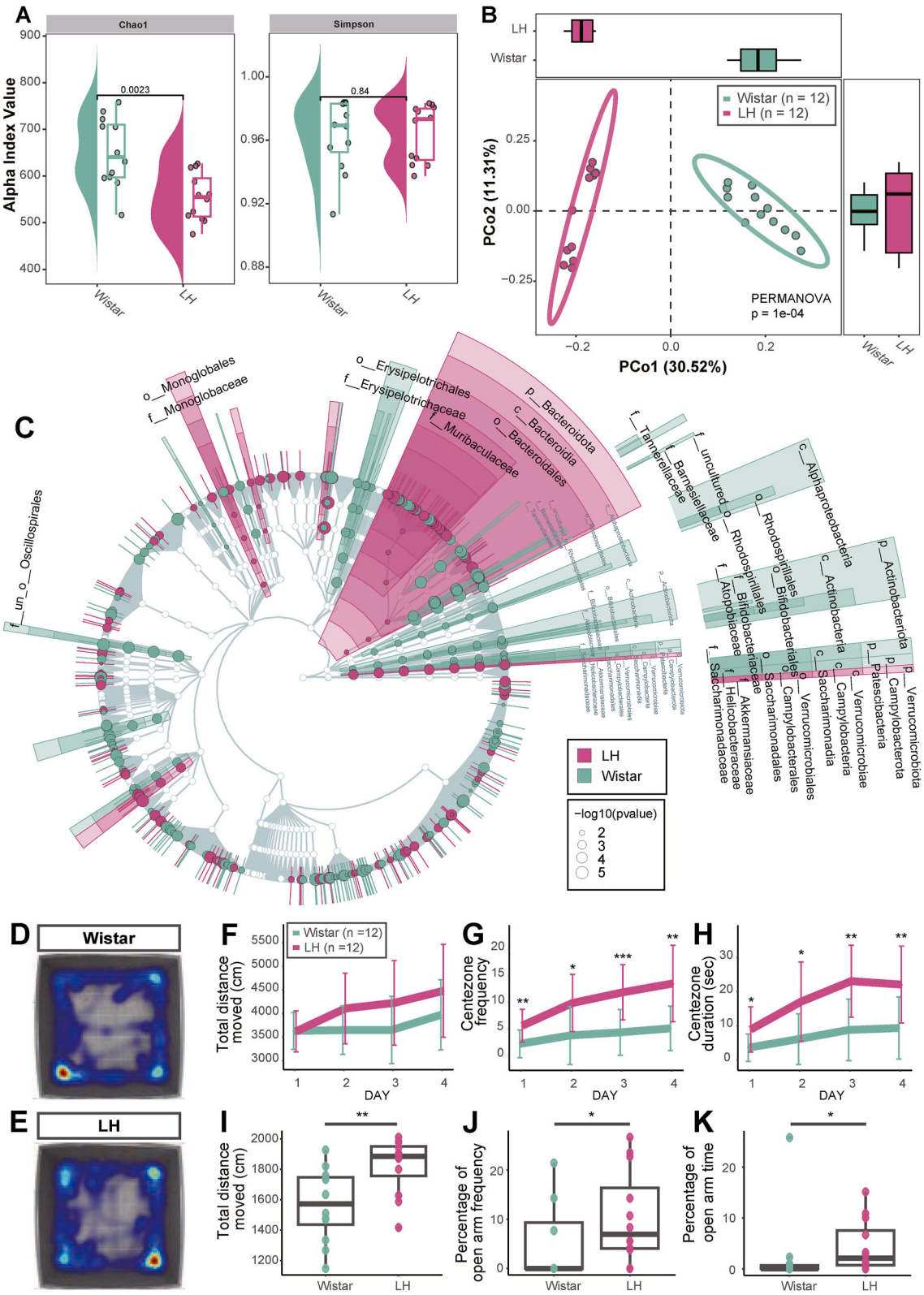

Fig. 1. Gut microbiota composition and behavior were different in ADHD model and Wistar rats. The gut microbiota of the two groups of rats were compared in 12 individuals in each group (6 weeks old at the time of the FMT procedure) using 16S rDNA analysis. Alpha diversity boxplots comparing Wistar and LH groups, with p-values calculated using the Wilcoxon rank-sum test, are presented (A). The PCoA plot produced using Bray-Curtis distances is shown in (B). The cladogram highlights clades representing differentially abundant species enriched in the respective groups (C). In the cladogram, colored circles indicate taxa with significant differences, with circle sizes scaled according to p-values from the Kruskal-Wallis test. Taxa at the family level and above are visualized. Taxa names written in small blue text are enlarged to the right for better visibility. Behavioral tests were conducted in the same rats at 8 weeks old. OFT results: Representative heatmaps for Wistar rats (D) and LH rats (E) are shown. Comparisons between Wistar and LH rats include total distance moved (F), frequency of entries (G), and time spent in the central zone (H). EPM results: Results include total distance moved (I) and the percentages of open arm entry frequency and time spent in the open arm for LH and Wistar rats (J, K). Data are expressed as mean ± SD. Statistical significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, using Welch's *t*-test.

