Biomedicine & Pharmacotherapy 165 (2023) 115100

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# Low and high doses of oral maslinic acid protect against Parkinson's disease via distinct gut microbiota-related mechanisms

Xu Cao [a], Zhong-Rui Du [b,c,d,e], Xin Liu [b], Xiong Wang [b], Chong Li [b,c,d], Sai-Nan Zhou [b,c,d], Jia-Rui Liu [a], Ping-Yi Xu [f], Jun-Li Ye [g], Qing Zhao [h], Fang Zhao [i], Ka-Hing Wong [b,c,d,*], Xiao-Li Dong [b,c,d,**]

[a] Department of Neurology, Shenzhen University General Hospital, Shenzhen University Clinical Medical Academy, Shenzhen, China
[b] Key Laboratory of Food Biological Safety Control, The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen, China
[c] Research Institute for Future Food, The Hong Kong Polytechnic University, Hong Kong, China
[d] Department of Food Science and Nutrition, The Hong Kong Polytechnic University, Hong Kong, China
[e] BioNanotechnology Institute, Ludong University, Yantai, China
[f] Department of Neurology, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China
[g] Department of Physiology and Pathophysiology, School of Basic Medicine, Qingdao University, Qingdao, China
[h] Department of Neurology, Linzi Maternal & Child Health Hospital of Zibo, Zibo, China
[i] BGI-Qingdao, BGI-Shenzhen, Qingdao, China

ARTICLE INFO

*Keywords:*
Parkinson's disease
Maslinic acid
Dose
Gut microbiota
Neurotransmitters
Short chain fatty acids
Neuroinflammation

ABSTRACT

The use of oral agents that can modify the gut microbiota (GM) could be a novel preventative or therapeutic option for Parkinson's disease (PD). Maslinic acid (MA), a pentacyclic triterpene acid with GM-dependent biological activities when it is taken orally, has not yet been reported to be effective against PD. The present study found both low and high dose MA treatment significantly prevented dopaminergic neuronal loss in a classical chronic PD mouse model by ameliorating motor functions and improving tyrosine hydroxylase expressions in the substantia nigra pars compacta (SNpc) and increasing dopamine and its metabolite homovanillic acid levels in the striatum. However, the effects of MA in PD mice were not dose-responsive, since similar beneficial effects for low and high doses of MA were observed. Further mechanism studies indicated that low dose MA administration favored probiotic bacterial growth in PD mice, which helped to increase striatal serotonin, 5-hydroxyindole acetic acid, and γ-aminobutyric acid levels. High dose MA treatment did not influence GM composition in PD mice but significantly inhibited neuroinflammation as indicated by reduced levels of tumor necrosis factor alpha and interleukin 1β in the SNpc; moreover, these effects were mainly mediated by microbially-derived acetic acid in the colon. In conclusion, oral MA at different doses protected against PD via distinct mechanisms related to GM. Nevertheless, our study lacked in-depth investigations of the underlying mechanisms involved; future studies will be designed to further delineate the signaling pathways involved in the interactive actions between different doses of MA and GM.

*Abbreviations:* AA, acetic acid; BBB, blood brain barrier; CNGB, China National Gene Bank; CNS, central nervous system; CT, comparative threshold cycle; DA, dopamine; DAB, 3′-diaminobenzidine; DMEM-H, Dulbecco's Modified Eagle Medium-High glucose; ENS, enteric nervous system; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GM, gut microbiota; GI, gastrointestinal; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, serotonin; HVA, homovanillic acid; IF, immunofluorescence; IHC, immunohistochemistry; IL-1β, interleukin 1β; LC-MS, liquid chromatography–mass spectrometry; LDA, linear discriminant analysis; L-Dopa, levodopa; LEfSe, Linear discriminant analysis Effect Size; MA, maslinic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SCFAs, short chain fatty acids; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TH-IR, tyrosine hydroxylase immunoreactive neurons; TNFα, tumor necrosis factor alpha.

* Correspondence to: Y809, Department of Food Science and Nutrition, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China.
** Correspondence to: Y817, Department of Food Science and Nutrition, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China.
*E-mail addresses:* kahing.wong@polyu.edu.hk (K.-H. Wong), xiaoli.dong@polyu.edu.hk (X.-L. Dong).

https://doi.org/10.1016/j.biopha.2023.115100
Received 12 May 2023; Received in revised form 20 June 2023; Accepted 28 June 2023
Available online 6 July 2023

## 1. Introduction

Maslinic acid (MA; 2α,3β-dihydroxyolean-12-en-28-oic acid) is a pentacyclic triterpene widely distributed in nature [1]. At least 50 plants, including many commonly consumed fruits and vegetables, have been identified as sources of MA, with the highest amount of MA found in olive oil [2]. There is substantial evidence of a variety of biological activities of MA, such as antioxidant [3], anti-inflammatory [4], anti-tumor [5] and even neuroprotective activities [6–8]. MA has also shown good therapeutic effects against multiple organ diseases, which along with its low toxicity, has led to great interest in its potential for clinical applications [9]. However, MA has low oral bioavailability [9, 10]. Moreover, like many other natural products, the activities of MA might also be dependent on the gut microbiota (GM) when it is taken orally [11].

There has been considerable interest in and enthusiasm for the study of GM on human health in recent years. A large number of studies demonstrating the vital role of GM in human physiological and biological processes have been published and GM dysbiosis has been found to be related to many human diseases [12,13]. Healthy GM can produce versatile enzymes that metabolize and transform natural products into secondary metabolites with better bioavailability or lower toxicity [11]. Conversely, natural products can modulate GM and change its composition and therefore, the metabolic processes in the intestine [14]. Interactions between GM and natural products can also result in alterations of GM metabolites, including but not limited to short chain fatty acids (SCFAs) and bile acids, to further mediate physiological processes in the host [15]. Although there has been no study on the interaction between MA and GM until now, it has been recently reported that oleanolic acid, which has chemical structure similar to that of MA, could alter the immune function of intestinal epithelial cells by optimizing GM in normal mice [16].

