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Exercise and probiotics attenuate the development of Alzheimer's disease in transgenic mice: Role of microbiome

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ABSTRACT

It has been suggested that exercise training and probiotic supplementation could decelerate the progress of functional and biochemical deterioration in APP/PS1 transgenic mice (APP/PS1^{TG}). APP/PS1^{TG} mice were subjected to exercise training and probiotic treatments and functional, biochemical and microbiome markers were analyzed. Under these conditions the mice significantly outperformed controls on The Morris Maze Test, and the number of beta-amyloid plaques decreased in the hippocampus. *B. thetaiotaomicron* levels correlated highly with the results of the Morris Maze Test ($p < 0.05$), and this group of bacteria was significantly elevated in the microbiome of the APP/PS1^{TG} mice compared to the wild type. *L. johnsonii* levels positively correlated with the beta amyloid content and area. Data revealed that exercise and probiotic treatment can decrease the progress of Alzheimer's Disease and the beneficial effects could be partly mediated by alteration of the microbiome.

1. Introduction

The gut microbiota consists of billions of cells and the microbiome (microbial genes) outnumber the host genes by about 150 to 1 (Hill et al., 2014). Moreover, this relatively plastic complex biomass can play a role in the development and progress of a variety of disorders and diseases outside of the gastrointestinal system (Gill et al., 2006; Honda and Littman, 2016; Human Microbiome Project, 2012; Pope et al., 2017). Direct bidirectional communication between the brain and the gut has been suggested (Dinan and Cryan, 2017; Rhee et al., 2009) as brain can alter gut function by influencing motility, secretion, blood flow and gut-associated immune function in response to psychological and physical stressors (Mayer, 2000). Additionally, the microbiome can modulate neuronal, immune, metabolic and endocrine pathways (Lynch and Pedersen, 2016). It has also been shown that aging, nutrition, and physical exercise can modify the microbiome (Voreades et al., 2014).

Aging has a significant effect on the human microbiome (Rampelli et al., 2013) and germ-free laboratory rats have longer life-spans than wild animals under laboratory conditions (Gustafsson, 1946). The blood brain barrier of germ-free mice has an increased permeability when compared to pathogen- free mice with a normal microbiome (Braniste et al., 2014), suggesting a complex effect of the microbiome on the brain.

Alzheimer's disease (AD), which is one of the most common, irreversible, neurodegenerative disorders of the central nervous system in the elderly, results in non-curable cognitive impairment and dementia. Accumulation of beta amyloid, which is one of the significant features of AD, involves an inflammatory response at the site of amyloid deposition in the brain (Hill and Lukiw, 2015) and the microbiome, at

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least in part, can be linked to this accumulation (Dinan and Cryan, 2017). Diverse microbes in human gut generate functional amyloids, including ones like those deposited in the brain of patients with AD (Hill and Lukiw, 2015). AD is associated with a decline in long-term potentiation (LTP), and it has been shown that when young and old rats were treated for six weeks with a probiotic mixture, the treated group was rescued from loss of LTP by the treatment (Distrutti et al., 2014). Another striking feature of AD is loss of memory. It has been shown that AD is associated with changes in the microbiome (Agahi et al., 2018; Junges et al., 2018; Nho et al., 2018), indicated by the differences in short chain fatty acid content of wild type of mice and mice from the AD model (Zhang et al., 2017). It has been shown that treatment with probiotics increases brain performance, as measured by a maze test and an altered microbiome environment (Athari Nik Azm et al., 2018; Leblhuber et al., 2018; Savignac et al., 2015). These results suggest that a treatment with probiotics might serve as a potential tool to retard the progress of AD.

Physical exercise can attenuate the incidence of AD via a complex mechanism including attenuation of oxidative stress, and increased metabolism of the brain (Radak et al., 2010). Exercise can up-regulate the activity of 8-oxoguanine DNA glycosylase-1 (OOG1), which not only decreases the damage to DNA but also is closely linked to inflammation (Ba et al., 2014; Boldogh et al., 2012).

Although it is well known that exercise alters the microbiome (Allen et al., 2018; Chen et al., 2018; Cook et al., 2016), it is not known whether exercise- mediated changes in the microbiome would affect the progress of AD.

The diversity of the microbiome is associated with cardiovascular fitness. The microbiome of individuals with higher levels of VO2max tends to produce greater amounts of butyrate (Estaki et al., 2016), which is an important short-chain fatty acid to suppress inflammation (Bachmann et al., 2017). It has also been observed that exercise induces a more diverse microbiome (O'Sullivan et al., 2015). This is an important observation even though exercise has not been thoroughly linked to gut integrity. Exercise normally reduces the risk of gastrointestinal cancer, reflux, and incidence of ulcers, fatty liver, irritable bowel syndrome, and diverticulitis (de Oliveira and Burini, 2009). In addition, exercise in older animals has been demonstrated to reduce expression of inflammatory mediators and apoptotic markers in intestinal lymphocytes, suggesting a protective role of exercise in intestinal health (Hoffman-Goetz et al., 2009; Packer and Hoffman-Goetz, 2012). Dietary probiotic bacteria, which are live microorganisms and considered to be beneficial for health, nowadays are widely used for maintaining or restoring gut microbiome in various disorders of the gastrointestinal tract (Wong et al., 2015). Chronic neurodegenerative diseases, including AD have a high rate of gastrointestinal comorbidities and it has been proposed that management of the gut microbiota by probiotics may prevent or alleviate the symptoms of these chronic diseases (Westfall et al., 2017). Recent clinical trials showed improvement of both bowel disorders and neuropsychiatric symptoms in irritable bowel syndrome (IBS) after uptake of a composition containing probiotic lysate, low-dose multivitamins and omega 3 fatty acids (FRAMELIM®) (Feher et al., 2014).

The aims of the present study were to test (i) the influence of exercise training and probiotic treatment on the development of AD in APP/PS1 transgenic mice and (ii) to observe whether beneficial effects would be associated with any changes in the gut microbiome.

2. Materials and methods

2.1. Experimental animals and training procedure

Thirty two male APP/PS1 transgenic mice (B6C3-Tg (APPswe,PSEN1dE9)85Dbo/Mmjax; APP/PS1^{TG}) were randomly assigned to control (APP/PS1^{TG}-C), exercise (APP/PS1^{TG}-Ex), probiotic treated (APP/PS1^{TG}-Pr) and combined (exercise and probiotic treated) (APP/PS1^{TG}-Ex-Pr) groups. For The open field test, The Morris Water Maze Test, the spontaneous alteration tests, and the microbiome investigation, we added wild mice of similar ages, from the same colony, as absolute controls (Wt). The investigation was performed according to the requirements of “The Guiding Principles for Care and Use of Animals, EU”, and was approved by the Semmelweis University Ethics Committee (No: PEI/001/2105–6/2014).

