Dietary lactoferrin intervention was unable to improve cognitive function from both young and middle-aged APPswe/PS1dE9 transgenic mice

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Abstract

Existing evidence suggest that lactoferrin might be beneficial for Alzheimer's disease. We aimed to determine the effects of lactoferrin intervention on cognitive function from APP/PS1 mice, and possible mechanisms involved in. Both young and middle-aged male APP/PS1 mice were divided into control and lactoferrin group with 16 weeks' intervention. Lactoferrin intervention had no effects on cognitive function from both young and middle-aged mice, and no key markers involved in Aβ, tau pathology, neuro-inflammation and synaptic plasticity were altered post lactoferrin intervention. In regards to gut microbiota profiles, in the young mice, lactoferrin elevated α diversity index including ACE and Chao 1, and reduced the relative abundance of the genera *Bacteroides* and *Alistipes* and elevated *Oscillibacter*, in addition, *Oscillibacter, Anaerotruncus, EF096579_g, EU454405_g, Mollicutes_RF39, EU474361_g, EU774448_g, and EF096976_g* were specifically abundant post Lf intervention via LEfSe analysis. In the middle-aged mice, the relative abundance of the phylum *Proteobacteria*, as well as the genera *Oscillospira, Coprococcus* and *Ruminococcus* was significantly reduced post Lf intervention, additionally, *S24_7*, *Bacteroidia*, *Bacteroidetes* and *Methylobacterium* were specific via LEfSe analysis post lactoferrin intervention. In conclusion, dietary lactoferrin might be beneficial for gut microbiota homeostasis although might have no effects on cognition.

Keywords: Alzheimer's disease; lactoferrin; cognitive function; gut microbiota; amyloid β

1. Introduction

Lactotransferrin or lactoferrin (LF), is a multifunctional, non-heme iron-binding glycoprotein, which belongs to the transferrin family [1]. Lf has been reported to possess multiple biological functions such as immuno-modulatory effects [2], antioxidant effects [3] and anticancer activities [4]. Lf had primarily two forms to exist, i.e. Fe^{3+} free/associated (apo-Lf) and Fe^{3+} saturated (holo-Lf) forms [5]. Over the past two decades, increasing evidence from both animal and population level suggest that Lf intervention might be protective against Alzheimer's disease (AD). To be specific, as early as in 1999, Fillebeen et al. [6] have reported that Lf was capable of crossing the blood-brain barrier via receptor mediated transcytosis. A recent open-label, randomized, controlled pilot study conducted in AD patients demonstrated that Lf administration (250 mg/d) for a total of 3 months could significantly improve cognitive function [7], this might be associated with its effects on Akt/PTEN pathway, consequently affecting key inflammatory and oxidative stress players involved in AD pathology. Similarly, intranasal human Lf (hLf) administration in APPSwe/PS1dE9 (APP/PS1) mice improved cognitive function [8]. However, there are also evidence indicating that Lf might contribute to AD pathology. For example, the presence of Lf had been detected in senile plaques and neurofibrillary tangles (NFTs) in the limbic system of APP transgenic mice [9], as well as in AD patients [10]. Additionally, some researchers postulated that presence of Lf in AD could be a counter-regulatory defense mechanism to fight against the inflammatory cascade normally existed in AD [11]. Nevertheless, the direct role of Lf in AD requires further exploration considering

the heavy burden of AD and no effective therapy at this time point [12].

The role of gut microbiota in the development of AD has been greatly appreciated in recent years [13]. Directly, gut microbiota could secrete quantities of amyloids and lipopolysaccharides, which might contribute to AD pathology such as neuro-inflammation and Aβ plaques [14]. Indirectly, imbalanced gut microbiota profiles are also associated with inflammation, obesity and type 2 diabetes [15], all of which are risk factors for AD. In the meantime, accumulating evidence confirmed that both human and bovine derived lactoferrin or bovine lactoferrin-derived lactoferricin (Lfcin) B are capable of modulating the fecal microbiome in different species such as in very low birth weight infants [16], suckling piglets [17] and Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 mouse model [18]. However, it remains unclear whether altered gut microbiome post Lf intervention might contribute to Lf's protective effects against AD.

Advanced age *per se* is the main risk factor for AD, age-related changes such as altered composition of the gut microbiota may be potentially involved in the onset of AD [13]. APP/PS1 transgenic mice, have been extensively used for AD research. Recent findings also suggest that age-related alterations including but not limited to hippocampal AD pathology [19] and microbiota diversity [20] existed in this AD mouse model. Therefore, it is likely that the beneficial effects of Lf on cognitive function in APP/PS1 mice might be age-dependent, and altered gut microbiota profiles, as well as AD pathology might be responsible for age-related improvement in cognitive function post Lf intervention. Consequently, we aimed to determine the

effects of Lf intervention on cognitive function by utilizing both young and middle-aged APP/PS1 mice as models, we also investigated alterations of the key makers involved in AD pathology (i.e. Aβ, tau phosphorylation, neuro-inflammation and synaptic plasticity related proteins), as well as the fecal microbiome post Lf intervention.

2. Materials and methods

2.1. Materials

AIN93-G standard and modified diet was purchased from Trophic Animal Feed High-Tech Company, Ltd. (Nantong, China). Lactoferrin of milk origin was purchased from Hilmar Cheese Company (CA, USA). The reagents, as well as molecular weight marker and nitrocellulose membranes for SDS-PAGE were purchased from Beyotime Institute of Technology (Jiangsu, China) and Bio-Rad (CA, USA), respectively. The antibodies as follows were obtained from ImmunoWay Biotechnology Company (DE, USA): phosphorylation of Tau at serine 396 (p-Tau serine396) (YP0263), p-Tau serine404 (YP0264). Anti-post synaptic density protein-95 (PSD95) (AJ1661a) was from Abgent (SD, USA). Antibodies directly against β-site APP cleaving enzyme 1 (BACE1) (5606), synaptophysin (5461), glial fibrillary acidic protein (GFAP) (3670) and β-Actin (4970) were from Cell Signaling (MA, USA). These following antibodies were obtained from Abcam (Shanghai, China): insulin degrading enzyme (IDE) (ab32216), cathepsin B (ab58802), and brain derived neurotrophic factor (BDNF) (ab108383). An antibody against Ibα1 (016-20001) was from Wako (Osaka, Japan).

2.2. Animals and intervention

All male APP/PS1 transgenic mice (B6C3F1 background, APPswe strain, cleanliness of SPF) were purchased from Nanjing Model Animal Center (Nanjing, China). All animal procedures followed the Guidelines in the Care and Use of Animals and were approved by the Soochow University Animal Welfare Committee (approval no. 201809A358). All mice were raised in standard plastic cages under specific pathogen-free conditions with appropriate temperature (20-25°C), humidity (55-60%) and a 12-h light-dark cycle. For experiment 1 in young APP/P1 mice, after one week of acclimatization, a total of 14 male APP/PS1 mice (10-week-old) were randomly assigned into two groups with either a standard AIN-93G diet served as control group (Young Tg control group, YTGCon) or AIN-93G diet supplemented with 0.8% lactoferrin (i.e. 800 mg lactoferrin dissolved into 100g AIN-93G diet) (YTGLf), 7 mice *per* group. Similarly, for experiment 2 in middle-aged APP/PS1 mice, the 24-week-old male APP/PS1 transgenic mice (N=14) were divided into middle-aged control group (ATGCon) and middle-aged lactoferrin diet group (ATGLf), respectively with the same diet intervention as described above. The total intervention duration was 16 weeks for both young and middle-aged mice. Over the intervention periods, the average daily dietary intake *per* mice was roughly 4 g/day, thus the average daily Lf intake was about 32 mg/d. The selection of the above dosage for Lf was based on findings from our [21-22] and other [23] research groups. Water and specific food were allowed for mice *ad libitum*. Body weight and food intake were measured on a weekly base over the duration of the study.