Parkinson's disease (PD) is a well-known and common neurodegenerative disorder characterized by motor symptoms, mainly resting tremors, bradykinesia, and muscle rigidity, as well as various non-motor symptoms, such as depression, sleep disorders, and gastrointestinal disorders [17]. A recent meta-analysis of 28 clinical studies reported that PD patients had a high degree of GM alterations compared with healthy controls [18], and GM is now increasingly being considered as one of the pathogenic factors involved in PD. Furthermore, we have previously shown that polymannuronic acid, a component of alginate can prevent dopaminergic neuronal loss via the brain-gut-microbiota axis in a mouse model of PD [19], indicating the possibility of using oral agents to treat PD via regulating the GM.

With regard to the underlying mechanisms, neuroinflammation, oxidative stress, and impaired autophagy have been implicated in the pathogenesis of PD [20–22]. Several natural triterpenoids and polyphenols, including ursolic acid, betulinic acid, chlorogenic acid, which have a similar structure to that of MA, have been proven to have neuroprotective effects against PD due to certain common effects, such as antioxidant and anti-inflammation activities [20,23]. Studies have also confirmed the neuroprotective effects of ursolic acid in rotenone- or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD animal models through enhancing autophagy [24] and suppressing neuroinflammation [25]. MA has been shown to have anti-inflammatory activity [4], and the results of our previous study also indicated that MA could promote autophagy in a neuron cell line by directly disrupting the interaction between Bcl-2 and Beclin 1 [26]. However, there has not been any animal model study on the effects of MA against PD.

In this study, our objective was to investigate the potential neuroprotective effects of oral MA against PD and to explore the underlying mechanisms, particularly with regard to the involvement of GM in its effects. We chose a classical chronic PD mouse model established by intraperitoneal (i.p.) injection of MPTP twice a week for 5 weeks. Thereafter, low or high dose MA, or levodopa (L-Dopa) as a positive control, was separately fed to PD model mice via oral gavage once daily for a total of 5 weeks. The potential neuroprotective effects of MA were then assessed on the basis of behavioral tests and biomedical parameters. The underlying mechanisms, especially those related to GM, were explored.

## 2. Materials and methods

### 2.1. Chemicals and reagents

MA was purchased from Sigma (USA). Its chemical structure is depicted in Supplemental Fig. 1. MPTP, L-Dopa, standards of neurotransmitters and their metabolites, and standards of SCFAs were also purchased from Sigma (USA). Primary and secondary antibodies were purchased from Millipore, Abcam, or Santa Cruz in the USA or Beyotime in China. The QIAamp DNA stool kit was purchased from Qiagen (USA). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor alpha (TNFα) and interleukin 1β (IL-1β) were purchased from RapidBio Lab (USA). Further details have been provided in the Supplemental file.

### 2.2. Animal experimental design

Forty male C57BL/6 J mice (weight, 22 ± 3 g; age, 8 weeks) were purchased from Beijing Vital River Laboratory (Beijing, China), housed in an air-conditioned room (at 22 ± 2ºC with 55 % ± 5 % relative humidity) under 12 h light/dark conditions, and provided with a standard diet (TD.94048, Purified Rodent Diet AIN-93 M, Harlan Laboratories Inc. USA) and distilled water. After acclimatization, the mice were randomly divided into five groups (n = 8 per group). The PD model was established by i.p. MPTP injection at a dose of 20 mg/kg twice a week for 5 weeks; normal mice received i.p. injections of the same volume of saline as control. L-Dopa (15 mg/kg/d) or low (50 mg/kg/d) or high (100 mg/kg/d) dose MA was fed to PD mice via oral gavage once daily for 5 consecutive weeks. The experimental timeline is shown in Fig. 1A. Further details have been provided in the Supplemental file. This animal experiment was reviewed and approved by Animal Ethical Committee of The Hong Kong Polytechnic University Shenzhen Institute with approval no: 180703.

### 2.3. Behavioral tests: grip strength test and open field test

The grip strength test and open field test were conducted as previously described [27]. The muscular strength of mice, specifically, the gripping ability, was assessed using a grip strength meter and expressed in terms of the peak force (g). The locomotor function of mice was assessed using an open field apparatus and expressed in terms of total distance traveled (cm) and activity index ($cm^2/s$) in a target area within a 5-min period.

### 2.4. Sample collection

After the behavioral tests, all mice were made to undergo overnight fasting before sacrifice. On the day of sacrifice, fecal samples were collected, and mice were euthanized by i.p. injection of pentobarbitone. Both sides of the substantia nigra pars compacta (SNpc) and striatum (n = 6) were harvested and stored at − 80 °C. Whole brains (n = 2) were also removed for immunohistochemistry (IHC) or immunofluorescence (IF) staining.

### 2.5. IHC and IF staining for tyrosine hydroxylase (TH) immunoreactive (TH-IR) neurons and microglia

Whole brains were post-fixed in 4 % paraformaldehyde and then dehydrated. The midbrain region containing the SNpc was acquired and serially cut into frozen coronal sections. These were then mounted on slides and treated with 1 % bovine serum to block non-specific binding

