Non-uinform gut microbiota alteration patterns associated with therapeutic outcomes in an Alzheimer's disease animal model

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Research

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Abstract

Background: Dysbiosis of gut microbiota is associated with the progression of beta-amyloid (Aβ) pathology in Alzheimer's disease (AD). We aimed to identify uniform Aβ-responsible gut microbiota status as possible guideline for gut microbiota manipulation and the prediction of outcomes of microbiota targeted treatments. Six months old APP/PS1 mice from the same genetic background, housing and feeding conditions were then daily gavage with Metformin, peptides WN5 or PW5 to manipulate the gut microbiota for 12 weeks. Aβ pathology and gut microbiota were then explored and compared. Results: Fecal microbiota transplantation (FMT) from a 16 month old APP/PS1 mouse reconstituted the gut microbiota towards the donor and increased Aβ pathology in APP/PS1 mouse model. Metformin, peptides WN5 and PW5 all attenuated Aβ-plaque formation in APP/PS1 mouse model but each was associated with distinct gut microbiota status. No uniform gut microbiota pattern associated with Aβ pathology was found among different gut microbiota-targeted treatments. Conclusion: We found no uniform gut microbiota status associated with Aβ pathology suggesting gut microbiota status is not a suitable biomarker for AD diagnosis and treatment predictions. Alteration of gut microbiota in itself may not be sufficiently directly related to functional outcomes and might only be a shadow of deeper molecular mechanisms not fully understood. The findings here strongly suggested that the significance of gut microbiota alteration in disease pathology and treatment may have so far been over claimed and that interpretation of gut microbiota data should be done with utmost caution.

Background

Decades of research findings point to a bidirectional communication between the gut microbiota and the central nervous system (CNS) through the gut-brain-axis [1, 2]. In recent times, the gut microbiota has become the focus of numerous studies in a plethora of disease conditions due to its immense impact on host physiology and metabolism. The recognition of the gut microbiota as a major regulator of human physiology has stirred tremendous wave of excitement within the scientific research community with exponential increase in microbiota-focused reports already linking the gut microbiota to conditions such as inflammatory bowel disease [3], type 2 diabetes [4], anxiety [5], autism, Parkinson's disease [6]. multiple sclerosis [7], and Alzheimer's disease (AD) [8].

In AD, dysbiosis of the gut microbiota is hypothesized to be associated with the disease progression [9]. Studies involving germ-free mice suggested that the gut microbiota is necessary for the progression of AD pathologies [10]. Given the suggested significance and role of the gut microbiota and its dysbiosis in AD progression, substances capable of exerting significant alterations in the gut microbiota can also potentially alter the course of the disease. In this regard, several studies have attempted to manipulate the gut microbiota to ameliorate AD pathophysiologies using diverse methods. For example, it has been shown that both short and long term antibiotic treatment leads to significant microbial dysbiosis with associated changes in AD pathophysiology [11, 12]. Alternative methods employed to show the effect of microbiota alteration in AD include drug agents such as GV-971 [13] and Metformin [14, 15]. In addition, several studies have also reported that diet and dietary components remains one of the most important

external factors that significantly impact the gut microbiota in AD. Probiotics, prebiotics and the Mediterranian diet [16-18] among other dietary patterns have all been shown to exert significant impact on cognitive health by possibly impacting the gut microbiota.

The question of interest has been whether there is any uniform signature or pattern of microbiota alteration responsible for specific AD pathologies. In spite of the numerous studies on gut microbiota dysbiosis and AD, there are no definitive data on which types of gut microbiota are altered and to what extent are the alteration in AD and therefore no standard indicators are available for the therapeutic manipulation of the gut microbiota in AD [12]. Each study reports a significantly different dysbiosis state but similar conclusion of Aβ pathology. As a matter of fact, studies have relied on comparative rather than absolute definition of microbial dysbiosis to determine success of individual methods with all studies using “in-house” vehicle control as the standard. These differences may be due to the differences in animal models used, vivarium housing, diet and feeding conditions.

In this study, we aimed to identify uniform Aβ-responsible gut microbiota status as possible guideline for gut microbiota manipulation and the prediction of outcomes of microbiota-targeted treatments. To minimize the effect of interference factors on the results, we used animal models from the same genetic background, housing, feeding and laboratory conditions in testing the hypothesis. Short-term cocktail antibiotics followed by FMT were employed to confirm that alteration in gut microbiota was associated with a significant change in amyloidoisis. We found that 1 week of FMT, from an aged-APP/PS1 mouse (16 months old), reconstituted the gut microbiota and significantly increased Aβ deposition. We then chose Metformin as a drug candidate because Metformin is a well-studied microbiota-manipulating chemical agent. Two peptides, PW5 (Pro-Pro-Lys-Asn-Trp), recently reported [19] by our lab to effectively reshaped the gut microbiota composition and reduced Aβ plaques in the same APP/PS1 mice model and WN5 (Try-Pro-Pro-Lys-Asn), were used as dietary nutrient candidates. Comparative analysis of mice treated with daily gavage of Metformin, PW5 and WN5 revealed that all treatments reduced hippocampal Aβ plaques significantly and reshaped the gut microbiota composition but with no robust uniform pattern. The findings warrant further studies to identify mechanisms underlying gut microbiota alterations in AD.

Results

FMT restructured the gut microbiota and increased Aβ plaque in APP/PS1 mice

Preliminary experiment showed an age-dependent gut microbiota disbiosis and increased in Aβ plaques in 4-6 months old APP/PS1 mice (Fig. S1 and Fig. S2). To evaluate whether a short term drastic alteration in gut microbiota is associated with changes in Aβ pathology, we treated 3 months old APP/PS1 mice with cocktail of antibiotics or saline by daily oral gavage for 2 weeks. Antibiotic-treated mice were then transplanted with a 16-month-old untreated APP/PS1 mice fecal microbiota (Antibiotic+FMT group) by daily gavage for one week or maintained on normal lab conditions (Antibiotic group). Non-antibiotic-treated APP/PS1mice were treated with saline and used as control (Saline group). Fecal pellets were

collected prior to treatment (baseline, C1), following antibiotic administration and before and after FMT treatment up to age 5 months (C2 – C11) (Fig. 1A). 16s rRNA gene sequencing analysis revealed a drastic reduction in observed microbial species from a mean of 26.6 ± 2.50 at baseline to 2.25 ± 0.63 upon 2 weeks of antibiotic-treatment (Fig. 1B, $P < 0.001$), showing that the gut microbiota was largely depleted by the antibiotic treatment. It was also shown that microbial species recovered gradually to close-to-baseline levels (27.0 ± 3.80) within 6 weeks post antibiotic treatment (5 months of age) without any significant change in Aβ pathology (Fig. 1G). Furthermore, microbial species in FMT-treated mice were reestablished to levels before treatment but within a shorter period compared to antibiotic group. In addition, PCA analysis showed that there were no significant differences in β-diversity among the experimental groups at C1 (prior to treatment, Fig. 1C) and C11 (final collection point, Fig. 1D). These suggest there was a high level of similarity among treatment groups at these two points. As seen in Fig. 1E, FMT enhanced the reconstitution of α-diversity of the gut microbiota. Remarkably, the gut microbiota composition of the Antibiotic+FMT group shifted towards the direction of the donor microbiota, over time (Fig. 1F) indicating that FMT was successful.