Treatments were carried out for 20 weeks starting at the age of 3 months, with the aim of decreasing the progress of AD development and functional impairment. Interval treadmill running was applied as the exercise regimen for the combined groups. Previously, animals were habituated on a motor driven treadmill (Columbus Inst. Columbus Ohio) at the assigned running speed for two weeks. Training was performed four times a week, for 60 min. Each training session lasted ten cycles, each cycle consisting of four minutes at high intensity (20 m/min) and two minutes at low intensity (10 m/min). Transgenic control and probiotic treated groups (APP/PS1^{TG}-C and −Pr) were placed on the treadmill and stayed there for five minutes/day on the stationary belt.

FRAMELIM® contains probiotics *Bifidobacterium longum* and *Lactobacillus acidophilus* lysates, vitamins A, D and omega 3 fatty acids in cod liver oil, as well as vitamins B1, B3, B6, B9, B12, and it was administered five times a week (120 mg/day) for 20 weeks along with rodent chow. We have daily monitored the food uptake of all mice and probiotic treatment did not alter the amount of food and water intake of animals.

After the 20 weeks of treatment, animals were exposed to two weeks of cognitive testing, but even during testing the probiotic treatments were continued. After the cognitive tests were completed, animals were anesthetized with an intraperitoneal injection of ketamine (Richter, concentration: 100 mg/ml) /xylazine (Produlab Pharma, concentration: 20 mg/ml) cocktail in a dose of 0.1 ml/10 g bodyweight and transcardially perfused with heparinized ice- cold saline. Brain was removed and measured, rapidly dissected in half along the corpus callosum. One hemibrain was postfixed in 4% paraformaldehyde (PFA) for immunohistological staining, and the other hemibrain was dissected into three parts (frontal, parietal and occipital), and the hippocampus was removed. All parts were collected, frozen in liquid nitrogen and stored at −80 °C until further biochemical analysis. Fecal samples were collected for microbiome analysis one day before the end of the experiment.

2.2. The open field test, The Morris Water Maze Test and spontaneous alteration tests

An open field arena 400 × 400 × 300 mm was used as the open field test. The arena was divided into 16 quadratic blocks, in which to measure animal physical activity. Each mouse was placed in the center of the arena and spontaneous ambulatory locomotion was recorded for 5 min. The number of times each mouse crossed the block separating lines and the number of rearings were counted. (Alfieri et al., 2016).

Brain function was evaluated by the Morris Maze Test on four consecutive days (four trials per day). The maze consisted of a circular tub (60 cm in height and 100 cm in diameter), in which a platform 6 cm in diameter was placed in the center of the northwest quadrant of a circular pool, 1 cm below the surface of the water. The water temperature was maintained between 22 and 23 °C throughout training and testing. Mice received four trials (up to 60 s) per day from each of four different start locations (north, south, west, or east). The mice were allowed to rest on the platform for 20 s between trials, and then were placed in a holding cage for 5 min between the two blocks. Swim paths were recorded by an overhead video camera and tracked by the automated software.

The Y-maze consisted ofa three-arm horizontal maze with an angle of 120°, which were 28 cm in length, 6 cm in width, and 18 cm in height. The maze floor and walls were constructed with white polyvinyl plastic.

Mice were initially placed in one arm, and then the sequence and number of arm entries were monitored for an eight-minute period. An actual alternation was defined when a mouse entered into all three arms on consecutive choices (i.e., ABC, BCA, or CAB, but not CAC, BAB, or ABA). The spontaneous alternation (%) was derived from the total number of alternations divided by the total number of arm entries minus two, which was multiplied by 100 as shown in the following equation: % Alternation = [(Number of alternations)/(Total number of arm entries −2)] × 100. The number of arm entries also served as an indicator for movement and locomotor activity (Alfieri et al., 2016).

2.3. Immunofluorescent labelling

After conservation in 4% PFA for 1 day, brain hemispheres were washed in 0.1% phosphate buffer (PB) at room temperature. Then, hemispheres were placed into a 15% sucrose solution for 4 h, then placed in a 30% sucrose solution overnight at 4° Celsius. Tissue was then embedded in cryoprotectant (Tissue Tek, Sakura Finetek Europe Ref. 4583) in liquid nitrogen. Brains were then sectioned in a Leica Sliding Microtome (Model SM2000R) to 40 μm dorsal coronal sections, and stored in PB with sodium-azide.

2.4. β-amyloid and OGG1 staining

Every sixth free-floating brain section of each group was immunostained for amyloid plaques (6E10, Anti-β- amyloid, 1–16 antibody, BioLegend #803015). Beta-amyloid was detected with an affinity-purified antiserum from a mouse immunized with human antigen. This antiserum (1:15000 for 3,3′-diaminobenzidine) was applied overnight at room temperature followed by incubation of the sections in biotinylated anti-mouse secondary antibody for 1 h (1:1000 Vector Laboratories #BA 2000) and then in avidin-biotin-peroxidase complex (1:500, VECTASTAIN Elite ABC-Peroxidase Kit, PK-6100) for 1 h. Subsequently, sections were treated with 0.06% diaminobenzidine (DAB, sigma) 0.08% nickel (II) sulfate and 0.003% H_2O_2 in Tris-hydrochloride buffer (0.1 M, pH 8.0) for 2.5 min, mounted and coverslipped. DNA repair enzyme (Anti-OGG1 Abcam #ab22766) was visualized using the same method described above. The antiserum was used in a dilution of 1:2000. A biotinylated anti-rabbit secondary antibody was used (Jackson ImmunoResearch #711–065-152).

2.5. Double labeling 6E10 and Iba1

Brain sections were processed for double labeling with 6E10 and Iba1. Every sixth free-floating section was first stained for Iba1 (AIF/Iba1 Novus biologicals #NB100–1028) by using FITC-tyramide amplification fluorescent immunocytochemistry. This antiserum (1:2500) was applied overnight at room temperature, followed by incubation of the sections in biotinylated anti-goat secondary antibody (1:1000, Vector Laboratories #BA9500), afterwards in ABC complex (1:500; Vector Laboratories) for 1 h. Then, sections were subsequently incubated with FITC-tyramide (1:8000, Sigma) and H_2O_2 in Tris hydrochloride buffer (0.1 M, pH 8.0) for 8 min. Sections were then incubated overnight in 6E10 (1:7000; BioLegend #803015) at room temperature. Following application of the primary antibody, sections were incubated in Alexa Fluor 594 anti-mouse secondary antibody (Invitrogen #A21203) for 2 h. After several washes, sections were mounted and coverslipped.

