**Lactobacillus plantarum** PS128 alleviates neurodegenerative progression in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse models of Parkinson’s disease

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**Abstract**

Evidence suggests that the Parkinson’s disease (PD) pathogenesis is strongly associated with bidirectional pathways in the microbiota-gut-brain axis (MGBA), and psychobiotics may inhibit PD progression. We previously reported that the novel psychobiotic strain, *Lactobacillus plantarum* PS128 (PS128), ameliorated abnormal behaviors and modulated neurotransmissions in dopaminergic pathways in rodent models. Here, we report that orally administering PS128 for 4 weeks significantly alleviated the motor deficits, elevation in corticosterone, nigrostriatal dopaminergic neuronal death, and striatal dopamine reduction in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse models. PS128 ingestion suppressed glial cell hyperactivation and increased norepinephrine and neurotrophic factors in the striatum of the PD-model mice. PS128 administration also attenuated MPTP-induced oxidative stress and neuroinflammation in the nigrostriatal pathway. Fecal analysis showed that PS128 modulated the gut microbiota. *L. plantarum* abundance was significantly increased along with methionine biosynthesis-related microbial modules. PS128 also suppressed the increased family *Enterobacteriaceae* and lipopolysaccharide and peptidoglycan biosynthesis-related microbial modules caused by MPTP. In conclude, PS128 ingestion alleviated MPTP-induced motor deficits and neurotoxicity. PS128 supplementation inhibited neurodegenerative processes in PD-model mice and may help prevent PD.

**Keywords:**

Parkinson’s disease

Microbiota-Gut-brain-axis

Psychobiotic

*Lactobacillus plantarum* PS128

Neuroinflammation

Oxidative stress

Gut dysbiosis

**1. Introduction**

Parkinson’s disease (PD) is a common neurodegenerative disorder occurring at a rate of ~1% in populations over 65 years old (Pringsheim et al., 2014). Degeneration and loss of dopaminergic neurons in the substantianigra (SN) of PD patients reduces dopamine (DA) signaling, resulting in motor deficits, including rigidity, resting tremors, gait impairment and bradykinesia (Cacabelos, 2017). The pathogenic mechanisms of PD may be associated with genetic factors (Nuytemans et al., 2010), toxic agents (Rokad et al., 2017), oxidative stress (Dias et al., 2013; Segura-Aguilar et al., 2014), neuroinflammation (Tansey and Goldberg, 2010), and metabolic disorders (Trost et al.,

**Abbreviations:**

BDNF, Brain-derived neurotrophic factor; DA, Dopamine; GFAP, Glial fibrillary acidic protein; GPx, Glutathione peroxidase; GSH, Glutathione; Iba1, Ionized calcium-binding adapter molecule 1; IL-1β, Interleukin-1β; IL-6, Interleukin-6; L. plantarum, *Lactobacillus plantarum*; L-DOPA, L-3,4-dihydroxyphenylalanine; LPS, Lipopolysaccharides; MGBA, Microbiota-gut-brain axis; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NBT, Narrow beam test; NE, norepinephrine; NGF, Nerve growth factor; PD, Parkinson’s disease; PT, Pole test; RTR, Rotarod test; SCFAs, Short chain fatty acids; SN, Substantia nigra; SOD, Superoxide dismutase; TH, Tyrosine hydroxylase; TNF-α, Tumor necrosis factor-α

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2019), which lead to misfolding and aggregation of proteins such as α-synuclein (Xu and Pu, 2016). Abnormal accumulation of α-synuclein aggregates form Lewy bodies, the pathological markers of PD in the central nervous system (CNS), are now found in the peripheral autonomic nervous system, and the enteric nervous system (ENS) (Botter et al., 2012; Gold et al., 2013). PD patients usually exhibit symptoms of gastrointestinal dysfunction and constipation years before motor symptom onset, which is strongly associated with α-synuclein-related neurodegenerative changes in the ENS (Cersosimo and Benarroch, 2012; Pfeiffer, 2011). The α-synuclein pathology may initiate in the neurodegenerative changes in the ENS (Cersosimo and Benarroch, 2012; Ulusoy et al., 2013). The concept of “gut-orignality” is now a key issue in PD pathogenesis research.

The gut microbiota plays important roles in gastrointestinal homeostasis, essential physiological processes and CNS function, and affects the gut-brain axis via neural, immune and endocrine pathways giving rise to the microbiota-gut-brain-axis (MGBA) (Cryan and Dinan, 2012; Foster et al., 2016). Combining the MGBA concept and the theory that PD is derived in the gut, researchers have studied the relationship between the gut microbiota and the PD neurodegenerative process (Felice et al., 2016; Mulak and Bonaz, 2015). Clinical studies have reported significant differences in the gut microbiota and its metabolites, such as short-chain fatty acids (SCFAs), between PD patients and healthy controls (Unger et al., 2016). Bacterial community abundances have been associated with motor symptom aggravation (Scheperjans et al., 2015), and proinflammatory microbiota dysbiosis has been reported in PD populations (Keshavarzian et al., 2015; Pietrucci et al., 2019). Using a germ-free PD-like mouse model, Sampson et al. showed that gut microbe and its SCFAs are heavily involved in the α-synuclein-related neurodegeneration and neuroinflammatory responses. Further, colonizing the gut microbiota from PD patients into germ-free PD-like mice induced enhanced motor dysfunction (Sampson et al., 2016). Choi et al. reported that orally administering Proteus mirabilis isolated from PD mice caused motor deficits and dopaminergic neuronal damage in normal mice (Choi et al., 2018). Sun et al. demonstrated that fecal microbiota transplantation successfully rescued the motor deficits and dopaminergic neuronal damage in MPTP-induced PD-like mice (Sun et al., 2018). These studies corroborate the important role of the gut microbiota on PD pathology and indicate that gut microbiota alterations may help ameliorate motor syndromes and deterioration into a disease state.

Probiotics are live microorganisms that confer health benefits on the host and can improve host physical and mental health by affecting MGBA homeostasis. Specific probiotic strains that modulate neurotransmitters, neurotrophic factors and behaviors are termed “psychobiotics” (Dinan et al., 2013), which may directly benefit the PD population (Cassani et al., 2011; Perez-Pardo et al., 2017). We previously reported that Lactobacillus plantarum PS128 (PS128), a novel psychobiotic strain that ameliorated anxiety-like behaviors and modulated DA and serotonin (5-HT) levels in the prefrontal cortices and striata of both naïve and germ-free mice (Liu et al., 2016a; Liu et al., 2016b). PS128 ingestion also reduced depression-like behaviors, monoamine declination, hypothalamic–pituitary-adrenal (HPA) axis dysfunction and inflammation in an early-life-stress mouse model (Liu et al., 2016b), and ameliorated tic-like behaviors and stabilized DA transmission by modulating MGBA in a 2,5-dimethoxy-4-iodoamphetamine-induced hyperactive rat model (Liao et al., 2019). Moreover, PS128 supplementation improved exercise performance and alleviated intensive exercise-induced oxidative stress and inflammation in a clinical study of triathletes (Huang et al., 2019). Thus, PS128 strengthens the host’s MGBA function, consequently improving CNS function, especially in the dopaminergic system.

Here, we assessed the neuroprotective properties of PS128 in an MPTP-induced PD-like mouse model and analyzed the neurodegeneration-associated factors in DA neuroprojection regions to study the underlying mechanisms. We also analyzed the fecal SCFA concentrations and gut microbiota compositions to further investigate the significance of the MGBA. Our findings indicate the potential for using psychobiotics such as PS128 to prevent and treat neurodegenerative diseases such as PD.

2. Materials & methods

2.1. Animals and experimental groups

Six-week-old male C57BL/6J mice (weighing 20 – 22 g) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan) and housed in a specific pathogen-free room at the Laboratory Animal Center of National Yang-Ming University. The room was kept at a constant temperature (22 ± 1°C) and humidity (55% – 65%) with a 12-hour light/dark cycle. Water and chow were provided ad libitum (LabDiet Autoclavable Rodent Diet 5010; PMI Nutrition International, Brentwood, MO, USA).

Mice were randomly divided into five groups (n = 18 mice/group).

1. Saline (untreated control) group: received oral gavage of saline only.
2. PS128 (untreated feeding control) group: received oral gavage of PS128 only.
3. Saline + MPTP group: received oral gavage of saline followed by MPTP treatment.
4. L-DOPA + MPTP (behavioral positive control) group: received oral gavage of saline immediately followed by an oral gavage of 1.3,4-dihydroxyphenylalanine (L-DOPA) plus benserazide and MPTP treatments (Guo et al., 2016; Ogawa et al., 1985).
5. PS128 + MPTP group: received oral gavage of PS128 followed by MPTP treatment. To further investigate the effect of PS128 on the MGBA, we used additional mice that were randomly divided into the Saline, PS128, Saline + MPTP, and PS128 + MPTP groups (n = 12 mice/group). Each group underwent the same experimental treatment as their respective previous groups except that additional fecal samples were collected at specified times. The Institutional Animal Care and Use Committee, National Yang-Ming University, approved all animal experimental procedures (IACUC No. 1050418).

