ABSTRACT

The development of new drug therapies for Alzheimer's disease (AD) is an important research topic today, but the pathogenesis of AD has not been thoroughly studied, and there are still several shortcomings in existing drug therapies. Therefore, this study aims to explore the molecular mechanism of lactoferrin in the treatments of AD and ulcerative colitis (UC) which are susceptible to AD, starting from the principle of “one drug, two diseases, and the same treatment.” This study used pathological staining and specific indicators staining to preliminarily evaluate the interventions of lactoferrin on UC injury and AD progression. And 16s RNA full-length sequencing was used to investigate the effect of lactoferrin on the abundance of intestinal microbiota in AD mice. Then, intestinal tissue and brain tissue metabolomics analysis were used to screen specific metabolic pathways and preliminarily verify the metabolic mechanism of lactoferrin in alleviating 2 diseases by regulating certain specific metabolites. Moreover, lactoferrin significantly changed the types and abundance of gut microbiota in AD mice complicated by UC. To conclude, this study proved the clinical phenomenon of AD susceptibility to UC, and verified the therapeutic effect of lactoferrin on 2 diseases. More importantly, we revealed the possible molecular mechanism of LF, not only does it enrich the cognitive level of lactoferrin in alleviating AD by regulating the gut microbiota through the brain gut axis from the perspective of the theory of “food nutrition promoting human health,” but it also provides a practical basis for the subsequent research and development of lactoferrin and drug validation from the perspective of “drug food homology.”

KEYWORDS: Alzheimer’s disease, ulcerative colitis, lactoferrin, intestinal microbiota, brain gut axis

INTRODUCTION

As a neurodegenerative disease commonly, the pathogenesis of Alzheimer’s disease (AD) is abnormal hydrolysis of β-amyloid precursor protein, which leads to the growth of amyloid β-protein (Aβ) and causes extracellular deposition of Aβ to form amyloid plaques. The alteration adds to the content of phosphorylated proteins and accumulates in neurons to form neurofibrillary tangles. The increasing levels of Aβ and tau protein (microtubule-associated protein tau, Tau protein) can activate microglia in the brain, further damaging neurons and synapses (Zhai and H.A., 2023; Zhang et al., 2017). As AD progresses, patients appear a range of symptoms, such as progressive cognitive decline and gradual loss of memory, socialization, and mobility (Duyckaerts et al., 2009; Reiman et al., 2020; Yamazaki et al., 2019). The genetic factor is one of the critical things influencing the development of AD, for example, the apolipoprotein E (APOE) gene, which is the most significant genetic risk factor that may contribute to AD (Holstege et al., 2022). Because of the rising aging population and incidence of Alzheimer’s disease, today’s society and healthcare system face a great challenge. According to relevant studies and surveys, the AD prevalence of 60 years and the older is 3.9% in China (Jia et al., 2020); between 2000 and 2019, the number of AD deaths in the United States increased by 145% (Alves et al., 2023). The high disability rate of AD reduces the quality of patients’ survival and puts patients’ lives at serious risk. Meanwhile, AD also brings serious economic and nursing care difficulties to patients’ families and society.

Compared with the healthy population, the AD patients are more susceptible to ulcerative colitis (UC). UC is classified as one of the modern intractable dis-
es by the World Health Organization (WHO) with complex etiology, and the common symptoms include diarrhea, bellyache, and so on (Wang et al., 2007). It is unclear about the specific pathogenesis of UC, for it cures difficultly, recurrence easily, and UC patients may be cancerous in severe cases. The composition of intestinal flora in patients with AD differs with healthy people, and this unfavorable alteration of intestinal flora raises the permeability of intestinal epithelial cells and releases pro-inflammatory cytokines into the circulatory system, further exacerbating the neuroinflammatory effects of AD. Holtzman’s team research has shown that short-chain fatty acids (SCFAs) secreted by the intestinal flora can modulate the AD pathological process because of Tau accumulation (Seo et al., 2003). Mice feeding SCFAs alone under sterile conditions promote their glial cell proliferation and Tau pathology. In studies of the intestinal flora of human AD or UC patients and AD model mice, the relative abundance of Proteobacteria and Bacteroidete adds and that of Firmicutes, Cyanobacteria, and Bifidobacterium descends. Many associated diseases can be triggered since the major anti-inflammatory bacterial group Firmicutes lessens and the pro-inflammatory group Bacteroidetes augments, generating an increased inflammatory response (Chen et al., 2022; Ding et al., 2017). Intestinal flora producing the anti-inflammatory substance butyrate contains a relatively lower percentage, or the associated flora (Bacteroides spp., Alistipes spp., Odoribacter spp., Barnesiella spp. etc.) shows reduced gene expression of encoding butyrate lyase, especially the anti-inflammatory P-glycoprotein pathway (Haran et al., 2023).