As expected, analysis of brain tissues at C11 by immunostaining revealed that there were no Aβ plaques in the hippocampus of the WT mice but, there were significantly higher Aβ depositions in the Antibiotic+FMT group (183.33 ± 40.19, $P < 0.05$) compared to Saline (57.33 ± 12.19) and Antibiotic (62.33 ± 14.68) groups (Fig. 1G, H). In addition, astrocytes but not the traditional Aβ-engulfing microglia was activated by FMT (Fig. S3). Taking together, the results confirmed that FMT-induced alteration in the gut microbiota from an aged APP/PS1 mouse is associated with the progression of Aβ pathology in APP/PS1mouse model.

Peptide WN5 attenuated Aβ plaque, ameliorated cognitive impairment and altered the gut microbiota in APP/PS1 mice

In vitro experiment revealed that peptide WN5 significantly inhibited Aβ plaque accumulation in HEK-293-E22G cell model, showing that peptide WN5 could be a potential anti-Aβ aggregation candidate (Fig. S4 and Fig. S5).

To confirm the above results, we explored the ability of WN5 to attenuate AD pathophysiology using APP/PS1 mouse model and wild-type (WT) littermates from the same genetic background. Mice were treated with WN5 or saline for 12 weeks. Immunohistochemistry (IHC) analysis of brain tissues of mice in the various treatment groups (Fig. 2A) showed that, no obvious positive Aβ aggregates were seen in the WT mice. Quantitative analysis (Fig. 2B) confirmed significantly reduced Aβ-plaques in the hippocampus of the WN5 group (17.6 ± 1.7) compared to the AD group (25.4 ± 1.7, $P < 0.009$). Cognitive function was tested by the Morris water maze and Shuttle box test. Krustal Wallis test (Fig. 2C) revealed that, there were significant differences in escape latency among the groups, (chi-square = 9.259, $P < 0.026$, df = 2) with a mean rank score of 3.75, 16.17 and 10.50 for the WT, AD, and WN5 groups respectively. Pairwise comparison analysis showed that the differences existed between the WT and AD groups ($P < 0.018$). In the shuttle box test, WT mice could remember the alarm sound and reacted often (13.58 ± 2.34 times) to

avoid the electric shock compared (Fig. 2D) to the AD group (5.59 ±0.91 times) ($P < 0.001$). Similarly, the number of active avoidance times in the WN5 (8.24 ± 1.04) mice was better than the AD model mice ($P < 0.05$). Correspondingly, the electric shock time (Fig. 2E) in the AD group (25.82 ± 2.18s) was longer than the WT group (6.1 ± 3.12s) and the WN5 group (16.55 ± 4.96s).

16s rRNA gene sequencing analysis of fecal pellets collected before and after treatment showed that, microbial α-diversity (Fig. 2F) was significantly reduced in the AD group compared to the WT and WN5 groups. Furthermore, β-diversity analysis (Fig. 2G) revealed that a wide separation between the fecal samples of mice treated with WN5 and AD groups implying significantly different gut microbiota. This was further confirmed by heatmap (Fig. 2H) of the gut microbiota of the various groups. At the genus level (Fig. 2I), WN5 was on one hand associated with reductions in *S24-7*, *Lactobacillus* and *Enterobacteriaceae* and on the other hand increases in the abundance of *Norank_o_Clostridiales*, *Norank_f_Lachnospiraceae*, *Acinetobacter*, *Oscillospira*, *Desulfovibrionaceae*, *Unclassified_f_Helicobacteraceae*, and *Unclassified_f_Ruminococcus;* all substantially different from the AD group. Altogether these results implied that peptide WN5 improved AD-associated learning and memory impairment, reduced Aβ plaque formation and reshaped the gut microbiota.

Metformin, WN5 and PW5 treatments achieved similar level of decreased in Aβ plaques and improvement in cognitive performance

Our research group recently published data showing that another walnut-derived pentapeptide PW5 (Pro-Pro-Lys-Asn-Trp), significantly altered the gut microbiota and attenuated Aβ aggregation also using our APP/PS1 mice model [19]. Using Metformin as a well-studied microbiota-manipulating chemical drug, and peptides WN5 and PW5, we sort to compare the differences in effectiveness in ameliorating Aβ pathology and cognitive impairment in APP/PS1 AD mouse model. As can be seen from Fig. 3A-C, Metformin, WN5 and PW5-administered mice performed better in cognitive and behavioral performance compared to the non-treated APP/PS1 mice (AD group). Interestingly, we found no statistically significant differences in cognitive performance among the Metformin, WN5 and PW5 treated mice. Apart from the WT group, Aβ-plaques were observed in all the other treatment groups, however, AD mice had significantly increased Aβ aggregates in the hippocampus compared to the Metformin, WN5 and PW5 treated mice (Fig. 3D-E). Surprisingly, no such differences were observed between the Metformin, WN5 and PW5-treated mice.

No uniform gut microbiota pattern among Metformin, WN5 and PW5 treatments

Since Metformin, WN5 and PW5 all attenuated Aβ pathology and improved learning and cognitive impairment to similar levels in same conditioned AD mouse model; we wondered if there is any uniform gut microbiota pattern responsible for the similar functional output. Diversity analysis (Fig. 4A) showed that there were no significant differences in α-diversity between the groups before treatment ($P > 0.05$). However after treatment analysis revealed that α-diversity in the Metformin group was significantly higher than the WN5 ($P < 0.049$) and the PW5 ($P < 0.024$) groups. Interestingly, diversity between the two peptide groups did not significantly differ ($P = 0.830$). PCA analysis (Fig. 4B) of the distances between fecal

samples showed obvious overlapping before treatment, indicating that the samples were similar. After the treatment period, a clear separation towards different directions was observed between the fecal samples in the different treatment groups (Fig. 4C); indicating that gut microbiota composition was significantly dissimilar between the treatment groups. These differences were further confirmed by taxonomic analysis (Fig. 4D). For example at the genus level, whiles the abundance of *S24_7* was 40.01 and 39.96% in the WN5 and PW5 treatment respectively; it was only 23.74% when treated with Metformin. Similarly, *norank_f_Ruminococcaceae* was much higher in the Metformin and WN5 group compared to the PW5 group. In addition, *norank_o_Clostridiales*, *Lactobacillus* and *norank_f_Rikenellaceae* were all significantly different among the groups. Consistent with the higher α-diversity in the Metformin group compared to the WN5 and PW5 groups, more species were detectable in the Metformin group compared to other groups. These suggest that, even though, Metformin, WN5 and PW5 all similarly reduced Aβ-plaques and ameliorated cognitive impairment, the associated gut microbiota were distinct.