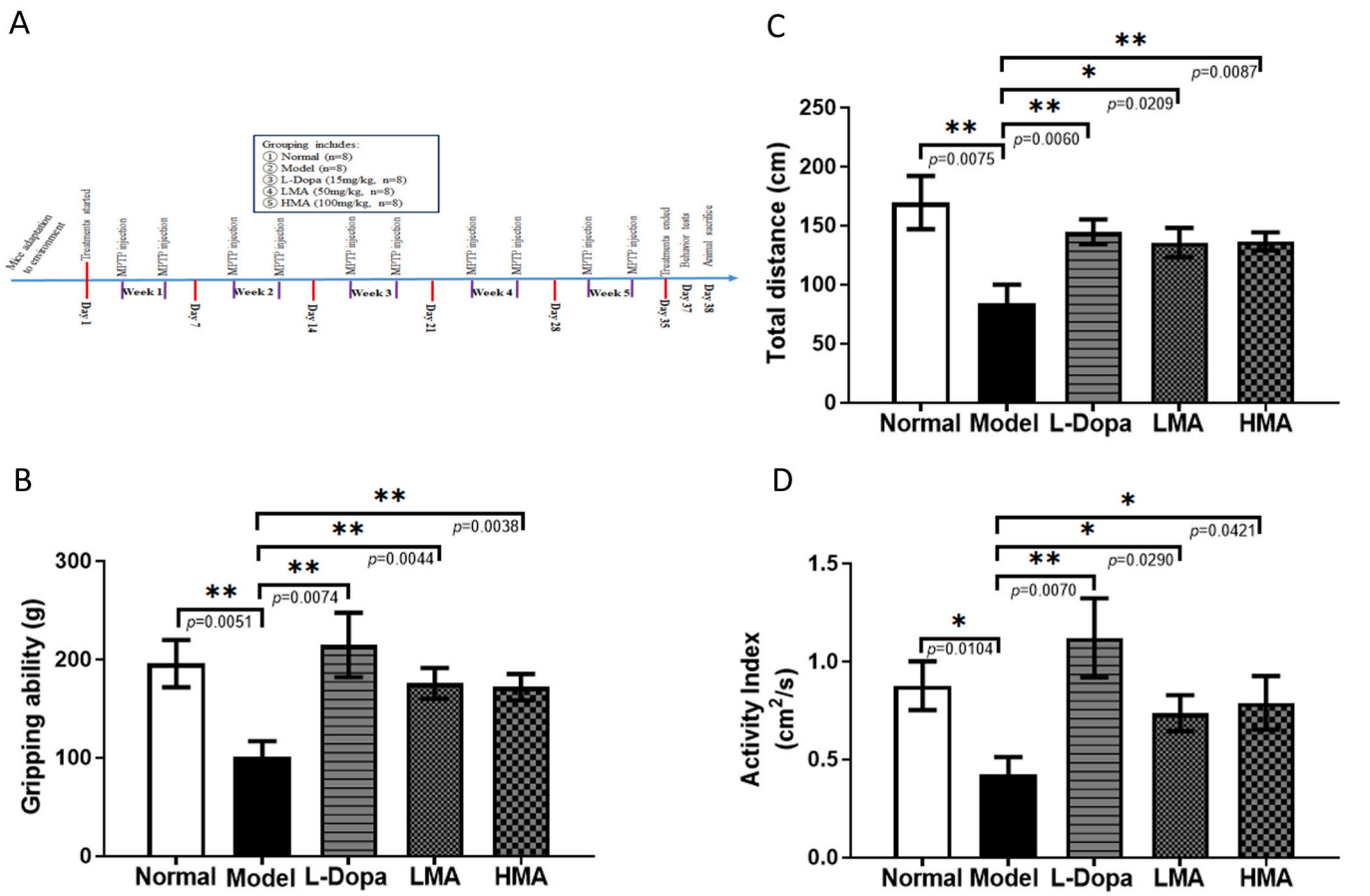



**Fig. 1. Timeline and behavioral tests.** (A) Experimental timeline. (B) Grip strength test to assess the gripping ability (g) of mice. (C, D) Open field test to assess total distance traveled (cm) (C) and activity index ($cm^2/s$) (D) within a 5-min period. n = 8 in each group. Data are expressed as mean ± SEM values. The unpaired t-test was used to compare values between two groups. The exact t-test *p* values are shown in the figures. * $p < 0.05$, * * $p < 0.01$ vs. Model group.

sites. For IHC, the sections were then incubated with primary (mouse anti-TH) and secondary (goat anti-mouse IgG) antibodies and stained with 3′-diaminobenzidine (DAB), after which TH-IR neurons were visualized under an ordinary microscope. For IF staining, the sections were incubated with primary (rabbit anti-TH or rabbit anti-Iba-1) and fluorescent secondary (Cy3-labeled donkey anti-goat IgG or Alexa Fluor 488-labeled goat anti-rabbit IgG) antibodies, after which fluorescent TH-IR neurons and microglia were visualized under a confocal microscope (Olympus, Japan).

### 2.6. Western-blot analysis

Half of the SNpc tissue was homogenized in ice-cold lysis buffer and then centrifuged. The protein concentrations in the samples were determined, and equal amounts of proteins were separated by SDS-PAGE. Thereafter, the proteins were transferred to PVDF membranes that were blocked using 5 % non-fat milk and then incubated sequentially with primary antibodies (mouse anti-TH and mouse anti-β-actin) and the corresponding secondary antibodies. Protein bands were developed using a chemiluminescence kit, and densitometry analysis was performed using ImageJ software.

### 2.7. Measurement of neurotransmitter and corresponding metabolite levels by liquid chromatography–mass spectrometry (LC–MS)

The freshly isolated striatum was weighed, suspended in water (1:9 wt/vol), and homogenized. Standards for dopamine (DA) and its metabolite homovanillic acid (HVA), serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA), and γ-aminobutyric acid (GABA) were freshly prepared and injected into the column for calibration. The levels of neurotransmitters and their metabolites in the striatum of mice were determined by LC-MS as previously described [19, 27].

### 2.8. Analysis of fecal SCFA levels

Mice fecal samples were thawed, and 0.2 g of sample from each mouse was mixed with distilled, deionized water. Samples were then processed as previously described [19,27] and injected into a gas chromatography instrument (Agilent Technologies, USA) to determine the concentrations of different SCFAs (acetic acid [AA], propionic acid [PA], and butyric acid [BA]).

### 2.9. 16S rRNA gene sequencing and data analysis

DNA from proximal colon feces samples was extracted using the QIAamp DNA stool kit (Qiagen, USA) and used to amplify the V3–V4 region of 16 S rRNA genes. The extraction, 16 S rRNA gene sequencing, and data analysis methods used have been described previously [27,28]. All raw sequence data were deposited in the China National Gene Bank (CNGB; project accession number: CNP0001329).

### 2.10. ELISA detection

Levels of TNFα and IL-1β in the SNpc of mice from each group were detected with commercial ELISA kits (RapidBio Lab., USA) according to the manufacturer's instructions. TNFα and IL-1β concentrations in brain tissues are expressed as pg/100 mg brain tissue.