2.6. Microscopy and image processing

An Olympus BX60 light microscope equipped with fluorescent epi-illumination and a dark-field condenser was used to examine the sections. Images were captured at 2048 × 2048 pixel resolution with a SPOT Xplorer digital CCD camera (Diagnostic Instruments, Sterling Heights, MI) using 10–20× objectives. Images were adjusted using the "levels" and "sharpness" commands in Adobe Photoshop CS5.1. Full resolution of the images was maintained until the final versions, which were adjusted to a resolution of 300 dpi. Images were analyzed with ImageJ Software version 1.48v.

2.7. Library preparation and identification of prokaryotic species

The DNA from stool samples was isolated by QIAmp Fast DNA stool mini kit (Quiagen). Fragment libraries were constructed from purified DNA using NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs) according to the manufacturer's instructions. Briefly, DNA was enzymatically digested and the fragments were end-repaired. Ion Xpress Barcode Adaptors (Life Technologies) were than ligated and the template fragments size-selected using Agincourt AMPure XP magnetic beads (Beckman Coulter). Adaptor ligated fragments were than PCR amplified, cleaned-up using AMPure beads, quality checked on D1000 Screen Tape and Reagents using Tape Station instrument (Agilent) and finally quantitated using the Ion Library TaqMan Quantitation Kit (Life Technologies). The library templates were prepared for sequencing using the Life Technologies Ion OneTouch protocols and reagents. Briefly, library fragments were clonally amplified onto Ion Sphere Particles (ISPs) through emulsion PCR and then enriched for template-positive ISPs. More specifically, PGM emulsion PCR reactions utilized the Ion PGM Hi-Q OT2 Kit (Life Technologies), and as specified in the accompanying protocol, emulsions and amplification were generated using the Ion OneTouch System (Life Technologies). Enrichment was completed by selectively binding the ISPs containing amplified library fragments to streptavidin- coated magnetic beads, removing empty ISPs through washing steps, and denaturing the library strands to allow for collection of the template-positive ISPs. For all reactions, these steps were accomplished using the Life Technologies ES module of the Ion OneTouch System. Template-positive ISPs were deposited onto the Ion 318 chips (Life Technologies); finally, sequencing was performed with the Ion PGM Hi-Q view OT2 Kit (Life Technologies).

2.8. Analytical methods

Bacterial genome annotation was carried out by uploading the FASTQ data files to the automated web-based metagenomics analysis server MG-RAST version 3.6 (Aziz et al., 2008). MG-RAST takes FASTQ data files as input, identifies open reading frames that are likely to be genes, and uses a series of subsystem techniques (the 'ST' in RAST) to compare these with a sophisticated database of genes and RNA sequences, producing a high-quality annotation of the assembly. The data files, annotated based on the RefSeq database, were downloaded for further analysis. The MG-RAST ids of the data sets are as follows: Wt (healthy control): mgm4672670.3; APP/PS1^{TG}-C: mgm4673335.3; APP/PS1^{TG}-Ex: mgm4682164.3; APP/PS1^{TG}-Ex-Pr: mgm4682165.3; APP/PS1^{TG}-Pr: mgm4682167.3.

2.9. Bioinformatics analysis

The data were filtered based on the following criteria of annotation quality: minimum alignment length: 30 base pairs; minimum percentage of identity: 60%; maximal *E*-value: 10^{-5}. Then, the annotated reads of each sample were permutated in random order, and were divided into 10 non-overlapping subsets, containing 10% of the original data. This process was performed using a Python script. The generated populations were used for calculating the relative abundances and standard deviations of selected microbial groups. The results were visualized in bar graphs. The significance of differences between the groups was tested with two-sample Kolmogorov-Smirnov test with $p < 0.001$ significance threshold. These analyses were carried out in MATLAB.

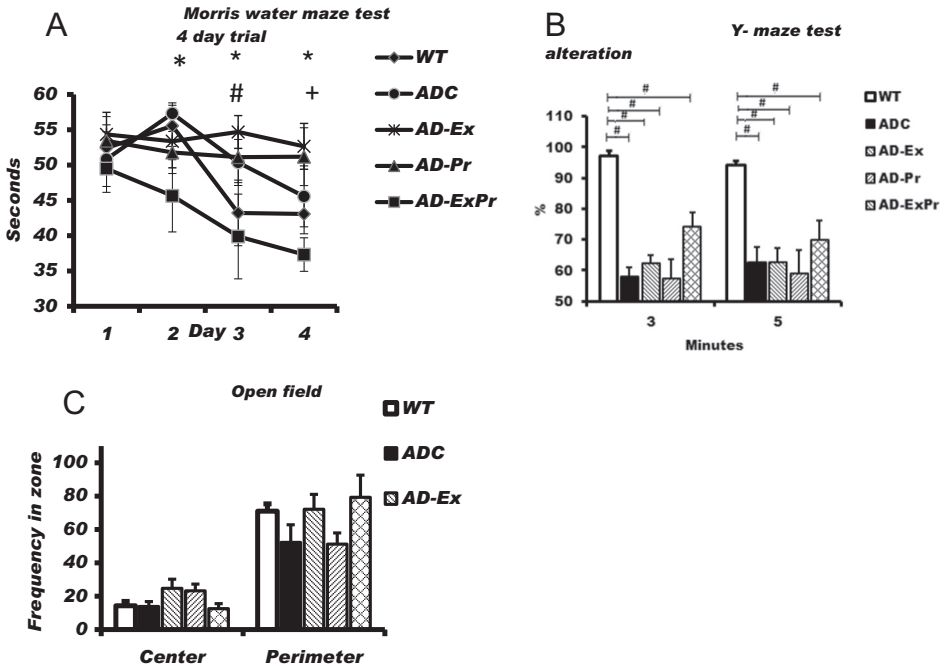


Fig. 1. Functional and behavioral tests of APP/PS1^{TG} mice.
The exercise and probiotic treatment improved spatial memory assessed by The Morris Maze Test (panel A). Similar trends have been observed in the Y-test (panel B). Open field test results also suggested that the probiotic and exercise training increased the exploratory activity of the mice. Results are expressed as mean ± SD. Groups: control (APP/PS1^{TG}-C), exercise (APP/PS1^{TG}-Ex), probiotic treated (APP/PS1^{TG}-Pr) and combined (exercise and probiotic treated (APP/PS1^{TG}-Ex-Pr) ($N = 8$)), * $p < 0.05$: APP/PS1^{TG}-C vs. APP/PS1^{TG}-Ex-Pr, # $p < 0.05$: APP/PS1^{TG}-Ex vs. APP/PS1^{TG}-Ex-Pr, + $p < 0.05$: APP/PS1^{TG}-Pr vs. APP/PS1^{TG}-Ex-Pr. Normality of variables was evaluated by the Shapiro–Wilk test, then repeated measures and ANOVA with post hoc Tukey test was used.