No uniform gut microbiota patterns associated with Aβ plaque pathology in APP/PS1 mouse model

Park et al reported of a distinctive gut microbiota state associated with Tg-APP/PS1 transgenic mice using bacteria-derived membrane vesicles in blood [20]. To confirm the non-uniform nature of the gut microbiota associated with Aβ pathology in APP/PS1 mouse model, we compared our data of the untreated APP/PS1 model with Park et al's data available online. Comparative analysis (Fig. 5) clearly revealed that even same APP/PS1 models have different gut microbiota patterns. For example our APP/PS1 model had more microbial species compared to Park et al's model.

Furthermore, we explored the differences in microbiota associated with disease progression and that associated with disease attenuation. We found that the gut microbiota associated with disease attenuation by Metformin, WN5 and PW5 treatments were not completely distinct from the microbiota associated with disease progression. For instance, whiles *g_norank_f_Desulfovibrionaceae* was significantly higher in PW5 and reduced in our APP/PS1 model, it was undetected in Park et al's (untreated APP/PS1 model), Metformin and WN5 treated groups. Similar patterns observed in terms of *g_norank_f_S24-7*, *g_unclassified_f_Lachnospiraceae*, *g_Pseudomonas* and *g_Acinetobacter* etc were all either disproportionately and mixed in abundance in the treated and untreated groups. Given that there are no clear patterns between microbiota of untreated AD models and treated (with improved AD status), it could be deduced that, there is no uniform pattern of gut microbiota structure associated with progression of Aβ pathology in APP/PS1 mouse model.

Discussion

Many studies have demonstrated a connection between gut microbiota dysbiosis and different disease conditions. Patterns of dysbiosis have often been assessed by changes in richness and diversity with variable findings in different disease conditions. For instance, the richness and diversity of gut microbiota was found to be reduced in arteriosclerosis, hypertension [21], diabetic patients [22] and animal model, whereas increased diversity was associated with atrial fibrillation [23] myocardial infarction, stroke and

transient ischemic attacks. Interestingly alterations associated with autism [24] and obesity [25] was found to be inconsistent. In AD, available evidence suggests an age-dependent dysbiosis of the gut microbiota [26] which is consistent with our findings. In addition, results of patterns of diversity across different treatments have been mixed with some studies reporting both high and low diversities in both AD patients and animal models compared with controls [27]. However studies using germ free mice, FMT, pro- and antibiotics [10, 12] have raised prospect of therapeutic manipulation to restore the integrity of the gut microbiota as treatment for AD. In the present study, we found using fecal sample from an aged APP/PS1 mouse that, FMT significantly reconstituted the gut microbiota towards the host microbiota with a corresponding increase in Aβ plaque deposition in 5 months old APP/PS1 transgenic mice. Interestingly, Kim et al also found that transfer of healthy gut microbiota reduced Aβ and tau pathology in an AD animal model [10]. These suggest that FMT could indeed be a potential therapeutic tool for AD. However this requires the identification of a standardized or healthy microbiota fingerprints to serve as a guide or measure of success.

Our findings suggest that, there is no uniform gut microbiota structure associated with improved Aβ pathology in AD. We found that Metformin, WN5 and PW5 were all associated with significant reduction in Aβ plaques in the hippocampus but different patterns of alterations in the gut microbiota. The structure of the gut microbiota is significantly influenced by both internal host and external factors [28, 29]. On the internal side, interspecies competition for available resources, symbiotic and mutualistic specie interactions and crosstalk and genetic factors shape the gut microbiota. On the external side, diet, antibiotics and other drug agents as well as physical exercises, exposure to sunlight and FMT are known to impact the gut microbiota. Given that Metformin, WN5 and PW5 treatments all reduced Aβ pathology and improved learning and memory impairment, it was expected that they may have similar gut microbiota patterns since it is hypothesized that gut microbiota alteration is associated with the progression of AD pathology. Interestingly, each treatment showed distinct microbiota fingerprints. For instance β-diversity analysis showed that the 3 treatments were each characterized by different microbiota states. Heterogeneity in the findings of similar study designs are usually attributed to inherent design differences such as model differences, environmental and feeding among others. For example, a recent review article concluded that there were not enough consistency in the association between obesity and gut microbiota profiles and attributed this observation to differences in methodologies and data management systems used across different studies [25]. In addition, Zhou et al recently showed that adding unsterile or sterile soil to bedding of mice either before birth or after weaning significantly influences the composition of the gut microbiota differently [30]. Furthermore, studies reported that age, housing, littering and feed access [31-33] also affect the composition of the gut microbiota. These findings suggest that genetic and environmental factors may drive microbiota differences across different studies. However all the animals including controls used in this study were from the same genetic background, raised in the same laboratory conditions with the same feed and feeding practices and of the same age. Therefore it could be deduced that, the observed differences in the gut microbiota composition across the different treatments were not due to inherent differences; implying that the underlying mechanisms beyond gut microbiota alteration needs to be further explored.

It is worth noting that, peptides WN5 and PW5 are both walnut-derived, of the same amino acid composition and only differs in the order of amino acids. This implies that upon the digestion of both peptides, the same amino acids are going to be obtained. Strikingly, though the administration of both peptides reduced Aβ pathology but produced different microbiota status. It is suggestive therefore that the mechanism of action may not necessarily and sufficiently be the effect of the constituent single amino acids on the gut microbiota; suggesting that upon digestion, they may still exist and function in different combinations of small peptides but not as single amino acids. Interestingly, reported APP/PS1 AD model [34] from different group also revealed different dysbiosis. It could be argued that alterations in the gut microbiota in itself might not be sufficient to explain the impact of various treatment agents in disease prevention and might only be a shadow of deeper molecular mechanisms still unknown. Li et al [35] recently reported that mild cognitive impairment has similar alterations as Alzheimer's disease in gut microbiota. However the authors rightfully stated that for comprehensive evaluation of effect, multi-model assessments of biomarkers including cognitive tests, neuroimage, and amyloid and/or tau markers are required. It is worthy of note that having similar microbiota status does not prove that it contribute to disease pathology especially when critical biomarkers of the pathology were not assessed. The challenge is compounded by the non-availability of definitive gut microbiota-related metabolomics biomarkers proven to be directly related to AD. We herein reported that different treatments resulting in similar amyloid pathology produced different gut microbiota signatures; as such gut microbiota status is not a suitable biomarker for AD diagnosis. The sophisticated mechanism underlying the dysbiosis of the gut microbiota and disease progression including AD is far more that we know now. The findings here also suggest that the significance of gut microbiota alteration in disease pathology and treatment may have so far been over claimed.

Given the bidirectional nature of the gut-brain axis theory [36], it is worth considering that the gradual loss of brain function may in turn reshape the gut microbiota, rather than the traditional theory that gut microbiota dysbiosis results in neuro-degeneration [37, 38]. Interestingly Ruggiero et al argued that the concept of brain dysbiosis should be evaluated, together with the concept of gut dysbiosis [39]. Therefore the question worth exploring is whether dysbiosis of the gut microbiota occur before neuro-degeneration or compromises in the integrity of the brain and nervous system precede gut microbiota dysbiosis. The blood-brain-barrier (BBB) "isolates" and confers an immune privilege status unto the brain [40, 41]. However the gradual breakdown of the BBB, the impairment of the meningeal lymphatic system, or the loss of intestinal epithelial barrier integrity may impact negatively on brain function which may in turn trigger downstream reactions including gut dysbiosis [41-43]. Therefore caution should be exercise in interpreting gut microbiota data especially in AD.