2.3. Glucose and Insulin Tolerance Test

At the end of the intervention, intraperitoneal glucose and insulin tolerance test were performed as described previously by our laboratory [24]. Briefly, mice were intraperitoneally (I.P.) injected with glucose (1.5 g/kg body weight) during the GTT in the morning (8 a.m.) after a total of 6 hours' fasting. The next day, mice were given an I.P. injection of insulin (0.5 IU/kg body weight) in the morning (8a.m.) without fasting. The blood glucose level was determined by tail vein sampling at the following time points (i.e. 0, 15, 30, 45, 60, 90 and 120 min post injection) via a hand-held glucometer. Changes in glucose over time were plotted and the total area under the curve (AUC) of glucose levels was calculated.

2.4. Morris Water Maze Test

Morris water maze (MWM) test was used to evaluate the spatial learning and memory ability of mice as described previously by our laboratory with small modifications [25]. Briefly, the test consists of a five consecutive days of navigation trial and a one day probe trial. During navigation trial, mice were allowed to find the platform within 80s, those who had found the platform within 80s were allowed to rest for 10s, otherwise would be forced to stay at the platform for 20s. The time that the mice required for reaching the platform (escape latency) was recorded to assess spatial learning ability. During the probe trial, the platform was removed to assess the retention of tasks of the mice. The number of crossing the platform area, the swimming distance and time spent in the targeted quadrant were recorded to evaluate the spatial memory capacity. Supermaze tracking software (Shanghai Xinsoft

Information Technology Co., Ltd., Shanghai, China) was used for data collection and analysis.

2.5. Sample collection and storage

Mice were fasted overnight, and sacrificed post-behavioral test. The brains were immediately removed, and the hippocampus and parietal-temporal cortex of the cerebral hemisphere were separated and frozen rapidly in liquid nitrogen and stored at -80℃ for further analysis. The other hemispheres were fixed in 10% formalin for immunohistochemistry and immunofluorescence staining. Cecal contents (150-200 mg) were also obtained and snap frozen in liquid nitrogen and then stored at -80°C for further 16S rRNA Gene Sequencing and Microbiota Analysis.

2.6. Western Blotting

Proteins of interest from the hippocampus and parietal cortex were determined by Western blotting as described previously by our laboratory [25]. In brief, a total of 20 μL samples were taken for SDS-PAGE gel electrophoresis, and then protein was transferred onto nitrocellulose membrane. After one hour of blocking by 5% skim milk powder solution, blots were incubated with respective primary antibodies rocking slowly overnight in the fridge. The next day, blots were incubated with proper secondary antibodies at room temperature for 1 hour, and imaged by Syngene chemi-imaging system (MD, USA) using Immobilon western chemiluminescent HRP substrate. Beta-Actin was used as internal control to normalize.

2.7. 16S rRNA Gene Sequencing and Microbiota Analysis

DNA was extracted by PowerMax extraction kit (MoBioLaboratories, CA, USA) and

stored at -20℃. The quantity and quality of DNA were determined by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Science, MA, USA). The V4 region of the bacteria's 16S ribosomal RNA (rRNA) gene was amplified by PCR system. The PCR reactions were conducted using the following program: 30 s of pre-denaturation at 98℃, 30 cycles of 15 s for denaturation at 98℃, 15 s for annealing at 58℃, and 15 s for elongation at 72℃, and a final extension at 72℃ for 1 min. PCR products were purified using AMPure XP Beads (Beckman Coulter, IN, USA) and quantified using PicoGreends DNA Assay Kit (Invitrogen, CA, USA). Quantitative sequencing was performed using Illlumina HiSeq4000pair-end 2×150 BP platform. The data of each sample was separated from the original data according to Barcode sequence and primer sequence. After truncating Barcode and primer sequence, Vsearch v2.4.4 was used to splice the reads of each sample to get raw tags. Then clean tags were obtained through a series of preprocessing, including the removal of low quality bases, ambiguous bases and adapter sequences and the detection of chimeric tags. Based on sequence identification, clean tags were clustered into operational taxonomic unit (OTU). Sequences that were more than 97% identical were assumed to be derived from the same bacterial species. Species annotation of the representative sequence was carried out through VSEARCH based on SILVA128 database [26]. QIIME software was used to calculate alpha diversity index of OTU level, including Chao1, ACE, Shannon Index and Simpson index, and the significance was determined using a student's *t* test within the same age group. Principal Coordinate Analysis (PCoA) was made to analyze β diversity of microbial community structure of different samples

based on UniFrac distance measurement [27]. Linear discriminant analysis with effect size (LEfSe) was performed to predict biomarkers specific abundant in each group. The cut off value was set as the absolute LDA score (log10) >2.0 [28].

2.8. Statistical Analysis

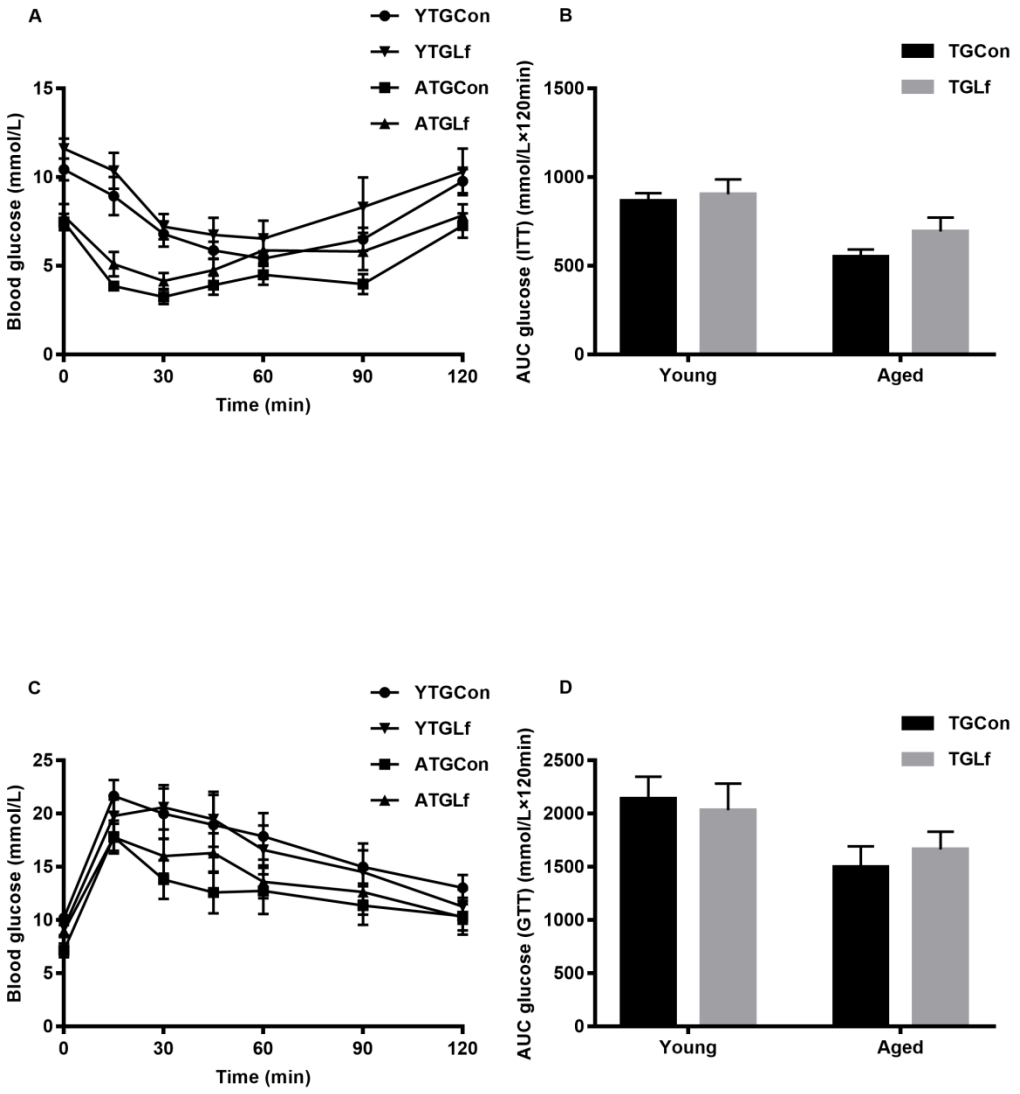
All data are presented as mean ± SEM. Except for data of gut microbiota, a student's *t* test were used for comparisons between YTGCon and YTGLf group, as well as ATGCon and ATGLf group because we conducted the two experiments separately. Statistical significance was established at a $p < 0.05$.