2.10. Statistics

For the evaluation of physiological and biochemical data, all dependent variables were performed using the Shapiro-Wilk normality test. Depending on the result, two- way analysis of variance (ANOVA) followed by Tukey's post hoc test or Kruskal-Wallis ANOVA with Dunn's post hoc test was used. The correlation between two variables was measured by Pearson coefficient. Significance level was set at $p < 0.05$.

3. Results

3.1. Exercise and probiotics increased spatial memory of APP/SP1 mice

Parallel groups of wild-type and APP/PS1 transgenic mice were subjected to exercise (Interval treadmill running) plus/minus probiotic supplementation for 20 weeks and cognitive functions, especially spatial memory, which is dependent on hippocampal function, were evaluated by The Morris Maze Test on four consecutive days. APP/PS1 transgenic control mice showed no improvement in performance during the four trial days (Fig. 1A). On the other hand, animals from APP/PS1^{TG}-Ex-Pr group could locate the hidden platform in a shorter period of time ($p < 0.05$) day by day, suggesting that the brain performance did not decline as much as in other groups in this study.

The Y-maze test results revealed that wild mice out-performed AD mice, and the combined effects of exercise training and probiotic treatment increased the number of alterations (Fig. 1B). Open field test results also suggested that the probiotic and exercise training increased the exploratory activity of the mice (Fig. 1C).

These data imply that cognitive performance that relies on hippocampal regions are severely altered in APP/PS1^{TG} mice. Exercise training and probiotics significantly improved cognitive functions.

3.2. Exercise and probiotics decrease histological changes in APP/PS1 expressing mice

Brains were sectioned every sixth free- floating dorsal coronal section from each group and were immunostained for amyloid plaques using anti-β-amyloid. The overall examinations showed that amyloid beta plaques are developed in brain regions but are most abundant in the hippocampal area. These results imply that ± exercise and probiotics did not alter distribution of β- amyloid plaques in treated vs non-treated APP/PS1^{TG} mice but show that the mean number of plaques in hippocampus is significantly lower in the APP/PS1^{TG}-Ex group (13.9 ± 6.7) than in the APP/PS1^{TG}-C group (31.7 ± 9.0) and in the APP/PS1^{TG}-Ex-Pr group (29.1 ± 6.7). The APP/PS1^{TG}-Pr group showed a mean number of 18.1 ± 5.4 (Fig. 2A). In addition to the number of plaques, the plaque-covered area was also measured. The mean % of the areas covered by plaques was significantly lower in all treated groups: APP/PS1^{TG}-Ex (0.25 ± 0.1), APP/PS1^{TG}-Pr (0.35 ± 0.1), APP/PS1^{TG}-Ex-Pr (0.39 ± 0.2) compared to the APP/PS1^{TG}-C group (0.67 ± 0.2) (Fig. 2B). Microglia occupied area was lowest in the APP/PS1^{TG}-C group (5.36 ± 2.4) and was highest in the APP/PS1^{TG}-Ex-Pr group 8.11 ± 1.3 while in APP/PS1^{TG}-Pr 6.88 ± 1.5 and in APP/PS1^{TG}-Ex 7.9 ± 2.7 unit were measured, which was covered by microglia (Fig. 3).

AD in the animal model and in humans is associated with decreased levels of OGG1 (Shao et al., 2008), while exercise modulates the levels of OGG1 in the brain (Sarga et al., 2013). Therefore, it was tested whether exercise induced adaptation would alter OGG1 content in this animal AD model. OGG1 levels were significantly higher in APP/PS1^{TG}-Ex 7951 ± 4085 group than the two other treated groups APP/PS1^{TG}-Pr 2947 ± 2222 and APP/PS1^{TG}-Ex-Pr 3358 ± 1127 in APP/PS1^{TG}-C group levels were 6306 ± 3001 (Fig. 4). The occupied area of OGG1 showed the exact same pattern: APP/PS1^{TG}-Ex (0.26 ± 0.1) APP/PS1^{TG}-Pr (0.1 ± 0.06) APP/PS1^{TG}-Ex-Pr (0.11 ± 0.04) while in APP/PS1^{TG}-C (0.2 ± 0.01) area was measured.

3.3. APP/PS1 overexpression changes gut microbiome: beneficial effects of exercise

To study whether APP/PS1 expression, exercise and the probiotics change gut microbiome and consequently cognitive function the stool samples of mice were measured. Additionally, the wild type (Wt) group was added into the microbiome analysis. DNA from fecal samples was isolated and species were identified (Materials and Methods).

Generally, in each group (Wt, APP/PS1^{TG} ± treatments) *Firmicutes* and *Bacteroidetes* species were the most abundant. *Proteobacteria* and *Actinobacteria* were insignificantly lower, while *Fusobacteria* and *Verrucomicrobia* species were minor contributors to the microbiome.

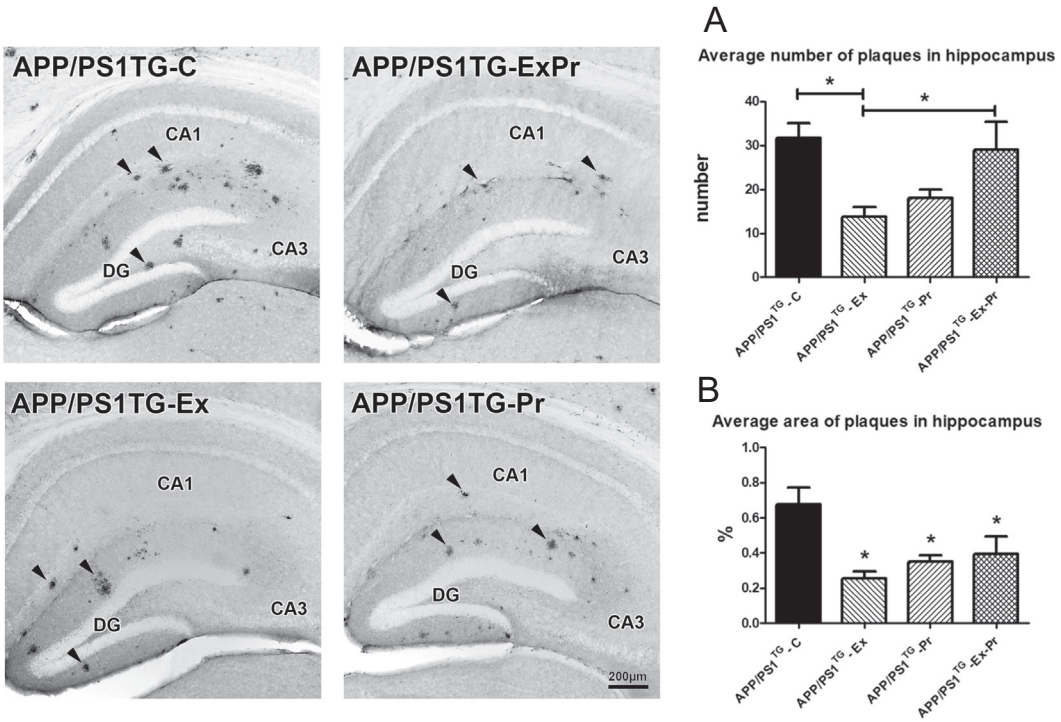


Fig. 2. Beta amyloid plaque number and hippocampus area.
Histochemical staining shows that exercise training reduced the plaque number compared to APP/PS1^{TG}-C group (A). The area of amyloid plaques decreases in all treated groups (B). Results are expressed as mean ± SD (N = 8), * $p < 0.05$. Data were evaluated by the Shapiro-Wilk normality test and then by the Kruskal-Wallis test.