Conclusions

In conclusion, we found no uniform gut microbiota patterns associated with improved Aβ pathology in APP/PS1 AD mouse model as possible guideline for gut microbiota manipulation and the prediction of outcomes of microbiota-targeted treatments. This study draws the attention of the research community that alteration of gut microbiota in itself may not be sufficiently directly related to functional outcomes;

as such the gut microbiota is not a suitable biomarker for AD diagnosis and treatment predictions. Therefore mechanisms underlying gut microbiota alterations should be taking into consideration to fully understand the contribution of gut microbiota to the physiology and pathology of disease conditions.

Methods

Materials and reagents

Ampicillin sodium salt (# 69-52-3), vancomycin hydrochloride from *Streptomyces orientalis* (# 1404-93-9), neomycin trisulfate salt hydrate (# 1405-10-3), gentamycin sulfate (# 1405-41-0), erythromycin (# 114-07-8), metronidazole (# 443-48-1), Sucrose (# WXBC7932V) and Triton™ X-100 (#SLBV4122) were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate buffer solution (PBS, # BF001) powder was purchased from Heart Biological Technology Co., Ltd (Xian, China). QIAamp Powerfecal DNA Kit (# 12830-50) was obtained from QIAGEN (Duesseldorf, Germany). Rabbit polyclonal antibody against GFAP (# Z0334) was purchased from Agilent Technologies, Inc (Santa Clara, CA). Mouse anti-β-Amyloid, 1-16 antibodies (# 9300-02) was obtained from BioLegend, Inc (San Diego, CA). Rabbit-anti-p2ry12 (# AS-55043A) was purchased from AnaSpec, Inc (Fremont, CA). Alexa Flour™ 555 donkey anti-mouse IgG (H+L) (# A31570) was obtained from ThermoFisher Scientific, Inc (Waltham, MA) and goat anti-rabbit IgG H&L (Alexa Flour® 488) (# ab150077) was purchased from Abcam, Inc (1:250, Abcam). 4’6-diamidine-2-phenylindole (DAPI) (# 10236276001) was obtained from Hoffmann-La Roche Ltd (Basel, Switzerland). Glycerol (# G8190) was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Paraformaldehyde (PFA) (# 30525-89-4) was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). The identification and purification of peptide WN5 was previously reported elsewhere [44]. In this study, WN5 was chemically synthesized by GL Biochem (Shanghai) Co., Ltd (Shanghai, China) using L-isomers of each amino acid by solid-phase synthesis via the fluorenylmethoxycabonyl (Fmoc) method. It was then stored at -20 ℃ for subsequent usage.

APP/PS1 transgenic mice

APP/PS1 transgenic mice were obtained from Nanjing Biomedical Research Institute of Nanjing University (SCXK 2017-0174). APP/PS1 transgenic mice contains the KM670/671NL Swedish mutation of human amyloid precursor protein (APP) and the L166P mutation of human presenilin 1 (PS1) under the control of the Thy-1 promoter that lead to greater aggregation of amyloid-β in the cerebral brain. Mice were on the C57BL/6J background and maintained in the specific pathogen free (SPF) facility at the Institute of Laboratory Animal, Jinan University. Mice were allowed access to autoclaved food and water ad libitum. Wild-type female C57BL/6J mice were crossed with APP/PS1 male double transgenic mice to generate the APP/PS1 double transgenic mice and WT littermates. The animal experiments were conducted following the guidelines established by the Chinese Committee on Experimental Animal Supervision.

HEK-293-E22G cells

HEK-293-E22G cells, a cell line constructed by Aβ42-mCherry plasmid, were provided by Prof. Alan Tunnacliffe (Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK) and were used as the model. Cells were cultured in DMEM supplemented with 10 % heat-inactivated FBS containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, 50 μg/mL of hygromycin B, 5 μg/mL of blasticidin S and 5 mM of L-glutamine at 37 ℃ under humidified air with 5 % CO_2. Cells in exponential growth phase were used for the experiment.

In vitro treatments

Cell viability measurement

The effects of WN5 on the viability of HEK-293-E22G cells were determined by MTT assay. In brief, cells were seeded at a density of 5×10^3 cells/mL in a 96-well plated for 24 h and then were cultured with different concentrations of WN5 (0.05 and 0.5 mM) for 48 h, respectively. After incubation, 20 μL of MTT was added to each well and the plates were further incubated for 4 h. The absorbance at 490 nm was measured by microplate ELISA reader.

Imaging and analysis

IncuCyte ZOOM live cell imaging system (Essen BioScience, MI, USA) was used to observe the Aβ-42 aggregates inside the cells. Cells pre-treated with WN5 (0.05 and 0.5 mM) were routinely propagated in culture medium. After 48 h, a solution of tetracycline was added to the cells and incubated at 37 ℃ for 72 h in the IncuCyte ZOOM apparatus and images of cells were recorded every 4 hours. We performed three identically prepared experimental replicates (n = 3) and 9-fields of vision were photographed in each hole. The Aβ aggregation rate was calculated according to formula 1. (see Formula 1 in the Supplementary Files)

Flow cytometry

In order to validate the above results, the Aβ aggregation in cells was quantified by ImageStreamx MKII imaging cytometer. The detection parameters were set up as follows: Channels 01 (bright field) and 04 (fluorescence channels). Magnification was 60x, providing a pixel size of 0.3 μm^3 and the laser 561 nm activated for fluorescence of mCherry. For simple enumeration of pre-prepared microparticle samples, the acquision cut-off was set to 10000. The aggregation rate was estimated using formula 2. (see Formula 2 in the Supplementary Files)

In *vivo* treatments

FMT protocols

During the antibiotics phase, four APP/PS1 mice (3 month old) received antibiotic cocktail for 2 weeks by gavage. Mice assigned to the FMT (n = 5) study also received 2 weeks antibiotic cocktail prior to FMT. Mice then received oral gavage of 200 uL of donor fecal matter for 7 consecutive days. Fecal pellets were

collected prior to antibiotic treatment (C1), following antibiotic exposure at days 7 and 15 (C2 and C3), during and after FMT at days 0, 4, 8, 15, 22, 30, 37 and 45 (C4, C5, C6, C7, C8, C9, C10, and C11). Gavage needles were cleaned with 70% ethanol and autoclaved after each experimental day.

Candidate treatments

After 1 week acclimatization, 6-month-old APP/PS1 mice were divided into four groups: vehicle treated wild-type mice group (n = 5, WT), vehicle treated APP/PS1 transgenic mice group (n = 5, AD), WN5-treated APP/PS1 mice group (400 mg/kg, n = 6, WN5), and Metformin-treated APP/PS1 group (500 mg/kg, n = 6, Metformin). Normal saline, peptide WN5 and Metformin were accordingly by oral administration for 12 weeks from the age of 6-months. Mice were sacrificed at the age of 9 months.