2.2. L. plantarum PS128 preparation

PS128 was cultured and prepared following previously published methods (Liao et al., 2019; Liu et al., 2016b). Cryopreserved PS128 was awakened by anaerobic culturing in de Man Rogosa Sharpe (MRS) broth (BDDiexo, Becton-Dickinson, Sparks, MD, USA) at 37°C for 18 h. The activated PS128 was further cultured anaerobically in MRS broth at 37°C for 18 h, then harvested by centrifugation at 6000 × g for 10 min. Bacterial pellets were re-suspended in MRS broth supplemented with 12.5% glycerol to a final concentration of 1010 colony-forming units per milliliter (CFU/mL), then aliquoted in freezertubes and stored at −80°C. Before oral administration, the cryopreserved PS128 was thawed in a 37°C water bath for 1 h, then centrifuged at 6000 × g for 10 min. The supernatants were removed, and the bacterial pellets were washed by saline then centrifuged at 6000 × g for 10 min. The supernatants were then removed, and the bacterial pellets were resuspended at an objective concentration with saline.

2.3. Experimental procedure and treatments

The PS128 and PS128 + MPTP groups were orally gavaged with PS128 (10⁶ CFU in 200 μL saline) daily (Liu et al., 2016a; Liu et al., 2016b), while the Saline and Saline + MPTP groups received saline (200 μL) throughout the entire experimental period (28 days). The L-DOPA + MPTP group was orally gavaged with saline (200 μL) from days 1 – 23, then administered L-DOPA (100 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) plus benserazide (25 mg/kg; Sigma-Aldrich) in saline from days 24 – 28. All MPTP-treated mice in the Saline + MPTP, L-DOPA + MPTP and PS128 + MPTP groups received daily intraperitoneal injections of MPTP (30 mg/kg; Sigma-Aldrich) from days 24 – 28, while the Saline and PS128 groups received daily
intraperitoneal injections of phosphate-buffered saline (PBS) from days 24 – 28.

2.4. Behavioral tests for motor functions

The pole test (PT), narrow-beam test (NBT), and rotarod test (RTR) were conducted to assess motor function. All mice underwent behavioral training once daily from days 20 – 22. Behavioral tests were performed 24 h after the last MPTP injection on day 29 (Fig. 1A). Both the PT and NBT were video recorded, and the video file names were converted into random-number codes and analyzed by at least two researchers blinded to the treatment condition.

The PT was used to evaluate agility and sensorimotor impairments (Ogawa et al., 1985). A 50-cm vertical pole (1-cm diameter) with a

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**Fig. 1.** Effects of *L. plantarum* PS128 on motor deficits in MPTP mice. (A) Experimental procedure time line. (B) Inversion time in the pole test. (C) Total descent time in the pole test. (D) Total walking time on the narrow-beam test. (E) Total retention time on the rotarod test. L-DOPA, L-DOPA (100 mg/kg) plus benserazide (25 mg/kg) treatment group. Data are expressed as means ± SEM and were analyzed via one-way ANOVA with Tukey’s post hoc test. ### *p* < 0.001, compared with the Saline group; *p* < 0.05, ###*p* < 0.001, compared with the Saline + MPTP group (n = 18/group).
rough surface was placed in a cage. On each training day, the mice were first placed in the pole cage for environmental familiarization, then placed head-upward on the pole 15 cm above the cage floor 3 times, followed by 30 cm and 50 cm above (top of the pole) for training. On the test day, each mouse was placed head-upward on the top of the pole, and the times taken to turn around to head-down position (T-turn) and to descend to the floor (T-total) were recorded for 60 s. If the mouse fell or jumped off the pole during the 60 s, the test was repeated. If the mouse could not turn head downward or kept falling off the pole, both the T-turn and T-total times were recorded as 60 s. Each mouse was tested 3 times (with 1-minute intervals between tests), and the average time was taken.

The NBT was used to evaluate motor coordination and balance (Luong et al., 2011). The beam apparatus comprised a 50-cm beam with a 0.8-cm-wide flat surface. The beam was set 50 cm above the table top on two poles. A black box was placed at one end of the beam as the finish point. Mouse chow was placed in the black box to attract the mouse. On each training day, mice were placed in the black box for 5 min for environmental familiarization, then placed on the beam 5 cm away from the box beam to train them walk towards the box 3 times. This was followed by 15-, 30- and 50-cm beam-walk training. On the test day, each mouse was tested 3 times (with 1-minute intervals between tests). The time required to cross the beam and enter the finish was recorded until completion or a maximum of 60 s. The test was redone if the mouse fell or turned back to the starting point halfway. If the mouse could not walk to the finish within 60 s or kept falling off the beam, a total time of 60 s was recorded. The average time from the 3 trials was taken.

The RTR was used to assess motor coordination (Shiotsuki et al., 2010). The RT-01 mouse rotarod (SINGA, Taiwan) containing automatic timers and falling sensors was used for both the training period and formal test. All mice were first trained at 10 rpm rotary speed for 180 ± 3 times on day 20, followed by 20 rpm rotary speed on day 21 and 30 rpm rotary speed on day 22. On the test day, each mouse was tested at 30 rpm rotary speed for 180 ± 3 times (1-minute intervals between tests); the latency time to fall was recorded, and the average time was taken.

2.5. Sample collection and tissue preparation

On fecal sample collection day, all mice were placed individually in empty autoclaved cages and allowed to defecate freely in the morning before treatment. Immediately after defecation, feces were collected in individual sterile tubes on ice then stored at − 80°C before use. After behavioral testing and fecal collection on day 29, all mice were sacrificed into 40-μm sections on a cryostat microtome. Optimal cutting temperature compound (Tissue-Tek, USA) and sec- tioned alternate sections from the bregma − 2.92 to − 3.8 mm using a laser confocal microscope (ZEISS LSM 700, ZEISS, Germany). The TH-positive cell number was counted in alternate sections from the bregma − 2.92 to − 3.8 mm using a Meta Wavelength Cell Scoring application in MetaMorph software (Molecular Devices LLC., San Jose, CA, USA).

2.6. Quantification of tyrosine hydroxylase-positive dopaminergic cells

The 40-μm cryostat sections of the midbrain were washed with washing buffer (0.1% Triton X-100, 0.01% sodium azide in PBS) for 10 min, then blocked with 3% donkey serum in PBS-Tween (PBST:0.3% Triton X-100, 0.01% BSA and 0.01% sodium azide in PBS) at room temperature for 1 h. Brain sections were then incubated with primary antibody (anti-tyrosine hydroxylase [TH], 1:300, Merck Millipore, Burlington, MA, USA) and Hoechst 33,258 (1:2000, Life Technologies, Thermo Fisher, MA, USA) in PBST overnight at 4 °C. After washing with PBST 3 times, the brain sections were incubated with the secondary antibody (FITC-conjugated AffiniPure donkey anti-mouse IgG, 1:300, Jackson ImmunoResearch, PA, USA) and Hoechst for 2 h at room temperature. Brain sections were then washed with PBST 3 times and mounted on slides with anti-fade mounting medium. Immunostained sections were imaged using a laser confocal microscope (ZEISS LSM 700, ZEISS, Germany). The TH-positive cell number was counted in alternate sections from the bregma − 2.92 to − 3.8 mm using a Multi Wavelength Cell Scoring application in MetaMorph software (Molecular Devices LLC., San Jose, CA, USA).

2.7. Quantification of monoamines and their metabolites via high-performance liquid chromatography - electrochemical detection (HPLC-ECD)

Monoamines and their metabolites were detected following previously published HPLC methods with slight modifications (Liao et al., 2019). Mouse striata were lysed by sonication in perchloric acid buffer (0.1% perchloric acid, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 0.1 mM Na2HPO4, Na2HPO4). After centrifuging at 12,000 × g for 10 min, the supernatants were collected and filtered through a 0.22-mm polyvinylidenedifluoride (PVDF) membrane (4-mm syringe filter; Milllex-GV, Millipore, USA). The HPLC-ECD system comprised a S1130 HPLC pump system (Strykam, Eresing, Germany), an on-line 55300 sample injector (Strykam), a DECADE II SDC electrochemical detector (Antec, Zoeterwoude, Netherlands), and a reversed-phase column (Kinnetex C18, 2.6 um, 100 × 2.1 mm I.D.; Phenomenex, CA, USA). The potential was set at + 700 mV for the glassy carbon working electrode respective of an Ag/AgCl reference electrode at room temperature (25 °C). To quantify the DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), and 5-HT, the mobile phase was pumped at a constant flow rate of 0.2 mL/min. The mobile phase contained 0.1 M NaH2PO4, 2 mM KCl, 0.74 mM 1-octanesulfonic acid (SOS, sodium salt), 0.03 mM EDTA and 8% methanol and was adjusted to pH 3.74 with H3PO4. To quantify the norepinephrine (NE) and 3-methoxy-4-hydroxyphenylglycol (MHPG), the mobile phase was pumped at a constant flow rate of 0.1 mL/min, containing 0.1 M NaH2PO4, 2 mM KCl, 1.8 mM SOS, 0.1 mM EDTA and 10% methanol and was adjusted to pH 3.0 with H2PO4. Diluted filtrates (20 μL) were injected into the chromatographic system, which had been prepared at the suitable mobile phase and analysis state. Monoamine and metabolite concentrations were interpolated via standard curves obtained from sample standards (Sigma-Aldrich) ranging from 1 to 100 ng/mL with DataApex Clarity chromatography software (version 5.0.3.180, DataApex Ltd., Prague, Czechoslovakia).

