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Intestinal endogenous metabolites affect neuroinflammation in 5×FAD mice by mediating "gut-brain" axis and the intervention with Chinese Medicine

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Abstract

Background Emerging evidence suggested the association between gut dysbiosis and Alzheimer's disease (AD) progression. However, it remained unclear how the gut microbiome and neuroinflammation in the brain mutually interact or how these interactions affect brain functioning and cognition. Here we hypothesized that "gut-brain" axis mediated by microbial derived metabolites was expected to novel breakthroughs in the fields of AD research and development.

Methods Multiple technologies, such as immunofluorescence, 16s rDNA sequencing, mass spectrometry-based metabolomics (LC-QQQ-MS and GC-MS), were used to reveal potential link between gut microbiota and the metabolism and cognition of the host.

Results Microbial depletion induced by the antibiotics mix (ABX) verified that "gut-brain" can transmit information bidirectionally. Short-chain fatty acid-producing (SCFAs-producing) bacteria and amino acid-producing bacteria fluctuated greatly in 5×FAD mice, especially the reduction sharply of the *Bifidobacteriaceae* and the increase of the *Lachnospiraceae* family. Concentrations of several Tryptophan-kynurenine intermediates, lactic acid, $CD4^+$ cell, and $CD8^+$ cells were higher in serum of 5×FAD mice, whilst TCA cycle intermediates and Th1/Th2 were lower. In addition, the levels of iso-butyric acid (IBA) in feces, serum, and brain of 5×FAD mice were increased compared with WT-M mice, especially in serum. And IBA in the brain was positively correlated with Aβ and proinflammatory factors.

Conclusion Together, our finding highlighted that the alternation in gut microbiota affected the effective communication between the "gut-brain" axis in 5×FAD mice by regulating the immune system, carbohydrate, and energy metabolism.

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Keywords Alzheimer's disease (AD), Neuroinflammation, Short-chain fatty acid (SCFA), Gut microbiome

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that accounts for 80% of dementia cases worldwide, particularly among individuals over the age of 60 [1]. According to projections made in 2016, more than 131 million people will suffer from AD by the year 2050, making it one of the major global health challenges in the future. Female sex is recognized as a major risk factor for AD. In the United States, approximately 5.3 million individuals over the age of 65 are diagnosed with AD, with women comprising 60% of population. The lifetime risk of developing AD at age 45 is nearly twice as high for women compared to men [2–4]. Work by the Alzheimer's Disease Metabolomics Consortium (ADMC) identified the role of the gut microbiome in cognitive decline and changes in the brain that are hallmarks of the disease. Patients with AD exhibited an imbalance of the gut microbiota that manifests as decreased fecal microbial diversity, lower abundance of some beneficial bacterial taxa (e.g., *Eubacterium rectale*, *Bifidobacterium*, *Dialister*), and higher abundance of potentially pathogenic microbes (e.g., *Escherichia-Shigella*, *Bacteroides*, *Ruminococcus*) [5–8]. A case study indicated that the memory and mood of AD patients improved significantly following fecal microbiota transplantation from a healthy donor, suggesting a novel treatment strategy based on gut microbiota modulation for AD [9]. Studies in germ-free animals and in animals exposed to pathogenic microbial infections, antibiotics, probiotics, or fecal microbiota transplantation suggest the gut microbiota is an essential regulator of the immune system's function and the nervous system and may be critical in the control of neuroinflammation. The gut microbiota can influence the local nervous system (e.g., enteric nerves, vagus nerve) to quickly transmit signals to the brain. Furthermore, accumulating evidence suggests that metabolites (e.g., short-chain fatty acids, neurotransmitters, and their precursors) produced by bacteria affect the levels of related metabolites in the brain through the blood circulation, thus regulating brain functions and cognition [10, 11]. Certain bacteria, including *E. coli*, *Bacteroides*, *Eubacterium*, and *Bifidobacterium* are implicated in the secretion of AD-associated neurotransmitters (acetylcholine, γ-Aminobutyric acid: GABA, and glutamate). This observation highlights a potential connection between gut dysbiosis and neurotransmitter dysregulation in AD pathogenesis [12, 13]. Previous studies have reported that the changes in the levels of SCFAs, specifically butyric acid (BA) and iso-butyric acid (IBA), in the gut of AD mice correspond with alterations in the brain, potentially inducing excessive deposition of Aβ by activating microglia [14, 15].

In this study, microbial depletion induced by ABX ameliorated neuroinflammation, cognition and anxiety in 5×FAD mice by modulating the interactions between Aβ, microglia, and astrocytes, thereby confirming the causal relationship between gut microbiota disturbance and AD-like lesions. The detection of SCFAs and amino acids in feces, blood, and brain, clarified that gut-derived metabolites serve as a communication bridge within the "gut-brain" axis. Additionally, SCFA and amino acids in feces exhibited an aged-dependent increase during the progression of AD, suggesting that variations in microbial metabolism could serve as predictors of AD. Therefore, investigating the dysregulation of gut-derived metabolites in both the central nervous system and peripheral organs may yield novel insights into the molecular basis of AD.

Results

AD progression was associated with the alteration of gut microbiota

Cognitive deficiency in 5×FAD mice

A comprehensive evaluation of three behavioral outcomes revealed that cognitive impairment, as assessed by the Y maze task and the Morris Water Maze (MWM; see Fig. 1a and c), was accompanied by increased anxiety (containing the open field arena, Fig. 1b) in Tg-M mice. In the MWM test, Tg-M mice required significantly more time than WT-M mice to locate the platform during the training days (5 days, 4 trials per day and per animal). Furthermore, on day 6 of the probe trial, Tg-M mice exhibited poorer performance compared to WT-M mice, as evidenced by shorter time spent and reduced distance traveled in the target quadrant, along with fewer crossings over the platform. In the Y Maze test, Tg-M mice spent slightly less time in the novel arm compare to WT-M mice. Tycically, animals with heightened anxiety levels tend to spend less time exploring the center zone and produce more fecal pellets in the open field arena [16, 17]. However, contrary to this expectation, we observed that the duratioin Tg-M mice in the center zone was 2.4 times longer than that of WT-M mice, aligning with previous reported trends [18]. Additionally, Tg-M mice excreted more fecal pellets in the open field test compared to WT-M mice ($P<0.001$). During the rearing period, we noted that female 5×FAD mice (Tg-F mice) had lower body weights than Tg-M mice and exhibited higher mortality rates (6/17) and irritability (8/17). The irritable Tg-F mice displayed aggressive behaviors in the open field arena (Fig. 1a (Tg-F(b)). Tg-F mice performed

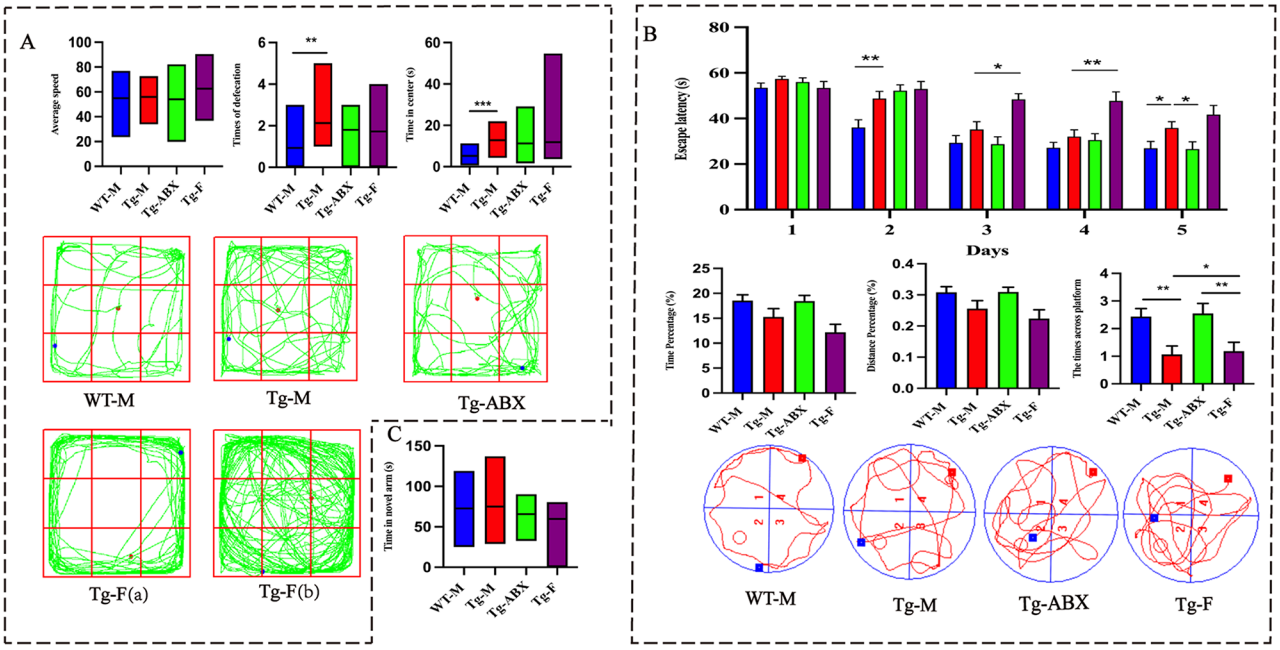

Fig. 1 Cognitive deficits and anxiety increased in AD mice. (**A**) Open field arena, (**B**) Morris Water Maze test, and (**C**) Y Maze, which were behavioral evaluation related to cognition and anxiety of 5×FAD mice. (**A**) Line at mean, floating from min to max. (**B**, **C**) values were expressed as mean ± SEM. WT-M: $n = 16$; Tg-M: $n = 16$; Tg-ABX: $n = 10$; Tg-F: $n = 11$. Differences were analyzed by Two-tested Student's t test and donated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

worse in a comprehensive evaluation of the three behavioral task, indicating that they exhibited greater cognitive deficits than Tg-M mice at the same age. Specifically, Tg-F mice demonstrated increased latency in the Morris Water Maze, longside shorter retention times and distance in the target quadrant.