Behavior test

Cognitive function was assessed by Morris water maze and shuttle box tests, as previously described [19] in mice after 12 weeks of WN5 and Metformin intervention.

Immunostaining

5 months old APP/PS1 mice after FMT were sedated with pentobarbital and well-perfused with PBS and 4% PFA. Brain matter was dissected and hemispheres fixed in 4% PFA for 24 h, followed by 10%, 20%, and 30% sucrose gradient dehydrated for 24 h, until the brain completely sank to the bottom. Brain tissues from prefrontal lobe to hippocampus were processed into 25 μm per sections for immunostaining. There were 12 brain tissue sections on each slide that covered different brain areas (3750 μm). Sections were stained with anti-Aβ42 (1:200), anti-p2ry12 (1:200) and anti-GFAP (1:200) over night at 4 ℃. Subsequently, these sections were stained with Alexa Flour™ 555 donkey anti-mouse IgG (H+L) (1:250) or Goat anti-rabbit IgG H&L (Alexa Flour® 488) (1:250) at room temperature for 2 h. Then sections were stained with DAPI for 5 min and then mounted with 60% glycerol, and imaged with a 4x/20x objective on a Zeiss fluorescence microscope. Aβ plaques were analyzed with Fiji software and the morphology of microglia and astrocytes were analyzed with Fiji software using Sholl analysis.

Immunohistochemistry

After the behavior tests, WN5 and Metformin treated APP/PS1 mice were sacrificed and brain tissue was processed into sections (4-μm) for immunohistochemistry. Aβ immunohistochemical staining was performed on coronal slices by rabbit anti-Aβ42 antibody (1:1500, Abcam). Primary antibodies were detected with biotinylated goat anti-rabbit IgG (1:200) in conjunction with the DAB kit coupled with diaminobenzidine substrate. The stained sections of the β-amyloid plaques were observed and captured with an Olympus IX-73 microscope. Quantification of the β-amyloid plaque was performed on the hippocampus and sub-regions of the hippocampus using ImageJ software.

DNA extraction and 16S rRNA gene sequencing

Bacterial genomic DNA was extracted from mouse fecal pellets using the QIAamp DNA Stool Mini Kit. The library was generated according to a previously reported method. The V3-V4 regions of the 16S rRNA gene were PCR amplified using Forword primer 5'-ACTCCTACGG GAGGCAGCA-3' and Reverse primer 5'-GGACTACHVGGGTWTCTAAT-3', purified and then sequenced using the Illumina MiSeq platform according to the standard protocols. The 16S rRNA genes of the gut microbiota were analyzed using QIIME (version 1.17). Raw FASTQ files were processed to remain high-quality sequences and further clustered into operational taxonomic units (OTU) at 97% similarity using the Silva reference data base (Release128, http://www.arb-silva.de). OTUs were further classified into six taxonomic ranks of phylum, order, class, family, genus, and species. Principal component analysis (PCA) was performed to visually evaluate the differences and similarities in bacterial communities between groups (β-diversity) using Bray-Curtis method. Alpha-diversity was assessed using the species diversity indices (Inverse Simpson, Shannon, or Simpson).

Statistics analysis

Excluding the microbiome population statistics mentioned above, data from other experiments were presented as mean ± SEM (standard error of the mean). Differences between two groups were determined using two-tailed, unpaired Student *t*-test with Welch's correction. Statistical significance among > 2 groups with only one variable was assessed using ANOVA followed by Bonferroni's post hoc test. Krustal-Wallis test on ranks was performed for data that failed the normality test. Differences were considered significant at $P < 0.05$.

List Of Abbreviations

AD: alzheimer's disease; FMT: fecal microbiota transplantation; Aβ: beta-amyloid; CNS: central nervous system; PW5: Pro-Pro-Lys-Asn-Trp; WN5: Try-Pro-Pro-Lys-Asn; APP: amyloid precursor protein; PS1: presenilin 1; SPF: specific pathogen free; DAPI: 4'6-diamidine-2-phenylindole; BBB: blood-brain-barrier; IHC: immunohistochemistry; OTU: operational taxonomic units; PCA: principal component analysis.

Declarations

Ethics approval and consent for participate

The study was approved by the Jinan University Experimental Animal Ethics Committee (permission number: 20170313184750 and IACUC-20190505-05).

Consent for publication

Not applicable.

Availability of supporting data and materials

The datasets used and analyzed during the current study is available upon reasonable request through the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization: JY. R., and MJ.Y; Investigation: M.W., JN.C., W.K.A. and XY. S.; Formal Analysis: M.W., W.K.A., JN.C., CC.G., and XY. S.; Writing – Original Draft: W.K.A., M.W., MJ.Y, and CC. G.; Review & Editing: JY.R., MJ.Y., M.W., JN.C., W.K.A., and CC. G: Funding Acquisition: JY. R. All authors discussed the results and approved the manuscript.

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Additional File Legends

Additional file 1: Immunostained brain tissues and quantification of Aβ-plaques in the hippocampus and cortex from 4, 5 and 6-month old APP/PS1 transgenic mice.

Additional file 2: Alterations of gut microbiota in 4, 5 and 6-month old APP/PS1 transgenic mice.

Additional file 3: Immunostained brain tissues and Sholl analysis of astrocytes and microglia after fecal microbiota transplantation.

Additional files 4: Images and quantification of Aβ42-aggregates in HEK-293-E22G cells after treatment with peptide WN5.

Additional files 5: Image cytometry analysis of Aβ42-aggregates in HEK-293-E22G cells after treatment with peptide WN5.