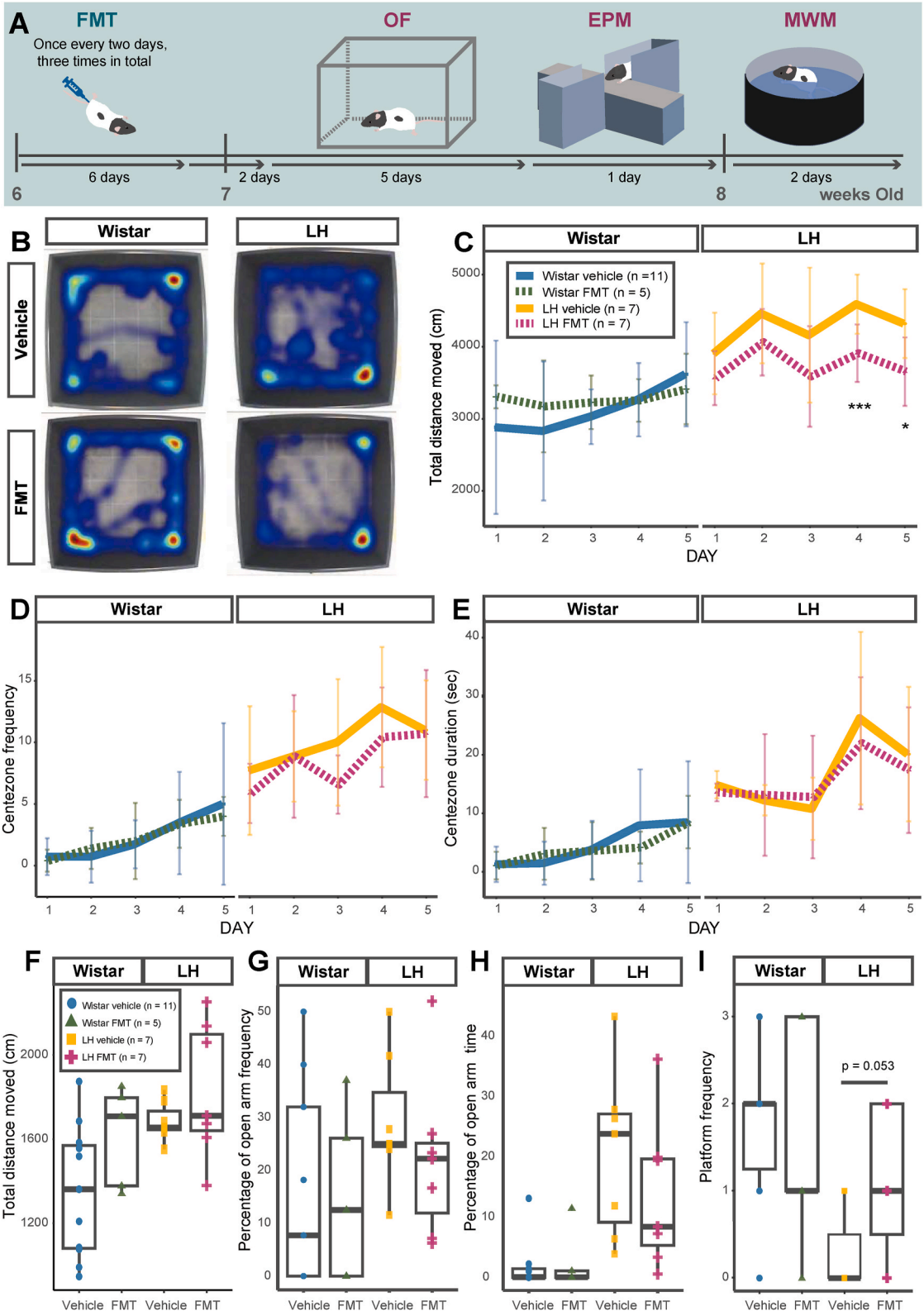

Fig. 2. FMT reduced activity levels in ADHD-like model rats, whereas no significant behavioral changes were observed in wild-type rats following FMT. Wistar and LH rats were each divided into two groups: the Vehicle group (control, receiving rectal administration of solvent three times every other day) and the Feces + Vehicle group (treatment, receiving rectal administration of fecal samples on the same schedule). The test was conducted according to the schedule outlined in Figure A. The results of the OFT are shown: representative heatmaps (B) for Wistar Vehicle (left-upper, n = 11), LH Vehicle (right-upper, n = 7), Wistar FMT (left-lower, n = 5), and LH FMT (left-upper, n = 7). Comparisons between the Vehicle and FMT groups include total distance moved, frequency of entries, and time spent in the central zone (C, D, E). The EPM results indicate total distance moved (F), and percentages of open arm entries and time spent in the open arm (G, H). Additionally, frequency of entries into the learned platform position in the MWM probe test for 90 s, assessing reference memory, is shown (I). Data are expressed as mean ± SD, and statistical significance was determined using Welch's *t*-test: *p < 0.05, **p < 0.01, ***p < 0.001.

significantly higher in the vehicle-treated LH group than in the vehicle-treated Wistar group (Fig. 2B and C). On the other hand, the total distance traveled was significantly decreased in the FMT-LH group compared with the vehicle-LH group from day 4 onwards (Fig. 2B and C). In contrast, no significant changes were observed between the two groups in Wistar rats (Fig. 2B and C). Furthermore, regarding anxiety indicators, there were no significant differences between the FMT and Vehicle groups in either LH or Wistar rats in terms of the number