At present, all drug therapies for AD can only ameliorate symptoms in a certain degree, but they cannot cure AD completely. Additionally, some medicines has even been proven to have possibly serious side effects. The main drug types currently ratified by the US Food and Drug Administration (FDA) are cholinesterase inhibitors and NMDA receptor antagonists (Wang et al., 2023). The monoclonal antibodies Lecanemab and aducanumab have been approved to market recently. As one of the principles of traditional Chinese medicine (TCM) (Wang et al., 2017), the meaning of 2-disease treatment is that when patients suffer from 2 or more diseases at the same time, and during the progression of these diseases, similar and significant symptoms appear in different diseases, they can achieve the effect of treating multiple diseases by adopting the same therapeutic method. But the specific metabolic pathways and molecular mechanisms have not yet been elucidated and need to be further investigated in this study.
MATERIALS AND METHODS

Animals model

APP/PS1 (presenilin 1, early onset Alzheimer’s protein 1) double transgenic mice were chosen as AD mice (3 mo, male). Twenty AD mice were randomly assigned into 4 groups: AD control group without any treatment, LF administration AD group, AD infected UC (AD+UC) group, and LF administration AD+UC group. The UC mice were given water containing 3.5% dextran sodium sulfate for 28 d to induce colitis. The LF treatment group received LF oral gavage treatment, 100 mg/kg body weight (b.w.), once a day for 28 d. On the 29th day, the mice were killed by cervical dislocation to attain brain tissue, intestinal tissue, and intestinal feces for subsequent experiments. The animal experiments in this study were approved by the Ethics Committee of the Sinoresearch Biotechnology Co., Ltd. (Beijing, China; ZYZC202302009S).

Inflammatory cytokines detection and intestine histopathological staining

Several inflammatory cytokines including IL-1β, IL-6, TNF-α and INF-γ in brain and colon tissue were measured by Elisa kits, according to the related protocols (Fan et al., 2021). After dehydration treatment, mice colon tissue sections were fixed with 4% paraformaldehyde and embedded in paraffin, then paraffin sections were stained with hematoxylin-eosin and sealed with neutral rubber, and the intestinal mucosa staining morphology was observed under an optical microscope (Fan et al., 2021).

Immunofluorescence double staining of glial fibrillary acidic protein/brain microglial cells (GFAP/ib-1) in the brain tissue

Blocking of brain tissues was performed with PBS containing a mixture of 5% NGS, 2% BSA, and 1.5% Triton. After processing, brain slices were incubated with the primary antibodies iba-1 or glial fibrillary acidic protein (GFAP) overnight at 4°C, then the goat anti-rabbit IgG or goat anti-mouse IgG conjugated with Alexa Fluor 568 was employed to visualize the immune markers using fluorescence. It was essential to use the same microscope setup and simultaneously process the slices to ensure accurate staining and imaging. Slices were excited with fluorescence and observed using a Nikon Eclipse 800 microscope equipped with an Olympus DP71 camera and the pictures were captured.