3. Results

3.1. Glucose and Insulin Tolerance Test

There was no significant difference for glucose levels at the detected timepoints, as well as no difference for incremental AUC between groups within the same age for both ITT (Figure 1A&B) and GTT test (Figure 1C&D). Overall, our results suggested that lactoferrin intervention in our study might have no effect on glucose and insulin tolerance in APP/PS1 mice.

Figure 1

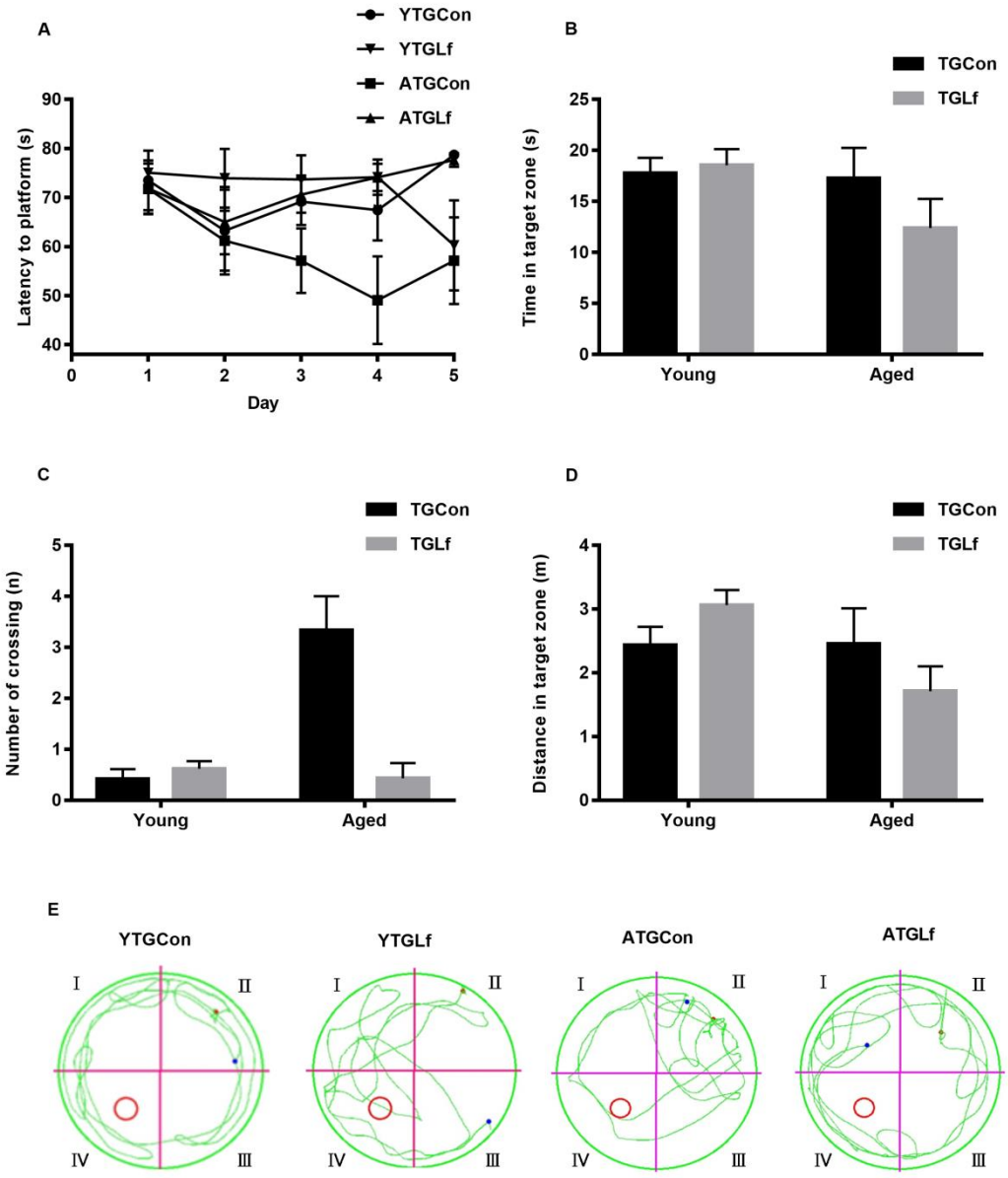


3.2. Behavioral Performance via MWM

As shown in Figure 2A, on the fourth day of navigation trial test, mice from ATGLf group had elevated escape latency in comparison with ATGCon group, while there was no difference for escape latency between YTGCon and YTGLf group at the test days of navigation trial. During the probe trial test, there was no significant difference for time in the target zone, number of crossing and distance in the target zone between TGCon and TGLf groups within the same age (Figure 2B-D). The swimming paths of representative mice in each group were shown in Figure 2E. Collectively, our results

suggested that 16-weeks' Lf intervention might have no effect on the spatial learning and memory abilities of both young and middle-aged APP/PS1 mice.