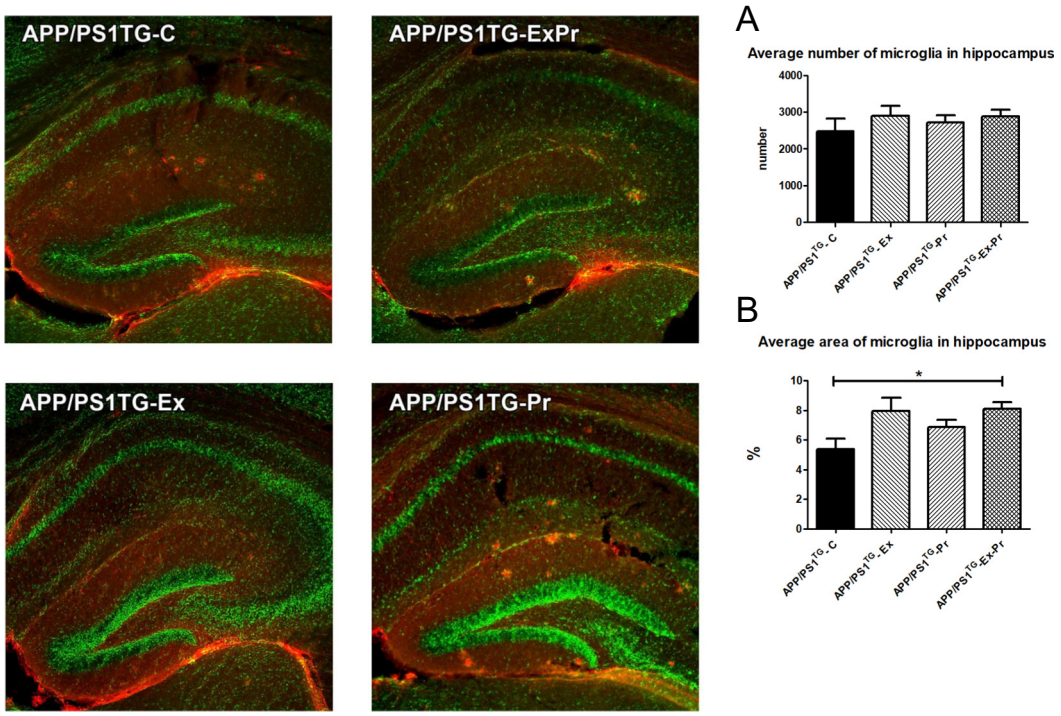
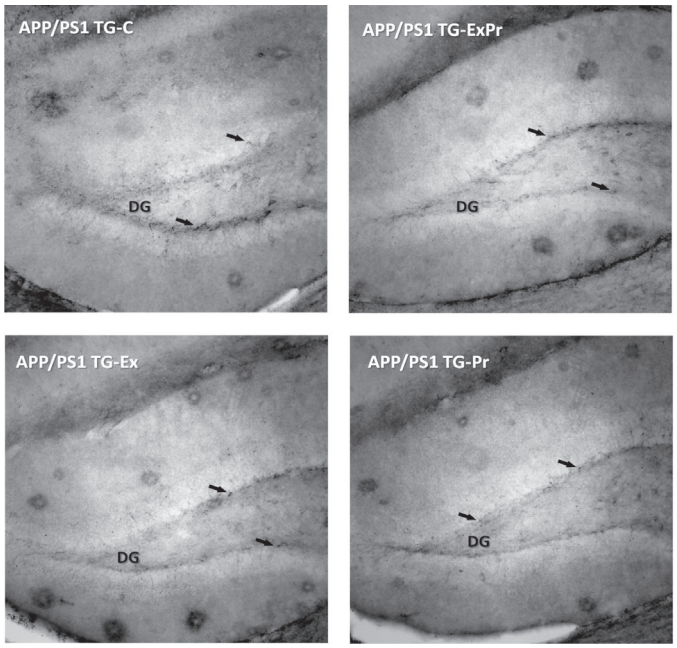


Fig. 3. Microglia number and area in the hippocampus.
The number of microglia was similar in each group (A). On the other hand, the area increased with the combined effects of exercise and probiotics (B). Moreover, microglia accumulated around the amyloid plaques, which could be a part of a protective mechanism. Results are expressed as mean ± SD (N = 8), * p < 0.05. Data were evaluated by the Shapiro-Wilk normality test and then by the Kruskal-Wallis test.



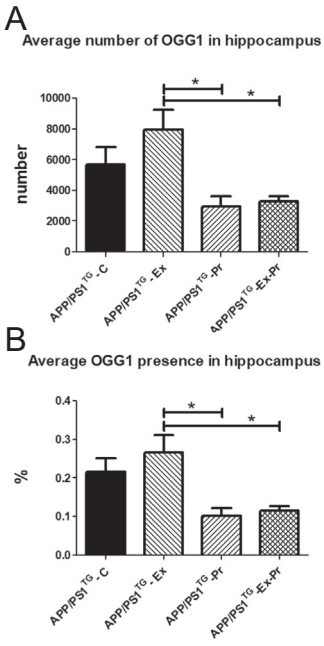


Fig. 4. OGG1 levels in the hippocampus of APP/PS1 transgenic mice. Exercise increased OGG1 number (A) and area (B) in the hippocampus, while probiotics treatment prevented this up-regulation. Results are expressed as mean ± SD (N = 8), * $p < 0.05$. Data were evaluated by the Shapiro-Wilk normality test and then by the Kruskal-Wallis test.

The ratio of the *Firmicutes/Bacteroides* was the lowest in the APP/SP1^{TG}-Pr group and highest in the Wt group without APP/PS1 overexpression. In the Wt control group we found an increased ratio of *Firmicutes* to *Bacteroides* species. There were no significant numbers of eukaryotic cells (yeast, fungi or single cell organisms) identified using the current approach. We found that the *B. thetaiotaomicron* levels correlated with poorer results in The Morris Maze Test ($p < 0.05$), and this group of bacteria was significantly elevated in the microbiome of all of APP/PS1^{TG} mice compared to wild type, while *L. johnsonii* levels positively correlated with beta amyloid content and area ($p < 0.05$).

