Inhibition of Rho Kinase by Fasudil Ameliorates Cognition Impairment in APP/PS1 Transgenic Mice via Modulation of Gut Microbiota and Metabolites

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Abstract

**Background:** Fasudil, a Rho kinase inhibitor, exerts therapeutic effects in the mouse model of Alzheimer’s disease (AD), a chronic neurodegenerative disease with progressive loss of memory. However, the mechanisms remain unclear. In addition, the gut microbiota and its metabolites have been implicated in AD.

**Methods:** Here, we examined the effect of fasudil on learning and memory using the Morris water-maze (MWM) test in APPswe/PSEN1dE9 transgenic (APP/PS1) mice (8 months old) treated (i.p.) with fasudil (25 mg/kg/day; ADF) or saline (ADNS) and in age- and gender-matched wild-type (WT) mice. Fecal metagenomics and metabolites were analyzed to identify novel biomarkers of AD and to elucidate the mechanisms whereby fasudil produced beneficial effects in AD mice.

**Results:** Morris water maze test showed significant improvement of spatial memory in APP/PS1 mice treated with fasudil as compared to ADNS. The metagenomic analysis revealed the abundance of the dominant phyla in all the three groups, including *Bacteroidetes* (23.7% - 44%) and *Firmicutes* (6.4% - 26.6%), and the increased relative abundance ratio of *Firmicutes/Bacteroidetes* in ADNS (59.1%) compared to WT (31.7%). In contrast, the *Firmicutes/Bacteroidetes* ratio was decreased to the WT level in ADF (32.8%). Lefse analysis results of metagenomics showed *s_Prevotella_sp_CAG873* as an ADF potential biomarker, while *s_Helicobacter_typhlonius* and *s_Helicobacter_sp_MIT_03-1616* as ADNS potential biomarkers. Metabolite analysis revealed the increment of various metabolites, including glutamate, hypoxanthine, thymine, hexanoyl-CoA, and leukotriene, were relative to ADNS or ADF microbiota potential biomarkers and mainly involved in the metabolism of nucleotide, lipids, sugars, and the inflammatory pathway.

**Conclusions:** Memory deficit in APP/PS1 mice was correlated with the gut microbiome and metabolite status. Fasudil reversed the abnormal gut microbiota and subsequently regulated the related metabolisms to normal in the AD mice. It is believed that fasudil can be a novel strategy for the treatment of AD via remodeling of the gut microbiota and metabolites. The novel results also provide valuable references for the use of gut microbiota and metabolites as diagnostic biomarkers and/or therapeutic targets in clinical studies of AD.

Introduction

Alzheimer’s disease (AD) is a complex heterogenous disease characterized by progressive impairment of memory and cognition; it is the most prevalent cause of dementia [1]. Decreases in expression of amyloid β (Aβ) peptides and phosphorylated Tau (pTau) protein are assumed to lower the probability of developing AD [2]. AD has emerged as a severe medical concern worldwide due to a lack of sensitive, accurate, and accessible biomarkers for detection, diagnosis, and monitoring of the disease progression [3, 4]. Therefore, it is of the utmost importance to develop the most suitable biomarkers for AD. Recently, the human gut microbiota, which plays a critical role in the maintenance of normal physiological
condition and health, has been demonstrated to modulates the physiological aspects of neurodegenerative disorders [5]. A plethora of evidence suggests that the gut microbiota is with AD progression and that changes in gut microbiome and its metabolites adversely affect cognition [6–8].

The gastrointestinal (GI) tract has $10^{13}$-$10^{14}$ microorganisms, most of which belong to the Bacteroidetes and Firmicutes phyla [9]. Clinical studies have shown that Bacteroides in AD patients are significantly decreased, suggesting the crucial role in health protection by providing intestinal barrier and blocking gut leakiness [10, 11]. Firmicutes are also known to increase as a risk factor in AD pathogenesis [12]. The association of Prevotella and Helicobacter in AD patients has been reported recently [13], the latter of which is significantly increased at the genus level, while Prevotella is significantly lower in APP/PS1 mice relative to WT controls [14]. Curcumin treatment has been shown to significantly change the abundance of Bacteroidaceae, Prevotellaceae and 8 metabolites in AD mice [15].

Clinical studies have demonstrated that elderly patients with dementia exhibit Aβ plagues with bacterial accumulation in the pathogenesis of cognitive impairment [16]. Helicobacter has been shown to induce Tau hyperphosphorylation in several AD-related Tau phosphorylation sites [17]. Accumulating data indicate that the gut microbiota communicates with CNS through the neural, endocrine, and immune pathways, thereby influencing brain function and behaviors [9]. Altered gut microbial composition is associated with fecal metabolome changes [18]. Metagenomics is a new method to study microbial diversity by taking all the genomes of the microbial population in samples and analyzing small molecules metabolites, which can help identify novel biomarkers [19–22].

APPswe/PSEN1dE9 transgenic (APP/PS1) mice express human amyloid precursor protein (HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) and are a widely used model of AD. It has been shown that APP/PS1 mice exhibit learning and memory impairment at the age of 8 months [23] and significant Aβ plaque accumulation in the hippocampus at 9 months [24, 25]. Our previous studies have demonstrated that fasudil, a selective Rho kinase (ROCK) inhibitor [26], decreases expression of ROCK-II in experimental autoimmune encephalomyelitis (EAE) mice [27]. Inhibition of ROCK by fasudil reverses Aβ$_1$-42-induced neuronal apoptosis, intracellular calcium overload, and decreases in mitochondrial membrane potential. Thus, ROCK inhibitors such as fasudil can be conferred as future therapeutic and preventive strategies for neuroinflammatory and neurodegenerative diseases [28]. It is not clear if the gut microbiota is involved in the cognition-enhancing effect of fasudil in AD mice. In the present study, we deciphered the close connection between the gut microbiota and AD through the microbiota-gut-brain axis. Gut microbiota could serve as a potential new target for therapeutic intervention in AD [29]. The brain-gut axis controls the interaction of biochemical molecules of brain and gut [30]. Therefore, we proposed that the gut microbiota and its metabolites trigger the neurodegenerative disorder through the brain-gut axis, which may be a potential mechanism of AD pathogenesis and the anti-AD effect of fasudil.