The CNS neuroinflammation in 5×FAD mice

The 5×FAD transgenic mice, characterized with severely accelerated cognitive impairment, amyloid deposition by the 2_{nd} postnatal month, synaptic degeneration by the 4_{th} postnatal month, and behavioural changes in the 6_{th} month [19], are widely utilized in AD study. In line with these findings, a rapid accumulation of Aβ plaque deposition was observed in the hippocampus and cortex of male 5×FAD mice (Tg-M) compared to aged-matched wild-type (WT-M) mice (Fig. 2a, b). Aβ multiplex ELISA reveals significant elevations in all three Aβ species ($Aβ_{38}$, $Aβ_{40}$, $Aβ_{42}$) in both soluble and insoluble brain lysates from Tg-M compared to WT-M (Fig. 2d). Immunostaining for allograft inflammatory factor 1 (IBA-1) and glial fibrillary acidic protein (GFAP), markers of microglial and astrocyte activation respectively, demonstrated an increased number of microglia and astrocytes in closely proximity to Aβ plaques in 5×FAD mice (Fig. 2a, b, c). Reactive astrocytes, along with reactive microglia, contributed to neuroinflammatory burden and blood-brain barrier (BBB) dysfunction. WB or RT-PCR showed that major histocompatibility complex II (MHC class II) and inflammatory cytokines (IL-1α, TNF-α and MCP-1) were overexpressed in the CNF of Tg-M mice (Fig. 2e). Additionally, cytokines such as IL-1α, MCP-1, IL-6 and TNF-α, were found to impair microglial clearance functions [20, 21]. These results illuminate that the neuroinflammation observed in the CNF of 5×FAD mice result from insufficient clearance of Aβ accumulation by microglia [22] (Fig. 2d). Compared with the Tg-M mice, Tg-F mice showed more severe neuroinflammatory deficits. Immunofluorescence results showed that Tg-F mice had higher levels of Aβ, IBA-1, and GFAP in the brain.

Peripheral immune responses in 5×FAD mice

In addition to innate immunity, our results indicate that 5×FAD mice exhibit dysfunction in the peripheral immune system. Specifically, the ratios of Th1 ($CD3^{+}CD8^{-}INFγ^{+}$), Th2 ($CD3^{+}CD8^{-}IL4^{+}$), and Th17 ($CD3^{+}CD8^{-}IL17α^{+}$) cells in the spleens of Tg-M mice were significantly higher than those in WT-M mice (Fig. 3). However, the frequency of Treg cells ($CD4^{+}CD25^{+}Foxp3^{+}$) decreased. Th1 cells are considered to be detrimental factor in AD. Elevated levels of Th1 cells have been identified in the brain of APP/PS1 mice [23] and are dependent on senescence [24], which correlates with glial activation, increased expression of inflammatory cytokines, impaired cognitive function, and disrupted synaptic plasticity [25]. Th2 cells can inhibit Th1 cells, and under normal conditions, Th1 and Th2 cells maintain a relatively balanced state. However, when the body functions abnormally, a Th1/Th2 imbalance occurs. Our findings demonstrated that the increase

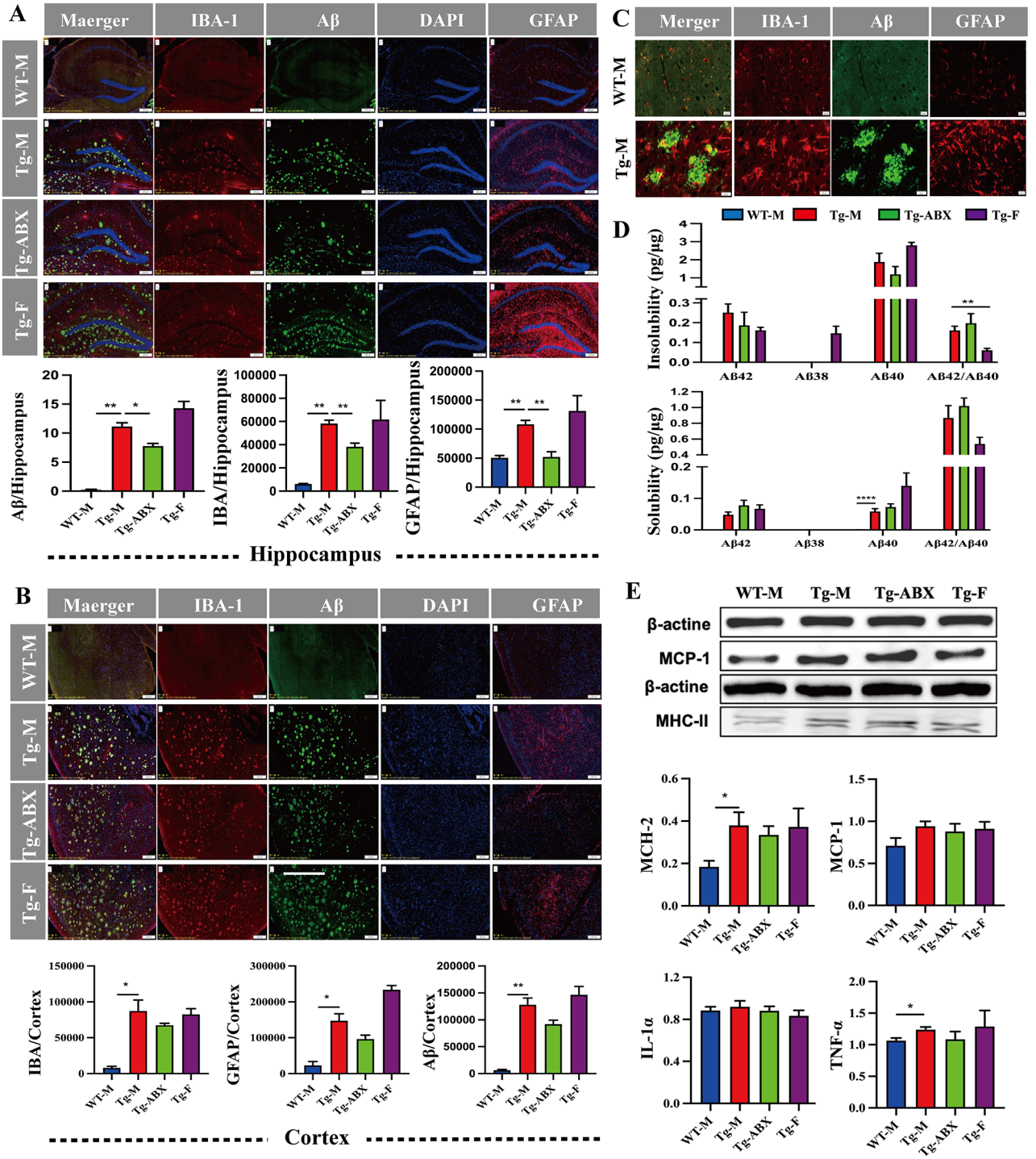

Fig. 2 Increased Aβ induces neuroinflammation. Representative images of microglia labeled with Iba-1 (red), reactive astrocytes labeled with GFAP (magenta), and senile plaques labeled with Aβ (green) in hippocampus (**A**) and cortex (**B**), $n = 3$; (**C**) Aβ plaques recruited many activated microglia and astrocyte, $n = 3$; (**D**) MSD Mesoscale® analysis of soluble and insoluble $Aβ_{38/40/42}$ levels in brain of 5×FAD mice, $n = 6–7$. (**E**) RT-PCR (IL-1α/TNF-α: $n = 6$) and Western-blot (MCH-2/MCP-1: $n = 4$). All values are expressed as mean ± SEM. Difference were analyzed by Two-tested Student's t test and donated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

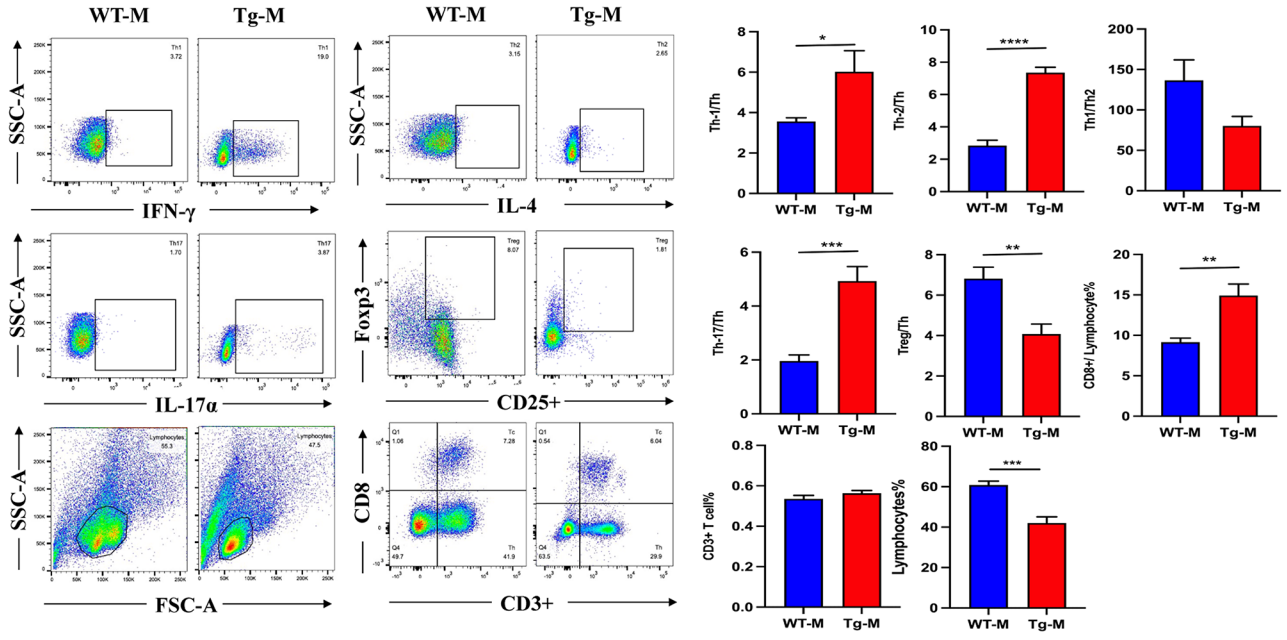

Fig. 3 Levels of immune cells in spleen of 5 x FAD mice. Differences were analyzed by Two-tested Student's t test and donated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 5

in Th1 cells levels in the peripheral spleens of Tg-M mice is accompanied by an increase in th2 cells, yet the Th1/Th2 ratio decreased compared to WT-M mice. Therefore, we hypothesize that Th1 cells in the peripheral spleens of 5×FAD mice promote an increase in peripheral inflammation, which subsequently stimulates excessive secretion of Th2 cells in an attempt to counteract the inflammation, leading to a Th1/Th2 imbalance in these mice. Treg cells serve as neuroprotective mediators in AD [26, 27]. Their transient depletion can accelerate cognitive impairment and facilitate the clearance of Aβ deposition by microglia [28, 29].