2.8. Serum corticosterone detection

Mouse serum corticosterone concentrations were determined using a commercial CORT EIAKit (Item No. 501320, Cayman Chemical, Ann Arbor, MI, USA). Mouse serum was collected and diluted on day 29 (Fig. 1A) and analyzed per the manufacturer's instructions. The corticosterone concentration was interpolated using the standards in the kit following the manufacturer's instructions.

2.9. Cerebral antioxidant detection

Superoxide dismutase (SOD), glutathione (GSH), catalase and glutathione peroxidase (GPx) levels were detected using assay kits according to antioxidant activity (SOD [Item No. 706002], GSH [No.
703002), catalase [No. 707002], and GPs [No. 703102] Cayman Chemical). Mouse brain tissues were lysed by sonication in PBS buffer. After centrifugation, the supernatant was collected and analyzed using assay kits for each manufacturer's instructions. Antioxidant levels in all mouse brain tissues were estimated based on antioxidant standard activity, then normalized to the protein concentration and expressed as enzyme units per milligrams protein.

2.10. Enzyme-linked immunosorbent assay (ELISA) and western blotting

Mouse brain tissues were lysed by sonication in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS; pH = 7.5) containing protease inhibitor (cComplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, Basel, Switzerland) and phosphatase inhibitor (Halt™ Phosphatase Inhibitor Cocktail, Thermo Fisher, MA, USA). Tissue lysate was homogenized by vortexing at 5-minute intervals for 30 min on ice, then centrifuged at 12,000 × g for 10 min. The supernatant was collected as the tissue protein extractions, and the protein concentration was determined.

To measure the cytokines, commercial ELISA kits (Invitrogen, Thermo Fisher) were used to detect the TNF-α (Cat# 8-7324, limitation: 8 – 100 pg/mL), IL-1β (Cat# 88-7013, limitation: 8 – 1000 pg/mL) and IL-6 (Cat# 88-7064, limitation: 4 – 500 pg/mL) concentrations in the mouse striata. All procedures were conducted per the manufacturer's instructions. Cytokine levels of all mouse brain tissues were normalized with the protein concentration and expressed as pg/mg.

For the western blot analysis, equal amounts of protein were loaded and fractionated on 15% SDS-PAGE gels, then transferred to PVDF membranes (Roche) via electrophoresis. The membranes were blocked for 1 h with blocking buffer (5% skim milk in tris-buffered saline containing 0.1% Tween-20, TBST) then were cut according to the size of target proteins. The cut membranes containing the target protein were sealed and incubated with the corresponding primary antibodies: anti-TH (1:1000; Cell Signaling, Danvers, MA, USA), anti-glial fibrillary acidic protein (GFAP; 1:1000; Cell Signaling), anti-ionized calcium-binding adapter molecule 1 (Iba1; 1:1000, GeneTex, Irvine, CA, USA), anti-brain-derived neurotrophic factor (BDNF; 1:1000, GeneTex), and anti-nerve growth factor (NGF; 1:1000, GeneFex) overnight at 4 °C simultaneously. After washing twice with TBST, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG 1:2000 and goat anti-mouse IgG 1:2000, Thermo Fisher). The antibody-protein complex was visualized using an enhanced chemiluminescence system (Immobilon™ Western Chemiluminescent HRP Substrate; Merck Millipore) and detected using Luminescent Image Analyzer (LAS-4000, FUJIFILM, Japan). The images were analyzed using ImageJ software. Protein expression levels are expressed as fold-changes compared with the saline control group after normalizing with β-actin.

16S rRNA gene amplicons generation

The 16S rRNA gene amplicons were prepared following the Illumina protocol for preparing the 16S metagenomic sequencing library. The V3 – V4 region of the bacterial 16S rRNA gene was amplified in the first PCR reaction with a bacterial universal primer set (341F: 5′-TCGTCG – V4 region of the bacterial 16S rRNA gene was amplified in the first PCR reaction were further used in the second PCR amplification with the Illumina SS/N7 Index primer set to introduce dual indices and sequencing adapters (Illumina Inc., San Diego, CA, USA). The reaction mixture (50 μL) contained 10–100 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 200 μM of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primer, and 0.625 U ExTaq HS (TaKaRa Bio Inc., Shiga, Japan). The first PCR conditions were 98°C for 2.5 min; 18 cycles of 98°C for 15 s, 58°C for 30 s, and 72°C for 20 s; and 72°C for 5 min. The DNA amplicons from the first PCR reaction were further used in the second PCR amplification with the Illumina S5/N7 Index primer set to introduce dual indices and sequencing adapters (Illumina Inc., San Diego, CA, USA). The reaction mixture (50 μL) contained 10–100 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 10 pmol of each primer, and 1.25 U Ex Taq HS. The PCR conditions were 98°C for 2.5 min; 13 cycles at 98°C for 15 s, 60°C for 30 s, and 72°C for 20 s; and 72°C for 5 min. The final PCR products were purified using a QiAquick PCR Purification Kit (Qiagen, Hilden, Germany) per the manufacturer's protocol.

2.12. DNA extraction from fecal samples

Bacterial DNA was extracted from the fecal samples and purified via previously published methods with slight modifications (Liao et al., 2019). Fecal samples (100 mg) were washed three times with PBS and re-suspended in extraction buffer (100 mM Tris-HCl, 40 mM EDTA, 1% SDS; pH 9.0). Glass beads (diameter, 0.1 mm, 300 mg) and buffer-saturated phenol (500 μL) were added, and the mixture was vortexed vigorously for 30 s using the FastPrep FP 120 homogenizer (Q-Biogene, CA, USA) at 5.0 m/s. After centrifugation at 12,000 × g for 5 min, the supernatant (400 μL) was collected. Subsequently, phenol–chloroform extractions were performed, and 250 μL of the supernatant was precipitated with 3 M sodium acetate (pH 5.4) and isopropanol, then air-dried. The DNA was dissolved in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then stored at −20°C.

2.13. 16S rRNA gene amplicons generation

The 16S rRNA gene amplicons were prepared following the Illumina protocol for preparing the 16S metagenomic sequencing library. The V3 – V4 region of the bacterial 16S rRNA gene was amplified in the first PCR reaction with a bacterial universal primer set (341F: 5′-TCGTCGCGAGCCTGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′; 805R: 5′-GTCGTGTAGCCGGGTGCTGAAATATTAGTGTAATAAGACGACAGACTACHVGGGTATCTAATCC-3′). The 25-μL reaction mixture contained 10 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 200 μM of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primer, and 0.625 U ExTaq HS (TaKaRa Bio Inc., Shiga, Japan). The first PCR conditions were 98°C for 2.5 min; 18 cycles of 98°C for 15 s, 58°C for 30 s, and 72°C for 20 s; and 72°C for 5 min. The DNA amplicons from the first PCR reaction were further used in the second PCR amplification with the Illumina SS/N7 Index primer set to introduce dual indices and sequencing adapters (Illumina Inc., San Diego, CA, USA). The reaction mixture (50 μL) contained 10–100 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 10 pmol of each primer, and 1.25 U Ex Taq HS. The PCR conditions were 98°C for 2.5 min; 13 cycles at 98°C for 15 s, 60°C for 30 s, and 72°C for 20 s; and 72°C for 5 min. The final PCR products were purified using a QiAquick PCR Purification Kit (Qiagen, Hilden, Germany) per the manufacturer's protocol.
2.16. Statistical analysis

All data are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism (GraphPad Prism, version 7, La Jolla, CA, USA), statistical analysis of metagenomic profiles (STAMP, version 2.0.9) or R software (version 3.5.3). Spearman’s rank correlation coefficient was used for the correlation analysis. Permutational multivariate analysis of variance (PERMANOVA) was performed using QIIME2 for multigroup significance tests and visualization. Between-group differences were analyzed via one-way analysis of variance (ANOVA) with Tukey’s post hoc test, Kruskal–Wallis test followed by Dunn’s post hoc test, the Wilcoxon t-test (alpha diversity), or the analysis of similarity test (beta diversity); p < 0.05 was considered statistically significant.

3. Results

3.1. L. plantarum PS128 improves MPTP-induced motor deficits

To assess the potential neuroprotective effects of PS128 on MPTP-treated mice, the motor functions of all mice were evaluated from the PT, NBT and RTR results. The saline + MPTP group showed significantly increased inversion and total descent times on the PT and total walking time on the NBT, and a significant decreased RTR latency (all p < 0.001) compared with the Saline group (Fig. 1B – E). Both L-DOPA and PS128 treatment significantly attenuated the MPTP-induced motor deficits on the PT, NBT and RTR compared with those of the Saline + MPTP group (Fig. 1B – E), suggesting that both L-DOPA and PS128 treatment alleviated MPTP-induced motor deficits. PS128 ingestion in naive mice did not affect motor performance on the PT or NBT but slightly elevated the RTR retention time compared with that of the saline group (Fig. 1E).