### 2.11. BV-2 microglia cell culture

The murine BV2 microglial cell line was obtained from the Cell Resource Center of IBMS, CAMS/PUMC (3111C0001CCC000063, Beijing China, RRID: CVCL_0182). BV-2 microglia were cultured in Dulbecco's Modified Eagle Medium-High glucose (DMEM-H; Life technology, USA) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin (Invitrogen, USA), and maintained in a humidified atmosphere with 5 % $CO_2$ at 37 °C, and medium changed every two days. A 70–80 % confluent monolayer was detached into a single cell suspension for further cell culture.

### 2.12. Quantitative real-time PCR (q-PCR)

BV2 microglial cells were treated with AA or MA or their vehicle for 1 h. Subsequently, N-Methyl-4-Phenylpyridinium Iodide ($MPP^+$) was added to the cell culture; after further incubation for 6 h, total RNA was isolated using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized and real-time PCR was performed using SYBR Premix Ex Taq (Qiagen, USA). The comparative threshold cycle (CT) method was used, and the $2^{-\Delta\Delta CT}$ formula was used to assess the changes in the mRNA levels of TNFα and IL-1β. Data were normalized to the 18 S rRNA levels in the samples, and mean expression for each gene was calculated relative to the group mean of the Control group. The PCR primer sequences used for TNFα, IL-1β, and 18 S rRNA were the same as those used in our previous study [28].

### 2.13. Statistical analysis

Animals were randomly assigned to each group, and experimenters were blinded for all analysis purposes. All data are reported as mean ± SEM values. Subsequently, one-way analysis of variance (ANOVA) was conducted using PRISM version 9.0 (GraphPad, USA). When significant variations were found using one-way ANOVA, the unpaired t-test as the post-test was used to compare two groups if there was no significant variance inhomogeneity ($p > 0.05$ in the F test), and the unpaired t-test with Welch's correction was used if there was significant variance inhomogeneity ($p < 0.05$ in F test). Differences were considered statistically significant if the corresponding $p$ value was less than 0.05. Differences in microbiome features between two treatments were assessed using the linear discriminant analysis (LDA) effect size (LEfSe) method, with the cutoff value of absolute $\log_{10}$ LDA score > 2.0. Only taxa with absolute $\log_{10}$ LDA scores > 3.0 in pairwise comparison and with relative abundance of phylum to genus > 1 % were compared and mentioned in the results.

## 3. Results

### 3.1. Effects of low and high dose MA treatment on the motor functions of PD mice

The grip strength test and open field test were employed to evaluate the motor functions of mice. PD mice had weakened muscle strength as shown by reduced gripping ability (t = 3.313, $p = 0.0051$; F=2.452, $p = 0.2596$) in the grip strength test (Fig. 1B, vs. Normal). PD mice also exhibited locomotor deficits in terms of reduced walking distance (t = 3.124, $p = 0.0075$; F=2.198, $p = 0.3206$) (Fig. 1C) and activity (t = 2.956, $p = 0.0104$; F=1.849, $p = 0.4360$) (Fig. 1D) in the open filed test (vs. Normal). L-Dopa treatment, as a positive control, could greatly improve the gripping ability (t = 3.130, $p = 0.0074$; F=4.585, $p = 0.0623$) (Fig. 1B), walking distance (t = 3.235, $p = 0.0060$; F=2.075, $p = 0.3563$) (Fig. 1C) and activity index (t = 3.159, $p = 0.0070$; F=4.884, $p = 0.0530$) (Fig. 1D) of PD mice in these behavioral tests (vs. Model). Oral administration of low and high doses of MA was also found to greatly ameliorate the motor functions of PD mice as indicated by elevation of the reduced gripping ability (Low: t = 3.393, $p = 0.0044$; F=1.036, $p = 0.9642$ and High: t = 3.462, $p = 0.0038$; F=1.308, $p = 0.7324$) (Fig. 1B), walking distance (Low: t = 2.602, $p = 0.0209$; F=1.512, $p = 0.5992$ and High: t = 3.045, $p = 0.0087$; F=3.839, $p = 0.0968$) (Fig. 1C) and activity index (Low: t = 2.433, $p = 0.0290$; F=1.016, $p = 0.9837$ and High: t = 2.237, $p = 0.0421$; F=2.246, $p = 0.3078$) (Fig. 1D, vs. Model). Moreover, the low and high doses of MA appeared to exert similar beneficial effects, without any dose-response relationship, on the motor functions of PD mice.

### 3.2. Effects of low and high dose MA treatment on preventing dopaminergic neuronal loss in PD mice

IHC was used to monitor changes in TH-IR neurons in the SNpc of mice (Fig. 2A and Supplemental Fig. S3). It seemed that PD mice had reduced numbers of dopaminergic TH-IR neurons in PD mice, and there were more TH-IR neurons in L-Dopa, low or high dose MA treated PD mice.

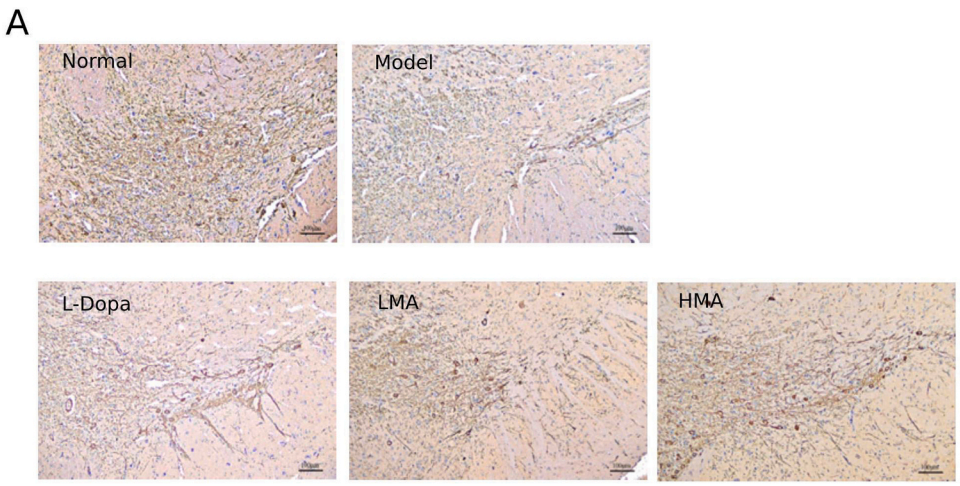
Western blotting was used to assess the levels of TH in the SNpc (Fig. 2B, Supplemental Fig. S2) of mice. The results indicated that MPTP injection resulted in a significant decline of TH level (t = 4.188, $p = 0.0019$; F=1.180, $p = 0.8602$) in the SNpc of mice (Model vs. Normal). L-Dopa (as a positive control) (t = 2.522, $p = 0.0303$; F=1.371, $p = 0.7378$) and low (t = 2.332, $p = 0.0419$; F=2.082, $p = 0.4400$) or high (t = 2.441, $p = 0.0348$; F=1.373, $p = 0.7364$) dose MA treatment could significantly enhance TH level in the SNpc of mice (vs. Model). Moreover, the effects of MA on TH level were also not dose dependent.