Figures

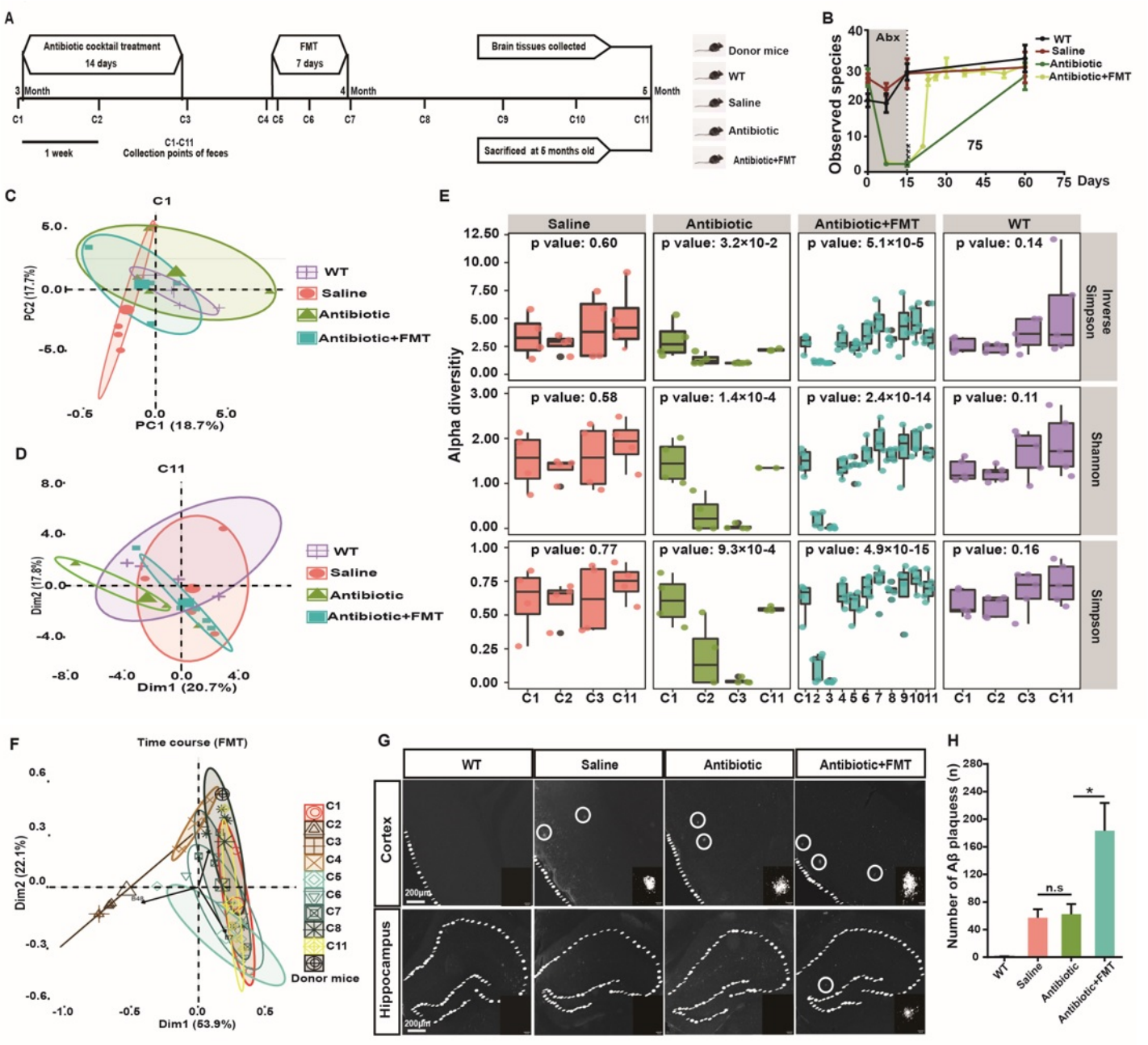

Figure 1

Fecal microbiota transplantation (FMT) increased Aβ position and reconstituted gut microbiota communities in post-antibiotic treated APP/PS1 mice. (A) Schematic design of FMT experiment and fecal collection time points (from C1 to C11). (B) Observed species depleted by antibiotic administration, but sharply restored following FMT. (C-D) β-diversity analysis revealed that no significant differences between C1 (prior to any treatment) and C11 (final collect point) in FMT group. (E) α-diversity analysis results showing FMT promoted the reconstitution of evenness and diversity of gut microbial. (F) PCA analysis showing microbiota composition in FMT mice shifted towards the direction of the donor microbiota communities over time. (G-H) Immunostained brain tissues and quantification showing the increase in

Aβ-plaques in the hippocampus and cortex after FMT. All data were presented as means ± SEM. Significant differences are indicated as follows: n.s, P > 0.05; * P < 0.05; ** P < 0.01 using ANOVA.

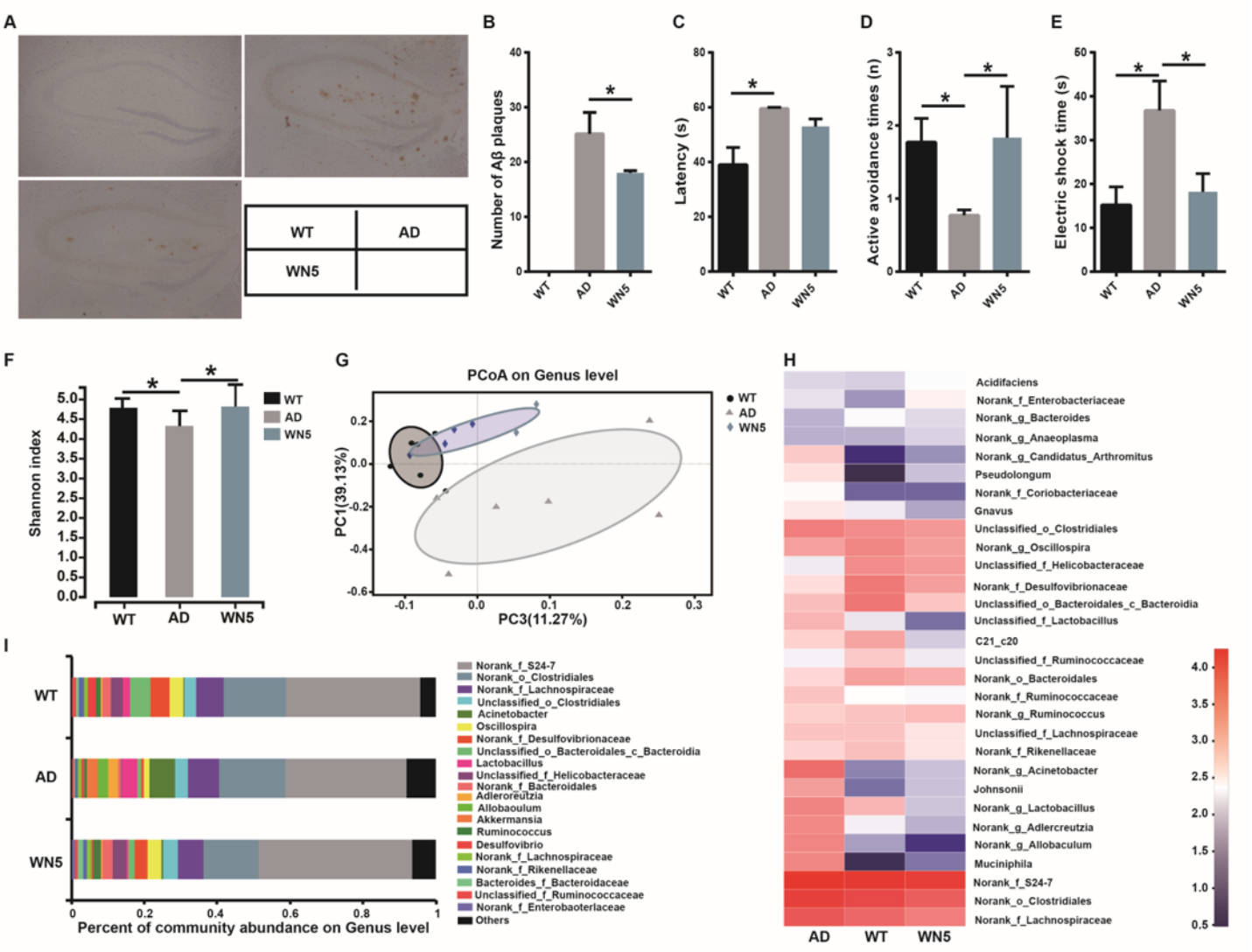

Figure 2

Peptide WN5 altered the gut microbiota and reduced Aβ plaque in APP/PS1 mice. (A) Images of Aβ42 immunohistochemistry in the hippocampus of brain sections. Wild-type mice treated with vehicle (WT), APP/PS1 mice treated with vehicle (AD), APP/PS1 mice treated with peptide WN5 (400 mg/kg, WN5). (B) Quantification results of immunohistochemistry in the hippocampus of mice brain section. (C) Latency in Morris water maze test (s). (D) Active avoidance times (n) in Shuttle box test. (E) Electronic shock time (s) in Shuttle box test. WT vs AD, *P < 0.05; WN5 vs AD, *P < 0.05. (F) Shannon index of alpha-diversity. (G) Principle coordinate analysis (PCoA) plot generated using OTU metrics based on the Bray-Curtis similarity. (H-I) The Heatmap and bar graph showing percent abundance of the major bacteria at genus level.