of entries and time spent in the center zone (Fig. 2B–D, E). Similarly, in the EPM, no significant differences were observed between the FMT and Vehicle groups in both Wistar and LH rats for the number of entries and the percentage of time spent in the open arms, both of which are indicators of anxiety (Fig. 2G and H). Additionally, there were no significant differences in the total distance moved in the EPM (Fig. 2F). In the MWM probe test, which assesses memory retention, the number of entries into the platform area was non-significantly higher in the FMT group (Fig. 2I, $p = 0.053$) without changes in activity levels (Fig. S1).

These results suggest that the gut microbiota may have influenced hyperactivity and memory retention in LH rats.

3.3. FMT altered some gut microbiota in LH rats and tended to increase dopamine levels in ADHD-related brain regions

To investigate changes in gut microbiota after FMT, we collected fecal samples the day after the final MWM session. Using 16S rDNA analysis, we compared the gut microbiota of the Vehicle and FMT groups in ADHD-like model (LH) rats (Fig. 3A–C). Alpha diversity analysis showed that the Chao1 and Simpson indices were similar between both groups (Fig. 3A). PCoA using Bray-Curtis distances revealed no significant differences in beta diversity (Fig. 3B, $p = 0.32$). Differential abundance analysis found no significant differences at the phylum, class, or order levels. However, at the family level, Oscillospiraceae was more abundant in the Vehicle group, whereas the FMT group exhibited higher levels of Bacteroidaceae and Erysipelatoclostridiaceae (Fig. 3C). At the genus level, taxa with significantly higher abundance were V9D2013_group ($p = 0.025$) in the Vehicle group, and Bacteroides ($p = 0.040$) and Marvinbryantia ($p = 0.0018$) in the FMT group.