### 3.3. Effects of low and high dose MA treatment on the levels of neurotransmitters in the striatum of PD mice

Striatal neurotransmitter levels were measured using LC-MS, and the results are summarized in Table 1. The results showed that the levels of striatal DA (t = 3.826, $p = 0.0033$; F=1.082, $p = 0.9330$), its metabolite HVA (t = 2.966, $p = 0.0141$; F=4.837, $p = 0.1086$) and GABA (t = 4.361, $p = 0.0014$; F=3.254, $p = 0.2212$) declined considerably in PD mice (vs. Normal). Oral administration of L-Dopa (DA: t = 2.995, $p = 0.0135$; F=1.804, $p = 0.5331$ and HVA: t = 5.076, $p = 0.0005$; F=2.438, $p = 0.3504$), and low (DA: t = 2.332, $p = 0.0419$; F=1.130, $p = 0.8964$ and HVA: t = 2.801, $p = 0.0188$; F=1.997, $p = 0.4661$) or high (DA: t = 2.320, $p = 0.0428$; F=1.587, $p = 0.6248$ and HVA: t = 3.209, $p = 0.0093$; F=2.884, $p = 0.2699$) doses of MA significantly increased the striatal levels of DA and HVA in PD mice (vs. Model). Furthermore, only low dose MA treatment was found to simultaneously enhance the levels of 5-HT (t = 3.102, $p = 0.0112$; F=4.271, $p = 0.1370$) and 5-HIAA (t = 3.459, $p = 0.0175$ with Welch's correction; F=93.82, $p = 0.0001$) and GABA (t = 2.397, $p = 0.0375$; F=3.458, $p = 0.1995$) in the striatum of PD mice (vs. Model).

### 3.4. Effects of low and high dose MA treatment on GM composition in PD mice

Based on 16 S rRNA gene sequencing data, alpha (Fig. 3A) and beta analysis (Fig. 3B) were conducted to assess the microbial diversity in different samples and compare it among different groups. Our results showed that PD mice had a higher degree of microbial richness and diversity, as indicated by a higher number of total species (t = 4.236, $p = 0.0008$; F=2.346, $p = 0.2830$) as well as Chao1 (t = 2.592, $p = 0.0213$; F=2.198, $p = 0.3204$), ACE (t = 2.951, $p = 0.0105$; F=2.325, $p = 0.2882$) and Shannon index values (t = 2.428, $p = 0.0293$; F=2.413, $p = 0.2680$), but lower Simpson index values (t = 2.540, $p = 0.0309$ with Welch's correction; F=5.841, $p = 0.0330$) in the alpha analysis compared to those in normal mice. L-Dopa treatment did not influence these alpha diversity analysis values, but both low and high dose MA treatment significantly reduced the increase in



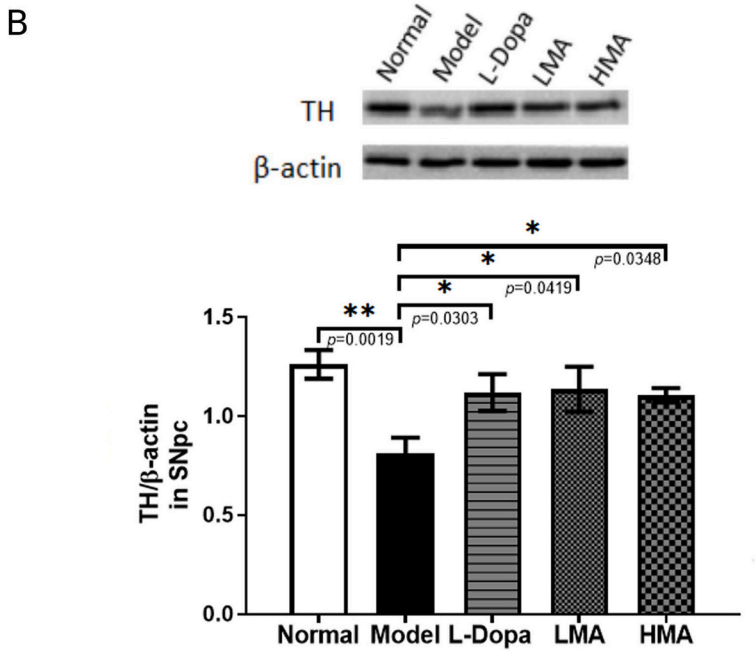




**Fig. 2.** IHC staining of TH-IR neurons and TH protein expression in the SNpc of mice. (A) IHC staining of TH-IR neurons in sections of the SNpc in each group (n = 2); One representative image from each group under 100 × magnification; scale bar represents 50 μm. (B) TH protein expressions in the SNpc of mice in each group (n = 6); One representative band for TH and endogenous β-actin from each group; and the following bar chart shows the ratio of TH/ β-actin in each group in the SNpc of mice. IHC, immunohistochemistry; TH, tyrosine hydroxylase; TH-IR, tyrosine hydroxylase immunoreactive; SNpc, substantia nigra pars compacta; Data are expressed as mean ± SEM values. The unpaired t-test was used to compare values between two groups. The exact t-test $p$ values are shown in the figures. * $p < 0.05$, * * $p < 0.01$ vs. Model group.