3.2. L. plantarum PS128 increases DA and NE levels in MPTP-treated mice

To investigate the underlying mechanism of the behavioral improvement, we further analyzed the effects of PS128 administration on neurotransmitters and their metabolites in the striata of MPTP-treated mice. The HPLC-ECD experiments showed that the MPTP treatment significantly reduced the DA, DOPAC, HVA, 5-HT, and MHPG levels (all p < 0.001) in the striata (Table 1). NE reduction in the striatum approached statistical significance in the MPTP-treated mice (Table 1).

| Levels of striatal monoamines and their metabolites. | MPTP (+) |
|-----------------------------------------------------|----------|
| Saline                                             | PS128    |
| DA 16365 ± 1401                                     | 17550 ± 1355 |
| DOPAC 1299 ± 68                                     | 1270 ± 109 |
| HVA 1670 ± 59                                       | 1486 ± 65 |
| 5-HT 794 ± 58                                       | 805 ± 32 |
| 5-HIAA 724 ± 54                                     | 715 ± 46 |
| NE 375 ± 47                                         | 278 ± 26 |
| MHPG 109 ± 8                                        | 50 ± 3*** |
| 2817 ± 128***                                       | 7076 ± 333*** |
| 173 ± 22***                                         | 691 ± 38*** |
| 246 ± 35***                                         | 1191 ± 53*** |
| 475 ± 33***                                         | 469 ± 35*** |
| 600 ± 67                                            | 488 ± 34* |
| 191 ± 31                                            | 419 ± 44* |
| 42 ± 2**                                            | 71 ± 5.*** |
| 4871 ± 278***                                       | 431 ± 39*** |
| 681 ± 37***                                         | 533 ± 48*** |
| 712 ± 63                                            | 459 ± 80** |
| 119 ± 14***                                         |          |

Concentrations (ng/g wet tissue) of monoamines and their metabolites are expressed as means ± SEM. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, serotonin or 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; L-DOPA, L-DOPA (100 mg/kg) plus benserazide (25 mg/kg) treatment group. Data were analyzed via one-way ANOVA with Tukey’s post hoc test. ***p < 0.001, compared with the saline group; *p < 0.05, **p < 0.01, ***p < 0.001, compared with the Saline + MPTP group (n = 10/group).

JCM 1649 T-type strain was cultured, and total bacterial cell counts from the bacterial cell counts. The specific primer set of En-lsu-3F (5′-CAGTGTTCAGTGTC-3′) was used to detect Enterobacteriaceae (E. coli) JCM 1649 1-type strain was cultured, and total bacterial cell counts were determined via 4,6-diamidino-2-phenylindole (DAPI) staining (Jansen et al., 1999). Total bacterial DNA was extracted from E. coli JCM 1649 1 using the bead-beating method and used to create the relative standards with DNA concentrations corresponding to 10–10 7 cells from the bacterial cell counts. The specific primer set of En-lsu-3F (5′-TGCCGTAACTTCGGGAGAAGGCA-3′) and En-lsu-3R (5′-TCAAGGAC CAGTTGTCACTGTC-3′) was used to detect Enterobacteriaceae in the fecal samples (Matsuda et al., 2009; Morita et al., 2015). Three serial 10-fold dilutions of extracted DNA samples were used in the RT-qPCR. Each reaction mixture (15 μL) contained 1 μL of DNA template, 10-fold dilutions of extracted fecal DNA samples were used in the RT-qPCR. Each reaction mixture (15 μL) contained 1 μL of DNA template, 10 μL of KAPA SYBR FAST qPCR Kit (Roche Sequencing and Life Science, MA, USA). The kits contained KAPA SYBR FAST DNA polymerase, reaction buffer, dNTPs, SYBR Green I dye, and MgCl2 at a final concentration of 2.5 mM. The reaction was performed on a StepOnePlus™ Real-Time PCR System (Thermo Fisher). Fluorescent products were detected in the last step of each cycle during the cycling stage. The melting curve was obtained by continuous fluorescence collection. The cycle threshold (Ct) of each sample was fitted to the standard curve generated in the same experiment to obtain the corresponding bacterial count in each sample.
Using L-DOPA treatment as a positive control significantly improved the DA (p < 0.05), DOPAC (p < 0.001), HVA (p < 0.001), NE (p < 0.05) and MHPG (p < 0.05) levels. PS128 ingestion also increased the DA levels and significantly improved the DOPAC (p < 0.05), HVA (p < 0.001), NE (p < 0.01) and MHPG (p < 0.001) levels in the MPTP-treated group (Table 1). The treatments did not change the MPTP-induced 5-HT reduction, while the 5-HIAA level in the L-DOPA + MPTP group was significantly reduced compared to that of the Saline group (Table 1; p < 0.05). Moreover, PS128 ingestion in naïve mice did not affect most striatal monoamines or their metabolites and only decreased the MHPG level (p < 0.001) compared with that of the Saline group (Table 1). These results suggest that PS128 ingestion partially restored the MPTP-induced reduction in DA and its metabolites and fully restored the NE and its metabolites to the saline control levels.

3.3. L. plantarum PS128 alleviates MPTP-induced dopaminergic neuronal death and corticosterone elevation

We examined the effects of PS128 on nigrostriatal dopaminergic neuron survival, including the numbers of TH + dopaminergic neurons in the SN and the striatal TH expression level. Immunofluorescence staining showed a ~50% reduction in TH + dopaminergic neurons in the SN after MPTP injection compared with that of the Saline group (Fig. 2A; p < 0.001). L-DOPA treatment could not rescue the MPTP-induced reduction in TH + cells, whereas PS128 ingestion significantly increased the number of TH + neurons in the SNs of MPTP-treated mice (Fig. 2A; p < 0.001 vs. Saline + MPTP group). Western blotting results showed that both the Saline + MPTP and L-DOPA + MPTP groups had markedly reduced striatal TH expressions compared with those of the Saline group (Fig. 2B; p < 0.001). Conversely, PS128 ingestion significantly reinstated striatal TH expression levels under MPTP toxicity (Fig. 2B; p < 0.001 vs. Saline + MPTP group). TH + cells numbers in the SN and striatal TH expression levels did not differ between Saline and PS128 groups (Fig. 2A – B). Thus, PS128 ingestion may have neuroprotective properties on dopaminergic neurons in the nigrostriatal pathway under MPTP-induced neurotoxicity. We also found that serum corticosterone was significantly elevated in the Saline + MPTP group (Fig. 2C; p < 0.001 vs. Saline group). Both L-DOPA and PS128 treatments significantly reduced the serum corticosterone levels in MPTP-treated mice (Fig. 2C). In addition, the serum corticosterone levels did not differ between the Saline and PS128 groups (Fig. 2C).

3.4. L. plantarum PS128 reduces MPTP-induced glial reactivity and increases the striatal neurotrophic factors

We further investigated whether PS128 ingestion could affect glial reactivity in MPTP-treated mice. Protein expression levels of the glial markers, GFAP and Iba1, in the striatum were quantified via western blotting. PS128 ingestion alone slightly elevated the striatal GFAP levels, but not significantly, compared with those of the Saline group. Striatal Iba1 expressions did not differ between these two groups (Fig. 3A – B). In the MPTP-treated groups, both striatal GFAP and Iba1 expression levels were markedly increased in the Saline + MPTP and L-DOPA + MPTP groups compared with those of the Saline group (Fig. 3A – B). However, PS128 ingestion significantly suppressed the MPTP-induced elevations of striatal GFAP (Fig. 3A; p < 0.05) and Iba1 (Fig. 3B; p < 0.01). We examined the mature striatal BDNF (m-BDNF) and NGF expression levels. Western blotting showed that PS128 ingestion significantly increased m-BDNF levels (Fig. 3C; p < 0.001) but not NGF levels (Fig. 3D) in naïve mice compared with those of the Saline group. In the MPTP-treated groups, the striatal m-BDNF and NGF levels did not significantly differ between the Saline, Saline + MPTP and L-DOPA + MPTP groups. Conversely, the PS128 + MPTP group showed elevated m-BDNF (Fig. 3C; p < 0.05) and NGF (Fig. 3D; p < 0.001) levels compared with those of the Saline + MPTP group. Interestingly, NGF expression in the PS128 + MPTP group was higher than that of the Saline and PS128 groups (Fig. 3D; p < 0.001). These results demonstrate the neuroprotective effects of PS128 on reducing glial activation and increasing neurotrophins in the striatum.