Figure 2

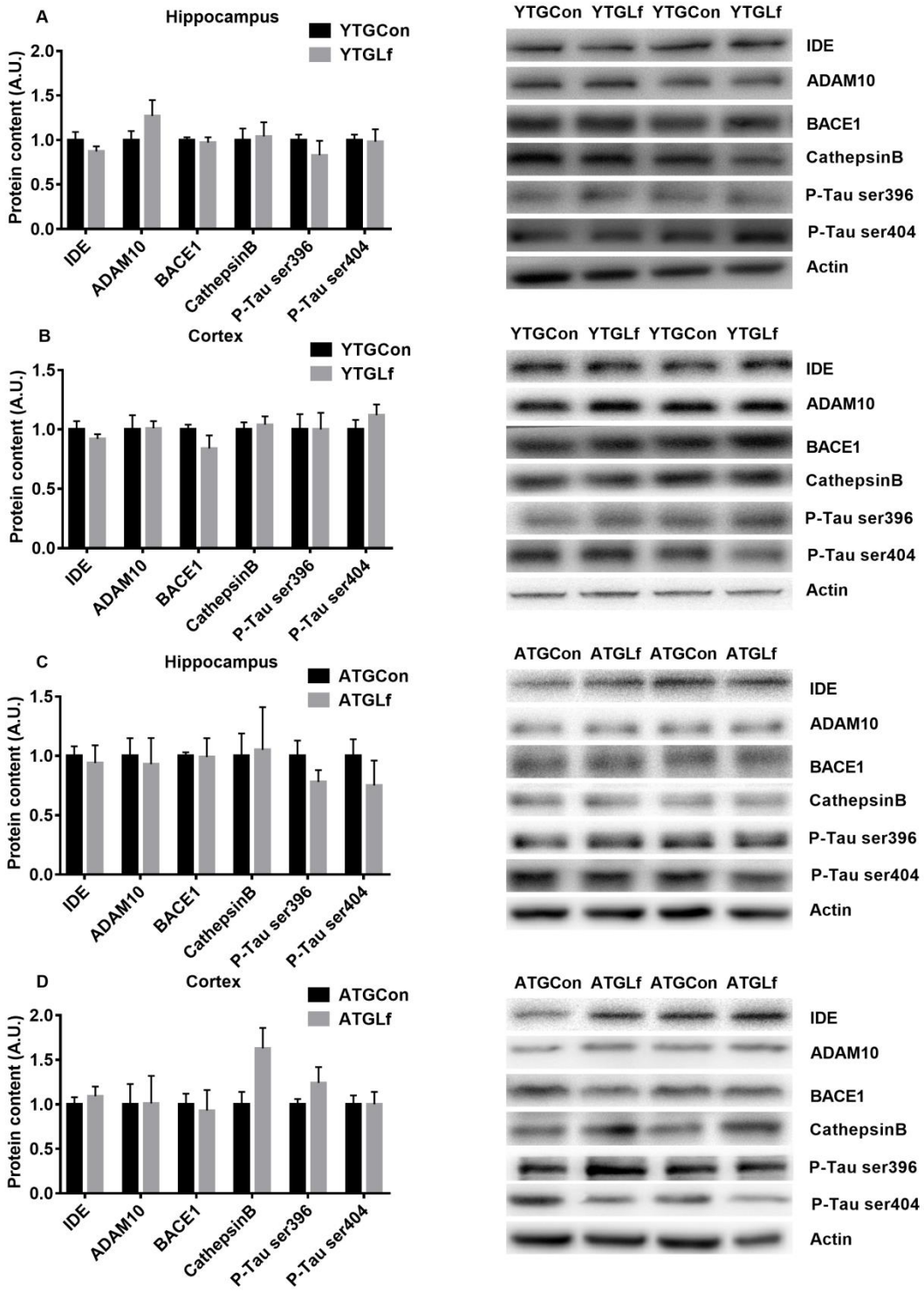


3.3. Proteins Involved in Aβ metabolism and Phosphorylation of Tau Protein

We further examined the protein expression of key indicators involved in Aβ pathology including IDE, ADAM10, BACE1 and cathepsin B, as well as p-Tau at serine396&404. No significant difference for IDE, ADAM10, BACE1, cathepsin B,

p-Tau ser396 and p-Tau ser404 protein expression were observed from both hippocampus and cortex between TGCon and TGLf groups for both young and middle-aged mice (Figure 3A-D).

Figure 3

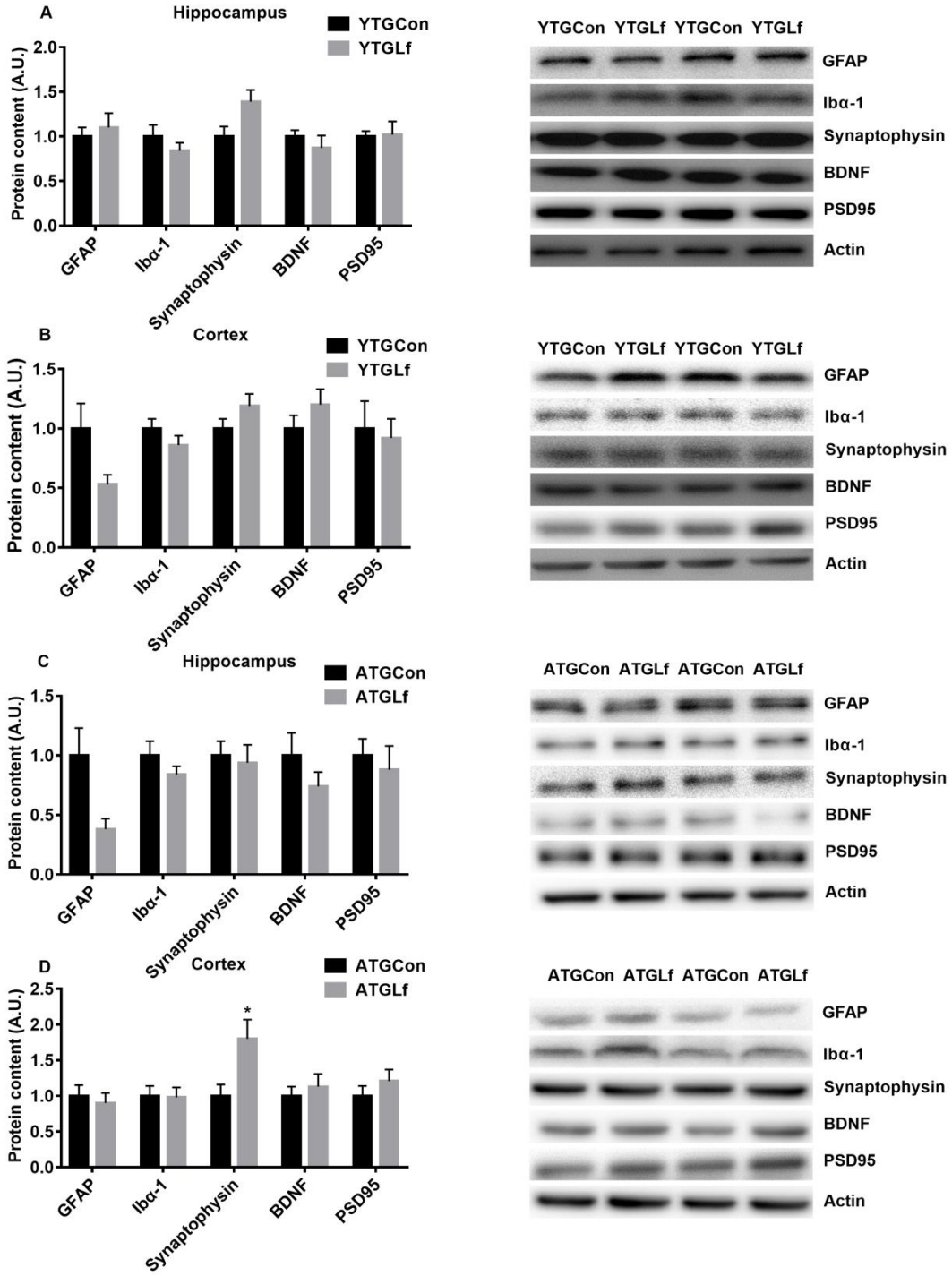


3.4. Proteins Involved in Neuro-inflammation and Synaptic Plasticity

For the young APP/PS1 mice, there was no significant difference for GFAP, Ibα1, synaptophysin, BDNF and PSD95 protein expression in both hippocampus and cortex

between YTGCon and YTGLf group (Figure 4A&B). For the middle-aged APP/PS1 mice, a significant elevation in synaptophysin protein expression was observed in cortex from ATGLf group relative to ATGCon, while there was no significant difference for GFAP, Ibα1, BDNF and PSD95 protein expression in both hippocampus and cortex, as well as synaptophysin in hippocampus between ATGCon and ATGLf group (Figure 4C&D).