**Table 1**
Neurotransmitters in the striatum of mice in different groups[1,2].

| Grouping | Neurotransmitters in the striatum | | | | |
|---|---|---|---|---|---|
| | DA (ug/g) | HVA (ug/g) | 5-HT (ug/g) | 5-HIAA (ug/g) | GABA (ug/g) |
| **Normal** | 9.24 ± 0.59 * *($p$=0.0033) | 26.0 ± 2.5 *($p$=0.0141) | 0.189 ± 0.036 | 1.49 ± 0.18 | 485.0 ± 45.3 * *($p$=0.0014) |
| **Model** | **6.12 ± 0.56** | **17.7 ± 1.2** | **0.142 ± 0.018** | **1.16 ± 0.04** | **259.1 ± 25.1** |
| **L-Dopa** | 8.95 ± 0.76 *($p$=0.0135) | 28.6 ± 1.8 * **($p$=0.0005) | 0.139 ± 0.027 | 1.08 ± 0.15 | 277.7 ± 36.2 |
| **LMA** | 8.04 ± 0.60 *($p$=0.0419) | 23.3 ± 1.6 *($p$=0.0188) | 0.273 ± 0.038 *($p$=0.0112) | 2.43 ± 0.36 *($p$=0.0175) | 386.2 ± 46.7 *($p$=0.0375) |
| **HMA** | 8.23 ± 0.71 *($p$=0.0428) | 25.0 ± 2.0 * *($p$=0.0093) | 0.130 ± 0.022 | 1.08 ± 0.13 | 231.0 ± 35.7 |

1) Values are expressed as mean ± SEM, n = 6. Unpaired T-test with or without Welch's correction was performed to compare values between either two groups and the exact p value from T-test was shown in this table. *p < 0.05, * *p < 0.01, * **p < 0.001 vs. Model group.
2) DA, dopamine; HVA, homovanillic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid; GABA, γ-aminobutyric acid.

total species number (Low: t = 5.635, $p < 0.0001$; F=1.504, $p = 0.6033$ and High: t = 6.088, $p = 0.0002$ with Welch's correction; F=8.577, $p = 0.011$) and Chao1 (Low: t = 4.553, $p = 0.0011$ with Welch's correction; F=5.034, $p = 0.0490$ and High: t = 3.811, $p = 0.0019$; F=2.662, $p = 0.2199$), ACE (Low: t = 4.128, $p = 0.0010$; F=1.631, $p = 0.5345$ and High: t = 4.792, $p = 0.0003$; F=3.917, $p = 0.0922$) and Shannon (Low: t = 3.150, $p = 0.0071$; F=4.590, $p = 0.0622$ and High: t = 3.500, $p = 0.0035$; F=1.467, $p = 0.6255$) index values, and