Materials And Methods
Animals and Treatment

Male APP/PS1 mice on the C57BL/6 background (8 months old) were purchased from Beijing Huafukang Bioscience CO., LTD (HFK, Beijing, China). Animals were housed in the pathogen-free facilities at the Institute of Brain Science, Shanxi Datong University and maintained in constant room temperature (25 ± 2°C) and humidity (50 ± 5%) in a 12-h light/12-h dark cycle. APP/PS1 mice were pre-screened based on the normal physiological behavior and randomly divided into two treatment groups: 1) vehicle-treated mice (ADNS), which were administered normal saline (the volume was adjusted similar to fasudil treatment); 2) fasudil-treated (ADF) mice, which received a daily injection of fasudil (Tianjin Chase Sun Pharmaceutical Co., Ltd.), 25 mg/kg/day, i.p., 16 wk; age- and gender-matched C57BL/6 (WT) mice were used as normal controls, which received saline in the same volume (n = 7 per group). Animals had ad libitum access to food and water. All the experiments were performed in compliance with the guidelines and regulations of the Administration Office of the International Council for Laboratory Animal Science. The experimental protocols were approved by the Animal Ethics Committee of Shanxi Datong University, Datong, China.

The Mouse Cognition Test

The Morris water maze (MWM) test was used to measure the spatial learning and memory abilities of APP/PS1 and WT mice. This was performed with a pool (140 cm in diameter) filled with water, which was mixed with milk and 1.5 cm above the platform; the water was maintained at a constant temperature (25 ± 2°C). The MWM, which was surrounded by a blue curtain with light in a fixed position, was divided into four quadrants: northeast (NE), northwest (NW), southeast (SE), and southwest (SW), and the MWM test was performed 1 week after the last treatment. During the 5-d acquisition training, mice were individually trained to locate the hidden platform from the starting point for 4 trials/day with the cut-off time 60 s. On each trial, the mice were placed in the water at different start locations (E, S, W and N), which were equally spaced from each other. The animal was allowed to locate the hidden platform by swimming for 60 s and to remain on the platform for at least 10 s. If the mouse was unable to locate the platform within 60 s, it was gently guided to the platform by the experimenter and allowed to remain on it for 10 s. In this case, its performance score (or escape latency) was marked as 60 s. The probe trial was performed on day 6 (24 hours after the last training trial) when the platform was removed, and the mouse was allowed to swim freely for 60 s. The position of the mice was tracked by a camera above the center of the pool and was connected to an automatic photographic recording and analysis system (SMART V3.0 system, Panlab, Barcelona, Spain). The escape latency (i.e. the time spent in locating the hidden platform), latency of the first entrance to the target zone, and the time spent in the target zone (% of the total time in all the four zones) during the 5-d acquisition training, the swimming paths, and the number of crossings into the target quadrant during the probe trial were all recorded using the SMART V3.0 system.

Bielschowsky Silver-plated Nerve Staining

The silver glycinate staining was performed following the procedures described previously [31–34]. In brief, after dehydrating with ethanol and staining with acidic formaldehyde, brain slices (pre-heated at
37°C) were placed in the silver glycine solution for 3–5 min, followed by clearing and mounting to reduce solution staining and dehydration. Microscopic examination, image acquisition, and analysis were performed.

**Metagenomics Sample Preparation and Analysis**

All fecal samples were frozen at −80°C before DNA extraction and analysis. Fecal DNA was extracted using PowerSoil DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA) following the instructions provided by the manufacturer. The purity and integrity of DNA were analyzed by agarose gel electrophoresis (AGE), and the accurate quantification of DNA concentrations was analyzed by Qubit. The DNA was sheared to 300 bp with the Covaris ultrasonic crusher. For sequencing library preparation, the fragments were treated by end repair, a tailing, and ligation of Illumina compatible adapters. After the construction, the library was initially quantified by Qubit 2.0, diluted to 2 ng/ul, and then the insert size of the library was detected by Agilent 2100. After the insert size met the expectation, the effective concentration of the library was accurately quantified by q-PCR (the effective concentration of the library was >3 nM) to ensure the library quality. DNA sequencing libraries were deeply sequenced on the Illumina Hiseq platform at Allwegene Company (Beijing, China). The software of MEGAHIT (v1.0.6) was used to assemble the sequencing samples, and the fragments below 500 bp in the assembly results were filtered out. Contigs were annotated with the Prodigal software to predict open reading frames (ORFs), and the CD-HIT software was used to construct the non-redundant gene set. Bowtie was used to compare the sequencing data with the non-redundant gene set, and the abundance information of genes in different samples was counted.

The high-quality sequences were compared with the NR database (i.e., the non-redundant protein sequence database) and classified into different taxonomic groups by the diamond BLAST tool. Clustering analysis and principal component analysis (PCA) were used to examine the similarities between different samples, based on the genus information from each sample. The gene function was annotated by and searched against the functional annotation database KEGG, GO and CAZyme.