Figure 4



3.5. Compositions and Overall Structure of Gut Microbiota

An average of 126425 clean tags was obtained from fecal samples of young APP/PS1 mice, and 90002 clean tags were obtained from fecal samples of middle-aged APP/PS1 mice. The ACE and Chao1 index were significantly increased from YTGLf

group compared to YTGCon group (Figure 5A&B), while Shannon and Simpson index showed no significant difference between groups within the same age (Figure 5C&D). By the taxonomic analysis of OTU representative sequences, the distributions of the top 10 bacteria at phylum and genus level in young and middle-aged mice were shown in Figure 5E-H, respectively. For the young APP/PS1 mice, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the dominant bacteria at the phylum level (Figure 5E), and *Lachnospiraceae_NK4A136_group*, *Ruminiclostridium*, and *Ruminiclostridium_9* dominated the microbiota at the genus level (Figure 5F). For the middle-aged APP/PS1 mice, the top 3 bacteria at the phylum level was *Firmicutes*, *Bacteroidetes*, and *Verrucomicrobia* (Figure 5G), at the genus level was *Akkermansia*, *Bacteroides* and *Oscillospira* (Figure 5H). We further analyzed the difference of gut microbiota at both phylum and genus level between each two groups within the same age by Mann-Whitney U test. As shown in table 1, for the young APP/PS1 mice, there was no significant difference for the top 10 bacteria between YTGCon and YTGLf group at the phylum level; while at the genus level, the relative abundance of *Bacteroides* and *Alistipes* of YTGLf group was lower than that of YTGCon group, and the relative abundance of *Oscillibacter* in YTGLf group was higher than that of YTGCon group. For the middle-aged APP/PS1 mice, the relative abundance of the phylum *Proteobacteria*, as well as *Oscillospira, Coprococcus* and *Ruminococcus* at the genus level from ATGLf group was significantly reduced compared to ATGCon group.

PCoA based on the abundance of OTUs demonstrated differences in the microbial

composition (Figure 6A&B). Specifically, an evidence clustering was identified between the TGCon and TGLf group for both the young and middle-aged mice. The observation suggested that significant difference in gut microbial community structure existed between the YTGCon and YTGLf, as well as ATGCon and ATGLf. The two principal component scores accounted respectively for 27.61% and 18.22% of the total variations for the YTGCon and YTGLf, also 22.33% and 16.34% of the total variations for the ATGCon and ATGLf. We used LEfSe analysis to compare the statistical differences in microbial communities between TGCon and TGLf group for both the young and middle-aged mice. As shown in Figure 6C, there were eight bacterial biomarkers (i.e. *Oscillibacter, Anaerotruncus, EF096579_g, EU454405_g, Mollicutes_RF39, EU474361_g, EU774448_g, and EF096976_g*) significantly abundant in YTGLf group in comparison with YTGCon group. Additionally, four bacterial biomarkers (i.e. *S24_7, Bacteroidia, Bacteroidetes and Methylobacterium*) were significantly greater in ATGLf group relative to ATGCon group (Figure 6D).