3.4. Distribution of prokaryotic species in experimental groups of animals

The differences among the Wt and the APP/PS1^{TG} groups (APP/PS1^{TG}-C, APP/PS1^{TG}-Ex, APP/PS1^{TG}-Pr, APP/PS1^{TG}-Ex-Pr) were particularly striking at the genus level (Fig. 5). The greatest differences between the APP/SP1 expressing and non-expressing (Wt control) were in the genera *Prevotella* and *Bacteroides* where *Prevotella* (Fig. 5A, B) was much more abundant in Wt control and *Bacteroides* was significantly higher in APP/PS1 expressing groups. The frequency of *Bacteroides* species was low in Wt controls compared to the APP/SP1^{TG} groups.

3.5. The effects of exercise and probiotics on butyrate producing microorganisms

Butyrate producer strains like *Eubacteria*, *Roseburia* and *Clostridia* were also more abundant in Wt compared to the APP/SP1^{TG}-C group (Fig. 5C, D, E). However, exercise in APP/PS1^{TG} increased the abundance of these genera in contrast to the APP/PS1^{TG} received probiotic ($p < 0.001$).

Differences were also observed, particularly at the species level, for other butyrate producers: including *Butyrivibrio proteoclasticus*, *Marvinbryantia formatexigens*, *Roseburia intestinalis* and *Roseburia inulinivorans* (Fig. 6A, B, C, D). APP/PS1^{TG}-C and APP/SP1^{TG}-Pr mice had significantly lower occurrences of *B. proteoclasticus* than Wt, APP/SP1^{TG}-Ex, and APP/SP1^{TG}-Ex-Pr groups. The levels of *Roseburia* spp. were significantly higher in Wt and APP/SP1^{TG}-Ex, APP/SP1^{TG}-Ex-Pr compared to APP/SP1^{TG}-C and APP/SP1^{TG}-Pr groups.

There was no significant difference in the butyrate production of microorganisms between Wt and APP/SP1^{TG}-Ex groups. However, the APP/SP1^{TG}-Pr group had the lowest frequency of butyrate producing strains.

3.6. Probiotic–induced changes in gut microbes

Gut bacteria can also produce all kinds of vitamins necessary for brain health, including vitamin B12. Studies confirm that a lack of vitamin B12 is an important risk factor for dementia. Low levels of vitamin B12 in serum are associated with an increased risk of AD (Quadri et al., 2004). *L. reuteri* is a known vitamin B12 producer (Leblanc et al., 2013) and in this study elevated levels of *L. reuteri* were observed in APP/PS1^{TG}-Ex and APP/PS1^{TG}-Ex-Pr groups (Fig. 6F). Omega 3, which was also in the probiotic complex increases the concentration of *Lactobacillus* spp. (Fig. 5F) and decreases the levels of *Clostridium* spp. (Foolad et al., 2013) which is in accordance with our data.

3.7. Exercise-mediated changes in Mucosa associated microbiota

Mucosal epithelium-associated bacteria are the following species: *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Bifidobacterium bifidum*, *Bacteroides fragilis*. There were no significant differences in abundance of *A. muciniphila*, *B. bifidum*. *Bacteroides fragilis* had similar abundance in Wt, APP/PS1^{TG}-Ex and APP/PS1^{TG}-Ex-Pr groups. There was significantly more *B. fragilis* in APP/PS1^{TG}-C and APP/PS1^{TG}-Pr groups (Fig. 6G). *B. thetaiotaomicron* was significantly lower in the Wt group and higher in APP/PS1^{TG} groups. However, among the APP/PS1^{TG} mouse groups *B. thetaiotaomicron* was significantly lower in APP/PS1^{TG}-Ex-Pr compared to APP/PS1^{TG}-Pr (Fig. 6H).

The abundance of butyrate producing bacteria increased in exercised (Fig. 5C, D, E) groups while the abundance of Lactobacillus was the lowest in APP/PS1^{TG}-Ex (Fig. 5F).

4. Discussion

Regular physical exercise and nutritional intervention have been proposed to decrease the incidence of AD. To test this hypothesis APP/PS1- expressing mice were subjected to regular exercise and probiotic treatments. The group which received both of these treatments significantly out-performed all other groups in the Morris Maze Test ($p < 0.05$) with decrease in size and beta-amyloid plaque number.