**Metabolomic and Metabolites Analysis**

The metabolites were detected by an ultra-high-performance liquid tandem chromatography quadrupole time of flight mass spectrometry (UHPLC-QTOFMS) at the Allwegene Company (Beijing, China), including basic and personalized data analyses. The data statistical analysis, which includes data preprocessing, Student’s t-test, PCA, and differential metabolite screening, was carried out by MetaX on the qualitative data of the metabolome for screening metabolites with significant differences. Generally, the threshold with $p < 0.05$ and VIP (Variable Importance in the Projection) > 1 was used to screen differential metabolites. The VIP values exceeding 1.0 were first selected as changed metabolites; the remaining variables were then assessed by Student’s t-test ($p < 0.05$). Personalized analyses included the KEGG analysis and metabolic pathway analysis of differential metabolites. Commercial database including KEGG (http://www.kegg.jp) and MetaboAnalyst (http://www.metaboanalyst.ca/) were utilized to search for the pathways of metabolites.
Association analysis of the metagenomic and metabolomic

Spearman algorithm was used to calculate correlation between metagenomic and metabolomic, and R language GGPlot package was used to draw correlation heat map.

Statistical Analysis

The SPSS software (International Business Machines Corporation, IBM, USA) was used for statistical analysis. All data were expressed as means ± SEM. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA), with post hoc comparisons of Dunnett tests. A value of $p < 0.05$ was considered statistically significant. Graphical presentations were performed with GraphPad Prism 5 software (GraphPad 122 Software, San Diego, IL, USA).

Results

**Fasudil improved cognitive function in APP/PS1 mice**

The MWM test was used to assess the effect of fasudil on cognitive function in APP/PS1 mice at the age of 12 months. During the 5-d training period, APP/PS1 mice treated with saline (ADNS) showed significant increases in the latency to locate the platform (Fig. 1A), latency of the first entrance into the target zone (SW) (Fig. 1B), and decreases in the time (%) spent in SW (Fig. 1C) when compared with WT + saline (WT) ($p < 0.05$ or $p < 0.01$). In contrast, APP/PS1 mice treated with fasudil (ADF) showed significant decreases in the two latencies and increases in the time (%) in the target zone, as compared with ADNS ($p < 0.05$ or $p < 0.01$; Fig. 1A-C). Representative raw traces of swimming in the probe trial test (Day 6) were shown in Fig. 1D. The traces of ADNS mice were much longer than WT, which was shortened in ADF. Similarly, the number of crossings over the target area was significantly lower in ADNS compared to WT, which was attenuated in ADF ($p < 0.01$; Fig. 1E). These results suggest that the impairment of learning and memory in the AD mice were reversed or attenuated by treatment with fasudil.

**Fasudil treatment decreased senile plaques and neurofibrillary tangles in the hippocampus of AD mice**

To identify Aβ plaques and neurofibrillary tangles in the hippocampus, we used Bielschowsky silver staining, the most commonly used method for examining senile plaques and neurofibrillary tangles [34, 35]. It was found that a large number of senile plaque deposition and neurofibrillary tangles were accumulated in the hippocampus of ADNS mice, but not in WT controls (Fig. 2A, B). This was remarkably attenuated as both senile plaques and neurofibrillary tangles were obviously decreased in the hippocampus in ADF relative to ADNS mice.

**Fasudil treatment reversed the specific alterations of gut microbial diversity in AD mice**
To assess the effects of fasudil on gut microbiota alterations related to the pathogenesis of AD, we used Principal Component Analysis (PCA) by variance decomposition to reflect the differences between groups using APP/PS1 mice and WT controls. PCA revealed based on species annotation abundance that ADNS showed different species abundance composition from WT and ADF, both of which had similar composition of species abundance (Fig. 3A). In addition, we performed species annotated relative abundance bar chart analysis, which showed the dominant phyla in all groups, including \textit{p-Bacteroidetes} (23.7\% – 44\%) and \textit{p-Firmicutes} (6.4\% – 26.6\%) (Fig. 3B and Additional file 1: the dominant phyla in all groups). In these two most dominant phyla, the relative abundance ratio of \textit{Firmicutes/Bacteroidetes} was increased in ADNS (59.1\%) compared to WT (31.7\%), while decreased in ADF (32.8\%) relative to ADNS (Fig. 3C, D), suggesting the progression of AD may be associated with a high proportion of \textit{Firmicutes/Bacteroidetes}, which can be lowered to normal following fasudil treatment. Further, we analyzed the data at the family levels and observed that \textit{f-Bacteroidaceae, f-Prevotellaceae, f-Lachnospiraceae, and f-Bacteroidaceae} were relatively the most abundant in all groups (Fig. 3E and Additional file 2: the level of family in all groups).