3.5. L. plantarum PS128 alleviates MPTP-induced neuroinflammation

We measured levels of proinflammatory cytokines, including TNF-α, IL-1β and IL-6, in the striatum and midbrain region containing the SN. ELISA showed that MPTP induction significantly increased the TNF-α (p < 0.001), IL-1β (p < 0.05) and IL-6 (p < 0.001) levels in the striatum of the Saline + MPTP group compared with that of the Saline group (Fig. 4A – C). PS128 ingestion successfully alleviated the elevated striatal TNF-α (p < 0.001), IL-1β (p < 0.01) and IL-6 (p < 0.01) in MPTP-treated mice (Fig. 4A – C). MPTP toxicity also elevated IL-6 in the midbrain region (p < 0.05). In the PS128 + MPTP group, TNF-α, IL-1β and IL-6 levels were all significantly lower than those of the Saline + MPTP group (Fig. 4D – F). Proinflammatory cytokine levels did not significantly differ between the PS128 and Saline groups (Fig. 4A – F).

3.6. L. plantarum PS128 increases antioxidants and reduces MPTP-induced oxidative stress

We examined antioxidant levels based on activity assays in the striatum and midbrain region in MPTP-treated and control mice. PS128 ingestion significantly increased the GSH levels in both the striatum (p < 0.05) and midbrain (p < 0.01) and catalase levels in the midbrain (p < 0.05) compared with those of the Saline group (Fig. 5B, F, G). MPTP treatment significantly reduced SOD and GSH levels and activity in both the striatum and midbrain, and PS128 ingestion reversed these effects (Fig. 5A, B, E, F). MPTP treatment did not significantly alter the catalase or GPx levels or activity in the striatum or midbrain (Fig. 5C, D, G, H), and PS128 ingestion increased the catalase levels in both the striatum and midbrain and GPx levels in the striatum under MPTP toxicity (Fig. 5C, D, G). GPx levels did not significantly differ in the midbrain regions among any experimental groups (Fig. 5H).

3.7. L. plantarum PS128 did not affect the MPTP-induced SCFA decline

We collected mouse feces from all experimental groups on day 0 as the untreated basal state, day 21 when mice had received saline or PS128 for 3 weeks, and day 29 following MPTP or vehicle treatment when mice had received saline or PS128 for 4 weeks (Fig. 1A). Fecal SCFA concentrations were detected via GC–MS analysis. All mice in both the Saline and Saline + MPTP groups presented significantly increased acetic acid, propionic acid, butyric acid, valeric acid (all p < 0.001) and isovaleric acid (p < 0.01) on day 21, resulting in markedly elevated total SCFA concentrations (p < 0.001; Fig. 5A – E, Supplementary Table 1). Although mice in both the PS128 and PS128 + MPTP groups also showed elevated fecal SCFAs on day 21, the increase was less than that of the saline-fed mice (Fig. 5A – E, Supplementary Table 1). The fecal butyric acid and total SCFAs (p < 0.05) were significantly lower (both p < 0.05) than those of the Saline group in both the PS128 and PS128 + MPTP groups on day 21 (Fig. 6C, E, Supplementary Table 1). On day 29, the fecal SCFAs, especially acetic acid and the total SCFAs, continued to increase in the Saline group (both p < 0.001; Fig. 6A, E, Supplementary Table 1). However, in the PS128 group, the SCFAs did not continue to increase, and the acetic acid (p < 0.001), butyric acid (p < 0.05), and total SCFAs (p < 0.001) were significantly lower than those of the Saline group (Fig. 6A, C, E, Supplementary Table 1). MPTP treatment induced a massive decrease in all SCFAs, resulting in a marked reduction in total SCFAs in the Saline + MPTP and PS128 + MPTP groups on day 29.
3.8. *L. plantarum* PS128 and MPTP treatment effect on fecal microbiota composition

We investigated how the experimental treatments affected the mouse gut microbiota. Microbial DNA was extracted from mouse feces and analyzed via 16S rRNA metagenomics. The ASVs were classified into known taxa (8 phyla, 12 classes, 15 orders, 31 families, 90 genera, and 115 species) and unclassified groups. At the phylum level, all experimental mice showed a continuous increase in *Actinobacteria*, which was more distinct in the PS128 + MPTP group than in the Saline group on day 29 (Table 2). Feeding PS128 for 4 weeks decreased the *Firmicutes* population and increased the *Bacteroidetes* population in both the PS128 and PS128 + MPTP groups, resulting in a reduced *Firmicutes/Bacteroidetes* ratio on day 29 (Table 2). However, phyla in the Saline and Saline + MPTP groups showed no significant changes on day 29 (Table 2).

At the family level, nine bacterial populations were predominant among all mouse groups (Fig. 7A). All groups showed a continuous decrease in *Erysipelotrichaceae*, which was a major population in the mouse feces before the experimental procedures started (Fig. 7A, B). On day 21, after feeding PS128 or saline for 3 weeks, *Erysipelotrichaceae* was noticeably decreased in PS128-fed mice compared with that in the saline-fed mice (Fig. 7B; \( p < 0.01 \)). All groups showed increases in *Lachnospiraceae*, *Lactobacillaceae*, *Ruminococcaceae*, *Atopobacteraceae* and *Bifidobacteriaceae* during the experimental period except *Lachnospiraceae* in the PS128 + MPTP group (Fig. 7C – G). Moreover,
MPTP treatment significantly decreased the Lachnospiraceae population in the Saline + MPTP group on day 29 (Fig. 7C; \(p < 0.001\)). PS128 ingestion for 3 weeks significantly increased the Lactobacillaceae population in the PS128-fed groups compared with those of the saline-fed groups on day 21 (Fig. 7A, D; \(p < 0.001\)). MPTP treatment caused steep increases in Lactobacillaceae and Bifidobacteriaceae (both \(p < 0.001\)) in both the Saline + MPTP and PS128 + MPTP groups on day 29 (Fig. 7D, G). The Atopobiaceae population was significantly increased in the PS128-fed groups compared with that in the saline-fed groups on day 29 (Fig. 7F; \(p < 0.05\)).

We further calculated the microbial diversity measures for family-level bacterial communities. Chao1 alpha-diversity indices showed a lower species richness (Fig. 8A; \(p < 0.001\)), while both Shannon (\(p < 0.05\)) and Evenness indices (\(p < 0.001\)) showed a higher species abundance in PS128-fed mice than in saline-fed mice on day 21 (Supplementary Fig. 2). All alpha-diversity indices in Saline + MPTP group were slightly lower than those in the Saline group on day 29 although not significantly (Fig. 8A, Supplementary Fig. 2). Both the PS128 and PS128 + MPTP groups showed lower Chao1 indices compared with the Saline and Saline + MPTP groups (Fig. 8A; \(p < 0.05\)). Weighted UniFrac analysis showed the microbial community structures by PCoA, and samples from saline-fed and PS128-fed mice differed significantly on day 21 (Fig. 8B; \(p < 0.01\)). Moreover, on day 29, microbial community structures differed significantly between the PS128 and Saline...
groups ($p < 0.01$), Saline + MPTP and Saline groups ($p < 0.01$), and PS128 + MPTP and Saline groups ($p < 0.05$) but not between the Saline + MPTP and PS128 + MPTP groups (Fig. 8C). Thus, the mouse gut microbiota was consistently influenced by long-term PS128 ingestion and significantly affected by MPTP treatment.

3.9. *L. plantarum* PS128 increased the occurrence of *L. Plantarum* in microbiota

Analyzing the species-level ASVs revealed a prominence of *L. plantarum* in PS128-fed but not saline-fed mice on day 21, although *L. plantarum* was undetected from the feces of any experimental mice on day 0 (Fig. 9A). *L. plantarum* remained prominent in both the PS128 and PS128 + MPTP groups on day 29, whereas only one mouse from the Saline + MPTP group exhibited *L. plantarum* (Fig. 9A). The relative abundance of *L. plantarum* was significantly decreased in the PS128 group ($p < 0.001$ vs. day 21) but not in the PS128 + MPTP group on day 29 (Fig. 9A). Further correlational analysis showed that the relative abundance of fecal *L. plantarum* in the PS128 + MPTP group on day 29 was negatively correlated with the total time spent on the PT (Spearman $r = -0.627$, $p < 0.05$) and the total walkthrough time on the NBT (Spearman $r = -0.639$, $p < 0.05$). *L. plantarum* occurrence was positively correlated with MPTP-induced motor deficit amelioration (Fig. 9B, C).
Interestingly, the relative abundance of synthesis, energy metabolism and fermentation pathways metabolism, nucleoside and nucleotide biosynthesis, fatty acid bio from those of saline-fed mice on day 21, including the amino acid functional pathways in the microbiomes of PS128-fed mice differed metagenomics data. According to metagenomic predictions, several metabolic functions of the mouse gut microbiota based on 16S rRNA benefits of PS128, and MPTP toxicity, we used PICRUSt2 to assess the exist after MPTP treatment 3.10. The influences of L. plantarum PS128 on gut microbial functions still exist after MPTP treatment.