Amyloid β protein (Aβ) staining of brain tissue

Aβ is a polypeptide comprising 39 to 43 amino acids, commonly generated from its precursor protein APP via β -and γ -secretase hydrolysis. It then bonds with β-amyloid molecules, allowing the amyloid to adhere to each other to form plaque deposition gradually. In this study, nanogold (AuNPs) was utilized as a colorimetric probe for detecting Aβ. The nucleic acid aptamer adsorbed by AuNPs (aptamer @ AuNP) is the combination components of Aβ40, and aptamer @ AuNP can aggregate to produce blue-purple substances under high salt conditions. Aβ could combine with the nucleic acid aptamer, forming a complex attached to the surface of AuNPs, thus enhancing its salt tolerance, maintaining a dispersed state, and making the solution pink. Quantification of Aβ content was performed by measuring the absorption intensity ratio of the solution before and after the addition of Aβ, with a detection limit of 10 nmol/L.

Nissl corpuscle staining of brain tissue

The brain tissue was immersed in xylene I and xylene II for 15 min in water to dewax and then dehydrated with a series of ethanol concentrations for 5 min each, including 100% (I), 100% (II), 95%, 90%, 80%, 70%, and 50%. The treated tissues were then rinsed with distilled water 3 times for 5 min, respectively, 1% toluidine blue was added and treated in a 60°C temperature chamber for 40 min. The sections were then washed off the dye in distilled water, dehydrated using 70%, 80%, 95%, and 100% ethanol with xylene, and sealed with neutral balsam.

16s RNA sequencing of intestinal flora

Fecal samples were collected using RNase-free tubes of more than 50 mg per tube, and all the experiments were performed according to the reference (Liu et al., 2023). These samples were immediately immersed in liquid nitrogen to fix and preserve the DNA and then stored at −80°C for future analysis. Fecal DNA was extracted using the E.Z.N.A. DNA Kit. The agarose gel electrophoresis was used to detect the concentration of the extracted DNA. Next, library construction began by amplifying the V1-V9 region of the 16s rRNA gene from the extracted DNA. This amplification was accomplished using PCR with specific primers: 5′-barcode-AGAGTTTGATCMTGGCTCAG-3′ and 5′-CRGTYACCTTGTACGACTT-3′. The amplified products were quantified using Qubit 3.0. DNA repair was subsequently performed and the SMRTbell templates were purified using magnetic beads to construct...
a PacBio sequencing library. The purified products were passed through a 1.2% agarose gel to obtain a 16S SMRTbell library of -1.5 kb in size. Finally, DNA gene sequencing used the PacBio sequel Ile platform. Raw data processing was performed by RS ReadsOfinsert.1. Operational Taxonomic Units (OTU) were clustered with a similarity threshold of 98.65%, and chimeric sequences were identified and removed using UCHIME. The phylogenetic relationships of each sequence were analyzed at a confidence threshold of 70% using the Silva database (Li, 2021). Species classification annotation was performed using the RDP classifier, taxonomic analysis of OTU representative sequences was performed by the uclust algorithm, and the community composition of each sample was assessed at the taxonomic level of phylum and species. Community richness was calculated using the Chao1 algorithm, from the following equation:

\[ S_{\text{chao}} = S_{\text{obs}} + \frac{n_1(n_1 - 1)}{2(n_2 + 1)} \]

where \( S_{\text{chao}} \) was the estimated number of OTUs; \( S_{\text{obs}} \) was the number of actually measured OTUs; \( n_1 \) was the number of OTUs with only one sequence; and \( n_2 \) was the number of OTUs containing only 2 sequences. Community diversity was calculated using ACE, Shannon, and Simpson.

ACE index was used to estimate the number of OTUs in the microbial community. It differed from the Chao1 algorithm. A higher value of the ACE index indicated a greater variety of species within the community. From the following equation:

\[ \text{ACE} = S_{\text{abund}} + \frac{S_{\text{rare}}}{C_{\text{ace}}} + \frac{F_1}{C_{\text{are}}} \gamma^2_{\text{ace}}. \]

where \( S_{\text{abund}} \) was the number of species with abundance threshold > n (abundant); \( S_{\text{rare}} \) was the number of species with abundance threshold ≤ n (rare); \( F_1 \) was the number of species containing only one individual; \( \gamma^2_{\text{ace}} \) was the estimate of the coefficient of variation for rare species.