Antibiotic-induced perturbations in gut microbiome influenced cognition and neuroinflammation

Previous studies have suggested that gut microbiota plays a role in triggering neuroinflammation in the brain [30, 31]. Results from 16S ribosomal RNA (rRNA) gene amplicon sequencing indicated that the α-microbial diversity in feces of Tg-M mice increased (Fig. 4a), as evidenced by a significant rise in Chao ($p < 0.05$). Using partial least squares discrimination analysis (PLS-DA, Fig. 4b) revealed a remarkable shift in the gut microbiota composition between Tg-M and WT-M. To investigate the cause-effects of microbiome dysbiosis on neuroinflammation, Tg-M mice were administered ABX [gentamicin (1 mg/mL), vancomycin (0.5 mg/mL), metronidazole (2 mg/mL), neomycin (0.5 mg/mL), ampicillin (1 mg/mL), kanamycin (3 mg/mL), colistin (6000 U/mL), and cefoperazone (1 mg/mL) diluted in physiological saline] once every two days for six months. ABX treatment in Tg-M mice resulted in a sharp drop in α-diversity, reflecting in the significantly decreased chao and Shannon values and the dramatically increased coverage index (Fig. 4a). PLS-DA showed that the Tg-ABX administration groups were relatively independent, showing no overlap with other groups(Fig. 4b), indicating that the structural homeostasis of the intestinal flora in Tg-M mice has been disrupted. Concurrently, the aggregated forms of Aβ were reduced in the Tg-ABX mice, as shown by additional quantification of Aβ species via ELISA (Fig. 2d). As Aβ plaques decreased, there was also a reduction in the expression of activated microglia and astrocytes surrounding them (Fig. 2a-c), suggesting that ABX-treatment positively influences the microglial clearance function in Tg-M mice. Furthermore, ABX-treatment decreased the expression of MHC class II and inflammatory cytokine (IL-1α, TNF-α, and MCP-1) in the brain of Tg-M mice (Fig. 2e). The suppression of inflammatory cytokine production appears to reset microglial phagocytosis in AD mice [22]. ABX exhibited significant ameliorative effect on the cognitive impairment and anxiety (Fig. 1), as shown by the enhanced spatial learning and memory performance of Tg-M mice in the Morris Water Maze (MWM) task and reduced fecal pellets in the open field test. These results demonstrate that the"gut-brain" axis can transmit information bidirectionally: from the brain to the gut (top-down) and from the gut to the brain (bottom-up). BugBase phenotype prediction analysis indicated that levels of aerobic bacteria (Fig. 4c), mobile elements, and gram-positive bacteria were increased in Tg-M mice compared to WT-M mice.

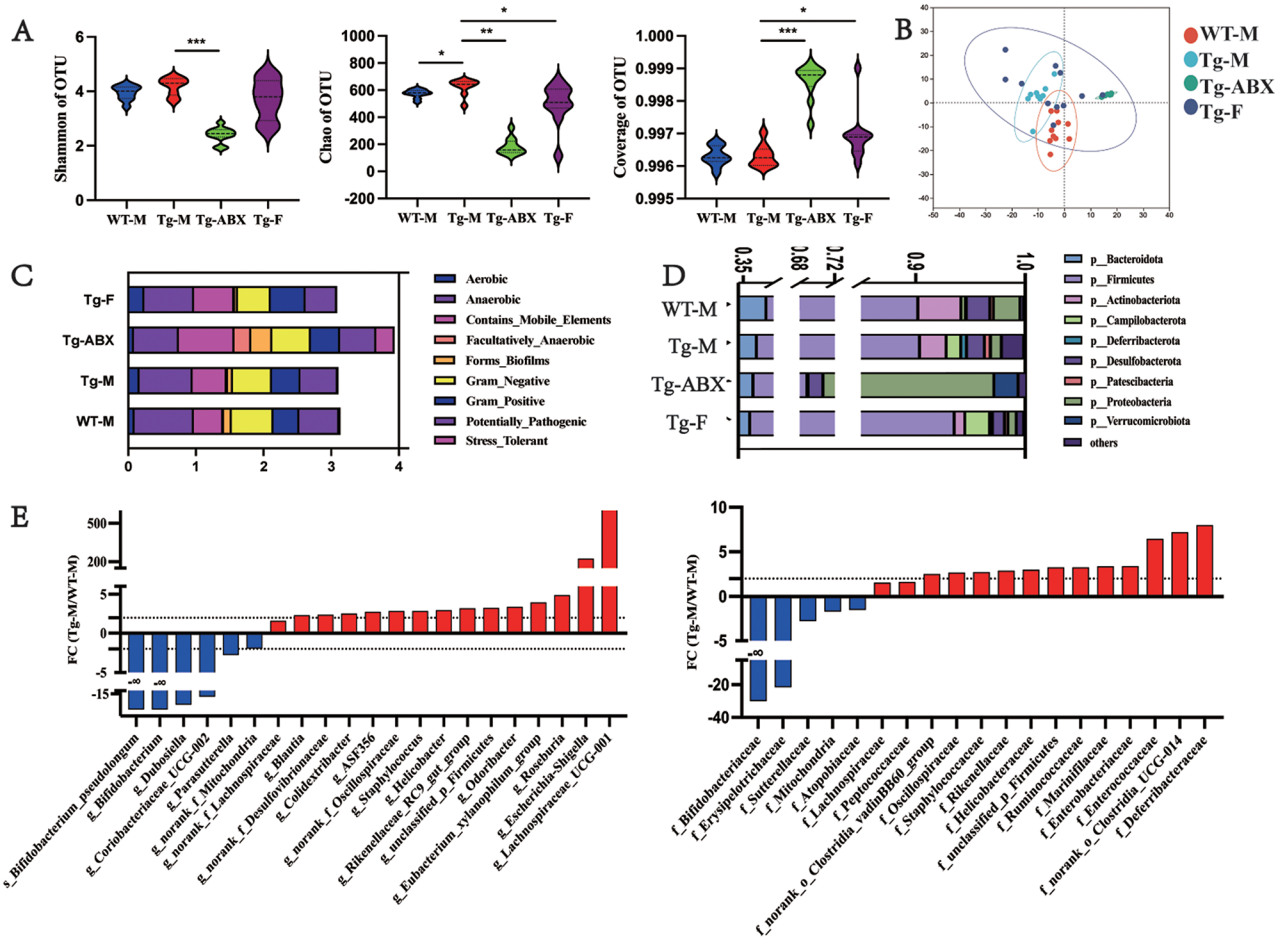

Fig. 4 Alterations in gut microbial diversity. (**A**) Diversity of fecal microbiota, including shannon, chao, coverage index. (**B**) PLS-DA plot. (**C**) BugBase phenotype prediction analysis. (**D**) Bacterias at phylum, (**E**) genus and family with relative abundance greater than 0.001%, red mean: Tg-M/WT-M > 1.5; blue mean: Tg-M/WT-M < 1.5. (**A**) Line at mean, floating from min to max. $n = 9–11$. Differences were analyzed by Two-tested Student's t test and donated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Additionally, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [32] revealed that the functional pathways in Tg-M mice were richer than those in WT-M mice, encompassing amino acid, carbohydrate and energy metabolism, as well as immune system function (Table 1).

Changes in gut-derived metabolites related to amino acid, carbohydrate, and energy metabolism

SCFAs, produced from fermentation of dietary fiber by the gut microbiota, have been suggested to modulate energy metabolism, and are believed to play a significant role in microbial-gut-brain interactions. Analysis of results based on OUT at the family and genus level revealed substantial fluctuations in the abundance of SCFAs-producing bacteria in the feces of Tg-M mice. Compared to WT-M mice (Fig. 4e), the proportion of *Erysipelotrichaceae*, *Bifidobacteriaceae*, *Bifidobacterium*, *Bifidobacterium_pseudolong*, *Parasutterella* in Tg-M mice decreased markedly (Fig. 3e). Conversely, the populations of *Odoribacter*, *Ruminococcaceae* (0.32-1.04%), and *Lachnospiraceae* (11.09-17.22%) were more abundant in Tg-M mice than in WT-M, particularly the family of *Lachnospiraceae*. GC-MS/MS results indicated a decrease in the level of Picolinic acid (PA) and an increase in the level of IBA in the feces of Tg-M mice compared to WT-M mice. Additionally, we conducted a longitudinal study of SCFAs in the feces of mice. The results showed that fecal-SCFAs in Tg-M mice aged 5 to 9 months fluctuated slightly over time but increased rapidly from 9 to 11 months (Fig. 5a). Studies have reported that the gut microbiota in AD model mice exhibits high dynamism during the growth process [22, 33, 34]. Therefore, the underlying rationale for the dynamic changes in SCFA in Tg-M mice feces can be attributed to the corresponding dynamic changes in intestinal microbes.