To further investigate the link between the gut microbiota, the benefits of PS128, and MPTP toxicity, we used PICRUS2 to assess the metabolic functions of the mouse gut microbiota based on 16S rRNA metagenomics data. According to metagenomic predictions, several functional pathways in the microbiomes of PS128-fed mice differed from those of saline-fed mice on day 21, including the amino acid metabolism, nucleoside and nucleotide biosynthesis, fatty acid biosynthesis, energy metabolism and fermentation pathways (Supplementary Fig. 3). Interestingly, the relative abundance of microbial modules related to methionine biosynthesis were markedly increased in the PS128-fed mice on day 21 (Supplementary Fig. 3; \( p < 0.0001 \)). On day 29, modules related to the superpathways of L-methionine biosynthesis (transsulfuration) and S-adenosyl-L-methionine biosynthesis and the pathways of L-methionine biosynthesis I and L-methionine biosynthesis II remained higher in the PS128 group compared with those of the Saline group (Fig. 9D). In addition, all methionine biosynthesis-related microbial modules were markedly increased in the PS128 + MPTP group compared with those in the Saline and Saline + MPTP groups (Fig. 9D).

3.11. L. plantarum PS128 alleviates MPTP-induced increase in Enterobacteriaceae and lipopolysaccharides and peptidoglycan related microbial modules

RT-qPCR showed that the number of family Enterobacteriaceae was \( < 4 \times 10^5 \) cells/g in fecal samples from all experimental mice on days 0 and 21 (Fig. 10A). MPTP treatment markedly increased the Enterobacteriaceae in the Saline + MPTP group (\( p < 0.05 \) vs. day 29 in the Saline group) but not in the PS128 + MPTP group (\( p < 0.05 \) vs. day 29 in the Saline + MPTP group). PS128 ingestion did not affect the amount of Enterobacteriaceae during the experimental period (Fig. 10A). Further correlational analysis showed that amount of fecal Enterobacteriaceae was positively correlated with TNF-\( \alpha \) (Spearman \( r = 0.430; \ p < 0.05 \)) and negatively correlated with SOD (Spearman \( r = -0.517, \ p < 0.05 \)) in the mouse midbrain region in the MPTP-treated mice (Saline + MPTP & PS128 + MPTP groups), suggesting a positive correlation between the increased Enterobacteriaceae and MPTP neurotoxicity in the brain (Fig. 10B, C).

Our metagenomic predictions showed that MPTP treatment significantly increased the microbial modules related to nucleoside and nucleotide biosynthesis and decreased several microbial modules related to amino acid biosynthesis in the Saline + MPTP group compared with that of the Saline group on day 29 (Supplementary Fig. 4). Notably, several microbial modules related to lipopolysaccharides (LPS) and peptidoglycan biosynthesis were increased in Saline + MPTP group (Fig. 10D, Supplementary Fig. 4). In contrast to the Saline + MPTP group, some microbial modules related to amino acids, nucleosides and nucleotide biosynthesis were restored in the PS128 + MPTP group (Supplementary Fig. 4), and the increases in LPS and peptidoglycan biosynthesis were significantly alleviated (Fig. 10D; \( p < 0.05 \)).

4. Discussion

In this study, we used MPTP-induced PD-like model mice to assess the neuroprotective potential of Lactobacillus plantarum PS128. PS128 ingestion demonstrated a neuroprotective effect by significantly attenuating motor deficits and protecting the dopaminergic neurons in the nigrostriatal pathway (Figs. 1, 2). PS128 ingestion also promoted endogenous neuroprotective factors in the mouse brain (Figs. 3, 5) and alleviated MPTP-induced neuroinflammation (Fig. 4), oxidative stress (Fig. 5), and the increased Enterobacteriaceae in fecal sample (Fig. 10).

The vulnerability and selective degeneration of the dopaminergic neurons originate from the oxidative stress caused by DA itself and its metabolic processes (Segura-Aguilar et al., 2014). Post-mortem studies revealed decreased mitochondrial complex I activity and an increased number of dopaminergic neurons with functional defects in the mitochondria in the brain tissue of PD patients (Bender et al., 2006), as well as \( \alpha \)-synuclein accumulation in the mitochondria of affected patients (Devi et al., 2008). Neurotoxicity due to neurotoxins such as MPTP, 6-OHDA and rotenone also involves mitochondrial dysfunction and oxidative stress, suggesting that these deficits play key roles in both \( \alpha \)-synucleinopathies and the environmental hypothesis of PD etiology (Blesa et al., 2015; Cacabelos, 2017; Duty and Jenner, 2011). Depletion of antioxidants in the nigrostriatal pathway is commonly observed in
MPTP animal studies, suggesting a concomitant increase in reactive oxygen species during neurodegenerative progression (Sriram et al., 1997; Sugumar et al., 2019; Tsai et al., 2010). Here, SOD and GSH capacities were also significantly reduced in the nigrostriatal pathway of MPTP-treated mice. In this regard, PS128 ingestion restored SOD and GSH antioxidant capacities and increased catalase and GPx under MPTP toxicity (Fig. 5). PS128-fed mice exhibited higher GSH levels in the nigrostriatal pathway than did saline-fed mice, suggesting that PS128 ingestion ameliorated the MPTP-induced superoxide toxicity partly by increasing the antioxidant levels and their activity. The levels of GSH or its related makers under both healthy and disease conditions could provide important information for overall neuronal health (Dias et al., 2013; Smeyne and Smeyne, 2013). Although GSH is not the only antioxidant affecting PD, GSH depletion occurs prior to other hallmarks of PD, such as decreased mitochondrial complex I activity and Lewy body formation, and appears to parallel the severity of PD (Pearce et al., 1997; Sian et al., 1994). Enhancing cerebral GSH is a potential therapy for PD, and some pilot studies have reported its positive effects (Cacciatori et al., 2012; Sechi et al., 1996). We also examined the serum GSH levels and found no significant differences among all mouse groups (data not shown), indicating that the elevated nigrostriatal GSH was in situ rather than exogenous. Although more studies are needed to clarify the exact mechanisms by which PS128 improves cerebral antioxidants, we found that PS128 ingestion enhanced the CNS antioxidant activities and helped mice cope with abnormal oxidative stress and alleviate superoxide toxicity.

**Table 2**

Relative abundances of phylum-level bacterial compositions in mouse fecal samples and ratios of bacterial communities at different experimental times.

| Day Groups | Day 0 Untreated | Day 21 Saline | PS128 Day 29 | PS128 Saline + MPTP | PS128 + MPTP |
|------------|----------------|--------------|--------------|---------------------|--------------|
| **Phylum level (relative abundance (%))** | | | | | |
| **Firmicutes** | 57.2 ± 3.0% | 54.2 ± 4.4% | 52.3 ± 2.0% | 57.3 ± 5.7% | 45.8 ± 1.8%<sup>##</sup> | 52.2 ± 1.9%<sup>##</sup> |
| **Bacteroidetes** | 38.9 ± 2.4% | 41.1 ± 3.4% | 41.7 ± 2.1% | 34.7 ± 4.0% | 45.2 ± 1.7%<sup>##</sup> | 39.3 ± 2.1%<sup>##</sup> |
| **Actinobacteria** | 1.8 ± 0.1% | 2.9 ± 0.5%<sup>##</sup> | 4.2 ± 0.4%<sup>##</sup> | 6.2 ± 0.9%<sup>##</sup> | 7.1 ± 1.0%<sup>##</sup> | 9.1 ± 1.0%<sup>##</sup> |
| **Verrucomicrobia** | 1.3 ± 0.2% | 0.7 ± 0.1% | 0.7 ± 0.1% | 0.6 ± 0.1% | 0.8 ± 0.2% | 0.7 ± 0.1% |
| **Proteobacteria** | 0.6 ± 0.1% | 0.8 ± 0.1% | 0.9 ± 0.1% | 1.1 ± 0.2% | 0.9 ± 0.1% | 0.5 ± 0.1% |
| **Others** | 0.1 ± 0.1% | 0.3 ± 0.1% | 0.3 ± 0.1% | 0.1 ± 0.1% | 0.1 ± 0.0% | 0.1 ± 0.0% |
| **Bacterial communities ratios** | | | | | | |
| **F/B ratio** | 1.5 ± 0.1 | 1.4 ± 0.2 | 1.3 ± 0.1 | 1.8 ± 0.2 | 1.0 ± 0.4<sup>##</sup> | 1.4 ± 0.1 | 1.3 ± 0.1<sup>##</sup> |