The Shannon index and Simpson index were used to estimate the microbial diversity in the samples. A higher value of the Shannon index indicated higher community diversity, and a higher value of the Simpson index valued lower community diversity. From the following equations:

\[ H_{\text{shannon}} = -\sum_{i=1}^{S_{\text{obs}}} \frac{n_i}{N} \ln \frac{n_i}{N}, \]

\[ D_{\text{simpon}} = \frac{\sum_{i=1}^{S_{\text{obs}}} n_i(n_i - 1)}{N(N - 1)}. \]

Brain and intestinal tissues were taken as samples after the mice were euthanized. The samples were then divided into 4 groups: AD control group, AD administration group with lactoferrin (LF, 100 mg/kg body weight), AD infection with UC group, and AD infection with UC followed by administration of LF (100 mg/kg body weight), which were labeled as groups G1, G2, G3, and G4, respectively.

The Pre-treatment of the collected samples was subjected as follows: 20 mg of sample was placed into a 2 mL centrifuge tube, and 900 μL of 80% methanol containing 0.1% formic acid was added. Then the tube was vortexed and shaken for 1 min and then ground with a high-throughput tissue grinder for 3 min at 4°C and 65 Hz. Afterward, the tube was removed after being placed at −20°C overnight (time >12 h). Following the overnight incubation, the supernatant was taken after centrifugation at 4°C and 12,000g for 15 min and placed in a refrigerator at 2~8°C for 12 h. Subsequently, the resulting supernatant was retrieved by centrifugation at 4°C and 12,000g for 15 min and transferred to a vial for further chromatographic analysis.

The ACQUITY UPLC I-Class chromatographic system was used for chromatographic analysis under the following conditions: ACQUITY BEH C18 1.8μm, 2.1 × 100mm column, column temperature of 40°C, sample chamber temperature of 4°C. The mobile phase A was a 0.1% formic acid aqueous solution, and the mobile phase B was a 0.1% formic acid acetonitrile solution. The injection volume was 10 μL. The chromatographic gradient program was found in Table 1. The data processing system employed was MassLynx 4.1.

The Synapt G2 HDMS mass spectrometry system was used, and the mass spectrometry analysis conditions were as follows: ionization mode was ESI (+)/ESI (−), the capillary voltage was 3.0 kV (positive mode) and 2.5 kV (negative mode), cone voltage was set to 30
V, collision energy ranged from 15 to 45 eV, desolvation gas temperature was 550°C, flow rate of desolvation gas was 1000 L/h, and the source temperature was maintained at 120. The collected data were subjected to multivariate statistical analysis using the PLS-DA model. Variance analysis (ANOVA) was performed to calculate the p-values, and metabolites with \( P \) < 0.05 and VIP > 1 (Variable Importance in Projection, a measure of variable weight) were selected as differential metabolites.

**Cell culture and cell viability assay**

Neuronal cells (PC12 cells) and colon epithelial cells (NCM460 cells) were cultured in RPMI1640 medium, supplemented with 10% serum and 1% double-antibody. Cells were passaged every 2–3 d. The cells were treated with different concentrations of lactoferrin (0, 10 \( \mu \)M, 100 \( \mu \)M, 1 mM, 10 mM, 100 mM), and the cell viability was assessed after 48 h. This step aimed to preliminarily screen the appropriate dose of lactoferrin that exhibits a suitable effect in the 2 cell models for subsequent experiments.

**Western blot experiment to verify the protein pathway**

Neuronal cells (PC12 cells) and colon epithelial cells (NCM460 cells) were selected to establish in vitro brain neuronal and colon epithelial cell models of lactoferrin intervention, respectively. Western blot experiment was performed according to the reference (Li et al., 2023). The cells were treated with 10 \( \mu \)M lactoferrin (bovine source, 98% purity) for 24 h. After that, the cells were collected and processed in cell lysis buffer for 10 min, and the Loading buffer was added to heat the samples at 95°C for 15 min. Protein samples were loaded onto a 12% SDS polyacrylamide gel for electrophoresis. Subsequently, all proteins were transferred to a nitrocellulose membrane using a reverse blotting kit, and the membrane was blocked in 5% BSA buffer for 1.5 h. The primary antibody and internal reference \( \beta \)-actin were added to the membranes and incubated separately for 2 h. The membranes were then washed 3 times with TBST buffer and followed by incubation with the secondary antibody for 60 min. Finally, the target proteins on the membranes were detected with an enhanced chemiluminescence reagent, and the protein bands were scanned and quantified using Quantity One software.