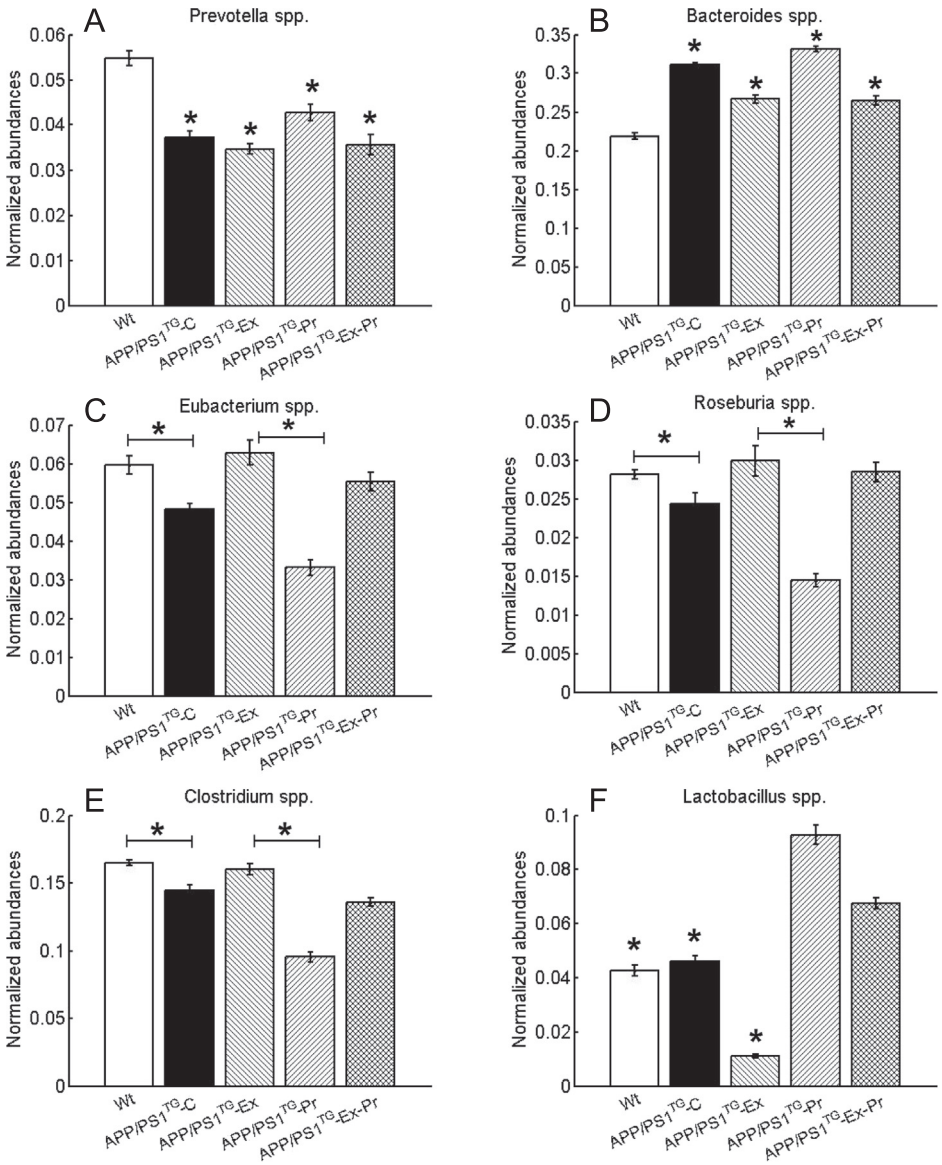


Fig. 5. The effects of exercise and probiotics on the microbiome.
Exercise and probiotics altered the levels of *Prevotella* (A) *Bacteroides* (B), *Eubacteria* (C), *Roseburia (D)*, *Clostridium* (E), *and Lactobacillus* (F) in the microbiome. Groups: wild type (WT), control (APP/PS1^{TG}-C), exercise (APP/PS1^{TG}-Ex), probiotic treated (APP/PS1^{TG}-Pr) and combined (exercise and probiotic treated (APP/PS1^{TG}-Ex-Pr) (N = 8). * $p < 0.001$. Results are expressed as mean ± SD from the two-sample Kolmogorov-Smirnov test.

Indeed, one of the main findings of this study was that exercise, probiotics and the combined effects of these two interventions decreased the number and area of amyloid plaque in the hippocampus of mice. The lower density of amyloid plaques was associated with better results in The Morris Maze Test only in mice with the combined treatment. This observation suggests that accumulation of beta-amyloids may not be closely related to impairment in spatial memory in this strain of transgenic mice (Fernandez-Murga and Sanz, 2016).

Microglia are important for brain development and provide structural and metabolic support for neurons, play a role in synaptic plasticity and are key regulators of injury and repair (Barres, 2008). Increased numbers of microglia were detected around amyloid plaques, which is in agreement with previous work (Kraft et al., 2013), and suggests that microglia could play a role in clearing dystrophic neurons (Kraft et al., 2013). In addition, our findings fit with previous results that microglia lead to glutamate receptor 3 activation against beta amyloid neurotoxicity and releases protective neurotrophins and induces amyloid removal from extracellular space by glia-mediated phagocytosis (Durand et al., 2017). Therefore, the effects of exercise and probiotics on the distribution of microglia are clearly neuroprotective.

The histological data of the measured proteins revealed that both treatments alone or combined decreased the accumulation of amyloid beta- activated microglia to repair the plaque and OGG-1 to better maintain the integrity of DNA.

4.1. *APP/PS1^{TG} model has pro-inflammatory microbiome*

Analysis of the microbiome revealed that decreased cognitive performance of APP/PS1^{TG} could be associated with leaky gut syndrome which releases LPS and cells and increases levels of *B. thetaiotaomicron* pili compared to Wt mice. Indeed, the higher levels of *B. thetaiotaomicron* correlated with poorer results in The Morris Maze Test ($p < 0.05$). This bacteria ferments glucose and lactate to propionate, acetate, and succinate. Unlike butyrate, acetate and succinate decrease mucin production. *B. thetaiotaomicron* have been shown to bind to