The species levels of \textit{s_Bacteroides_dorei_CAG222} (\( p < 0.05 \)), \textit{s_Bacteroides_bacterium_OLB8} (\( p < 0.05 \)), \textit{s_Prevotella_sp_CAG1031} (\( p < 0.05 \)), and \textit{s_Prevotella_sp_CAG873} (\( p < 0.01 \)) in ADF were significantly higher compared to ADNS, which exhibited significantly lower abundance compared to WT (\( p < 0.05 \)), suggesting that fasudil treatment reversed these species abundance (Fig. 4 upper panels and Additional file 3: the level of species in all groups). Similarly, the abundance levels of some species in ADNS were significantly lower compared to WT, including \textit{s_Alistipes_finegoldii} (\( p < 0.01 \)), \textit{s_Alistipes_sp_CAG53} (\( p < 0.05 \)), \textit{s_Alistipes_sp_CAG435} (\( p < 0.05 \)), and \textit{s_Butyricimonas_synergistica} (\( p < 0.01 \)) (Fig. 4 middle panels and Additional file 3: the level of species in all groups). The abundance levels were increased after fasudil treatment, but with no significant difference between ADF and ADNS. In contrast, ADNS showed significantly more abundance in \textit{s_Helicobacter_saguini}, \textit{s_Helicobacter_typhlonius}, and \textit{s_Helicobacter_sp_MIT_03-1616} compared to WT (\( p < 0.05 \)) (Fig. 4 lower panels and Additional file 3: the level of species in all groups), which was reduced in ADF, with statistical significance in \textit{s_Helicobacter_saguini} (\( p < 0.05 \)), suggesting that fasudil treatment blocked AD-induced increases in the abundance of these species, especially \textit{s_Helicobacter_saguini}. Finally, we used Lefse (LDA Effect Size, linear discriminant analysis) combined with the statistical analysis to screen key biomarkers [36, 37]. The LDA scores (\( \log_{10} > \pm 3 \)) indicating more abundance at the species level in ADNS and ADF are shown in Fig. 5. The results of metagenomics demonstrated that \textit{s_Prevotella_sp_CAG873} was identified as an ADF potential biomarker, while \textit{s_Helicobacter_typhlonius} and \textit{s_Helicobacter_sp_MIT_03-1616} were identified as ADNS potential biomarkers in the fecal of APP/PS1 mice (Fig. 5).

**Fasudil treatment altered gut metabolites in APP/PS1 mice**

To determine the effect of fasudil on changes in gut metabolites associated with AD, we carried out metabolomic analysis using UHPLC-QTOFMS and PLS-DA cluster analysis, the latter of which was used to further determine the trends of metabolic changes in ADNS, relative to WT or ADF. All samples were analyzed with a 95\% confidence interval (Hotelling’s t-squared ellipse; Fig. 6A). The results from the
screening of different metabolites were visualized in the form of volcano plots (Fig. 6B, C). There were 295 different metabolites in ADNS vs. WT, including 117 downregulated and 178 upregulated metabolites (Fig. 6B); 335 different metabolites in ADNS vs ADF, including 185 downregulated and 150 upregulated metabolites (Fig. 6C). Further clustering analysis of differential metabolites in each group revealed that ADNS had a different heat map from WT and ADF, both of which were presented similarly (Fig. 6D). In addition, the 295 differential metabolites in ADNS vs. WT were enriched in 28 signaling pathways (Fig. 6E and Additional file 4: the differential metabolites in ADNS vs. WT were enriched in 28 signaling pathways), focusing on the metabolisms of pyruvate, glycolysis/gluconeogenesis, fructose and mannose, citrate cycle (TCA cycle), amino sugar, and nucleotide sugar; the 335 differential metabolites in ADNS vs. ADF were concentrated in 20 signaling pathways focusing on the metabolisms of pyrimidine, purine, glycolysis/gluconeogenesis, glycerophospholipid, and fatty acid degradation (Fig. 6F and Additional file 5: the differential metabolites in ADNS vs. ADF were concentrated in 20 signaling pathways).

**Association analysis of the metagenomic and metabolomic profiles**

To examine the correlation between metagenomic and multiple metabolites in AD, we analyzed the metabolites from the gut microbiota and the host in APP/PS1 mice. By taking the intersection of ADNS-WT and ADNS-ADF, 83 important, differential metabolites were obtained and narrowed down to 60 different metabolites by adjustment of $p < 0.04$ (Additional file 6: the 60 differential metabolites by adjustment of $p < 0.04$), among which 20 important, different metabolites were screened as per available literatures (Additional file 7: the correlation analysis of the species and 20 differential metabolites ($p$ value)).

Correlation analysis of genus levels in top 30 abundance of gut microbiota and 20 different metabolites were presented in Fig. 7A. Some gut microbiotas were correlated with a single metabolite, including *g_Clostridium*, which had a positive correlation with dTDP-4-oxo-2,3, 6-trideoxy-d-glucose ($p < 0.05$), and *g_Faecalibaculum*, which showed a positive correlation with DG (22:4(7Z,10Z,13Z,16Z)/24:0/0:0) ($p < 0.01$). Others were associated with a variety of metabolites (Additional file 7: the correlation analysis of the species and 20 differential metabolites ($p$ value)).

Furthermore, we examined 20 different metabolites correlation with the ADNS biomarkers (*s_Helicobacter_typhlonius, s_Helicobacter_sp_MIT_03-1616*) or the ADF biomarker (*s_Prevotella_sp_CAG873*). In addition, it was shown that *s_Bacteroides_dorei_CAG222* and *s_Bacteroidetes_bacterium_OLB8* were significant lower in ADNS compared to WT; this was reversed in ADF ($p < 0.05$; Fig. 4 upper; Additional file 3: the level of species in all groups). The correlation analysis revealed that the two species were correlated with 14 metabolites, including 9-ribofuranosyl hypoxanthine, leukotriene C5, thymine, dTDP-4-oxo-2,3,6-trideoxy-D-glucose, alpha-amino-4-carboxy-3-furanpropanoic acid, L-dopachrome, UDP-4-dehydro-6-deoxy-D-glucose, prolyl-gamma-glutamate, 2-hydroxy-3-[4-(sulfooxy)phenyl]propanoic acid, CDP-DG(18:0/18:0), leukotriene F4,
TG(22:5(4Z,7Z,10Z,13Z,16Z), TG(22:1(13Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z), and hexanoyl-CoA, which were widely related to a variety of metabolisms, such as carbohydrate metabolism and fatty acid metabolism. (Fig. 7B, Additional file 7: the correlation analysis of the species and 20 differential metabolites (p value)).