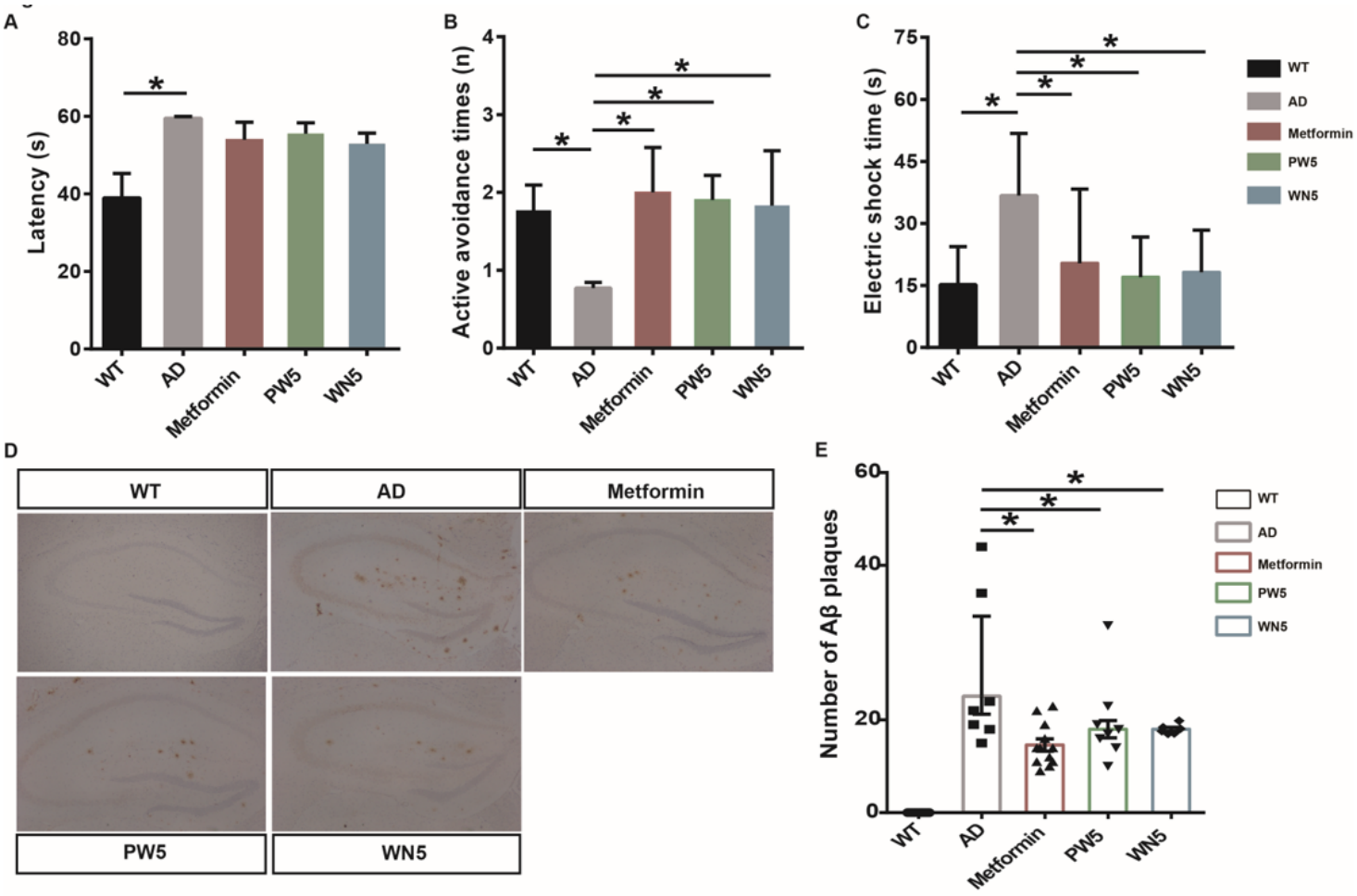

Figure 3

Metformin, WN5 and PW5 treatments achieved similar level of decreased in Aβ plaques and improvement in cognitive performance. (A) Latency in Morris water maze test (s). (B) Active avoidance times (n) in Shuttle box test. (C) Electronic shock time (s) in Shuttle box test. WT vs AD, *P < 0.05; WN5/PW5/Metformin vs AD, *P < 0.05. There were no significant differences between WN5, PW5 and Metformin. (D) Images of Aβ42 immunohistochemistry in the hippocampus of brain sections. (E) Quantification results of immunohistochemistry in the hippocampus of mice brain section. WN5/PW5/Metformin vs AD, *P < 0.05. There were no significant differences between WN5, PW5 and Metformin.