Phyla with relative abundances > 0.5% of the total population are presented. F/B ratio, Firmicutes/Bacteroidetes ratio. Data were analyzed via Kruskal–Wallis test followed by Dunn’s post hoc test. <sup>##</sup><sup>p</sup> < 0.001, compared with day 0, untreated mice; <sup>##</sup><sup>p</sup> < 0.001, compared with day 0, untreated mice; <sup>##</sup><sup>p</sup> < 0.001, compared with day 21 saline-fed mice; <sup>##</sup><sup>p</sup> < 0.001, compared with day 21 saline-fed mice; <sup>##</sup><sup>p</sup> < 0.001, compared with day 21 saline-fed mice; <sup>##</sup><sup>p</sup> < 0.001, compared with day 29 Saline group. Day 0, untreated mice (n = 42); day 21, saline-fed mice and PS128-fed mice (n = 21/group); day 29, Saline and PS128 group (n = 10/group); Saline + MPTP and PS128 + MPTP groups (n = 11/group).
proinflammatory mediators activates glial cells, causing neuronal damage via oxidative mediator, which further damages neurons and produces more proinflammatory mediators, creating a self-perpetrating neurotoxicity. Post-mortem studies revealed numerous activated microglia surrounding the degenerated dopaminergic neurons in the SN (Braak et al., 1995; McGeer et al., 1988). Several inflammation-associated enzymes, such as inducible nitric oxide synthase and cyclooxygenase 2, were detected in the brains of PD patients (Hunot et al., 1996; Knott et al., 2000). Moreover, elevated proinflammatory cytokines, such as IL-1β, TNF-α, IL-2, IL-4 and IL-6, have also been found in the post-mortem brains, and serum and cerebrospinal fluid of PD patients (Boka et al., 1994; Mogi et al., 1994a; Mogi et al., 1994b), suggesting inevitable neuroinflammation during neurodegenerative processes (Tansey and Goldberg, 2010). We also observed glial hyper-activation and increased proinflammatory cytokines in the nigrostriatal pathway similar to those found in other MPTP mice studies (Khan et al., 2013; Sun et al., 2018; Yasuda et al., 2008; Zhou et al., 2019). These MPTP-induced inflammatory responses were alleviated in PS128-fed mice (Fig. 3A – B, 4). PS128 ingestion also improved MPTP-induced peripheral corticosterone elevation (Fig. 2C), which is consistent with our previous findings that PS128 improved HPA axis dysfunction (Liu et al., 2016b). Hyperactivation of HPA axis was originally considered a consequence of chronic stress due to motor syndromes in PD patients and PD-model animals (Charlett et al., 1998; Djamshidian et al., 2011;
Hartmann et al., 1997; Hemmerle et al., 2012; Kaku et al., 1999). Glucocorticoids are important modulators of microglial reactivity. Glucocorticoid dysregulation might lead to sustained inflammation and cause dopaminergic neuronal injury (Ros-Bernal et al., 2011; Sierra et al., 2008). Although glucocorticoids can suppress inflammatory reactions, elevated glucocorticoids induced by chronic stress can increase microglial proliferation and prime the microglia to proinflammatory stimuli, thus augmenting neuroinflammation in the CNS (Frank et al., 2014; Nair and Bonneau, 2006; Vyas et al., 2016). We believe that PS128 ingestion may help alleviate inflammation-mediated neurodegeneration and improve psychiatric disturbances in PD patients.

HPLC analysis showed that MPTP-induced reductions in striatal DA and its metabolites were restored in PS128-fed mice (Table 1). In addition, norepinephrine levels were markedly elevated in the PS128-fed

Fig. 8. *L. plantarum* PS128 and MPTP treatment affected the mouse fecal microbiomes. (A) Alpha diversities of bacteria communities in individual samples from each group shown by Chao-1 estimator analysis. Data are expressed as means ± S.E.M. and analyzed via the Wilcoxon t-test. *p < 0.05, **p < 0.01, ****p < 0.0001 comparison between indicated groups. (B) Principal coordinate analysis (PCoA) profiles based on weighted UniFrac metrics of bacterial diversity across all samples on days 21 and (C) 29. Samples of mice from different groups are highlighted with different colors and analyzed via PERMANOVA; *p < 0.05. The percentages of variation explained by PC1 and PC2 are indicated on the axes. Day 0, untreated mice (n = 42); day 21, saline-fed mice and PS128-fed mice (n = 21/group); day 29, Saline and PS128 group (n = 10/group); Saline + MPTP and PS128 + MPTP groups (n = 11/group).
Loss of norepinephrine neurons in the locus coeruleus likely contributes to the neuropathological progression and both motor and non-motor syndromes in PD patients (Remy et al., 2005; Zarow et al., 2003). In addition to its role as a neurotransmitter, norepinephrine also provides tonic anti-inflammatory action via β2-adrenergic receptor (β2-AR) signaling which suppresses glial cell activation and inhibits production of inflammatory mediators (Dello Russo et al., 2004; Hinojosa et al., 2013). Animal studies have suggested that norepinephrine depletion exacerbates DA neuron loss and motor deficits (Marien et al., 1993; Rommelfanger et al., 2007). Norepinephrine shown to downregulate α-synuclein in a NE-β2-AR-dependent manner (Mittal et al., 2017). Norepinephrine also promotes the synthesis of neurotrophic factors including NGF and BDNF (Counts and Mufson, 2010; Juric et al., 2008). Interestingly, PS128-fed mice exhibited increased striatal m-BDNF and striatal NGF in MPTP-treated mice (Fig. 3C – D). BDNF is now widely considered to be closely related to development of the dopaminergic nervous system (Baquet et al., 2005; Bothwell, 2014; Hempstead, 2015). In addition to promoting neuronal survival, BDNF modulates the neuroinflammatory response via the m-BDNF-trkB pathway (Bothwell, 2016; Hempstead, 2015; Lu et al., 2005). NGF, on the other hand, modulates the cholinergic system and acetylcholine signaling via TrkA activation, which stimulates parasympathetic nerves to inhibit inflammation and activate anti-inflammatory pathways (Auld et al., 2001; Minnone et al., 2017; Pavlov and Tracey, 2017). Therefore, the elevated striatal neurotrophic factors in PS128-fed mice may also contribute to anti-inflammatory functions (Figs. 3, 4). L-DOPA treatment during the MPTP injection period also restored the motor deficits and increased striatal

**Fig. 9.** *L. plantarum* PS128 increased the *L. plantarum* proportions and microbial modules related to methionine biosynthesis in mouse fecal microbiota. (A) Relative abundances of fecal *L. plantarum* in each experimental group on days 0, 21, and 29. Data are expressed as means ± SEM and analyzed via one-way ANOVA with Tukey’s test. ***p < 0.001 compared with the Saline group at each experimental time; **p < 0.01 compared with the day 21-PS128 group. (B) Negative correlation between fecal *L. plantarum* abundances and total time spent on pole test and (C) narrow-beam test performance in the PS128 + MPTP group on day 29 (n = 11). Regression line and the Spearman’s correlation coefficient are presented. p < 0.05 was considered statistically significant. (D) Microbial modules related to methionine biosynthesis in the fecal microbiomes of all mouse groups on day 29. Data are expressed as means ± SEM and analyzed via one-way ANOVA with Tukey’s test and Benjamini-Hochberg false discovery rate multiple-test correction. #p < 0.05, ##p < 0.01, compared with the Saline group; **p < 0.01, compared with the Saline + MPTP group. Saline and PS128 groups (n = 10/group), Saline + MPTP and PS128 + MPTP groups (n = 11/group).
norepinephrine levels (Fig. 1, Table 1). However, L-DOPA failed to alleviate MPTP-induced dopaminergic neuronal loss and glial overactivation and did not affect striatal neurotrophic factor expression (Fig. 2 A – B, 3). Thus, PS128 ingestion may synergistically animate multiple nervous systems that exerted more beneficial effects than did L-DOPA treatment.

Mounting evidences showed that microbiota-derived SCFAs might be the major mediator to regulate multiple pathways especially those related to immune system functions in the host MGBA (Erny et al., 2015; Huuskonen et al., 2004; Soret et al., 2010). Although the role of microbiota-derived SCFAs in PD remains controversial, SCFAs directly affect T cells such as in CD4+ effector T-cell activation and may promote inflammation in the CNS (Park et al., 2015; Park et al., 2019). Sampson et al. (2016) demonstrated that microbiota-derived SCFAs are essential for α-synuclein-mediated neuroinflammation, glial cell hyperactivation and motor symptoms. Sun et al. (2018) reported that elevated SCFAs in mouse feces 1 week after subchronic MPTP induction were correlated with higher glial activation levels in the SN. In this

Fig. 10. Effects of *L. plantarum* PS128 on the MPTP-induced increase in *Enterobacteriaceae* and microbial modules related to lipopolysaccharide and peptidoglycan biosynthesis. (A) Total level of fecal family *Enterobacteriaceae* in each experimental group on days 0, 21, and 29. Bacterial amounts were analyzed via RT-qPCR and normalized to the fecal sample wet weight (cells per g fecal sample). Data are expressed as means ± SEM and analyzed via one-way ANOVA with Tukey’s post hoc test. ***p < 0.001 compared with the Saline group and **p < 0.001 compared with the Saline + MPTP group at each experimental time. (B) Positive correlation between fecal *Enterobacteriaceae* amount and TNF-α level in the midbrain in all MPTP-treated mice on day 29. (C) Negative correlation between *Enterobacteriaceae* amount and SOD level in the midbrain in all MPTP-treated mice on day 29 (Saline + MPTP and PS128 + MPTP groups, n = 11/group). Regression line and Spearman’s correlation coefficient are presented. *p < 0.05 was considered statistically significant. (D) Microbial modules related to LPS and peptidoglycan biosynthesis in the fecal microbiomes of all mice on day 29. Data are expressed as means ± SEM and analyzed via one-way ANOVA with Tukey’s post hoc test and Benjamini-Hochberg false discovery rate multiple-test correction. *p < 0.05, compared with the Saline group; **p < 0.05, compared with the Saline + MPTP group. Saline and PS128 groups (n = 10/group), Saline + MPTP and PS128 + MPTP groups (n = 11/group).
study, however, fecal SCFA concentrations were markedly decreased in MPTP-treated mice with or without PS128 feeding. Since PS128 ingestion alleviated motor deficits and neural damages without restoring the MPTP-induced SCFAs decline, these beneficial effects of PS128 are probably not mediated by the microbiota-derived SCFAs (Figs. 1, 2, 6).