Figure 5A-D

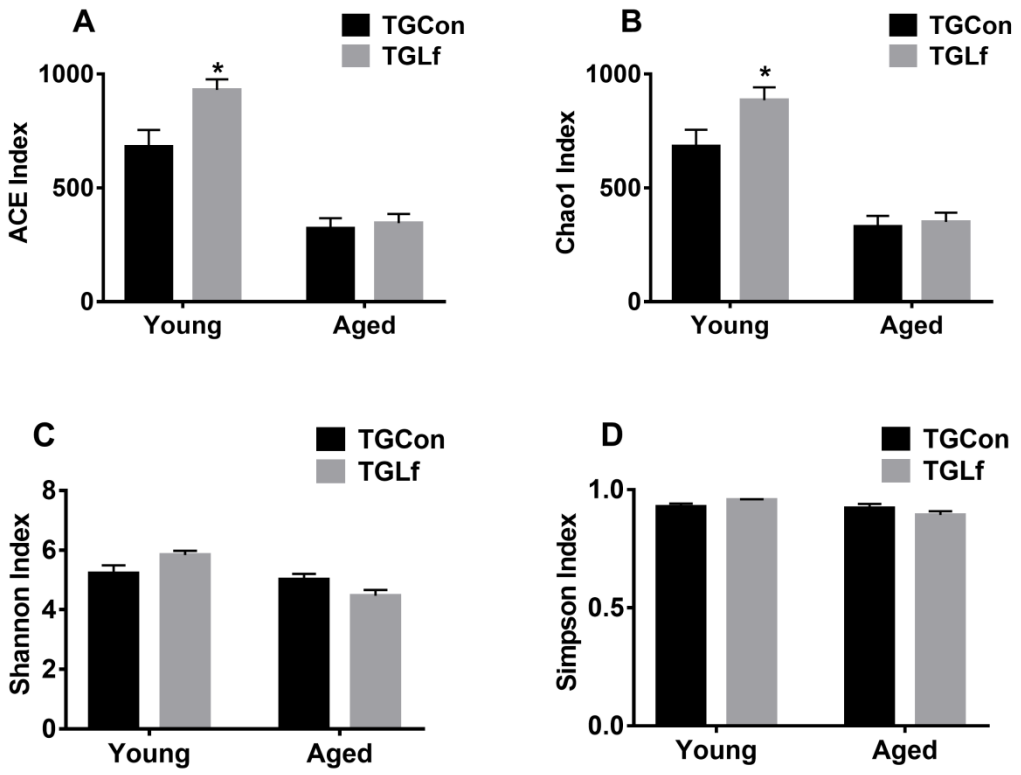


Figure 5E

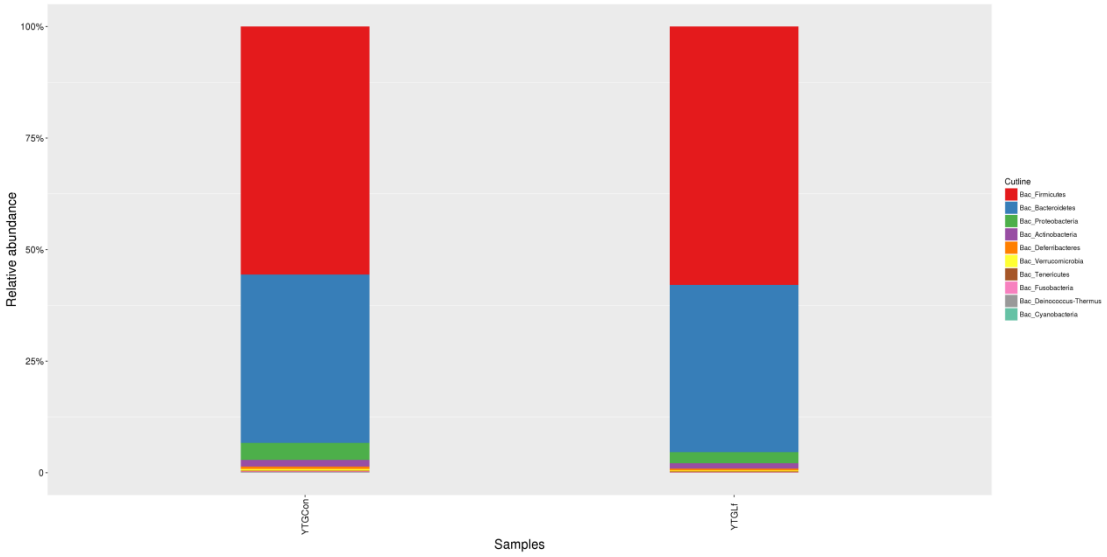


Figure 5F

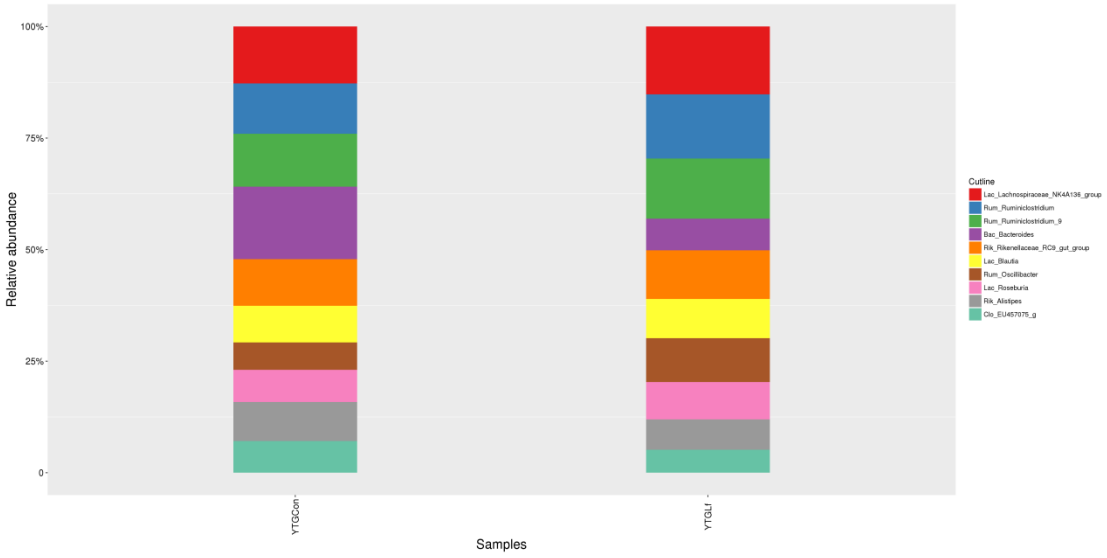


Figure 5G

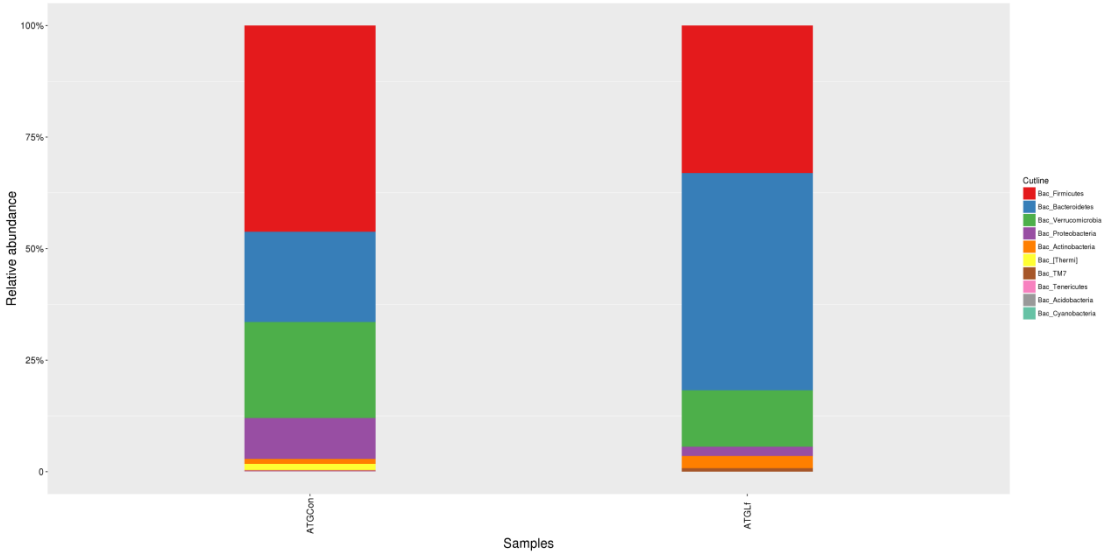


Figure 5H

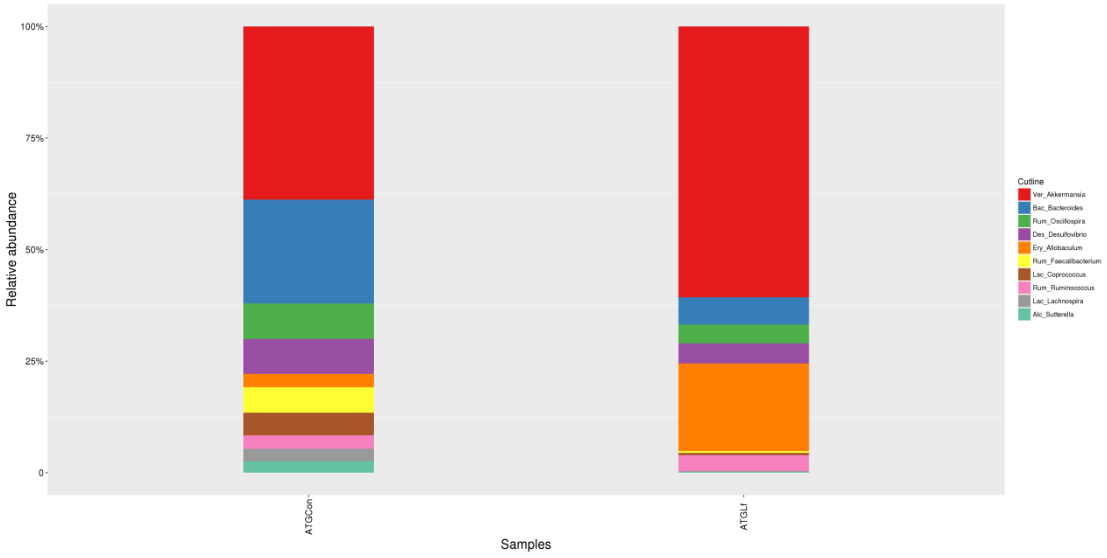


Figure 6

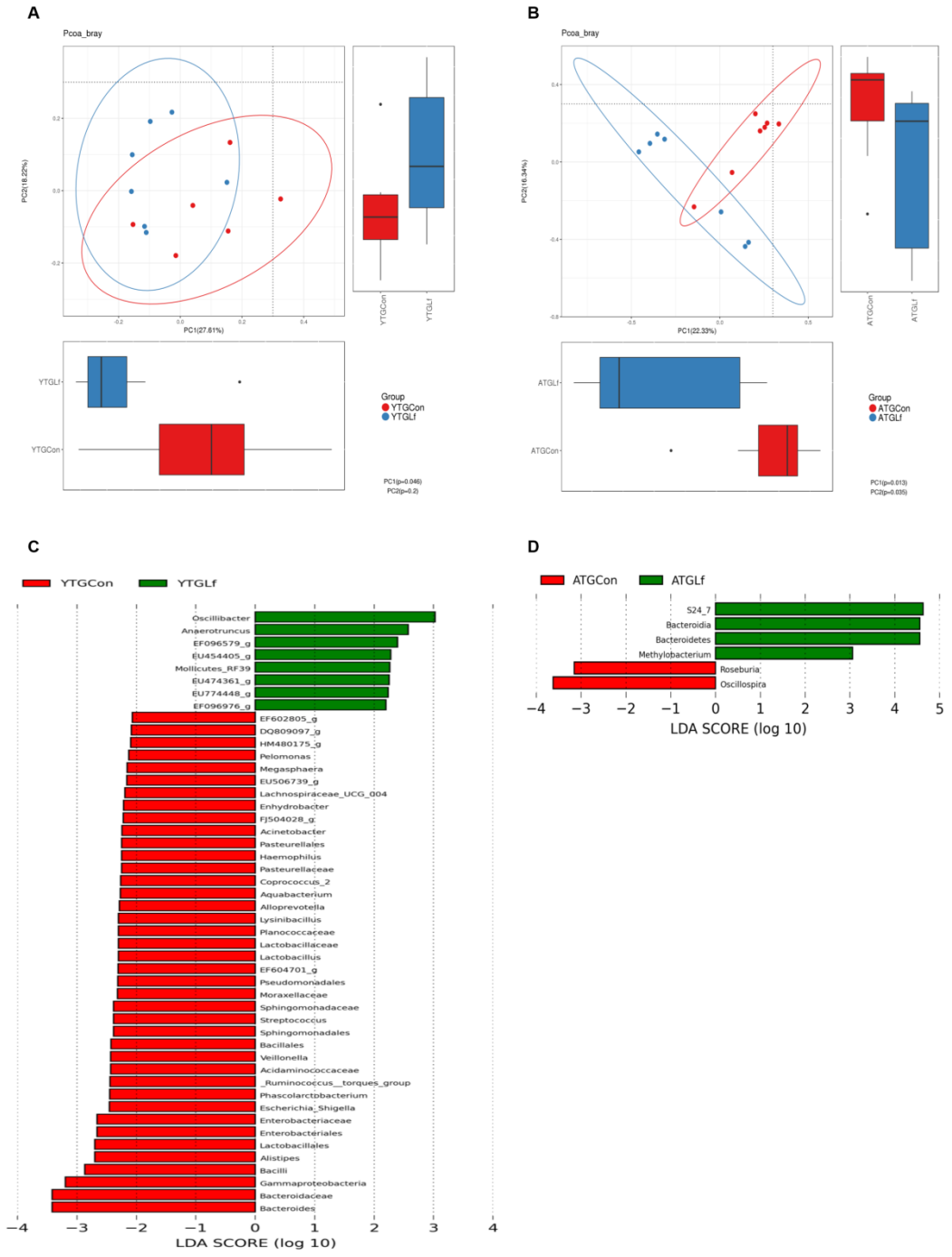


Table 1 The relative abundance of the top 10 gut bacterial genera at the phylum and genus level (%) from both young and middle-aged APP/PS1 mice

level	YTGCon	YTGLf	level	ATGCon	ATGLf
phylum			**phylum**		
Firmicutes	55.43±4.00	57.74±4.18	Firmicutes	17.19±5.70	8.30±1.64
Bacteroidetes	37.62±4.77	37.37±4.17	Bacteroidetes	7.54±5.72	12.21±4.69
Proteobacteria	3.80±0.66	2.49±0.18	Verrucomicrobia	8.00±5.47	3.17±2.60
Actinobacteria	1.48±0.24	1.17±0.23	Proteobacteria	3.42±1.69	0.52±0.12*
Deferribacteres	0.54±0.05	0.44±0.04	Actinobacteria	0.41±0.16	0.68±0.29
Verrucomicrobia	0.36±0.09	0.19±0.05	[Thermi]	0.52±0.51	0.00±0.00
Tenericutes	0.19±0.08	0.21±0.06	TM7	0.05±0.03	0.19±0.12
Fusobacteria	0.17±0.10	0.05±0.03	Tenericutes	0.05±0.02	0.01±0.00
Deinococcus-Therms	0.07±0.05	0.01±0.01	Acidobacteria	0.03±0.03	0.00±0.00

Cyanobacteria	0.05±0.04	0.01±0.01	Cyanobacteria	0.01±0.01	0.00±0.00
genus			**genus**		
Lachnospiraceae_NK4 A136_group	3.05±0.26	3.68±0.45	Akkermansia	8.00±5.47	3.17±2.60
Ruminiclostridium	2.69±0.18	3.48±0.61	Bacteroides	4.79±4.36	0.32±0.20
Ruminiclostridium_9	2.83±0.18	3.27±0.30	Oscillospira	1.64±0.56	0.22±0.07*
Bacteroides	3.88±0.75	1.71±0.29*	Desulfovibrio	1.62±1.56	0.24±0.11
Rikenellaceae_RC9_gut_group	2.49±0.48	2.64±0.32	Allobaculum	0.61±0.29	1.03±0.57
Blautia	1.96±0.21	2.13±0.20	Faecalibacterium	1.18±1.16	0.02±0.02
Oscillibacter	1.47±0.15	2.37±0.28*	Coprococcus	1.03±0.77	0.03±0.01*
Roseburia	1.72±0.24	2.03±0.28	Ruminococcus	0.63±0.15	0.19±0.04*
Alistipes	2.09±0.14	1.64±0.08*	Lachnospira	0.57±0.57	0.01±0.01

EU457075_g	1.69±0.21	1.25±0.17	Sutterella	0.53±0.51	0.01±0.01

N=7 animals *per* group. * $p<0.05$ versus YTGCon or ATGCon group, respectively by Mann-Whitney U test.

4. Discussion

We demonstrated that 16 weeks of lactoferrin intervention in both young and middle-aged male APP/PS1 mice might have no effect on cognitive function, consistently, no alterations in key markers involved in Aβ, tau pathology, neuro-inflammation and synaptic plasticity were observed post Lf intervention. However, we demonstrated that Lf intervention could broadly affect gut microbiota profiles, and the effects might be different for young and middle-aged mice. To be specific, in the young APP/PS1 mice, Lf elevated α diversity index including ACE and Chao 1, and reduced the relative abundance of the genera *Bacteroides* and *Alistipes* and elevated *Oscillibacter*, in addition, *Oscillibacter, Anaerotruncus, EF096579_g, EU454405_g, Mollicutes_RF39, EU474361_g, EU774448_g, and