Heatmap was used to show the correlation results in Fig. 7B, which showed a specific positive correlation between the ADNS biomarkers (s_Helicobacter_typhlonius, s_Helicobacter_sp_MIT_03-1616), and 5 metabolites, including leukotriene F4, CDP-DG (18:0/18:0), TG (22:5 (4Z,7Z,10Z,13Z,16Z), TG (22:1(13Z)/22:6 (4Z,7Z,10Z,13Z,16Z,19Z), hexanoyl-CoA (p < 0.01). All the 5 metabolites were significantly higher in ADNS than in WT, significantly lower in ADF than in ADNS, and returned to the WT levels in ADF (Additional file 7: the correlation analysis of the species and 20 differential metabolites (p value)). These 5 metabolites are mainly involved in lipid metabolism, indicating that fasudil might protect neurons by decreasing hexanoyl-CoA, a short-chain fatty acyl-CoA precursor. Notably, fasudil also significantly decreased leukotriene F4, which is involved in the proinflammatory pathways.

There were 8 metabolites specifically correlated with the ADF biomarker (s-Prevotella sp_CAG873), four of which were significantly higher in ADNS compared to WT or ADF, including alpha-amino-4-carboxy-3-furanpropanoic acid, prolyl-gamma-glutamate, 2-aminoadenosine, and 2-hydroxy-3-[4-(sulfooxy) phenyl] propanoic acid; in other word, ADF reduced ADNS-induced high contents of these metabolites to the WT levels (Additional file 8). The other four metabolites were significantly lower in ADNS relative to WT or ADF, including 9-Ribofuranosyl hypoxanthine, L-dopachrome, UDP-4-dehydro-6-deoxy-D-glucose, and TG (22:0/o-18:0/22:0); in other words, ADF increased these metabolites in ADNS to the WT levels (Additional file 7: the correlation analysis of the species and 20 differential metabolites (p value)). The 8 metabolites were highly related to carbohydrate, nucleotide, and fatty acid metabolism.

It was noted that two of the metabolites, i.e., leukotriene C5 and thymine, were correlated with both the ADF biomarker (s_Prevotella sp_CAG873) and the ADNS biomarkers (s_Helicobacter_typhlonius, s_Helicobacter_sp_MIT_03-1616). They were significantly higher in ADNS compared to WT or ADF; in other words, ADF decreased the two metabolites in ADNS to the WT levels (Fig. 7B, Additional file 7: the correlation analysis of the species and 20 differential metabolites (p value)).

**Functional annotations of association metabolites with genes of biomarkers of ADF or ADNS**

In the enrichment analysis of functional annotations of association of metabolites with genes of biomarkers of ADF or ADNS, we observed significant pathways mainly involved in amino sugar, nucleotide sugar, pyrimidine metabolisms. As shown in Table 1, UDP-4-dehydro-6-deoxy-D-glucose was correlated with the ADF biomarker (s_Prevotella sp_CAG873), while thymine was correlated with both the ADF biomarker (s_Prevotella sp_CAG873) and the ADNS biomarker (s_Helicobacter_sp_MIT_03-1616). Both the ADNS and ADF biomarkers contained genes involved in signaling pathways of the pyrimidine metabolism (ko00240), encoded various enzymes, and further influenced the production of thymine. More specifically, the thioredoxin reductase gene (trxB) in the ADNS biomarker (s_Helicobacter_sp_MIT_03-1616) encoded the enzyme thioredoxin-disulfide reductase (EC:1.8.1.9) (blue
rectangles, Fig. 8; Table 1), likely affecting the refolding of post-translation protein. In the ADF biomarker (s-Prevotella sp CAG873), genes such as the RNA polymerase subunit C gene (rpoC), cytidine deaminase gene (cdd, CDA), thymidine kinase gene (tdk), holA gene-encoded one subunit of DNA polymerase III holoenzyme (holA), dUTPase gene (dut), and uridine diphosphate gene(udp) respectively encoded the following enzymes: DNA-directed RNA polymerase (EC:2.7.7.6), cytidine deaminase (EC:3.5.4.5), thymidine kinase (EC:2.7.1.21), DNA-directed DNA polymerase (EC:2.7.7.7), dUTP diphosphatase (EC:3.6.1.23), and uridine phosphorylase (EC:2.4.2.3) (pink rectangles, Fig. 8; Table 1).

### Table 1

| Name                                      | KO_id  | Gene_name       | EC       | metabolites                      |
|-------------------------------------------|--------|-----------------|----------|----------------------------------|
| s._Prevotella sp. CAG:873 ko00520(Amino sugar and nucleotide sugar metabolism) | K07106 | murQ            | EC:4.2.1.126 | UDP-4-dehydro-6-deoxy-D-glucose |
|                                          |        |                 |          |                                  |
|                                          | K01835 | pgm             | EC:5.4.2.2 |                                  |
|                                          | K12373 | HEXA_B          | EC:3.2.1.52 |                                  |
|                                          | K02472 | wecC            | EC:1.1.1.336 |                                  |
|                                          | K01443 | nagA, AMDHD2    | EC:3.5.1.25 |                                  |
| s._Prevotella sp. CAG:873 ko00240(Pyrimidine metabolism) | K03046 | rpoC            | EC:2.7.7.6 | Thymine                          |
|                                          | K01489 | cdd, CDA        | EC:3.5.4.5 |                                  |
|                                          | K00857 | tdk, TK         | EC:2.7.1.21 |                                  |
|                                          | K02340 | holA, DPO3D1    | EC:2.7.7.7 |                                  |
|                                          | K01520 | dut, DUT        | EC:3.6.1.23 |                                  |
|                                          | K00757 | udp, UPP        | EC:2.4.2.3 |                                  |
| s_Helicobacter sp. MIT 03-1616 ko00240(Pyrimidine metabolism) | K00384 |trxB            | EC:1.8.1.9 | Thymine                          |

**Discussion**

Accumulating clinical studies support, Aβ and pTau as important biomarkers of AD [38–40]. It has been demonstrated that alteration of gut microbial community is associated with AD and that the gut microbiota is changed even before the onset of AD[41]. Diet plays an important role in determining the composition of the gut microbiota, which is known to impact metabolic functions as well as immune responses [42]. A specific anti-AD therapy is usually combined with lifestyle interventions [43] (Fig. 9). In the present study, we demonstrated the amelioration of impairment of learning and memory and revealed
the relationship between the gut microbiome and metabolites in APP/PS1 mice following treatment with the ROCK inhibitor fasudil.