To investigate the effects of FMT on the brain, we measured neurotransmitter levels in ADHD-related brain regions using HPLC on tissue samples collected 2 days after conducting the MWM (Fig. 3D–F). Fig. 3D, E, and F show the p-values for the rate of change of each monoamine in each brain region. When comparing the changes in each brain region of the representative monoamines norepinephrine, dopamine, and 5-HT, only the p-value for dopamine levels in the PrL was $p = 0.057$, and judging from the high p-values ($p = 0.13$–0.99) of the other monoamines and other brain regions, there was a tendency for DA to increase in the PrL in the FMT group (Fig. 3E).

4. Discussion

Recently, FMT has gained attention as a promising treatment for mental and neurological disorders. Its efficacy has been demonstrated in both clinical and animal studies for ASD, a neurodevelopmental disorder. However, the potential benefits of FMT for ADHD, another neurodevelopmental disorder, remain largely unexplored. Therefore, this study aimed to assess the effects of FMT in an ADHD-like rat model. This study revealed that the gut microbiota of ADHD-like model (LH) rats differed from that of healthy (Wistar) rats, contributing to hyperactivity. The Chao1 index, an indicator of within-individual diversity, was significantly lower in LH rats, and β-diversity calculated from Bray-Curtis distances also showed significant differences between the groups. Additionally, the abundance of several taxa varied significantly between the groups. Moreover, fecal transplantation from Wistar rats into LH rats improved hyperactivity and showed a tendency towards enhanced cognitive function. There was also a trend toward increased dopamine levels in ADHD-related brain regions of the ADHD-like model rats after FMT, although the difference was not statistically significant. Although these findings could not prove a causal relationship, they may be the result of differences in the gut microbiota between ADHD-like model rats and healthy rats influencing behavioral characteristics via the dopaminergic system.

4.1. Gut microbiota differences between ADHD-like model and healthy rats as a potential contributor to behavioral abnormalities

The diversity and composition of the gut microbiota were significantly different between LH and Wistar rats (Fig. 1A–C). The alpha diversity (Chao1 index) was significantly reduced in LH rats, and there were also significant differences in beta diversity. Significant changes in both within-individual and between-individual diversity have been reported in various neurological and psychiatric disorders, suggesting a potential link to these conditions.[36] Several taxa showed significant differences in abundance between LH and Wistar rats. These taxa included those previously reported to differ significantly between ADHD patients and healthy individuals, or in ADHD-like animal studies, such as the phyla Actinobacteriota and Bacteroidota,[36] and the genera *Bifidobacterium*,[15] *Parabacteroides*,[36] *Desulfovibrio*,[37,38] and *Turicibacter*.[39,40]

These rats exhibited significant behavioral differences during the behavior tests (Fig. 1D–K). The total distance traveled in the OFT tended to be increased in the LH rat group compared with the Wistar group, although this was not significantly different, and the LH rats showed a significant increase in this measure in the EPM. Anxiety-related indicators, including the frequency of entries into and time spent in the center zone of the OFT, as well as the percentage of entries into and time spent in the open arms of the EPM, were significantly higher in LH rats. These results suggest that LH rats are more hyperactive and less anxious, consistent with previous studies.[20,22] Moreover, the increased frequency of center zone entries and time spent in the center zone in the OFT, along with similar measures in the open arms of the EPM, may reflect not only reduced anxiety but also heightened risk-taking behavior, which can be interpreted as higher impulsivity.[41,42] Thus, these results also suggest increased impulsivity in LH rats.

FMT from Wistar to LH rats did not significantly affect anxiety or impulsivity in the OFT or EPM, but the total distance moved, an indicator of hyperactivity in the OFT, significantly decreased. In the MWM probe test, which assesses memory retention, LH rats after FMT showed a tendency to enter the platform location more frequently than Vehicle group. FMT had no significant effect on Wistar rats, suggesting that differences in gut microbiota between Wistar and LH rats may influence hyperactivity and cognitive function.