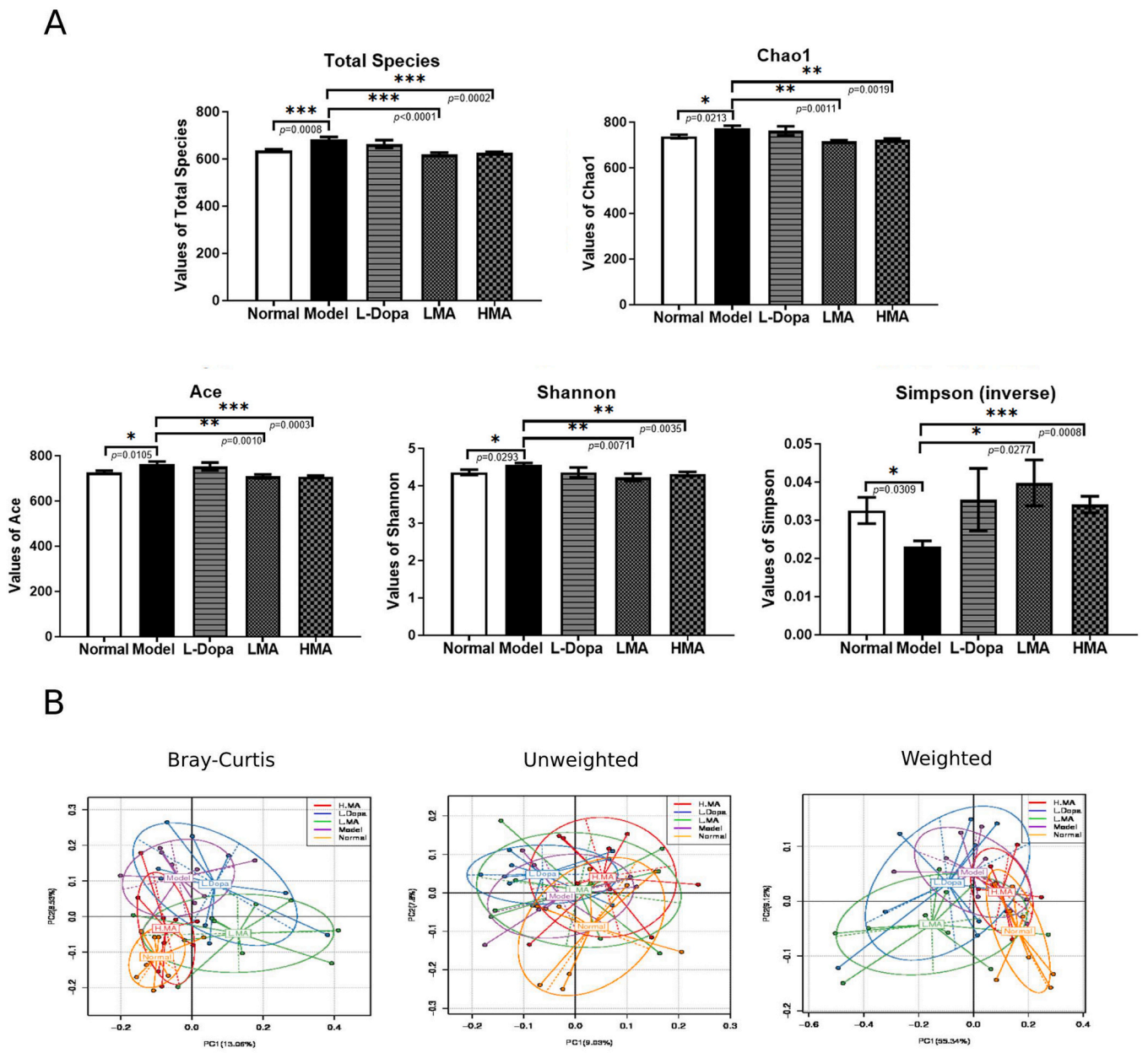



**Fig. 3.** Microbial alpha and beta diversity analysis. (A) Alpha diversity indices in different groups. (B) Principal coordinates analysis (PCoA) derived from Bray-Curtis distances, unweighted and weighted UniFrac among samples of five groups. Data are expressed as mean ± SEM values. The unpaired t-test, with or without Welch's correction, was used to compare values between two groups. The exact t-test *p* values are shown in the figures. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ vs. Model group.

increased the decreased Simpson index values (Low: $t = 2.703$, $p = 0.0277$ with Welch's correction; $F=17.90$, $p = 0.0011$ and High: $t = 4.236$, $p = 0.0008$; $F=2.346$, $p = 0.2830$) in PD mice (vs. Model). Notably, beta analysis did not reveal any significant contrast in microbial diversity among different groups.

Phyla *Firmicutes* and *Bacteroidetes* are the two most common components of the GM, constituting over 90 % of the GM in mammals. In this study, only the taxa with absolute $\log_{10}$ LDA scores $> 3.0$ in pairwise comparison and relative abundance of phylum to genus $> 1$ % were further compared. As shown in Fig. 4A, the abundance of phylum *Firmicutes* and its subordinate class *Erysipelotrichi*, order *Erysipelotrichales*, family *Erysipelotrichaceae*, and genus *Allobaculum* increased but that of phylum *Bacteroidetes* and its subordinate class *Bacteroidia*, order *Bacteroidales*, and family *S24_7* decreased in PD mice compared to those in normal mice. PD mice also had higher abundance of some other strains (such as phyla *Verrucomicrobia*, *WWE1*), but their relative abundance was extremely low. Low dose MA treatment was found to significantly influence GM composition in PD mice (Fig. 4B). Surprisingly, high dose MA and L-Dopa failed to induce any significant changes of GM composition and cladogram could not be constructed. Low dose MA treatment further decreased phyla *Bacteroidetes* abundance, which aggravated major GM changes in PD mice. Meanwhile, the abundance of *Bacilli* (class) under *Firmicutes* (phylum), and its subordinate *Lactobacillales* (order), *Lactobacillaceae* (family), *Lactobacillus* (genus) increased, but the abundance of *Ruminococcaceae* (family) with its genus *Oscillospira*, *Rikenellaceae* (family), *Ruminococcus* (genus) decreased in response to low dose MA treatment in PD mice.