**Statistical analysis**

The data were presented as mean ± standard deviation (SD), which was analyzed using the SPSS 18.0 and GraphPad Prism 6.0 software. The statistical analyses between 2 groups (control group vs. treatment groups, single treatment group vs. 2 treatment groups) were performed using a Student’s t-test. \( P \) < 0.05 was considered to be statistically significant.

**RESULTS**

**Inflammatory cytokines in brain and colon tissue**

As Figure 1 demonstrated, the pro-inflammatory factors IL-1\( \beta \), IL-6 and TNF-\( \alpha \) decreased significantly and the level of INF-\( \gamma \) increased with LF treatment (\( P \) < 0.05), both in AD group and in AD/UC group (Figure 1). When compared with the single AD group, the levels of IL-1\( \beta \), IL-6 and TNF-\( \alpha \) in AD/UC group were even higher (\( P \) < 0.05), and the level of INF-\( \gamma \) was obviously higher (\( P \) < 0.05), indicating that inflammation deteriorated in the combination of 2 diseases, and LF could alleviate the inflammatory damage in brain and colon tissue (Figure 1).

**Analysis of pathological staining results of intestinal tissue**

The HE staining of mouse colon tissue showed that the nucleus was stained with hematoxylin dye and appeared to be blue, the cytoplasm was stained with eosin dye and appeared red, and inflammatory cells appeared as blue dots. This study mainly observed the morphological changes of colonic tissue lesions and determined the progression of ulcerative colitis. After LF administration to AD mice, there were no significant changes in the morphology and structure of the colon tissue compared with AD mice (Figure 2A). After infection with UC in AD mice, villous structure destruction could be found in the colon tissue, severe epithelial wall damage, occasional edema and bleeding areas, and inflammatory cell aggregation could be observed (Figure 2A). When compared with AD/UC mice, the colon tissue in LF

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treatment group was repaired to some extent, and the colon wall structure was relatively intact, the fingertip structure was initially reconstructed and reproduced, but inflammatory cell infiltration was still visible (Figure 2A). The results suggested that the colon tissue structure in AD/UC group was severely damaged, and lactoferrin could inhibit this damage lesion obviously.

**Analysis of Immunofluorescence Double Staining Results of Astrocytes/Brain Microglia (GFAP/iba-1)**

The main function of astrocytes is to support and segment neuronal cells, forming the blood-brain barrier, and GFAP is an activation marker for astrocytes and participates in the formation of the cytoskeleton. Microglia are macrophages resident in the central nervous system, and iba-1 is a product of their specific expression, which is also a characteristic of their activation. When neuronal function is impaired, inflammatory reactions occur, or neuropathy occurs in brain tissue, the function of astrocytes and microglia is activated, and the expression level of specific indicators GFAP/iba-1 increases. Therefore, in this study, detecting and observing the distribution and overall expression of these 2 indicators can preliminarily evaluate the degree of AD disease development and the role of lactoferrin in AD and UC. As shown in Figure 2B, the nucleus was stained blue with DAPI_350, astrocytes were stained green with FITC 488, and microglia were stained red with Cy3.5 594. The experimental results showed that there was no significant difference in the red and green signal intensities in the brain tissues of AD group and AD+LF group mice, indicating that there was no significant difference in the expression level of GFAP/iba-1 indicators. The red and green signal intensities in the brain tissues of AD/UC group mice increased significantly, indicating a significant increase in the expression levels of the 2 indicators. After LF treatment, their expression levels were downregulated (Figure 2B). These preliminary findings suggested that Alzheimer’s disease worsened in AD/UC mice, and lactoferrin could inhibit the development of AD disease.