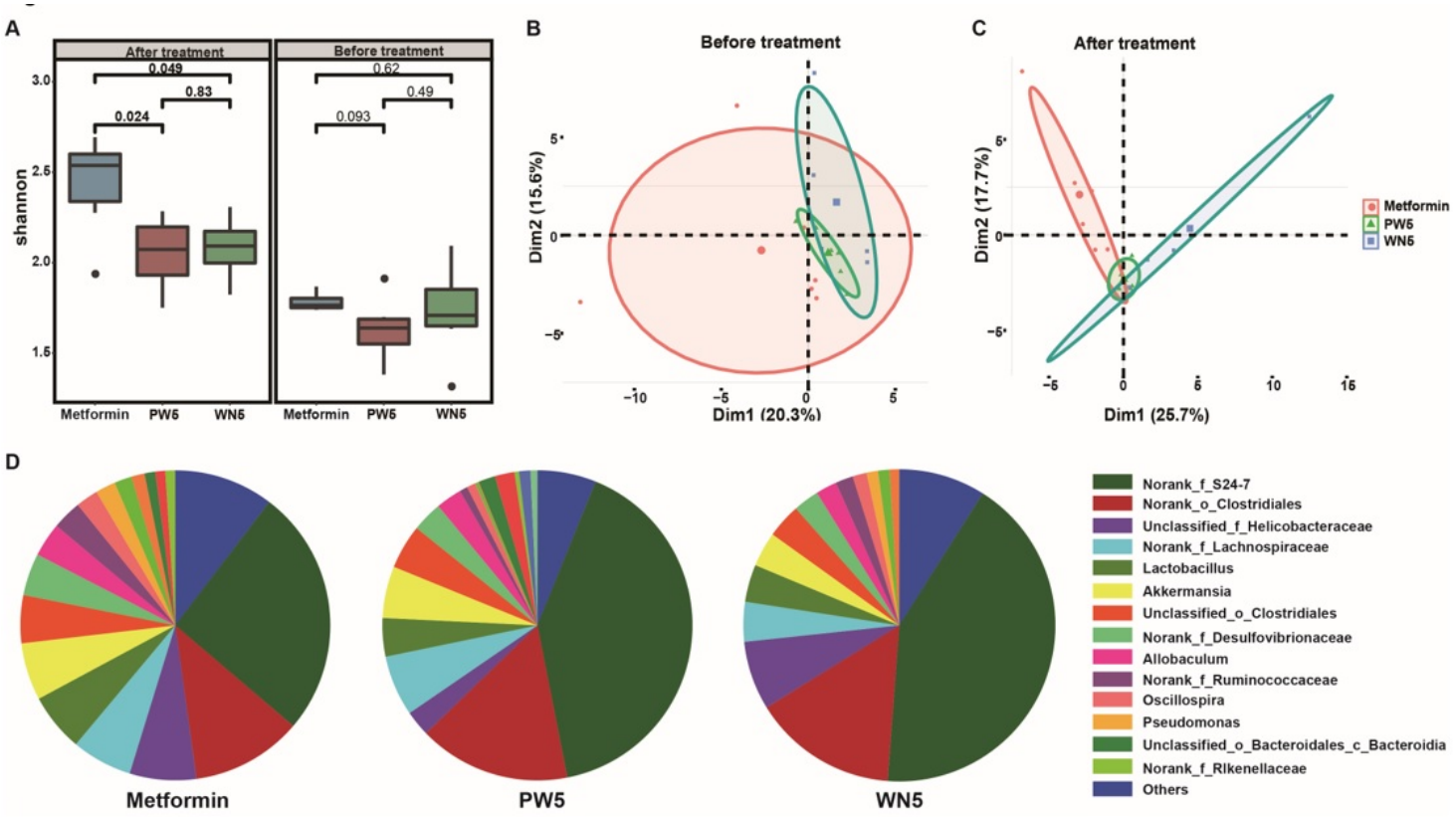

Figure 4

No uniform gut microbiota pattern among Metformin, WN5 and PW5 treatments. (A) α-diversity analysis results showing no differences among WN5, PW5 and Metformin intervention in evenness and diversity of the reconstituted gut microbial. (B-C) PCA analysis showing significantly different microbiota composition among WN5, PW5 and Metformin treated mice. (D) Pie chart showing the relative abundance of the dominant microbiota genera of WN5, PW5 and Metformin treated mice.

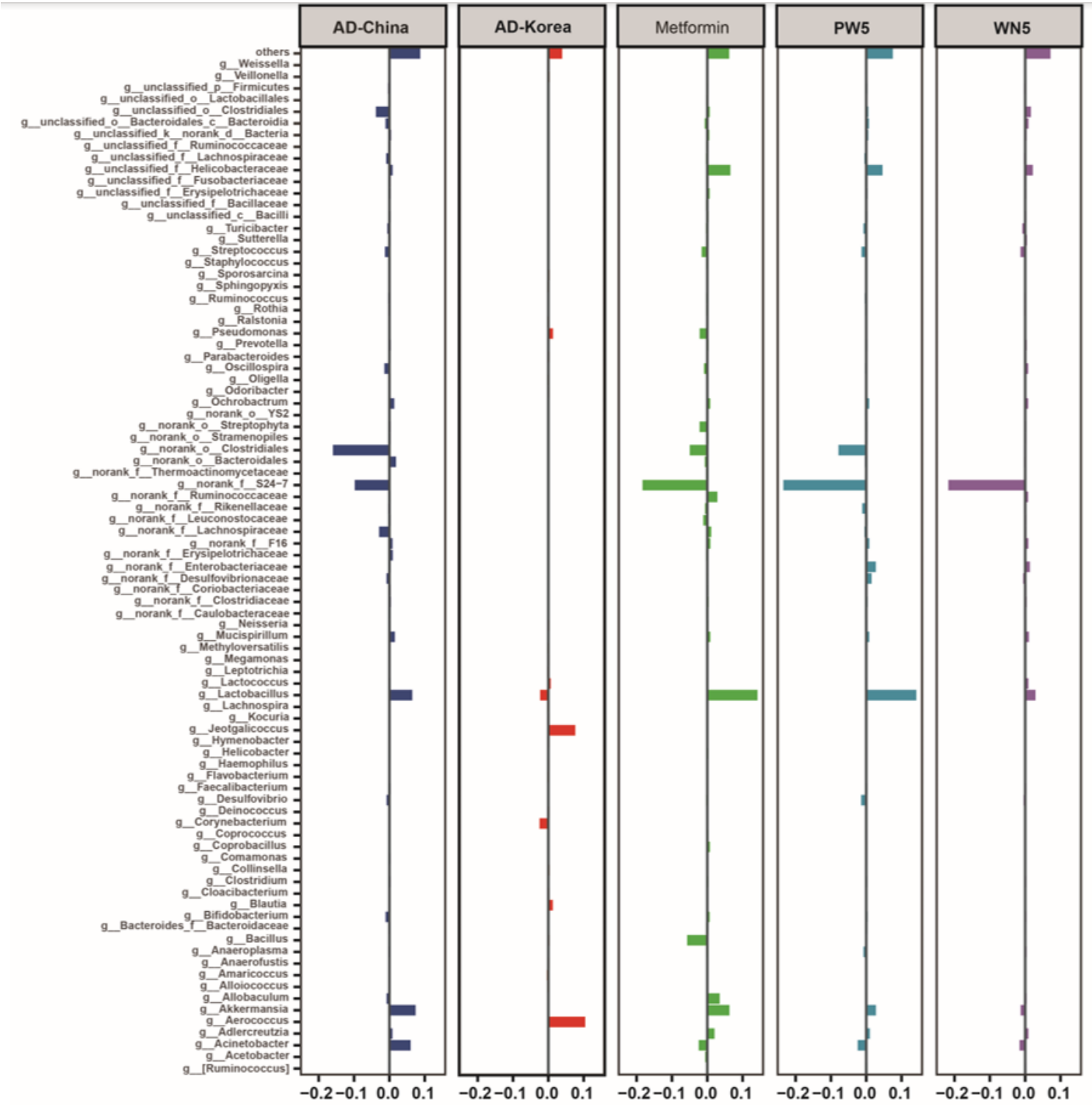

Figure 5

No uniform gut microbiota patterns associated with Aβ plaque pathology in APP/PS1 mouse model. Comparison of the gut microbiota compositions of our untreated APP/PS1 mouse model and untreated APP/PS1 mouse model of Park et al showed no uniform pattern. Metformin, WN5 and PW5 treated gut microbiota were not completely distinct from that of untreated APP/PS1 model.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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