Gut microbiota affected the bioavailability of amino acids to the host. SCFAs primarily originate from the fermentation of carbohydrates that escape digestion in the gut. However, on conditions where insoluble fiber

Table 1 PICRUSt analysis. The generated OTU table was imported into the PICRUSt and the Kyoto Encyclopaedia of genes and genomes (KEGG) database was used to predict the functional gene content of the various microbial communities represented in the Greengenes database of 16 S rRNA gene sequences

	FC (/Tg)		
	WT-M	Tg-ABX	Tg-F
Amino acid metabolism	0.85	1.94	1.07
Arginine and proline metabolism	0.86	2.45	1.05
Arginine biosynthesis	0.83	2.13	1.06
Cysteine and methionine metabolism	0.81	1.69	1.10
Lysine biosynthesis	0.82	1.48	1.13
Glycine, serine and threonine metabolism	0.83	1.73	1.11
Lysine degradation	0.87	2.30	1.04
Valine, leucine and isoleucine biosynthesis	0.82	2.06	1.03
Valine, leucine and isoleucine degradation	0.87	2.27	1.08
Alanine, aspartate and glutamate metabolism	0.90	1.85	1.09
Tryptophan metabolism	0.86	3.06	1.02
Tyrosine metabolism	0.84	1.90	1.12
Carbohydrate metabolism	0.85	2.10	1.15
Propanoate metabolism	0.75	1.97	1.15
Butanoate metabolism	0.85	1.86	1.09
Pyruvate metabolism	0.82	1.80	1.11
Pentose and glucuronate interconversions	0.81	3.51	1.17
Citrate cycle (TCA cycle)	0.85	2.37	1.00
C5-Branched dibasic acid metabolism	0.83	2.12	1.05
Ascorbate and aldarate metabolism	0.80	3.15	1.09
Glycolysis / Gluconeogenesis	0.83	1.71	1.17
Pentose phosphate pathway	0.86	2.00	1.16

	FC (/Tg)		
	WT-M	Tg-ABX	Tg-F
Endocrine system	0.87	1.78	1.14
Insulin signaling pathway	0.75	1.62	1.10
Prolactin signaling pathway	0.76	1.10	1.24
Glucagon signaling pathway	0.82	1.46	1.22
Energy metabolism	0.86	2.04	1.06
Sulfur metabolism	0.78	2.84	0.98
Carbon fixation pathways in prokaryotes	0.85	2.14	1.04
Methane metabolism	0.84	2.18	1.14
Oxidative phosphorylation	0.87	1.96	1.03
Immune system	0.77	1.62	1.06
NOD-like receptor signaling pathway	0.72	1.31	1.07
RIG-I-like receptor signaling pathway	0.29	1.56	1.45
Lipid metabolism	0.86	1.83	1.12
Fatty acid biosynthesis	0.76	1.68	1.08
Fatty acid degradation	0.85	1.80	1.13
Glycerolipid metabolism	0.80	1.50	1.19
Glycerophospholipid metabolism	0.87	1.92	1.13
Neurodegenerative disease	0.82	1.94	0.93
Alzheimer disease	0.81	1.33	1.01

The generated OTU table was imported into the PICRUSt and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database was used to predict the functional gene content of the various microbial communities represented in the Greengenes database of 16S rRNA gene sequences.

or resistant starch is deficient, amino acids can also act as precursors for the SCFA synthesis by bacteria [23], suggesting an interplay between microbial activity and homeostasis of host amino acids and SCFA. Consequently, 22 types of neurotransmitters (including amino acid and choline) were detected in the feces of Tg-M mcie using LC-QQQ-MS. Overall, the levels of neurotransmitters in Tg-M mice at 11 months were higher than those in WT-M mice (Fig. 6a), particularly for phenylalanine, methionine, tyrosine, leucine, proline, tryptophan, threonine, aspartic acid, histidine, arginine, and L-cysteine ($p<0.05$). However, the concentration of GABA in the feces of Tg-M mice at 11 months was lower than that in WT-M mice. Correlation analysis (Fig. 6b) showed that most of the amino acids in feces were positively correlated with SCFAs, such as IBA, BA, isovaleric acid (IVA), valeric acid (VA) and hexanoic acid (HA).

A longitudinal study demonstrated that the levels of essential amino acids (lysine, methionine, tryptophan, threonine), semi-essential amino acids (arginine,

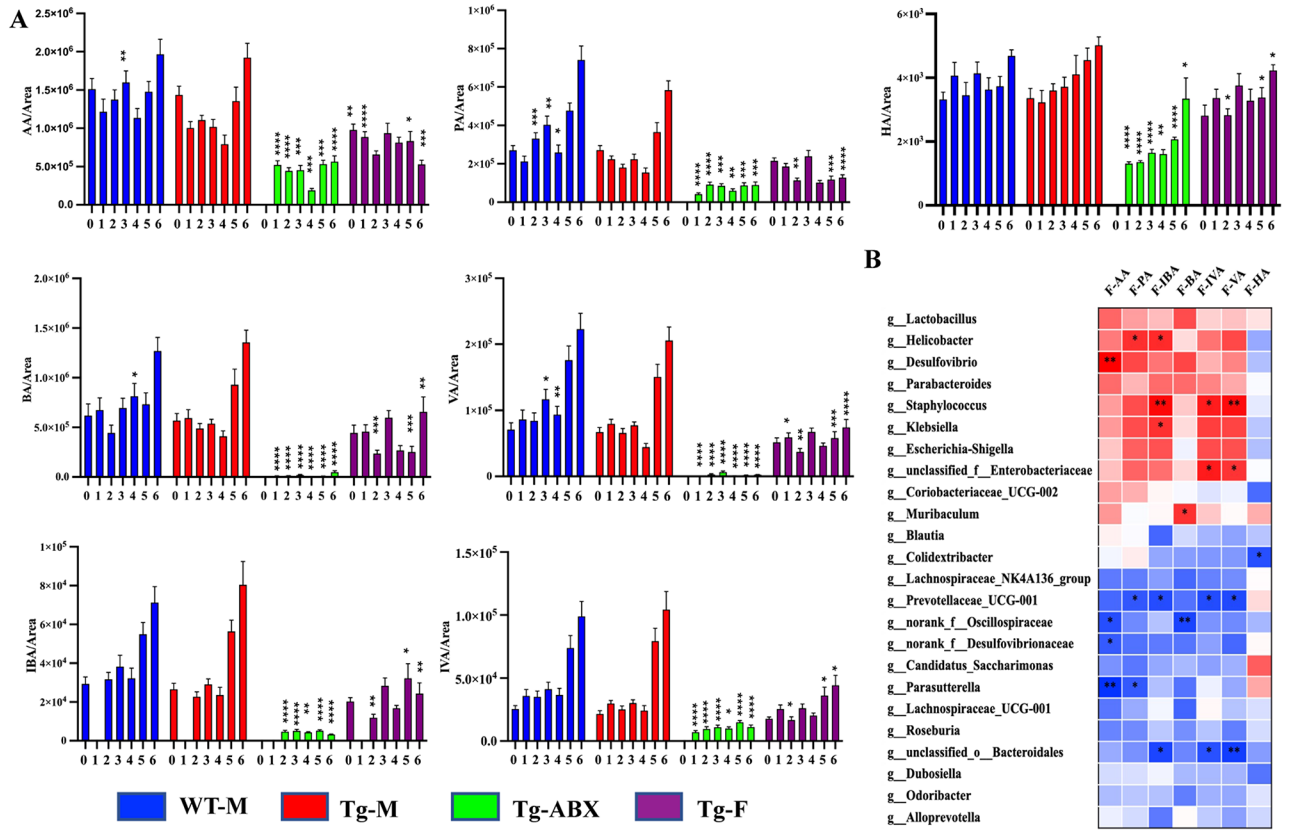

Fig. 5 Alterations of SCFAs in feces of 5 x FAD mice with age. (**A**) SCFAs of feces changed in a senescence dependent manner. Differences were analyzed by Two-tested Student's t test and donated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$. n = 10–15. Acetic acid: AA; Propionic acid: PA, Butyric acid: BA; Valeric acid: VA; Iso-valeric acid: IVA; Hexanoic acid: HA; Isobutyric acid: IBA

histidine), excitatory amino acids (aspartic acid, glutamic acid), aromatic amino acids (tyrosine) and other amino acids (proline, serine) in the feces of Tg-M mice aged 5 to 11 months were higher than those in WT-M mice. Furthermore, these levels increased gradually compared to age-matched WT-M mice (Fig. 6a). In contrast, the content of taurine in the feces of Tg-M mice was lower than that of WT-M mice, although it gradually approached the levels observed in WT-M mice as the aged. The level of inhibitory amino acid (GABA) in feces of Tg-M mice was lower than that of WT-M mice and reached the highest concentration in 7th and 9th month, respectively.

Gut-derived metabolites served as intermediaries for communicating the brain-gut axis

Gut-derived metabolites can enter the blood stream and be transported to various parts of the body, leading to alterations in brain function and influencing cognition in neurological diseases such as Alzheimer's disease [35]. Thus, GC-QQQ-MS and LC-QQQ-MS were used to quantify the levels of SCFAs and neurotransmitters in the CNS and peripheral blood of Tg-M mice (Fig. 7, Fig. S1a and b). Although the magnitude of SCFAs variation in Tg-M mice was not unexpected, it is noteworthy that the SCFAs in the brain of Tg-M mice exhibited a positive correlation with microglia activation in hippocampus and cortex. Interestingly, the levels of IBA were increased in the feces, serum, and brains of Tg-M mice compared to WT-M mice. Furthermore, the concentration of IBA in the brains of Tg-M mice was highly correlated with Aβ in the hippocampus and cortex, as well as pro-inflammatory factors (MCH-2, IL-1α, and TNF-α) in the brain (Fig. s1c). Additionally, we observed that the levels of alanine ($p<0.001$), tryptophan ($p<0.05$), 5-HIAA, xanthurenic acid (XA), kynurenine (Kyn), kynurenic acid (KA), and taurine ($p<0$ 0.001) in the peripheral blood of Tg-M mice were higher than those in WT-M mice. Meanwhile, the concentrations of alanine, methionine, choline, proline, iso-leucine and GABA in the brain of Tg-M mice were also significantly increased compared to WT-M mice.