Clinical studies have shown that patients with *Helicobacter pylori* infection exhibit symptoms of AD. *Helicobacter pylori*, which may cross the blood-brain barrier (BBB) and contribute to amyloid deposition [13, 44], damages the BBB and subsequently cause direct interactions of gut metabolites with enteric neurons in the brain [45]. Microbial metabolites also influence the peripheral immune response, consequently affecting the BBB [9]. Neurofibrillary tangles induced by Aβ and pTau can cause blood vessel abnormalities and BBB breakdown, which contribute to cognitive decline in APOE4 carriers independent of AD pathology [46]. Fasudil retains the BBB integrity by up-regulating expression of tight junction proteins ZO-1 and occludin as demonstrated in our previous studies [26, 47–50]. The favorable effects of fasudil may also be contributed by preventing intestinal mucosal barrier disruption, decreasing the release of amyloid peptides and lipopolysaccharides (LPS), and inactivating inflammatory signaling induced by cytokines [51, 52].

Glutamatergic neurons in the hippocampus are closely associated with AD pathogenesis [53, 54]. Abnormal increases in glutamate, an important excitatory neurotransmitter in neurons, can cause cell death [55]. Understanding the molecular mechanism of glutamate would help develop novel and effective targets for treatment of AD [56]. For instance, anti-glutamatergic drugs such as memantine produce beneficial effects in AD treatment [57]. Aβ-induced cognitive impairment has been proven to be mediated by glutamatergic neurons [58]. Consistent with these, demonstrated in the present study that fasudil treatment reduced prolyl-gamma-glutamate and improved cognition in APP/PS1 mice.

It was found that 9-ribofuranosyl hypoxanthine was significantly lower while thymine was higher in ADNS than WT or ADF, i.e., both changes were reversed by fasudil treatment. This is supported by the finding that hypoxanthine, a purine derivative and a product of DNA metabolism following apoptosis and cell lysis [59], is decreased at the early stages of AD-related pathology [60]. Deficiency of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) re-directs gene expression from neuronal to glial functions [61], leading to increased microglia-mediated neuroinflammatory responses that contributes to AD-associated neurodegeneration [62, 63]. Fasudil has been shown to inhibit macrophages/microglia (M1) and astrocytes (A1), while increasing expression of macrophages/microglia (M2) and astrocytes (A2) in the peripheral and central immune systems [64] (Figure 9). Thymine is related to the metabolism of isoleucine, which promotes both differentiation and proliferation of peripheral pro-inflammatory T helper 1 (Th1) cells [64].

Leukotrienes are a family of major pro-inflammatory lipid mediators produced in leukocytes by the lipoxygenase (LOX) pathway of the arachidonic acid metabolism [65]. Over-activation and/or overexpression of 5-LOX in AD are typically indicative of an inflammatory basis for AD pathobiology [66]. The leukotriene pathway can be a potential target to reduce the inflammation and ameliorate various aspects of AD pathology [67]. There is restoration of homeostasis and promotion of tissue healing by increasing anti-inflammatory cytokine levels and decreasing pro-inflammatory mediators [68]. These
support our data that fasudil decreased leukotrienes in AD mice, which showed increases in leukotrienes and hexanoyl-CoA. Fasudil also decreased hexanoyl-CoA in AD mice. It is not clear whether AD is mediated by hexanoyl-CoA, which is the main factor in the synthesis of cannabinoids and is associated with allergies. Hexanoyl-CoA is one of the potential target molecules for intervention of spleen deficiency syndrome[69].

Alteration of cerebral glucose metabolism plays vital role in pathogenesis of AD [70]. Brain glucose uptake is impaired in AD [71]. The disruption of homeostasis in lipid and glucose metabolism might aggravate neurodegeneration and/or cognitive dysfunction through the accumulation of Aβ and pTau and/or by impairing of neuronal integrity [72]. High glucose concentrations-induced reduction of glycolytic flux in the brain is associated with the severity of AD pathology [73]. UDP-4-dehydro-6-deoxy-D-glucose is mainly generated by glycolysis gluconeogenesis and participates in the N-glycan biosynthesis process [74]. Increased activities of glucose-6-phosphate dehydrogenase have been found in AD tissues due to elevated brain peroxide metabolism [75]. This is consistent with our results that UDP-4-dehydro-6-deoxy-D-glucose was significantly lower in AD mice relative to WT and was returned to the WT level after treatment with fasudil.

In conclusions, the present study demonstrated that the gut microbiota composition and metabolites were altered in APP/PS1 mice. Fasudil reversed the abnormal gut microbiota and subsequently regulated the related metabolisms to normal in the AD mice. Modulation of gut microorganisms through personalized diet or beneficial microbiota intervention may be a potential preventive treatment strategy for AD. The alteration of the gut microbiota composition caused changes in microbiome state, further influencing the pathway of lipid metabolism, amino acid metabolism, glucose metabolism, and nucleotide metabolism. Therefore, fasudil can be a novel strategy for the treatment of AD via remodeling of the gut microbiota and metabolites. The novel results also provide valuable references for the use of gut microbiota and metabolites as diagnostic biomarkers and/or therapeutic targets in clinical studies of AD.