**Amyloid in brain tissue β-Protein (Aβ) Analysis of staining results**

Aβ staining is an important criterion for judging the progression of AD, Aβ complex is commonly stained blue, and the size and color depth of the blue patches represent the expression level of Aβ. The darker and the larger of blue patches, the higher expression level of Aβ. As shown in Figure 2C, there was no significant change in the size and color depth of the blue plaques in the AD group and AD+LF group, indicating that there was no significant difference in the expression level of Aβ complex. However, the blue plaques of AD/UC mice significantly increased and deepened when compared with the control group, indicating that the expression level of Aβ significantly increased, and LF downregulated the expression level of this type of protein in the brain tissue of AD/UC mice to a certain extent, further suggesting that UC might worsen the progression of Alzheimer’s disease.
AD, and LF had an inhibitory effect on the progression of AD mice infected with enteritis (Figure 2C).

**Analysis of Nissl corpuscle staining results in brain tissue**

The number of nissl corpuscles is positively correlated with the progression of AD, therefore, by calculating the approximate number of nissl corpuscles, it is possible to preliminarily infer the worsening trend of AD disease. In this study, we found that when compared with AD control mice, the number of nissl corpuscles in the brain tissue of AD treated mice significantly decreased, while in the brain tissue of AD/UC mice, the number of nissl corpuscles increased, and the number of nissl corpuscles significantly decreased through LF intervention (Figure 2D). Further evidence suggested that infection with UC disease could exacerbate the progression of AD disease, and LF might indirectly regulate neuronal function in brain tissue by acting on intestinal tissue, thereby inhibiting the malignant development of AD disease.

**Analysis of 16sRNA sequencing results of gut microbiota.**

By comparing the abundance of gut microbiota in G1 (AD mice) and G2 (AD+LF mice), G3 (AD/UC mice), and G4 (AD/UC+LF mice) groups, a preliminary exploration was conducted on the changes in gut microbiota in AD/UC model mice after LF treatment.

![Pathological changes in colon tissue and special indicators in brain tissue.](image)

**Figure 2.** Pathological changes in colon tissue and special indicators in brain tissue. (A) HE staining of mouse intestinal tissue (200 × microscope); (B) Double immunofluorescence staining of astrocyte/brain microglia (GFAP/iba-1) in mouse brain tissue (200 × microscope); (C) IHC Staining of amyloid β-protein in brain tissue (200 × microscope); (D) Staining of nissl corpuscle in brain tissue (400 × microscope).
This study focused on comparing the taxonomic levels of phylum, genus and species, for the differences at these 3 taxonomic levels were more significant and representative compared with other taxonomic levels.

By comparing the differences in gut microbiota species between the G1 and G3 groups, we found that the gut microbiota species in AD/UC mice significantly increased compared with AD mice (Figure 3A, 3D, 3E, 3F), with the majority being pathogenic bacteria. Through the differences in gut microbiota species between the G1 and G3 groups of mice (Figure 3A, 3D, 3F, 3G, P < 0.05), the heat map in Figure 3F is shown in blue, indicating a significant increase in Beta diversity in the G3 group compared with the G1 group, confirming a significant difference in diversity between the 2 groups. It was further confirmed that the relative abundance of various harmful microbiota in the gut of AD/UC mice, including Romboussia ilealis, Megamonas funiformis, Streptococcus alactolyticus, Planctomycotes, and Proteobacteria, significantly increased, while the relative abundance of beneficial microbiota, Seminibacterium, Ligilactobacillus, Variovorax, and Chitinophaga decreased (Figure 3B, 3C, 3E, P < 0.05).

Compared with the G3 group, the diversity of gut microbiota species in AD/UC+LF mice (G4 group) was significantly reduced (Figure 4A, 4D, 4F, 4G, P < 0.05). Among them, the number of harmful bacteria Romboussia ilealis, Megamonas funiformis, Streptococcus alactolyticus, pathogenic bacteria Planctomycotes, and Proteobacteria decreased significantly, while the relative abundance of Candidatus Saccharibacteria, Duncanella, and Muribaculum increased significantly (Figure 4B, 4C, 4E, P < 0.05). The results indicated that LF treatment might have an impact on the abundance of gut microbiota in AD/UC mice, manifested by a significant decrease in the abundance of harmful microbiota.