Cerebral glucose hypometabolism, characterized by impaired glucose uptake and utilization related to brain insulin resistance [36, 37], along with progressive mitochondrial dysfunction associated with aging [38] has recently been associated with AD. PICRUSt predicted energy metabolism disorders in Tg-M mice based on intestinal microbiota function. Therefore, LC-QQQ-MS was used to further investigate the metabolites involved

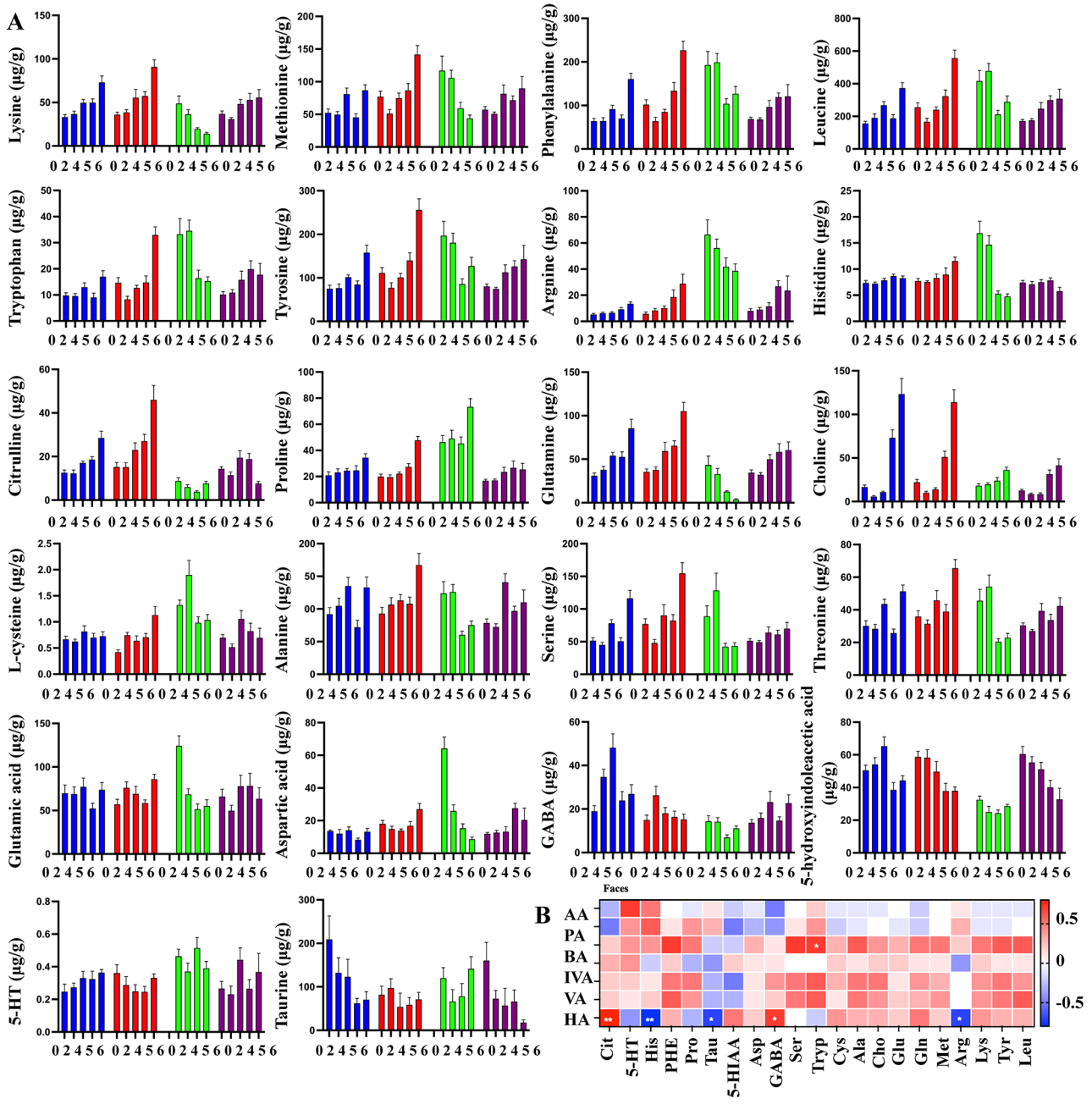

Fig. 6 Alterations of gut-derived metabolites in feces of 5×FAD mice with age. (**A**) gut-derived metabolites of feces changed in a senescence dependent manner. (**B**) Correlation analysis between gut-derived metabolites and SCFAs. $n = 6–9$. Difference was analyzed by Two-tested Student's t test and donated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. 5-OH indoleacetic acid: 5 HIAA; 5-hydroxytryptophan: 5 HT; Acetic acid: AA; Alanine: Ala; Arginine: Arg; Aspartic acid: Asp; Butyric acid: BA; Citrulline: Cit; Choline: Cho; Glutamic acid: Glu; Glutamine: Gln; Histidine: His; Hexanoic acid: HA; Iso-valeric acid: IVA; Iso-butyric acid: IBA; Lysine: Lys; Leucine: Leu; L-Cysteine: L-Cys; Methionine: Met; Propionic acid: PA; Phenylalanine: PHE; Proline: Pro; Serine: Ser; Taurine: Tau; Tryptophan: Trp; Tyrosine: Tyr; Valeric acid: VA

in the central and peripheral TCA cycle of Tg-M mice. As illustrated in Fig. 7a, the levels of pyruvic acid significantly increased in the brain of Tg-M mice compared to WT-M mice, whereas the concentrations of citric acid and succinic acid were significantly decreased. In terms of TCA metabolites in peripheral blood, the levels of pyruvic acid, lactic acid, and malic acid were higher than those observed in WT-M mice, while the content of succinic acid was lower. Notably, the trends in the levels of pyruvic acid, lactic acid, succinic acid, and malic acid in the periphery were consistent with those in the brain. Previous studies have indicated that impaired mitochondrial function exacerbated by hypoxia in the brain, can result in increased lipid peroxidation [39], which