EF096976_g* were specifically abundant post Lf intervention via LEfSe analysis. In the middle-aged APP/PS1 mice, the relative abundance of the phylum *Proteobacteria*, as well as the genera *Oscillospira, Coprococcus* and *Ruminococcus* was significantly reduced post Lf intervention, additionally, *S24_7*, *Bacteroidia*, *Bacteroidetes* and *Methylobacterium* were specific via LEfSe analysis post Lf intervention.

Although still inconsistent [9-10], existing evidence suggest that Lf might exert beneficial effects on AD [7-, [29]. For example, Carro et al. [29] reported that healthy individuals with low salivary lactoferrin levels had a higher likelihood (more than 77%) to develop AD. Lf administration (orally or intranasally) could improve cognitive function in both AD patients [7] and APP/PS1 mice [8]. Our study was the very first to explore whether Lf intervention on cognition might be age-dependent by

using both young and middle-aged APP/PS1 mice. To our surprise, we observed no protective effects of 16 weeks' Lf intervention on cognitive function from both young and middle-aged APP/PS1 mice, we further demonstrated that almost no key markers involved in Aβ metabolism (IDE, ADAM10, BACE1, cathepsin B), Tau phosphorylation (p-Tau ser396&404), neuro-inflammation (GFAP and Ibα1) and synaptic plasticity (BDNF and PSD95) were altered post Lf intervention at both young and middle-aged APP/PS1 mice. This is also in contradictory to the study by Guo et al. [8], who reported that intranasal human derived lactoferrin improved cognitive function in APP/PS1 mice. We postulate that the following reasons might explain the absence of beneficial effects of Lf on cognition. First of all, we only set one single dosage of Lf, although previously similar dosages of Lf with 8 weeks intervention had been reported to demonstrate beneficial effects in lipid metabolism [21] in high fat/cholesterol fed mice from our laboratory, we can't exclude the possibility that the beneficial effects of oral administration of Lf on cognitive function might require higher dosage than the current study or longer intervention duration than 16 weeks. Secondly, Liu et al. [30] has demonstrated that apo-Lf might exert greater efficacy of neuroprotective effects on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinson's disease mouse model, compared with holo-Lf [30]. The Lf used in our study had an iron content of 0.028% [22], which means it is a form of Lf existed between holo-Lf and apo-Lf, therefore, different forms of Lf might results in variable effects on cognitive function. Nevertheless, further studies are still required to explore the effects of Lf

intervention on cognition via different AD mouse models with specific emphasis on different forms of Lf.