Analysis of cross metabolomics results in brain and intestinal tissues.

This part compared and analyzed the specific metabolites in brain and intestinal tissues of AD and AD/UC model mice through chromatography and mass spectrometry, and compared the specific metabolic pathways involved before and after LF treatment, to identify the possible mechanism of LF action. Figures 5A and 5B demonstrated the differential metabolites in brain and intestinal tissues of different groups, respectively. The green color in the Wayne diagram shown in Figures 5C and 5D represented specific metabolic pathways in intestinal tissue, while the blue color represented specific metabolic pathways in brain tissue. Compared with normal mice, there were significant changes in several metabolites in the brain and intestinal tissues of AD/UC mice and LF treated AD/UC model mice. There were 19 metabolites with abnormal expression levels in the brain tissue (Figure 5C), and 12 metabolites with abnormal expression levels in the intestinal tissue (Figure 5D). The common metabolites found in the intersection of the 2 types showed that they were markers of abnormal expression in both diseases (Figure 6A). The results showed that after co-analysis of brain tissue metabolites and intestinal tissue metabolites, 16 Deacetylgeyerline, as a common marker for the simultaneous treatment of UC and AD, was found to have a lower content in AD mice. After AD complicated with UC, its expression level significantly decreased, while after LF treatment, its expression level significantly increased, indicating that it plays a positive regulatory role in this study model. The treatment of both diseases has certain effects (Figure 6B).

Protein pathway analysis by Western blot experiment.

In neuronal cells and colonic epithelial cells, specific metabolites 16-Deacetylgeyerline and LF were selected for intervention, and it was found that protein kinase C (PKC) and protein tyrosine kinase expression levels increased, with a more prominent effect when combined (Figure 6C, 6D). It was suggested that 16-Deacetylgeyerline and LF could activate membrane receptors and downstream special signaling pathways to activate protein kinases such as PKC and PTK, thereby regulating cell growth factor signal transduction, maintaining normal physiological function of neuronal cells, promoting neuronal cell proliferation and regeneration, and alleviating the progression of AD.

DISCUSSION

As a highly harmful neurodegenerative disease, AD has a complex pathogenesis and often involves changes in the expression of various proteins and signaling pathways. At present, the specific pathogenesis of AD has not been fully elucidated, and there is still a lack of safe and effective treatment methods for AD. AD patients are more prone to ulcerative colitis compared with the normal population, and the confirmed mechanism is that the gut microbiota of AD patients leads to the lacking of anti-inflammatory factors, like butyrate, which directly results in the occurrence of colitis through the P-glycoprotein pathway (Li et al., 2021). There have also been reports of cases of ulcerative colitis complicated by other types of dementia including Wernicke encephalopathy, vasculitis and acute confusional state, mainly attributing to the common molecu-
Lactoferrin, as a multifunctional protein that regulates immune, anti-inflammatory, and antioxidant functions, has been shown to play an important role as a neuroprotective mechanism in the brain tissues of AD patients and transgenic mice (Wang et al., 2011). However, the therapeutic effect of LF on mice with both diseases is not yet known. Thus, this study analyzed...
and compared different groups of mice with or without AD/UC and treated with LF to explore the role of LF in alleviating the pathological manifestations of AD and UC and its impact on the abundance of gut microbiota. Furthermore, specific metabolites and metabolic pathways were screened and verified, to elucidate the particular mechanism.