4.2. Behavioral changes after FMT may be related to an increase in specific gut microbiota, resulting in activation of the dopaminergic system

In the LH rat FMT group, the numbers of the families *Erysipelatoclostridiaceae* and *Bacteroidaceae*, and the genera *Bacteroides* and *Marvinbryantia* were significantly increased. Although little is known about *Erysipelatoclostridiaceae* in the context of ADHD, this family has been associated with Alzheimer's disease genes and cognitive decline,[43] and its abundance has been reported to decrease in mouse models of fluoride-induced memory impairment.[44] Similarly, *Marvinbryantia*, though not reported in ADHD-related studies, has been negatively correlated with amyloid status, a marker of Alzheimer's disease risk.[45] The Bacteroidaceae family and *Bacteroides* have been negatively correlated with cognitive decline and Alzheimer's risk.[46] Additionally, their abundance has been shown to increase following interventions such as FMT[47] and probiotics.[48] *Bacteroides ovatus* supplementation ameliorated spatial working memory deficits in ADHD-like model rat.[49] Both *Bacteroides* and *Marvinbryantia* have been linked to SCFA production,[50,51] which has anti-inflammatory effects,[52] and its administration improved cognitive function and reduced activity in the OFT in mouse studies.[53] The improvements in hyperactivity and memory retention observed in