**Declarations**

**Conflict of interest statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author contributions**

YQY and YG designed the study, carried out the animal tests, data analysis, outlined and drafted the manuscript. GK and KS helped in revision the manuscript. QLF, NPZ, HLY and LJS participated in animal tests and data analysis. YQY, GY, GK, HTZ and CGM revised and finalized the manuscript. JHL, YNZ, JXS, JWW, LHZ performed the animal treatment experiments. All authors read and approved the final manuscript.
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Availability of data and materials

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

Ethics approval and consent to participate

All the experiments were performed in compliance with the guidelines and regulations of the Administration Office of the International Council for Laboratory Animal Science. The experimental protocols were approved by the Animal Ethics Committee of Shanxi Datong University, Datong, China. Efforts were made to minimize the number of animals used.

Consent for publication

Not applicable.

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**Figures**

![Figure 1](image_url)

**Figure 1**

Effects of fasudil on learning and memory of APP/PS1 mice in the Morris water maze test. (A-C) Changes in escape latency (A), latency of first entrance to the target zone (B), and the total time (%) spent in target zone (C) during the 5-d acquisition training in WT, APP/PS1 mice treated with saline (ADNS) or fasudil (ADF). (D) Representative swimming paths of WT, ADNS, and ADF mice in the probe trial. (E) The number of crossings into the border of the target zone in the probe trial. Data presented are the means ± SEM; n = 7; *p < 0.05, ** p < 0.01 vs. WT; # p < 0.05, ## p < 0.01 vs. ADNS.
**Figure 2**

Senile plaques and neurofibrillary tangles in the mouse hippocampus with Bielschowsky silver-plated nerve staining. (A) Representative images of hippocampal sections from WT and APP/PS1 mice treated with saline (ADNS) or fasudil (ADF). The neurofibrillary tangles (red arrow) were densely distributed in ADNS, but not in WT and only sparsely observed in ADF. (B) Magnification of the representative images (squared areas) of the hippocampal sections from WT, ADNS, and ADF mice. A large number of senile plaques (black arrow) and neurofibrillary tangles (blue arrow) were visible in ADNS, but not in WT and only occasionally in ADF. Scale = 200 µm (A) or 50 µm (B).

**Figure 3**
Microflora alteration in WT and APP/PS1 mice treated with fasudil. (A) Comparison of microbiota based on species composition by principal component analysis (PCA) in WT (red triangles) and APP/PS1 mice treated with saline (ADNS, purple circles) or fasudil (ADF, blue squares). The colored ellipses indicate 0.95 confidence interval (CI) ranges within each tested group. The scale of the horizontal and vertical axes refers to a relative distance. PC1 and PC2 represent the suspected influencing factors for the deviation of the species composition of samples. The closer the distance between the two points represents the smaller the difference of species abundance composition between the two samples. The data indicate ADF is closer to WT than ADNS. (B) The dominant representation in phyla. WT, ADNS, and ADF were colored at the phylum level on a stream graph. Bacteroides (green) and Firmicutes (purple) were the two most abundant bacteria at the phylum level. (C, D) The two most abundant phyla of Bacteroidetes and Firmicutes. The columns indicate the abundance of Bacteroidetes (green) and Firmicutes (purple) (C). The ratio of Firmicutes/Bacteroidetes was increased in ADNS compared to WT, which was reversed in ADF (D), indicating an alteration in the types of bacteria. (E) The dominant levels of bacterium families in WT, ADNS, and ADF mice, which were colored on a stream graph. The order of relative abundance is: Bacteroidaceae > Prevotellaceae > Lachnospiraceae > Lactobacteriaceae; n = 5.
Species less abundant in ADNS

Comparison of bacterial species in WT, ADNS and ADF mice. Each box plot represents the median, interquartile range, minimum, and maximum values for the relative abundance of individual species. Species with less abundant in ADNS compared to WT (p < 0.05), which was reversed in ADF, included (upper): s_Bacteroides_dorei_CAG222 (p < 0.05), s_Bacteroidetes_bacterium_OLB8 (p < 0.05) (both in orange), s_Prevotella_sp_CAG1031 (p < 0.05), and s_Prevotella_sp_CAG873 (p < 0.01) (both in blue).

Species more abundant in ADNS

Figure 4
Species with less abundant in ADNS compared to WT, which was not significantly blocked in ADF, included (middle): s_Alistipes_finegoldii (p < 0.01), s_Alistipes_sp_CAG53 (p < 0.05), s_Alistipes_sp_CAG435 (p < 0.05) (all in purple), and s_Butyricimonas_synergistica (p < 0.01; in green).

Species with more abundant in ADNS compared to WT (p < 0.05), which was significantly blocked in ADF, included (lower left) s_Helicobacter_saguini (p < 0.05; in pink); species with increased abundance in ADNS relative to WT (p < 0.05), which was not significantly attenuated in ADF, included (lower middle and right) g_Helicobacter (s_Helicobacter_typhlonius and s_Helicobacter_sp_MIT_03-1616); n = 5.