Existing evidence suggest that Lf could modulate gut microbiota profiles in different species [16-18]. For example, in very low birth weight infants, two daily recombinant human lactoferrin intake from day 1-28 of life reduced *Enterobacter* and *Klebsiella*, while increased *Citrobacter* in feces [16]. Zhang et al. [18] reported that oral administration of Bovine Lactoferrin-Derived Lactoferricin (Lfcin) B could efficiently maintain gut microbiota homeostasis in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 mouse model. Our study is the very first to demonstrate that 16 weeks intervention of Lf might affect gut microbiota profiles differently in young and middle-aged APP/P1 mice.

(1) Effects of Lf on gut microbiota profiles in young APP/PS1 mice

For the young APP/PS1 mice, Lf could elevate richness of gut bacteria as demonstrated by elevated ACE and Chao1 index compared to age-matched control group. Meanwhile, at the genus level, the relative abundance of *Bacteroides* and *Alistipes* were reduced while *Oscillibacter* was elevated compared to control group. *Oscillibacter* has been negatively associated with body mass index or postprandial glucose levels in human. The elevated *Oscillibacter* post Lf intervention in young APP/PS1 mice could suggest Lf might be beneficial for metabolic parameters, which have been reported by our laboratory previously [21-22]. However, it should be realized our GTT and ITT results didn't support the beneficial effects of Lf at least on glucose and insulin metabolism. Elevated *Bacteroides* genus has been reported in

nonalcoholic steatohepatitis patients [31]. Members of genus *Bacteroides*, i.e. *Bacteroides fragil* have been reported to excrete a serious of complex neurotoxins that can boost inflammation including surface lipopolysaccharide (LPS) and toxic proteolytic peptides [32]. Elevated *Alistipes* has also been reported to be associated with improved gut microbiota composition after dietary intervention for HFD animals [33]. Additionally, Ma et al. [34] reported that genera *Bacteroides* and *Alistipes* were negatively associated with the maintenance of intestine redox in tea polyphenols treated HFD fed mice. The reduction of *Bacteroides* and *Alistipes* post Lf in young mice might also suggest these specific bacteria play function roles in the oxidative stress and anti-inflammatory response. The LEfSe analysis demonstrated that *Oscillibacter, Anaerotruncus, EF096579_g, EU454405_g, Mollicutes_RF39, EU474361_g, EU774448_g, and EF096976_g* were specifically abundant in young APP/PS1 mice post Lf intervention. The functional roles of these abundant bacteria post Lf intervention require further exploration.

(2) Effects of Lf on gut microbiota profiles in middle-aged APP/PS1 mice

Increased abundance of phylum *Proteobacteria* has been proposed to be a potential diagnostic signature of gut dysbiosis [35]. The genera *Oscillospira* is generally considered as anti-inflammatory bacteria, and is positively associated with leanness and health [36]. The genus *Coprococcus* has usually been reported to be beneficial for maintaining intestinal stability [37]. Elevated abundance of genera *Ruminococcus* is associated with irritable bowel syndrome [38], and is usually implicated in negative health outcomes including AD [39]. The reduction in phylum *Proteobacteria*, and

genera *Ruminococcus* post Lf intervention suggest that Lf might improve gut microbiota profiles in middle-aged APP/PS1 mice, while the functional roles of reduction in *Oscillospira* and *Coprococcus* post Lf intervention require further exploration. The LEfSe analysis demonstrated that *S24-7*, *Bacteroidia*, *Bacteroidetes* and *Methylobacterium* were specifically abundant post Lf treatment from middle-aged APP/PS1 mice. Similar to those in young APP/PS1 mice, the functional roles of these abundant bacteria post Lf intervention in middle-aged APP/PS1 mice also require further exploration.

In summary, we demonstrated 16 weeks' Lf intervention had no effect on cognitive function, and key AD related markers including Aβ, tau pathology, neuro-inflammation and synaptic plasticity from both young and middle-aged APP/PS1 mice; while Lf might exert diversified effects on gut microbiota profiles for APP/P1 mice with different ages. Our findings could indicate that dietary Lf might be beneficial for gut microbiota homeostasis although might have no effects on cognition.

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Figure legends

Figure 1 Glucose and Insulin Tolerance Test.

Glucose levels (A) at 15 min, 30 min, 45 min, 60 min, 90 min, 120 min and the total area under the curve (AUC) (B) during ITT. Glucose levels (C) at different timepoints and AUC (D).

Figure 2 Behavioral Performance via MWM

(A) Mean escape latency to hidden platform on five days of navigation test. During probe trails, time spent in the target quadrant (B), number of crossing the previous hidden platform (C), and swimming distance in the target quadrant (D) were recorded. (E) Representative motion tracking of the mice in the different groups. All values were presented as means ± SEM. * $p<0.05$ versus age-matched TGCon group.

Figure 3 Aβ aggregation and phosphorylation of Tau Associated Protein Expression

In young APP/PS1 mice, protein expression of IDE, ADAM10, BACE1, Cathepsin B, P-Tau ser396 and P-Tau ser404 in hippocampus (A) and cortex (B) were detected by western blotting. In middle-aged APP/PS1 mice, the above proteins were shown in hippocampus (C) and cortex (D) via western blotting. All values were presented as mean + SEM. A.U. means arbitrary units. Representative blots were shown at right panel in A-D.

Figure 4 Neuro-inflammation Related Markers and Synaptic Plasticity

In young APP/PS1 mice, protein expression of GFAP, Ibα-1, synaptophysin, BDNF, PSD95 in hippocampus (A) and cortex (B) were detected by western blotting. In

middle-aged APP/PS1 mice, the above proteins were shown in hippocampus (C) and cortex (D) via western blotting. All values were presented as mean + SEM. A.U. means arbitrary units. Representative blots were shown at right panel in A-D.

Figure 5 Alterations in Gut Microbiota Composition

The alpha diversity of ACE index (A), Chao1 index (B), Shannon index (C), Simpson index (D) displays the microbial diversity of each group. Relative abundance of top 10 microbes in young APP/PS1 mice at the rank of phylum (E) and genus (F), and in middle-age APP/PS1 mice at the rank of phylum (G) and genus (H) were shown.

Figure 6 PCoA based on the abundance of OTUs and LEfSe analysis

Principal Coordinate Analysis (PCoA) for gut microbial composition in the young (A) and middle-aged (B) APP/PS1 mice were shown, with the first two principal components were plotted to visualize UniFrac distances of fecal samples. Samples from TGCon and TGLf groups were depicted as red and blue, respectively for both the young and middle-aged mice. The bar graph indicated the taxa that discriminate between TGCon and TGLf group in young (C) and middle-aged (D) APP/PS1 mice, respectively via LEfSe analysis. The statistical test was performed using the LDA effect size method. Only absolute LDA (log10) scores >2.0 were considered statistically significant.