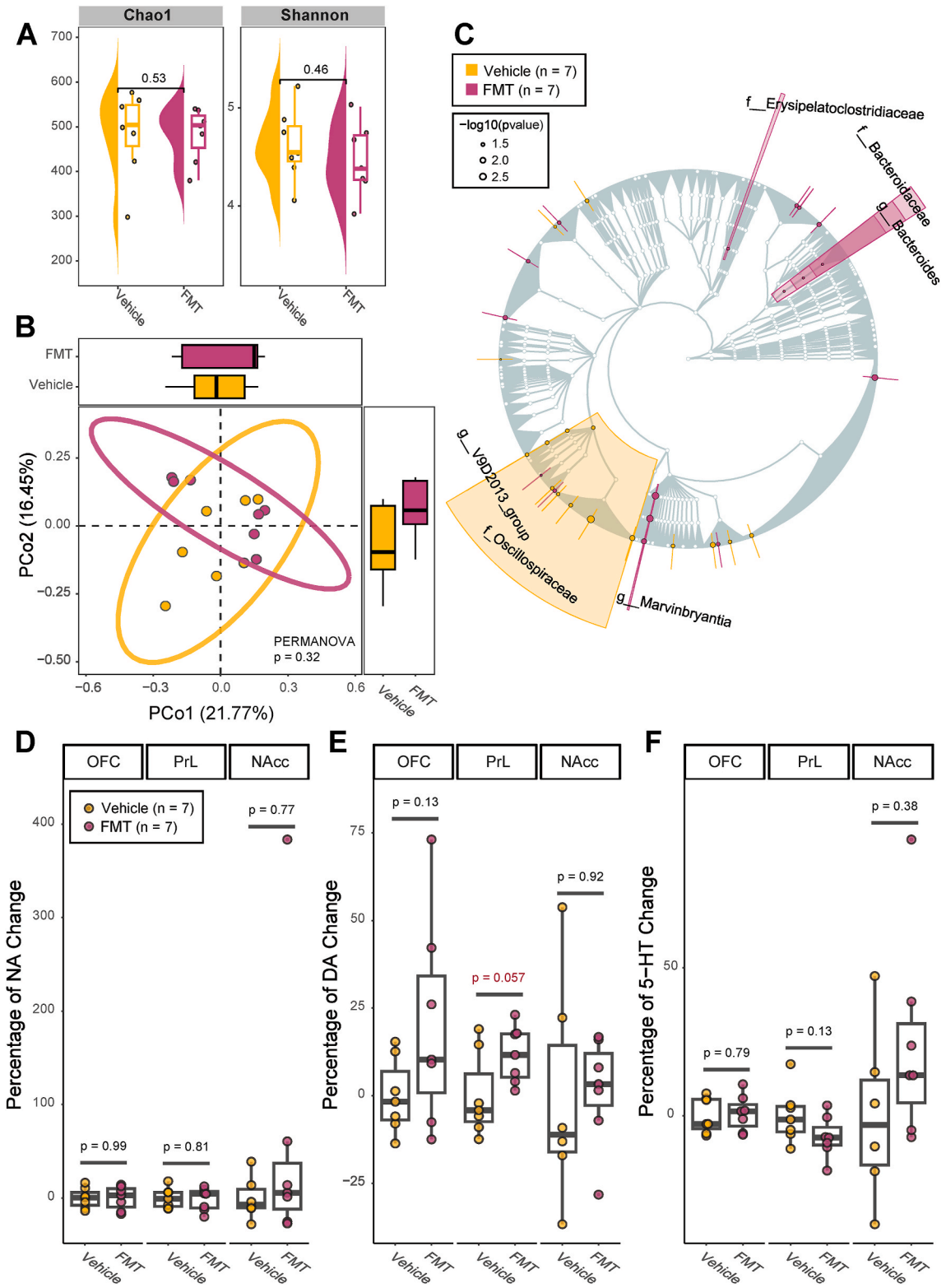

Fig. 3. Differences in intestinal microbiota and neurotransmitter levels in ADHD-related brain regions between FMT and Vehicle groups in an ADHD-like rat model. The results of the 16S rDNA analysis in ADHD-like model (LH) rats after FMT or Vehicle administration are shown in Figures A–C. Figure A presents an alpha diversity boxplot comparing the groups, with p-values calculated using the Wilcoxon rank-sum test. Figure B shows the PCoA plot calculated using Bray-Curtis distances. Figure C depicts a cladogram highlighting clades with differential abundance between the Vehicle and FMT groups. Taxa with significant differences are represented by colored circles, with circle sizes proportional to the p-values from the Kruskal-Wallis test. Only taxa at the family level and above are shown. Figures D–F are boxplots showing the levels of neurotransmitters in ADHD-related brain regions (orbitofrontal cortex [OFC], prelimbic cortex [PrL], nucleus accumbens [NAcc]) as measured using HPLC. The results for NA are shown in D, for DA in E, and for 5-HT in F. The vertical axis values represent the percentage change relative to the mean values of the control group (n = 7 for each group). Statistical significance was determined using Welch's *t*-test. NA: noradrenaline, DA: dopamine, 5-HT: serotonin.

this study may have been mediated by SCFAs. Although both are speculative, it is possible that the significantly increased gut microbiota taxa, in association with short-chain fatty acids (SCFAs), contributed to behavioral changes such as improved hyperactivity and memory retention, but further validation is required.

Bacteroidaceae and *Bacteroides* may act compensatory on ADHD-like symptoms through the dopaminergic system. Several studies have also connected ADHD with Bacteroidaceae and *Bacteroides*, which are often reported to be increased in ADHD patients.[36] In individuals with ADHD taking psychostimulant medications, *Bacteroides stercoris* CL09T03C01 abundance is reported to be lower compared with that in non-medicated ADHD patients,[54] and individuals who abuse methamphetamine show a decrease in Bacteroidaceae.[55] In a previous animal study, methylphenidate was found to increase *Bacteroides* in ADHD-like model rats.[40] The primary action of current ADHD medications is to increase dopamine activity and levels in the brain. Previous studies have shown that Bacteroidaceae and *Bacteroides* are more abundant in ADHD patients with impaired dopaminergic systems,[56] whereas lower abundance has been found in ADHD patients taking dopamine-enhancing medications and individuals who abuse stimulant drugs. These previous findings suggest that when dopamine activity is reduced, the abundance of Bacteroidaceae and *Bacteroides* increases, and when dopamine activity is elevated, their abundance decreases. Our study demonstrated an increase in Bacteroidaceae and *Bacteroides* alongside behavioral improvements following FMT, with a trend towards elevated dopamine levels in ADHD-related brain region (PrL, the prelimbic region of the medial prefrontal cortex). On the basis of the results described above, Bacteroidaceae and *Bacteroides* may regulate increased dopamine levels. A previous study reported that administration of *Bacteroides uniformis* CECT 7771 to a food addiction model rat significantly increased dopamine levels in the nucleus accumbens and elevated the expression of D1 and D2 receptors in the prefrontal cortex.[57] However, the mechanisms by which *Bacteroides* regulate dopaminergic activity remain unclear and warrant further investigation.