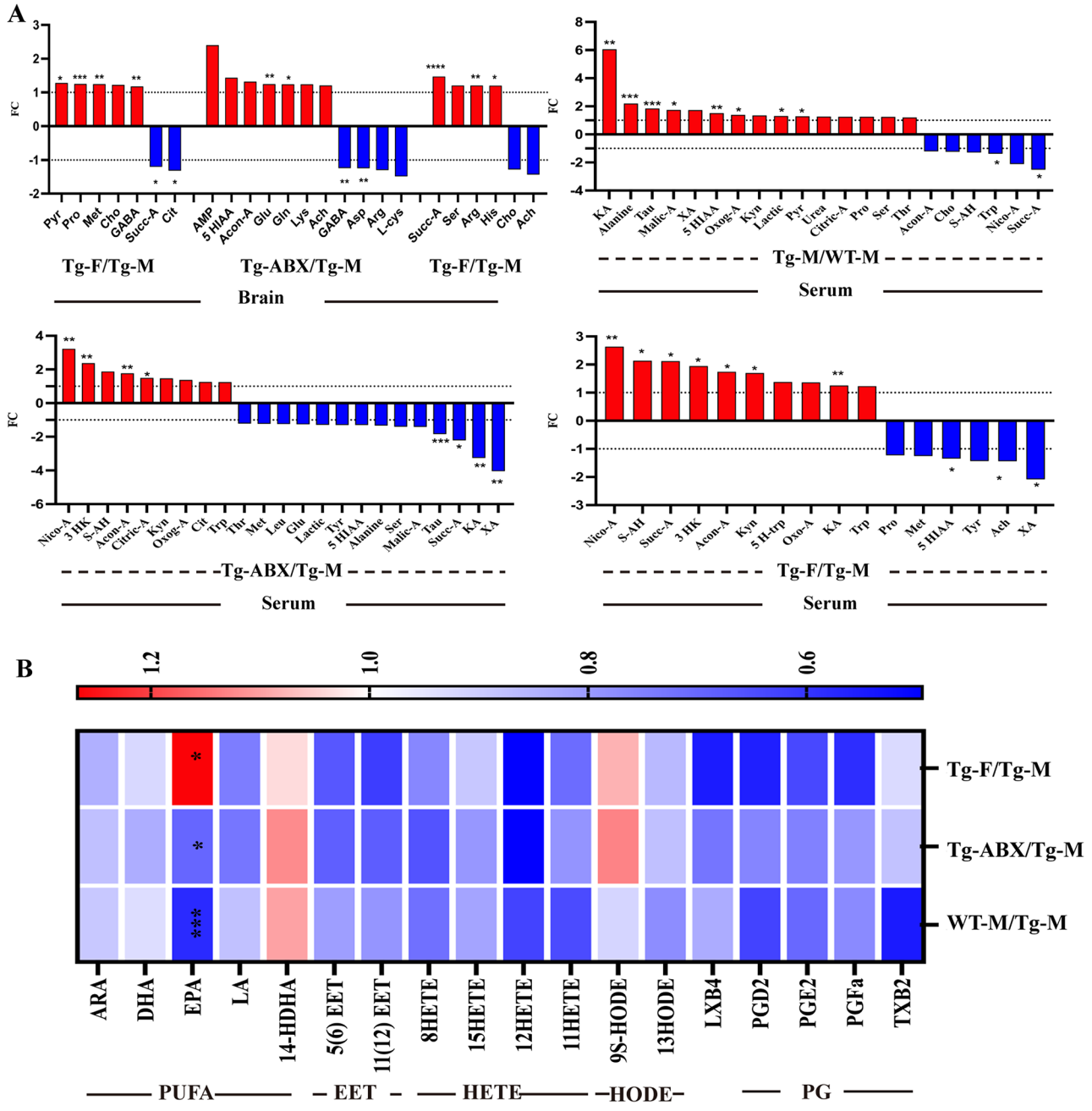

Fig. 7 Changes of endogenous metabolites in peripheral blood and brain of 5×FAD mice. (**A**) Gut-derived metabolites (including amino acid, TCA, neurotransmitters) with FC > 1.2 in the brain and serum. (**B**) Changes in PUFA and lipid peroxide in the brain. $n = 6–8$. Difference was analyzed by Two-tested Student's t test and donated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 5-Hydroxy-DL-tryptophan: 5 H-trp; 5-OH indoleacetic acid: 5 HIAA; Acetyl choline: Ach; Aconitic acid: Acon-A; Arginine: Arg; Arachidonic: Ara; Adenosine monophosphate: AMP; Asparaginate: Asp; Citrulline: Cit; Choline: Cho; Citric acid: Citric-A; Docosahexanoic: DHA; Eicosapentaenoic acid: EPA; Epoxy eicosenotricoic acid: EETs; Glutamic acid: Glu; Glutamine: Gln; Histidine: His; Hydroxyeicosaenoic acids: HETEs; Hydroxyoctadecadienoic acid: HODE; Kynurenine: Kyn; Kynurenic acid: KA; L-3-hydroxykynurenine: 3 HK; Lysine: Lys; Leucine: Leu; L-Cysteine: L-Cys; Malic acid: Malic-A; Methionine: Met; Nicotinic acid: Nico-A; Oxoglutaric acid: Oxog-A; Phenylalanine: PHE; Picolinic acid: PA; Proline: Pro; Pyruvic acid: Pyr; Prostaglandins: PGs; S-adenosyl homocysteine: S-AH; Succinic acid: Succ-A; Serine: Ser; Taurine: Tau; Tryptophan: Trp; Threonine: Tre; Tyrosine: Tyr; Xanthurenic acid: XA; γ-Aminobutyric acid: GABA

aligns with ourding of elevated hydroxyeicosaenoic acids (HETEs), prostaglandins (PGs) and epoxy eicosatrienoic acid (EETs) in Tg-M mice (Fig. 7b).

Huanglian Jiedu decoction (HLJDD) alleviated neuroinflammation by shaping the intestinal microbiome metabolism

The essential role of gut microbiota metabolome in AD progression revealed herein may suggest the therapeutic implications by the intervention of gut microbiota. To test this hypothesis, male 5×FAD mice, aged 5-month-old, were administered with HLJDD (H-L: 172 mg/kg/day; H-H: 344 mg/kg/day) for six months until 11-month-old. Comprehensive analysis of survival status, behavioral evaluation and biochemical indicators revealed significant differences in AD-like symptoms between males and females. Studies with gender differences may result in relatively discrete data, which cannot accurately reflect the efficacy of drugs. Therefore, male 5×FAD mice with relatively stable survival state were selected as the model mice used in this part study. HLJDD exhibited ameliorative effect on the cognitive impairment and anxiety of 5×FAD mice (Fig. 8), as evidenced by the enhanced spatial learning and memory performance in the MWM test, as well as reduced defecation in the open field arena. Forthermore, HLJDD alleviated neuroinflammation (Fig. 9) in 5×FAD mice by reducing the secretion of inflammatory factors (IL-1α and INF-γ), which were induced by the dysregulation of the interaction between Aβ, and innate immune cells (astrocytes and microglia). The effects of Ber, Bai, and Mix in improving cognition and reducing neuroinflammation in 5×FAD mice were like to those of HLJDD.

Our previous study involving APP/PS1 mice indicated that the potential therapeutic mechanism of HLJDD in AD is closely associated with the "gut-brain" axis [40]. The present study demonstrated that HLJDD remodeled the intestinal microbial structure of 5×FAD mice (Fig. s2), although it did not affect α-diversity. Notably, α-diversity in the Ber, Bai, and Mix groups was lower than that in HLJDD groups, particularly in the Ber group. Furthermore, we found HLJDD could regulate SCFA-producting bacteria in the feces of 5×FAD mice (Table S3-4), such as increasing the abundance of *f_Butyricicoccaceae*, *f_Mitochondria*, *g_Prevotellaceae_UCG_001* and *g_Bifidobacterium*, and decreasing the levels of *g_Odoribacter*, *g_Parabacteroides*, *g_Rikenellaceae_RC9_gut_group* and *g_Blauria*. To explore whether HLJDD improves neuroinflammation by regulating microbial derived metabolites, SCFAs and other gut-derived metabolites in the feces, blood, and brain of 5×FAD mice were detected by GC-MS and LC-MS respectively. HLJDD down-regulated the overall level of SCFAs in the feces of 5×FAD mice in a dose-dependent manner (Fig. s3a). The effect of the Ber group on reducing SCFAs in feces was more pronounced than in the other groups. Correlation analysis showed that the levels of SCFAs in feces of HLJDD group were negatively correlated with *g_Lactobacillus*, and positively correlated with *g_Lachnoclostridium* and *g_Parabacteroides* (Fig. s3b). Previous studies have found

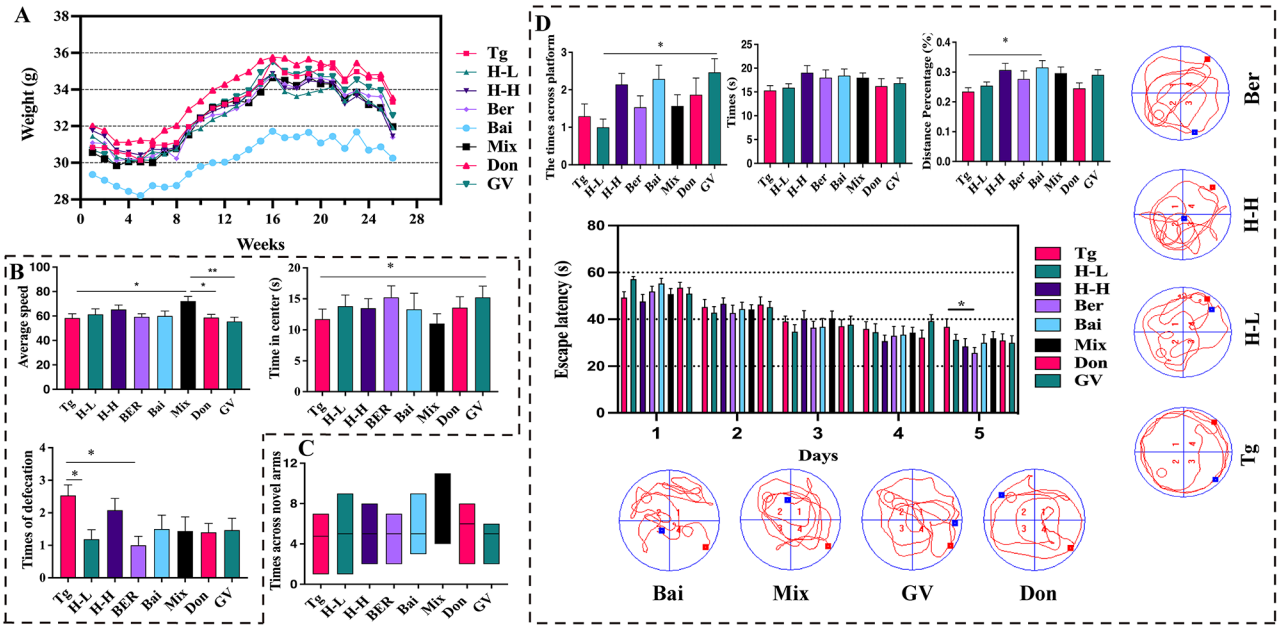

Fig. 8 HLJDD ameliorates cognitive deficiency in APP/PS1 mice. (**A**) Changes in body weight of 5×FAD mice during drugs intervention. (**B**) Open field arena, (**C**) Y Maze, and **D**) Morris Maze Water test, which were behavioral evaluation related to cognition and anxiety of 5×FAD mice. $n = 14–17$. All the values were expressed as mean ± SEM. All data were analyzed by one-way ANOVA with Dunnett-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