First, through HE staining and specific indicator staining of pathological sections of intestinal and brain tissues, the therapeutic effect of LF on 2 diseases, simple AD and AD/UC, was preliminarily evaluated, and we proved that LF could alleviate the damages in both UC and AD, suggesting that UC and AD might own the same or similar pathological basis. Subsequently, this study compared the types and abundance of gut microbiota between different groups through 16sRNA full-length sequencing, and found similar changes in the expression levels of gut microbiota in 2 groups. Moreover, specific metabolites and metabolic pathways were screened and verified, to elucidate the particular mechanism.
disease model mice. Results demonstrated an increase in the abundance of harmful gut microbiota *Romboutsia ilealis, Macromonas monocytogenes, Streptococcus non-lactolyticus, Mycobacteria* and *Proteobacteria* in 2 disease model mice, further indicating that these types of harmful microbiota could result in the progression of...
Figure 6. Specific protein expression results in brain neuron cells/colon epithelial cells. (A) The common metabolite analysis in both diseases, 16-Deacetylgerberline. (B) The expression level of 16-Deacetylgerberline in brain tissue and colon tissue. (C) The expression levels of protein kinase C (PKC), protein tyrosine kinase (PTK) and Macrophage myristoylated alanine-rich C kinase substrate proteins (MARCKs) in neuronal cells treated by 16-Deacetylgerberline and LF. (D) The expression levels of PKC, PTK and (MARCKs) in colonic epithelial cells treated by 16-Deacetylgerberline and LF. n = 5.
both UC and AD. Particularly, LF treatment reduced the types and quantities of harmful bacteria, indirectly regulating the expression level of gut microbiota metabolites, and to some extent inhibiting the progression of AD, validating that LF suppressed AD and UC through affecting gut microbiota.

Furthermore, cross metabolomics analysis was conducted on brain and intestinal tissues, and it was found that 16-Deacetylgeyerline was highly expressed in normal mice, significantly reduced in AD mice and significantly increased after LF treatment, which indicated that 16-Deacetylgeyerline might be a sensitive biomarker for simultaneous treatment of UC and AD. Moreover, Western blot analysis in brain neuron cells and colonic epithelial cells showed that both 16-Deacetylgeyerline and lactoferrin intervention could increase the expression levels of protein kinases such as PKC and PTK, indicating that LF might exert therapeutic effects on AD and UC by upregulating the beneficial metabolite 16-Deacetylgeyerline and activating the molecular signaling pathway of the “two disease treatment” common molecular basis, which might play key roles in clinical treatments.

Additionally, LF treatment was proved to regulate APP metabolism toward non-amyloid pathways, resulting in Aβ decreased production and improved cognitive decline symptoms in AD model mice (Guo et al., 2017). Similar positive changes were also observed in this study, verifying that the potential anti-oxidation bioactivity of LF in AD model. After LF treatment, pathological and inflammatory indicators in the brain and intestinal tissues of AD and UC mice improved, and neuronal function recovered, which also confirm the anti-inflammation effect of LF in the present models. Considering the above findings, these data preliminary suggested that LF maintained normal neuronal physiological function and promoted neuronal proliferation and regeneration, achieving the effects of alleviating the progression of AD.

In future research, we need to investigate the pathways in which LF promotes the production of 16-Deacetylgeyerline by regulating the gut microbiota, as well as the specific mechanisms of LF and 16-Deacetylgeyerline’s therapeutic ability in neurons and intestinal tissues, to clarify the therapeutic effects of LF on AD and UC. At the same time, further understanding the specific mechanisms of other brain gut axis regulation and the causes of comorbidity of AD and UC may be helpful for new drug development or other therapies.

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AUTHORSHIP CONTRIBUTION STATEMENT: Hui-ying Li conceived the study and supervised all the procedures. Long-yi Ran and Jia-rui Shi wrote the raw text. Yi-nan Lin and Zheng-kun Han performed the animal experiments. Chen-lin Xu amended the revised manuscript and adjusted all the figures in the text. Sen Tian and Xiao-yang Qin performed the cell experiments. Qin-jin Li and Tai-yu Zhang analyzed the data. Yu Zhang amended the manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

DATA AVAILABILITY OF STATEMENT: The datasets used and/or analyzed of this study are from corresponding author upon reasonable request.

ETHICS APPROVAL The animal experiments in this study were approved by the Ethics Committee of the Sinoresearch Biotechnology Co., Ltd. (Beijing, China; ZYZC2023020098).

DECLARATION OF COMPETING INTEREST No known conflicts of interest associated with this publication.

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