Figure 5

Linear discriminant analysis (LDA) effect size (LEfSe) analysis of microbiota in ADF (negative score) relative to ADNS (positive score). The LDA scores (log10) > ±3 were more abundant at the species level in ADNS compared to WT. s_Prevotella_sp_CAG873 was identified as a potential biomarker in response to fasudil treatment in APP/PS1 mice (ADF), while s_Helicobacter_typhlonius and s_Helicobacter_sp_MIT_03-1616 were identified as potential biomarkers in APP/PS1 mice (ADNS); n = 5.
Intestinal metabolite alteration of APP/PS1 mice treated with fasudil. (A) The PLS-DA score plots of metabolic profiles in WT (blue) and APP/PS1 mice treated with saline (ADNS, in green), or fasudil (ADF, in red). The separation trend of metabolic changes was observed in ADNS, WT and ADF; all samples were analyzed with 95% confidence interval (CI). (B, C) Volcano diagram of the changes in metabolites in WT, ADNS, and ADF mice. There were 295 metabolites differentially expressed in ADNS vs WT (B), including 117 metabolites significantly downregulated (green) and 178 metabolites significantly upregulated (red) (metabolites with non-significant differences are shown in gray). In addition, there were 335 metabolites differentially expressed in ADF vs. ADNS (C), including 185 downregulated metabolites and 150 upregulated metabolites. Each point in the volcano diagram represents a single metabolite. The scatter color represents the final screening result. (D) The heat map by the Hierarchical Clustering Analysis for different comparison combinations with significant changes. ADNS (middle 1-5) was presented in a different color pattern relative to WT (upper 1-5), which was similar to ADF (lower 1-5). (E, F) Analysis of the top 20 metabolic pathways in comparison combinations according to the impact factors (bubble plot). The results of the metabolic pathway analysis were presented as bubble plots. The bubble color represents the p value of the enrichment analysis, while the size of the point represents the number of different metabolites enriched in the pathway. Compared to WT, ADNS was focus on the metabolisms of pyruvate, glycolysis/ gluconeogenesis, fructose, mannose, citrate cycle (TCA cycle), amino sugar, and
nucleotide sugar; compared to ADNS, ADF was focused on the metabolisms of pyrimidine, purine, glycolysis/gluconeogenesis, glycerophospholipid, and fatty acid degradation; n = 5.

Figure 7

Correlation between gut microbiotas and metabolites. (A) Correlation analysis at the genus level of the 30 most abundant gut microbiotas and 20 different metabolites. Some microbiotas were correlated with specific metabolites, including g_Clostridium, which showed a positive correlation with dTDP-4-oxo-2,3,6-trideoxy-d-glucose (p<0.05), and g_Faecalibaculum, which had a positive correlation with DG (22:4(7Z,10Z,13Z,16Z) (p < 0.01). (B) The heat map of the most abundant species and 20 different metabolites. The correlation analysis revealed that both of s_Bacteroides_dorei_CAG222 and s_Bacteroidetes_bacterium_OLB8 were correlated with 14 different metabolites, including 9-ribofuranosyl hypoxanthine, leukotriene C5, thymine, dTDP-4-oxo-2,3,6-trideoxy-D-glucose, alpha-amino-4-carboxy-3-furanpropanoic acid, L-dopachrome, UDP-4-dehydro-6-deoxy-D-glucose, prolyl-gamma-glutamate, 2-hydroxy-3-[4-(sulfooxy)phenyl]propanoic acid, CDP-DG(18:0/18:0), leukotriene F4, TG(22:5(4Z,7Z,10Z,13Z,16Z), TG(22:1(13Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z), and hexanoyl-CoA, all of which were widely related to a variety of metabolisms, such as carbohydrate metabolism and fatty acid metabolism. * p < 0.05, ** p < 0.01; positive correlation is indicated in red and negative correlation is in blue; n = 5.
Figure 8

Correlation between thymine and the biomarkers for ADF and ADNS. The enzyme EC:1.8.1.9 (in blue rectangles) encoded by the gene trxB was correlated with the ADNS biomarker (s_Helicobacter_sp_MIT_03-1616), while the enzymes EC:2.7.7.6, EC:3.5.4.5, EC:2.7.1.21, EC:2.7.7.7, EC:3.6.1.23, and EC:2.4.2.3 (in pink rectangles) were encoded by the genes rpoC, cdd/CDA, tdk/TK, holA/DPO3D1, dut/DUT, udp/UPP, respectively; they were correlated with in the ADF biomarker (s_Prevotella_sp_CAG873) (also see Table 1 for the genes), n = 5. EC:1.8.1.9, thioredoxin-disulfide reductase; EC:2.7.7.6, DNA-directed RNA polymerase; EC:3.5.4.5, cytidine deaminase; EC:2.7.1.21, thymidine kinase; EC:2.7.7.7, DNA-directed DNA polymerase; EC:3.6.1.23, dUTP diphosphatase; EC:2.4.2.3: uridine phosphorylase; trxB, thioredoxin reductase gene; rpoC, RNA polymerase subunit C gene; cdd, cytidine deaminase gene; tdk, the thymidine kinase gene; holA, holA gene encoded one subunit of DNA polymerase III holoenzyme; dut, dUTPase (DUT) gene; udp, uridine diphosphate gene.
Figure 9

Schematic diagram of the gut-brain axis in AD treated with or without fasudil. Changes in the gut microbiota in the mouse model of AD (ADNS) cause abnormal production of metabolites, which aggravate peripheral inflammation, leading to increases in the brain infiltration of immune cells. Microglia M1 and astrocytes A1 cells are then activated in the brain, resulting in Aβ deposition, tau phosphorylation, and cognitive impairment. Treatment with fasudil (ADF) reconditions the gut microbiota, normalizes disordered metabolites, reduces the peripheral immune cell infiltration to the brain, ameliorates neuroinflammation, and lowers the accumulation of Aβ deposition and pTau, leading to ultimate improvement of cognitive functions. Blue arrows represent ADNS-related changes, while the red arrows...
refer to ADF-related changes. BBB, blood brain barrier; LPS, lipopolysaccharide; IL-1, interleukin-1; TNF-α, tumor necrosis factor -α; ZO-1, zonula occluden-1.

**Supplementary Files**

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