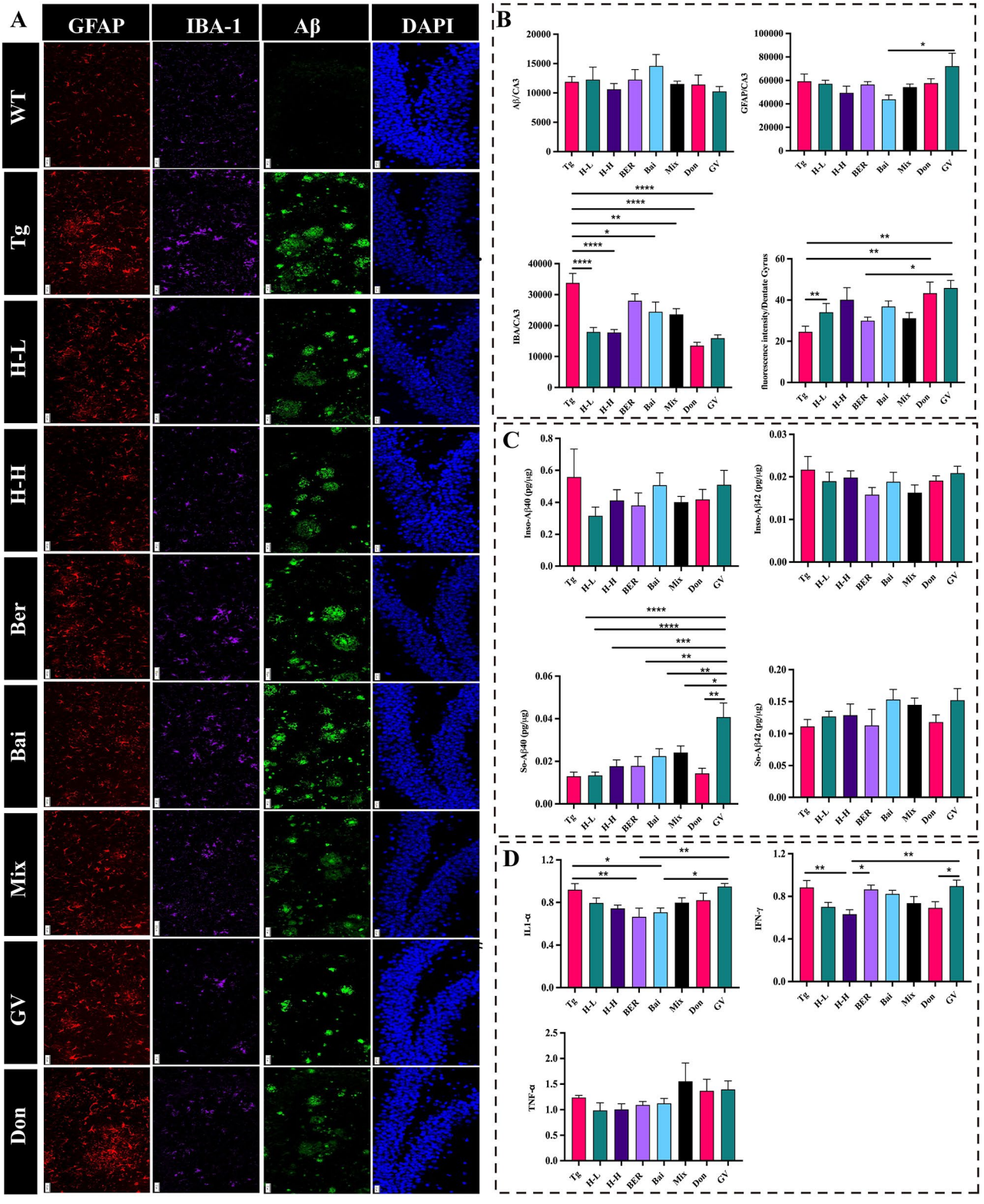

Fig. 9 HLJDD alleviated neuroinflammation in 5×FAD mice. (**A**) Representative images of microglia labeled with Iba-1 (purple), reactive astrocytes labeled with GFAP (red), and senile plaques labeled with Aβ (green) in hippocampus, $n=4$. (**B**) The levels of dentate gyrus, Aβ, Iba-1, and GFAP in the hippocampus were quantified by detecting the value of Integrated option density (IOD), $n=4$. (**C**) MSD Mesoscale® analysis of soluble and insoluble $A\beta_{38/40/42}$ levels in brain of 5×FAD mice, $n=6$–9. (**D**) RT-PCR (IL-1α, TNF-α, INF-γ), $n=6$. All the values were expressed as mean ± SEM. All data were analyzed by one-way ANOVA with Dunnett-test; *$p<0.05$, ** $p<0.01$, ***$p<0.001$

that IBA was positively associated with neuroinflammation, and HLJDD could reverse the levels in feces, peripheral blood, and brain of 5×FAD mice. HLJDD increased the levels of choline in 5×FAD mice feces and decreased the contents of Trp, GABA, Taurine, and leucine (Fig. s4). The levels of gut-derived metabolites in feces of the Mix group were highest compared with the others. After HLJDD intervention, the levels of endogenous metabolites in the brain of 5×FAD mice showed little fluctuation. However, HLJDD reduced the activity of Trp-Kyn metabolism in the peripheral of 5×FAD mice, specifically by reducing Trp, KA, XA. In addition, HLJDD also played a great regulatory role in peripheral immunity of 5×FAD mice by decreasing Th1 and Th2 cells and increasing Treg cells (Fig. s5).

Discussion

The interaction between the intestine and the brain can occur through the nervous system or via chemical substances crossing the BBB. Previous studies have indeed demonstrated this correlation, although they did not establish a direct cause-effect [40, 41]. Our study revealed that the overexpression of Aβ and neuroinflammation in Tg-M mice disrupted the structure of microflora. A marked reduction in microbial abundance in 5×FAD mice's gut caused by antibiotic mix (ABX) in Tg-M mice in turn alleviated the cognition and anxiety. Meanwhile, ABX ameliorated neuroinflammation of Tg-M mice by modulating the interactions between Aβ, microglia, and astrocytes, suggesting that alterations in intestinal flora structure could influence host innate immunity mechanisms that impact Aβ amyloidosis. These results verified that"gut-brain" could transmit information bidirectionally: from the brain to the gut (top-down) and from the gut to the brain (bottom-up). It has been proposed that AD lesions may arise from the propagation of toxic, misfolded Aβ protein from the gut to the brain. Therefore, understanding the metabolic "code" of gut microbiota provides a novel perspective for elucidating the mechanisms underlying the "gut-brain" axis in AD, thereby paving the way for new drug development strategies.

Dysbiosis of the gut microbiota was required for the infiltration of peripheral immune cells to the brain

Dynamic shift of gut microbiota composition during AD progression is significantly correlated with the elevation of Th1 cell infiltration, which further promotes Th1/M1 microglia-mediated neuroinflammation [42]. Gut-initiated adaptive immune response can impair brain function via circulating IL-17 [43]. *Bifidobacteriaceae* and *Lachnospiraceae* may be key bacteria that promote peripheral immune system dysregulation due to their dramatic decline in the feces of 5×FAD mice. *Bifidobacteriaceae* Feedings (*B. longum*) regulated the body's immune function by regulating the Th1/Th2 and Th17/Treg balance, and the expression of tight junction protein (Claudin1 and Occludin) [44]. *Lachnospiraceae* are robust butyrate-producers [45, 46] and this may induce alterations in T-cell differentiation [47, 48] and microglial function [49]. In addition, gut-derived metabolites played crucial roles in the balance between intestinal immune tolerance and microbiota maintenance. SCFAs have been shown to maintain intestinal homeostasis through protecting epithelial barrier integrity [50], promoting B-cell IgA production [51], and regulating T-cell differentiation [52]. Our study revealed that the abundances of alanine, methionine, phenylalanine and proline were increased in the feces and blood of Tg-M mice compared to that of WT-M mice. Functional assessment both in vitro and in vivo revealed the role of phenylalanine in promoting both differentiation and proliferation of peripheral inflammatory Th1 cells [42]. L-methionine-enriched diet causes neurotoxic effects in vivo and might contribute to the appearance of Alzheimer's-like neurodegeneration [53]. Accumulation of lactate in the tissue microenvironment is a feature of inflammatory disease. An increased in central and peripheral lactate of Tg-M mice may act specifically on $CD4^+$ T cells to induce a reshaping of their effector phenotype, resulting in increased IL17 production via nuclear PKM2/STAT3 and enhanced fatty acid synthesis [54]. In addition, PICRUSTs analysis thought that the disorder of intestinal flora affected immune system, carbohydrate and energy metabolism. Therefore, we speculated that disorders of gut microbiota deteriorated neuroinflammation by disrupting immune balance in the periphery and accelerating the infiltration of Th1 and Th17 cell.

Intestinal microbes were responsible for neuroinflammation through the fermentation of indigestible carbohydrate into SCFAs

SCFAs, as a messenger characterized with penetrating the BBB, have recently received unprecedented attention in the pathogenesis of AD. This study reports that IBA was prone to act on Aβ and was expected to be a marker for predicting AD lesions. The levels of IBA in feces, serum, and brain of Tg-M mice were increased compared to WT-M mice, particularly in serum. Furthermore, IBA levels in the brain exhibited a positive correlation with Aβ and pro-inflammatory factors. Additionally, other SCFAs in the brain of Tg-M mice were positively correlated with microglia activation in hippocampus and cortex. Clinical studies suggested that a correlation between amyloid load and blood SCFAs concentration [55]. Supplementation with SCFAs results in increased microglial activation [15]. Antibiotic ABX induced SCFAs deletion reduced microglial activation and Aβ plaque load, which was in accordance with previous study [15]. Thus, we proposed

that SCFAs mediate the signal transmission between the "gut-brain" axis and accelerates neuroinflammation of AD by activating microglia. It was surprising that antibiotic ABX dramatically reduced fecal SCFAs in Tg-M mice, but regulated SCFAs in peripheral blood and brain much less than in the gut. The gut lumen is the major site of production but the concentration gradient falls from the lumen to the periphery [56]. SCFAs can cross the BBB, their penetration is limited. Therefore, even subtle changes in SCFAs levels in the brain of male 5×FAD mice hold significant implications [57].

L-tryptophan played crucial roles in the balance between gut microbiota maintenance and neuroinflammation

Tryptophan hydroxylase (TH1) serves as a key rate-limiting enzyme in the synthesis of 5-HT, which can be stimulated by SCFAs [58, 59]. Our findings revealed that Trp-5 HT metabolites in the feces of Tg-M mice correlate with SCFAs-producing bacteria and SCFA, highlighting the critical role of gut microbiota in regulating enteric 5-HT production and homeostasis via SCFAs [58]. Preclinical evidence underscores the significance of the Trp-5-HT pathway in addressing cognitive decline and neuropathological changes associated with AD [7, 59]. In AD, there is a notable reduction in 5-HT neurons, which corresponds with decreased levels of 5-HT and its metabolite 5-HIAA [60]. Interestingly, 5-HIAA has been shown to stimulate neprilysin activity/expression, counteracting Aβ peptide-induced neurotoxicity [60]. Compared to WT-M mice, Tg-M mice exhibited a slight decrease in brain levels of 5-HT and 5-HIAA, while peripheral blood levels of 5-HIAA significantly increased, and Trp levels markedly decreased. This discrepancy might be attributed to increased BBB permeability in AD, facilitating the influx of neurotoxic substances, including 5-HIAA, from the blood into the brain, triggering inflammatory and immune responses [61].