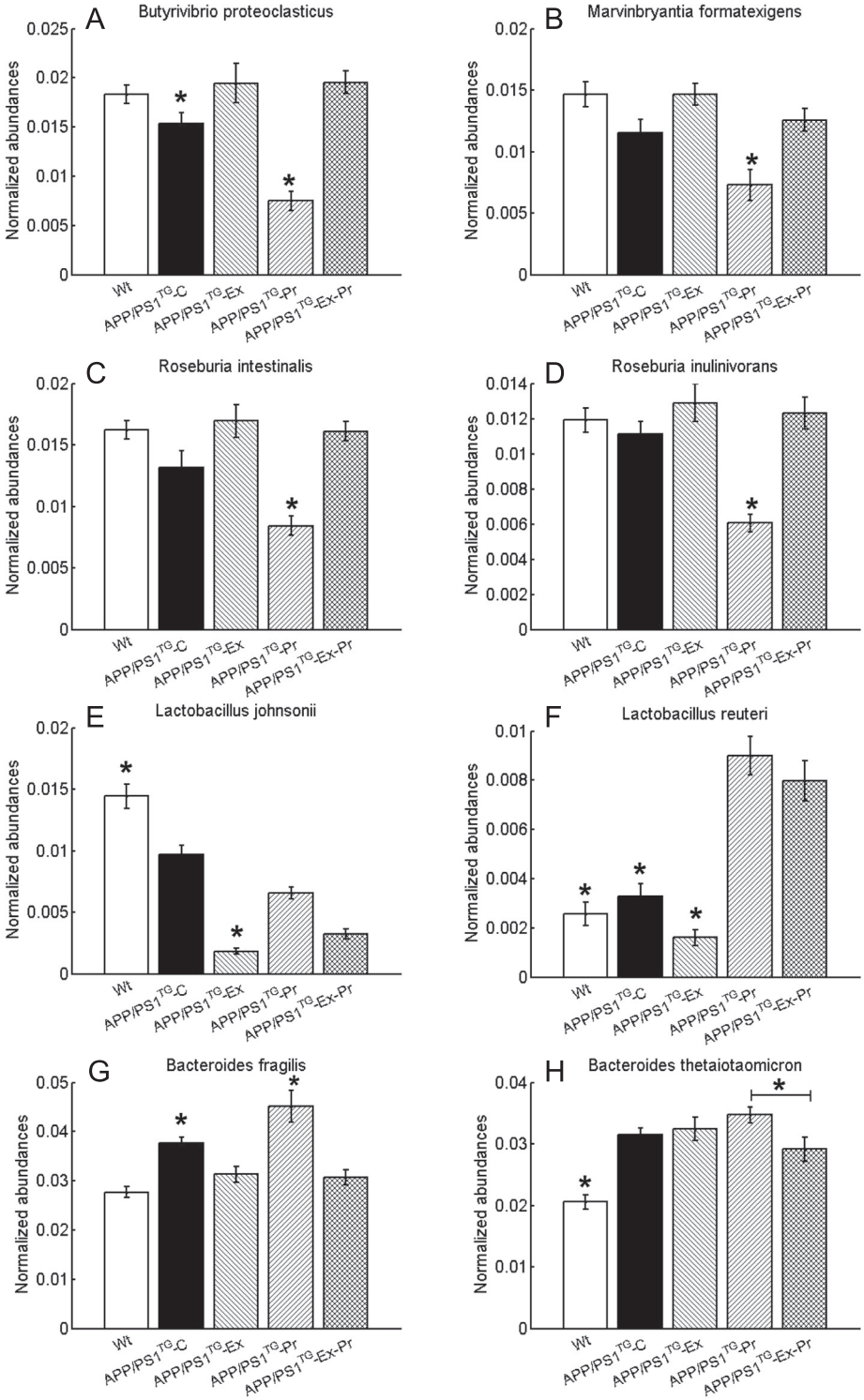


Fig. 6. Bacterial changes in the microbiome by exercise and probiotics.
Butyrivibrio proteoclasticus (A) *Marvinbryantia formatexigens* (B) *Roseburia intestinalis* (C) and *inulinivorans* (D) as well as *Lactobacillus johnsonii* (E) and *reuteri* (F) were modified in the transgenic model, by exercise and probiotics. Groups: wild type (WT), control (APP/PS1^{TG}-C), exercise (APP/PS1^{TG}-Ex), probiotic treated (APP/PS1^{TG}-Pr) and combined (exercise and probiotic treated (APP/PS1^{TG}-Ex-Pr) (N = 8). * $p < 0.001$. Results are expressed as mean ± SD, from the two-sample Kolmogorov-Smirnov test.

polysaccharides with their outer membrane receptor system (pili) before pulling the polysaccharides into the periplasm for monosaccharide degradation. This technique may help insure that the monosaccharides are not utilized by other intestinal organisms or lost in the intestines by diffusion. We hypothesize that LPS, a cell wall component of almost all Gram-negative bacteria, and the unique pili of *B. thetaiotaomicron* may be key substances, together responsible for AD. Indeed, it has been shown that LPS injection in APPs we transgenic mice resulted in neuroinflammation, and caused intracellular accumulation of β- amyloid (Sheng et al., 2003).

In contrast to natural microbiomes, the low number of *Prevotella* observed in APP/PS1^{TG} groups suggests a lack of physiological mucin

generation on the epithelial layer of intestines and may be a sign of future or current gut permeability. Butyrate induces mucin production and has an anti-inflammatory effect by inhibiting cytokine release, increasing expression of tight-junction proteins and consequently by decreasing the diffusion of LPS into the circulation. In contrast to a healthy microbiome, the relatively low number of *Prevotella* found in APP/SP1^{TG} mice suggests a lack of mucin on the epithelial layer of intestines and may have a diagnostic value on the state of gut permeability. *Prevotella* has been shown to have positive effects of mood on humans. Hence, AD- associated psychological upset could be linked to the decreased levels of *Prevotella*, underlining the complex role of the microbiome on the organism.

4.2. Exercise shifts the microbiome of APP/PS1^{TG} mice

There were significant differences between Wt and APP/PS1^{TG}-Ex groups in the butyrate producing species, consequently in butyrate levels. Suppression of inflammation has been shown to decrease the levels of beta amyloids (Liu et al., 2015; Ries et al., 2016), which actually occurred in our treated groups. Here we report that *Butyrivibrio proteoclasticus* and *Marvinbryantia formatexigens*, which are involved at butyrogenesis, are induced by exercise training as other *Clostridium*, *Eubacterium* and Roseburia species. A decrease in inflammatory processes can prevent changes in gut permeability and the flow of LPS into the circulation. It is known that SCFAs, such as acetate, propionate, and butyrate, are produced in the gut by microbial-mediated fermentation of indigestible carbohydrates. While the majority of the SCFAs in portal circulation are metabolized by the liver, SCFAs produced in the distal colon bypass portal circulation and reach the brain through circulation and neuroprotective effects (Smith, 2015).

The diversity of the microbiome is associated with cardiovascular fitness and the microbiome of individuals with higher levels of VO2max tend to produce greater amounts of butyrate (Estaki et al., 2016), which is an important SCFA acid that suppresses inflammation (Bachmann et al., 2017). It has also been observed that exercise induces a diverse microbiome (O'Sullivan et al., 2015). For the complexity of the effects of exercise another example is *L. johnsonii* which have been found to generate excessive H_2O_2 and our data show that exercise training decreases the levels of *L. johnsonii*. This observation might add to our understanding of why exercise decreases the incidence of colon cancer.

4.3. The combined effects of framelim and exercise on the microbiome

The decreased levels of beta-amyloids in APP/PS1^{TG} mice with exercise training and probiotic treatment could be partly due to an alteration of the microbiome. Exercise decreases the levels of those microorganisms which are involved in disease exacerbation and increases the amount of those microorganisms which produce SCFAs that have anti-inflammatory roles and beneficial effects on mood. Probiotic treatment increased the levels of *Lactobacillus* spp. which are correlated with the life-span in the caloric restricted model (Zhang et al., 2013). Exercise training and probiotic supplementation differently alter the microbiome of the gut, but the combined effects result in improved brain function and a decelerated progress of AD in the transgenic mice model. However, it should be kept in mind that our probiotic formulation contains probiotic lysate, i.e. heat-killed and fragmented bacteria. Consequently, certain compounds of microbes are responsible for the benefits of probiotics in this model of AD but not in live bacteria. Thus, this “probiotic paradox” well-known from several other studies needs further investigation in relation to AD (Adams, 2010).

In conclusion, currently there is no cure for AD, but experimental and epidemiological data suggest that physical exercise and proper nutrition can decrease the incidence of AD. We have shown that exercise or probiotic treatments alone are not effective in improving spatial memory. But, the combined effects of these treatments do increase brain performance, suggesting that the exercise and probiotic-induced modulation of the microbiome is important to attenuate the progress of AD. Results also showed that exercise training increased the levels of microorganisms involved in butyrogenesis. This finding is one of the first demonstrations that exercise and probiotic treatment decrease the progress of AD and that the beneficial effects are partly mediated by alteration of the intestinal microbiome. However, it has to be mentioned that the primary limitation of this study was a lack of a measure of inflammation and that future studies should investigate this possible mechanism.

Authors' contributions

DA, DS, IB, AD, MC, JJ, LB performed the analysis of samples. DA, JF, SP, GLS, MCG-C, JV, MH, KS, IB ZR all participated in the research design and the drafting, writing and revisions of the manuscript. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interest

The authors declare that they have no competing interests.

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