4.2.1. FMT methodology considerations

In this study, we performed fecal transplants from normal Wistar rats into ADHD-like model rats, and the ADHD-like symptoms of the ADHD model mice were alleviated. However, the effect was limited. This may be because we chose a method that would place as little strain on children as possible, assuming that this method would ultimately be applied mainly to children. In other words, the FMT method we performed this time did not use antibiotics, and transplanted the feces into the rectum once every two days for a total of three times to verify the effectiveness. On the other hand, for example, a clinical study on ASD by a research group at the University of Arizona combined FMT with vancomycin, administering the antibiotic vancomycin to children with autism spectrum disorder for two consecutive weeks, followed by intestinal irrigation with polyethylene glycol (PEG), and then FMT (two days of enema administration, 50 days of oral administration). As a result, it has been reported that approximately 50% of children with severe ASD transitioned to the moderate, mild, or normal group two years after FMT.[58]; [59]

In animal experiments, Wang et al. administered an antibiotic cocktail of ampicillin, vancomycin, metronidazole, and neomycin to valproic acid-induced ASD model mice from 14 to 28 days after birth, then performed FMT (21 days of oral administration), and behavioral tests from the 52nd day. As a result, ASD-like behaviors (such as lack of sociality and anxiety-like behaviors) caused by valproic acid were significantly improved.[60] In addition, as an antibiotic-free FMT, Goo et al. orally administered 200 μl of fecal sample supernatant once a day for four weeks and confirmed that ASD-like symptoms (lack of sociality, anxiety-like behavior, etc.) were alleviated in Fmr1 KO mice.[61]

These FMTs all involve higher doses and higher frequency than our method.

Therefore, if we had chosen another method, including the above, for our ADHD-like model rats, the effect may have been clearer.

4.2.2. Limitations of this study

Throughout the study, we would like to state that all the results obtained this time are merely a list of phenomena, and further detailed experiments are required to determine whether there is actually a causal relationship and whether the biological parameters are related to behavior. In other words, the role of each microorganism is currently only a guess based on previous studies, and the levels of short-chain fatty acids were not measured. There is also no data comparing the monoamine levels of LH rats and Wistar rats. Therefore, it is not possible to conclude from the current data whether the monoaminergic system in the brain is related to the behavior of LH rats observed here, and even to a causal relationship. Therefore, further research is needed to clarify these points one by one.

5. Conclusion

The current findings provide new insight into the potential efficacy of FMT as a treatment for ADHD. The diversity and composition of the gut microbiota differ between healthy rats and ADHD-like model rats, potentially contributing to behavioral variations. FMT from healthy rats to ADHD-like model rats improved hyperactivity. Similar differences in gut microbiota have been observed between healthy individuals and ADHD patients in clinical studies, suggesting that FMT may also be effective for treating ADHD in humans.

CRediT authorship contribution statement

Wakana Harigai: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kanata Mikami:** Writing – review & editing, Methodology. **Mohammed E. Choudhury:** Writing – review & editing, Methodology. **Hiroto Yamauchi:** Writing – review & editing, Formal analysis. **Chisato Yajima:** Writing – review & editing, Methodology. **Shin Shimizu:** Writing – review & editing, Resources. **Noriyuki Miyaue:** Writing – review & editing. **Masahiro Nagai:** Writing – review & editing. **Madoka Kubo:** Writing – review & editing, Formal analysis. **Junya Tanaka:** Writing – review & editing, Supervision, Resources, Methodology. **Taiichi Katayama:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Data sharing and data accessibility

16S rDNA sequences were deposited in the DDBJ (BioProject: PRJDB18931).

Declaration of generative AI in scientific writing

The authors used ChatGPT during the preparation of this work to improve the readability of the English text. The generated content was carefully reviewed and revised by the authors, who take full responsibility.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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Appendix A. Supplementary data

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