Moreover, over 90% of Trp is metabolized via the kynurenine pathway (KP), with epidemiological evidence linking KP activation to an elevated risk of dementia [62]. Our study aligns with clinical observations in AD and mild cognitive impairment (MCI), revealing increased serum kynurenine levels and kynurenine-to-tryptophan (K: T) ratios in 5×FAD mice [63, 64]. Unexpectedly, we observed an increase in neuroactive metabolites downstream of the KP, such as xanthurenic acid (XA) and kynurenic acid (KYNA), in the peripheral blood of Tg-M mice, which deviates from conventional reports [65, 66]. KYNA is believed to be a neuroactive substance that may protect cognitive function through its anti-inflammatory and antioxidant activities [67, 68]. However, there are still different voice. Pocivavsek et al. reported that KYNA levels in AD patients were positively correlated with cognitive deficits, suggesting that this may represent a compensatory protective mechanism [69].

In Alzheimer's disease (AD), a shift from aerobic respiration to less efficient anaerobic fermentative metabolism was accompanied

Pyruvate, the end-product of glycolysis, can subsequently be oxidized to CO2 and water through the TCA cycle for energy production, or converted to lactate under anaerobic conditions by lactate dehydrogenase [70]. Our study revealed a significant increase in pyruvate with levels in both the brain and peripheral blood of Tg-M mice compared to WT-M mice, which was accompanied by an elevation in lactate and a decrease in TCA cycle metabolites. Lactate, serving as an alternative neuronal energy substrate under conditions unfavorable for aerobic metabolism, can cross the BBB via the monocarboxylate transporter 1 (MCT1), emphasizing its critical role in neuronal energy supply and memory formation [71, 72]. The observed increase in lactate levels in both of the brain and periphery of Tg-M mice reflects trends seen in aging models [73, 74] and in AD patients [75]. Furthermore, the reduction in TCA cycle metabolites, including citrate and succinic acid, suggests compromised mitochondrial respiration and TCA cycle activity in Tg-M mice [76, 77], as evidenced by decreased activities of enzymes such as succinate dehydrogenase (SDH) and the pyruvate dehydrogenase complex (PDC) [78]. The shift from aerobic to anaerobic metabolism marked a pivotal metabolic reorientation in Tg-M mice, indicating a decrease in glucose utilization [79, 80]. The study further elucidates that hypoxia-induced mitochondrial dysfunction in the brain of 5×FAD mice escalates lipid peroxidation, with hydroxyeicosaenoic acids (HETEs), prostaglandins (PGs), and epoxyeicosenotricoic acids (EETs) as critical mediators of inflammation, neurotoxicity, and cell death [81, 82]. Collectively, our investigation into the metabolic landscape of male 5×FAD mice reveals a transition from glucose-centric bioenergetics to an alternative strategy that relies on lactic acid metabolism and lipid peroxidation.

Certain variations in gut microbes are thought to be predictors of longevity in humans

Previous research suggested that mechanisms leading to AD may originate from dysbiosis of the intestinal microbiome, which subsequently triggers local and systemic inflammation and disrupts the "gut-brain" axis [83]. Give that age is the primary risk factor for AD, investigating age-related changes in intestinal metabolites could provide valuable insights into the mechanisms linking gut dysbiosis with AD pathogenesis. However, the specific alterations in enterogenous metabolites during the transition from adulthood to senescence remain poorly

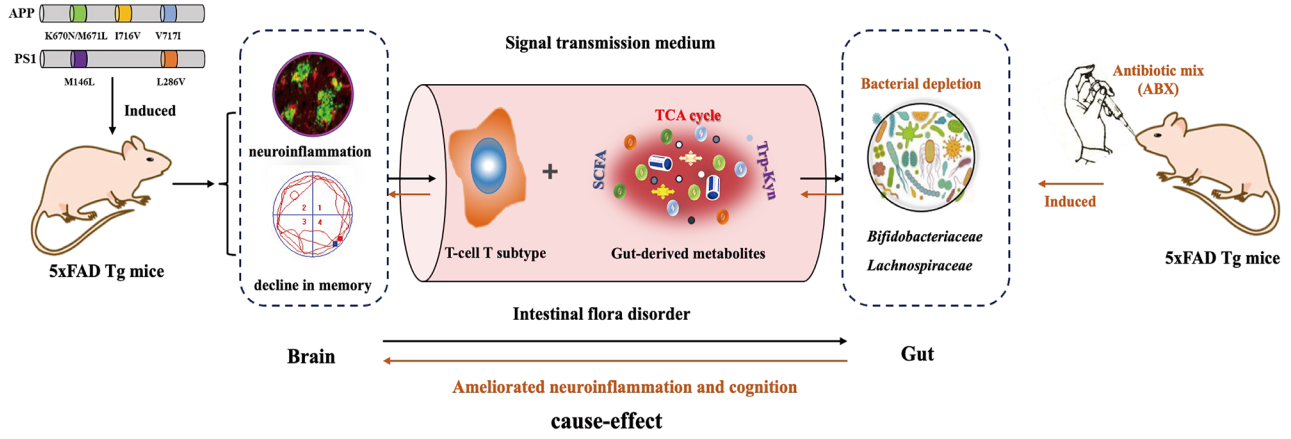

Fig. 10 Gut-derived metabolites served as intermediaries for communicating the "gut-brain" axis. Overexpressed Aβ and neuroinflammation in 5×FAD mice disrupted the microflora structure. Microbial depletion induced by ABX in turn alleviated neuroinflammation by modulating the interactions between Aβ and microglia and astrocytes. It proved that "gut-brain" could transmit information bidirectionally: top-down from the brain to the gut and bottom-up from the gut to the brain

understood. Notably, carbohydrate metabolism is a prominent feature of aging-related changes, with half of the 20 most critical characteristics identified through random forest classification associated with this metabolic pathway. Longitudinal analyses of fecal compositions in Tg-M mice revealed age-dependent fluctuations in SCFAs and amino acids, with a significant increase in fecal SCFAs observed between 9 and 11 months of age. This observation aligns with findings that, while microbial SCFAs typically diminish with age, they persist in abundance among centenarians, hinting at a protective role of SCFAs against aging [84]. Furthermore, the study found elevated fecal levels of essential, semi-essential, and aromatic amino acids in Tg-M mice compared to WT-M mice, with levels increasing as aging progressed. The aged-type microbiome was characterized by an overall increase in proteolytic functions [85]. Wu et al. reported that the metabolism of two aromatic amino acids, tryptophan and phenylalanine, is closely associated with aging, followed by the metabolism of other amino acids such as tyrosine, valine, and lysine [85]. Additionally, a study found that plasma levels of arginine, citrulline, ornithine, glutamate and GABA were higher in APP/PS1 transgenic mice than in WT mice, with levels increasing with age [86].

Limitation

While our study has made significant strides in establishing the cause-effect between gut microbiota disturbances and AD-like lesions, as well as illustrating the pivotal role of gut-derived metabolites in mediating interactions among the metabolic, peripheral immune, and central nervous systems via the gut-brain axis, further research is essential to identify the key bacterial strains and functional enzymes responsible for the changes in metabolites and neuroinflammation. The outbreak of COVID-19 in 2020 delayed the initiation of treatment, resulting in AD mice missing the optimal treatment period, which hindered HLJDD's ability to reverse AD-like lesions. Future studies addressing these critical issues may enhance our understanding of the pathogenesis of AD and explore the broader therapeutic potential of HLJDD in clinical treatments.

Conclusion

Our research illuminated the profound impact of alterations in gut microbiota on the functionality of the "gut-brain" axis in 5×FAD mice, primarily through the modulation of the immune system and changes in carbohydrate and energy metabolism (Fig. 10). We have noted significant elevations in serum concentrations of tryptophan-kynurenine intermediates, lactic acid, $CD4^+$ cells, and $CD8^+$ cells in 5×FAD mice, juxtaposed with reductions in TCA cycle intermediates and the Th1/Th2 ratio. Furthermore, a positive correlation was identified between IBA-1 levels in the brain and concentrations of Aβ and pro-inflammatory factors, suggesting a link between gut microbiota changes and neuroinflammatory responses. The administration of HLJDD demonstrated potential in mitigating gut dysbiosis and neuroinflammation, effectively modulating the Trp-Kyn metabolic pathway and restoring immune homeostasis in the periphery. This finding underscored the therapeutic promise of HLJDD in influencing the gut-brain communication axis, providing new insights into treatment strategies for AD.

Abbreviations

5 H-trp	5-Hydroxy-DL-tryptophan
5 HIAA	5-OH indoleacetic acid
AD	Alzheimer's disease
ABX	Combinatorial antibiotics

AA	Acetic acid
Ara-A	Arachidonic
BBB	Blood-brain barrier
BA	Butyric acid
GFAP	Glial fibrillary acidic protein
HA	Hexanoic acid
HODE	Hydroxyoctadecadienoic acid
HETEs	Hydroxyeicosaenoic acids
IBA-1	Ionized calcium binding adapter molecule 1
IBA	Isobutyric acid
IL-1α	Interleukin-1α
IVA	Isovaleric acid
DHA	Docosahexanoic
EPA	Eicosapentaenoic acid
EETs	Epoxy eicosenotricoic acid
Kyn	Kynurenine
KP	Kynurenine pathway
KA	Kynurenic acid
MHC class II	Major histocompatibility complex II
MCP-1	Monocyte chemoattractant protein-1
PLS-DA	Partial least squares discrimination analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PA	Picolinic acid
PGs	Prostaglandins
SCFA	Short chain fatty acid
TNF-α	Tumor necrosis factor
Trp	Tryptophan
VA	Valeric acid
MWM test	Water morris maze test
XA	Xanthurenic acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-024-01587-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions
Conceived and designed the experiments: Haiyu Zhao, Baolin Bian and Miaoxuan Fan. Wrote the paper: Xinru Gu, Miaoxuan Fan, Performed the experiments: Xinru Gu, Yanyan Zhou Linna Wang, and Wenya Gao. Analysed the data: Yan Zhang, Tao Li, Hongjie Wang and Nan Si,. Contributed to reagents/materials/analysis tools: Xiaolu Wei, Hongjie Wang. All authors read and approved the final manuscripts.

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

Declarations

Ethics approval and consent to participate
All experiments and animals care in this study were conducted in accordance with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publishcations NO.8023, revised 1978), and the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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