MPTP treatment significantly altered the gut bacteria (Fig. 7, 10A). Interestingly, other PD and PD-like rodent studies have also reported decreased *Lactobacillaceae* and increased *Lactobacillales* and *Bifidobacteriaceae* (Hill-Burns et al., 2017; Keshavarzian et al., 2015; Lai et al., 2018; Pietrucci et al., 2019; Sun et al., 2018; Unger et al., 2016). MPTP produces toxic effects on dopaminergic neurons in the ENS that causes intestinal motility and stool frequency changes, thus could affect the gut microbiota (Anderson et al., 2007; Lai et al., 2018). The increases in family *Lactobacillaceae* and genus *Lactobacillus* are reported in both PD and constipation-type IBS (Malinen et al., 2005), suggesting the changes in *Lactobacillaceae* might be a consequence associated with constipation. Nonetheless, further studies are required to clarify whether changes in *Lactobacillaceae* is the cause or consequence of Parkinsonian pathology and the physiological roles of such change. Our fecal sample analysis revealed a substantial presence of *L. plantarum* only in PS128-fed mice, which was positively correlated with attenuated MPTP-induced motor deficits (Fig. 9A–C). In addition, *L. plantarum* was not present in any experimental mice before the experimental procedures. In our past studies, PS128 improved behavioral deficits in germ-free mice (Liu et al., 2016a). We believe that PS128 passes through the digestive system and directly influences the gut-brain axis of host. PS128-fed mice presented lower *Firmicutes/Bacteroidetes* ratios and microbial diversity regardless of MPTP treatment (Table 2, Fig. 8A). These effects were similar to those of our previous study on rats (Liao et al., 2019). PS128 ingestion also led to altered microbial functions, particularly increased microbial modules related to methionine biosynthesis, which was even greater after MPTP treatment (Fig. 9D). Thus, PS128 consistently influences the gut microbiota, and the effect persists even after microbiota disturbances caused by MPTP (Fig. 7) or other adverse agents (Liao et al., 2019). Methionine and S-adenosyl methionine, both involved in GSH biosynthesis, could contribute to the improvement in depression, dementia, cognitive deficits and cerebral injury (Dash et al., 2016; Karas Kuzelicki, 2002; Mischoulon and Fava, 2002; Sharma et al., 2017). In our previous study on triathlete, plasma methionine was slightly increased and plasma branched-chain amino acids levels were substantially increased in the PS128-supplement group (Huang et al., 2019). Therefore, increasing the supplement of essential amino acids might be other important ways for the neuroprotective effects of PS128 such as increasing GSH level to counter oxidative stress (Figs. 5 & 9D). We speculate that symbiosis of sufficient live PS128 in the gastrointestinal tract may directly and indirectly benefit the MGBA, thus contributing to the neuroprotective effects of PS128 on the CNS. Further studies are needed to determine the exact mechanisms.

On the other hand, increased *Enterobacteriaceae*, which is strongly suspected to be involved in both PD and Alzheimer’s disease (AD), was also observed in our MPTP-treated mice (Fig. 10A) (Choi et al., 2018; Keshavarzian et al., 2015; Lai et al., 2019a; Pietrucci et al., 2019; Unger et al., 2016). Similarities in the fluctuations of specific gut bacterial populations between MPTP rodent studies and clinical studies highlight the gut dysbiosis caused by changes in bowel habits in the early stages of PD (Keshavarzian et al., 2015; Minato et al., 2017; Pietrucci et al., 2019; Unger et al., 2016). Our predictive metagenomic analysis revealed MPTP-induced alterations in gut microbial functions, especially in increased LPS and peptidoglycan biosynthesis (Fig. 1D). Elevated microbial LPS biosynthesis was also demonstrated in fecal microbiomes from PD patients via predictive metagenomic analysis (Keshavarzian et al., 2015; Pietrucci et al., 2019). PD patients also present increased intestinal penetrability and LPS-binding proteins in the plasma (Forsyth et al., 2011; Hasegawa et al., 2015). The role of microbiota-derived LPS is still controversial, some of them like LPS from *Bacteroides dorei* could be beneficial (Vatanen et al., 2016), whereas LPS from *E. coli* is detrimental and have been used to simulate the pathological progress from leaky gut to CNS neurodegeneration in PD-like animal (Gorecki et al., 2019; Liu and Bing, 2011). Orally administering *Proteus mirabilis* and its LPS caused gut leakage and inflammatory reactions, which further lead to dopaminergic neuronal damage and several PD-like pathological changes such as inflammation and α-synuclein aggregation in the nigrostriatal pathway (Choi et al., 2018). Liu et al. (2019) recently reported progressively enriched *Enterobacteriaceae* and microbial molecules related to glycan biosynthesis and metabolism in pre-onset stage AD patients. These findings alluded the possibilities of LPS from *Enterobacteriaceae* and *Morganellaceae* as triggers for pathogenesis of neurodegenerative diseases. Although in the current study PS128 feeding did not correct MPTP-induced fluctuations in the gut microbiota, it still countered the MPTP-induced elevation in *Enterobacteriaceae* and microbial molecules related to LPS and peptidoglycan biosynthesis (Fig. 10). Therefore, we believe that symbiosis of PS128 in GI tract of the host could disrupt some pathological factors in gut microbiota.

The MPTP model for PD research is widely used in both pathophysiological and therapeutic studies (Lai et al., 2018; Meredith and Rademacher, 2011). Our results showed that mice under subchronic MPTP administration presented neuroinflammation and oxidative stress in the nigrostriatal pathway along with HPA axis dysfunction and gut dysbiosis similar to clinical observations seen with MGBA impairments in the early stages of PD (Lubomski et al., 2019; Mulak and Bonaz, 2015; Nair et al., 2018). PS128 ingestion reduced these MGBA impairments, revealing its novel neuroprotective effects, which could reduce the pathophysiological changes seen in PD patients. Based on MGBA theory and past findings in psychobiological research, researchers believe that microbiota-gut axis modulators, such as fecal transplantation, dietary intervention, prebiotics and probiotics, may enable effective therapeutic management of neurodegenerative disorders (Fang et al., 2019; Srivastava et al., 2019; Sun et al., 2018; Zhou et al., 2019). Although probiotic administration has beneficial effects in PD, AD and multiple sclerosis patients, the field is in its infancy (Akbari et al., 2016; Leblhuber et al., 2018; Tamaia et al., 2019; Tankou et al., 2018). To date, PS128 is the first psychobiotic strain that may support dopaminergic pathways by strengthening host MGBA functions (Huang et al., 2019; Liao et al., 2019; Liu et al., 2016a; Liu et al., 2019b; Liu et al., 2016b). According to our past studies and current results, we believe that ingesting the psychobiotic strain PS128 may help amend neurodegeneration, hence displaying great potential for preventing PD and other neurodegenerative diseases.

5. Conclusion

We demonstrated that PS128 ameliorated MPTP-induced motor deficits, elevated corticosterone, dopaminergic neuronal death, and neurotransmitter reduction in mice. PS128 also improved NE, neurotrophic factors and antioxidant levels and reduced glial hyperactivation, neuroinflammation, and oxidative stress in the nigrostriatal pathway. Fecal sample analysis showed that MPTP-induced upregulating of *Enterobacteriaceae* and microbial modules related to LPS and peptidoglycan biosynthesis, which had been widely reported in clinical studies of neurodegenerative diseases, were alleviated in PS128-fed mice. Although PS128 treatment did not affect the MPTP-induced SCFA decline and microbial fluctuations, PS128-fed mice still exhibited ameliorations of MPTP neurotoxicity, suggesting a direct protective effect on the CNS by PS128. Our studies may provide new insight into using psychobiotics to reduce neuronal damage in neurodegenerative diseases such as PD, which could serve as a potential treatment or add-on treatment alternative.

6. Relevant conflicts of interests/financial disclosures

Y.F.C and C.H were employed by Bened Biomedical Co., Ltd. at
the time of the study. None of the other authors had a personal or financial conflict of interest.

7. Funding agencies

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8. Author's contribution statement

J.F.L., S.W. and Y.C.T. conceived and organized the study. J.F.L., Y.F.C., S.T.Y., and W.C.K. performed the experiments. C.C.H. and J.J.C. performed the Illumina MiSeq sequencing analysis. J.F.L., Y.F.C., and J.J.C. statistically analyzed the data. J.F.L. wrote the first draft of the manuscript and Y.F.C., C.W.H., J.J.C., H.M.H-L., S.W. and Y.C.T. contributed to writing the manuscript. All authors read, edited and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbbi.2020.07.036.

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