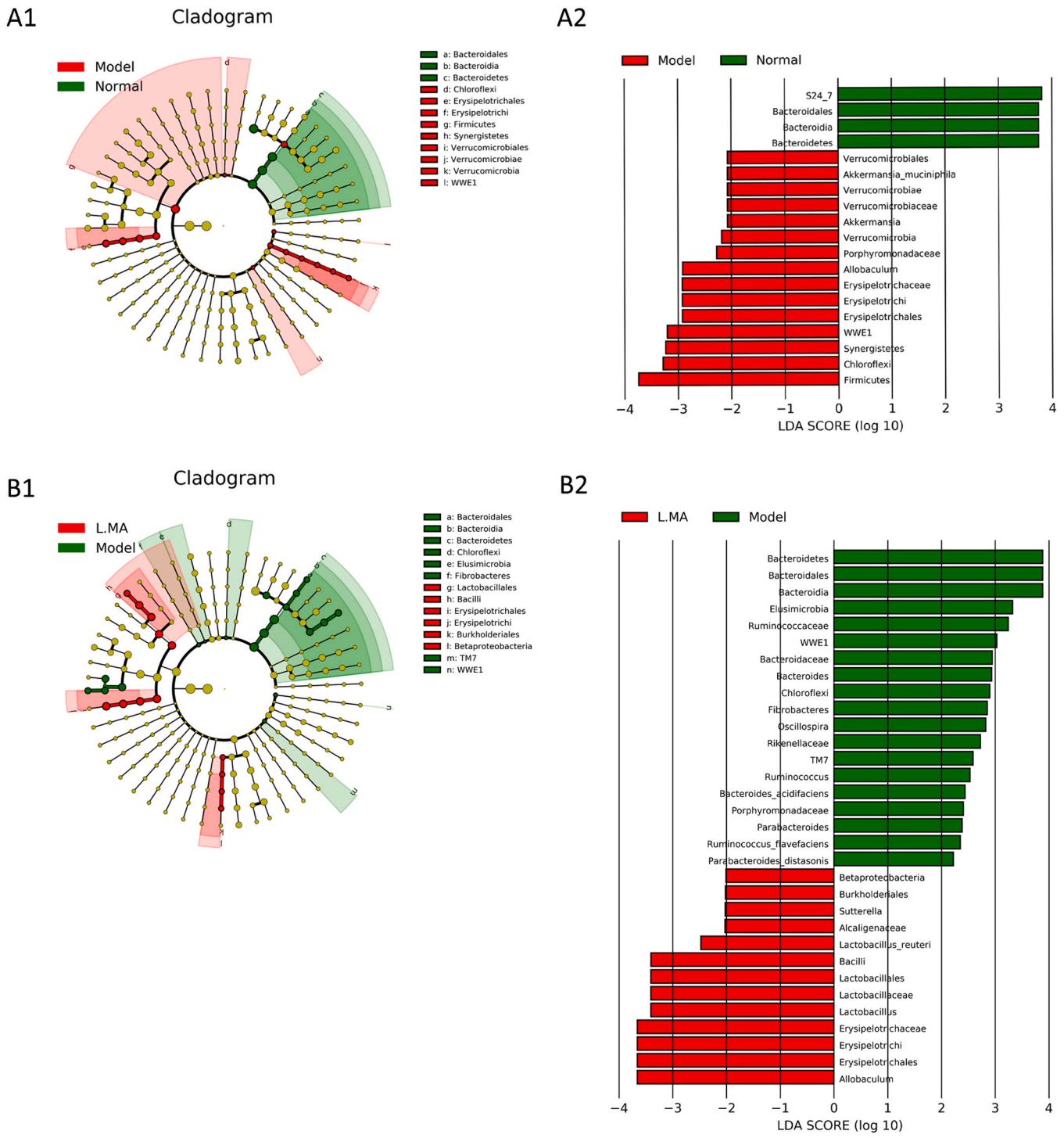



**Fig. 4.** Microbial composition analysis based on 16S rRNA gene sequencing. Cladograms and histograms displaying the taxa with significantly different abundance in any two groups marked with green or red. (A) Model vs. Normal; (B) L.MA vs. Model. Pairwise comparison was conducted and only the taxa with absolute $\log_{10}$ LDA scores > 2.0 are displayed. The higher abundance of bacteria was marked with green or red color to indicate its group in each figure.

### *3.5. Effects of low and high dose MA treatment on fecal SCFA levels in PD mice*

Next, we measured the levels of the most abundant SCFAs, AA, PA, and BA, in the feces of PD mice, and the results are summarized in Table 2. The levels of total SCFAs ($t = 4.479$, $p = 0.0005$; $F=4.069$, $p = 0.0840$), AA ($t = 4.149$, $p = 0.0010$; $F=2.715$, $p = 0.2110$) and PA ($t = 2.578$, $p = 0.0328$ with Welch's correction; $F=14.22$, $p = 0.0024$) in particular, were significantly lower in the feces of PD mice (vs. Normal). L-Dopa treatment greatly suppressed fecal SCFAs levels ($t = 2.349$, $p = 0.0340$; $F=2.884$, $p = 0.1857$), especially fecal PA ($t = 4.236$, $p = 0.0008$; $F=1.905$, $p = 0.4146$) and BA ($t = 3.356$, $p = 0.0047$; $F=1.037$, $p = 0.9627$) levels in PD mice (vs. Model). Neither low nor high dose MA could greatly influence the total SCFA levels. However, low dose MA treatment downregulated fecal PA ($t = 2.160$, $p = 0.0486$; $F=1.135$, $p = 0.8718$) and BA levels ($t = 2.638$, $p = 0.0195$; $F=1.051$, $p = 0.9493$), whereas high dose MA treatment significantly upregulated fecal AA levels ($t = 2.741$, $p = 0.0159$; $F=1.700$,

**Table 2**
Fecal short chain fatty acids levels in mice from different groups[1,2].

| Grouping | Fecal SCFAs (nmol/g wet feces) | | | |
|---|---|---|---|---|
| | Total SCFAs | Acetic acid (AA) | Propionic acid (PA) | Butyric acid (BA) |
| **Normal** | 5.05 ± 0.52* **($p$=0.0005) | 2.28 ± 0.28 * *($p$=0.0010) | 2.06 ± 0.39 *($p$=0.0328) | 0.72 ± 0.11 |
| **Model** | **2.46 ± 0.26** | **0.90 ± 0.17** | **1.03 ± 0.10** | **0.53 ± 0.04** |
| **L-Dopa** | 1.76 ± 0.15 *($p$=0.0340) | 0.93 ± 0.07 | 0.49 ± 0.07 * **($p$=0.0008) | 0.34 ± 0.04 * *($p$=0.0047) |
| **LMA** | 2.16 ± 0.16 | 1.02 ± 0.06 | 0.70 ± 0.11 *($p$=0.0486) | 0.43 ± 0.05 *($p$=0.0195) |
| **HMA** | 3.25 ± 0.69 | 1.68 ± 0.22 *($p$=0.0159) | 0.99 ± 0.30 | 0.58 ± 0.17 |

1) Values are expressed as mean ± SEM, n = 8. Unpaired T-test with or without Welch's correction was performed to compare values between either two groups and the exact p value from T-test was shown in this table. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Model group.
2) SCFAs, short chain fatty acids; AA, acetic acid; PA, propionic acid; BA, butyric acid.

$p = 0.5004$) in PD mice (vs. Model).

### 3.6. Effects of low and high dose MA on neuroinflammation in PD mice

As indicated in Fig. 5A, PD model mice had seemingly obvious dopaminergic neuronal loss (assessed using TH staining) and relatively higher numbers of activated microglial cells (assessed using Iba-1 staining) in the SNpc as compared to those in normal mice. However, the levels of the pro-inflammatory cytokines of TNFα and IL-1β (Fig. 5B, **C**) were not significantly elevated in the SNpc of PD mice (vs. Normal). Only high dose MA treatment was found to greatly suppress TNFα (t = 2.849, $p = 0.0341$ with Welch's correction; F=43.05, p = 0.0008) and IL-1β (t = 2.441, $p = 0.0348$; F=2.609, $p = 0.3161$) (Fig. 5B, C) levels in the SNpc (vs. Model). As shown in Fig. 5A, it appeared that low dose MA treatment group had more dopaminergic neurons (vs. Model) and more activated microglia (vs. Normal); while the high dose MA treatment group had more dopaminergic neurons and less activated microglia in the SNpc (vs. Model).

### 3.7. Effects of MA or AA alone or in combination on inflammation in BV2 microglial cells

As our results showed that high dose MA treatment could inhibit neuroinflammation in the SNpc and simultaneously increase the fecal AA level in the colon of PD mice, to determine whether the inhibitory effects of MA on neuroinflammation were direct or SCFA-mediated, BV-2 cells were pre-treated with different concentrations of MA or AA separately or in combination for 1 h, followed by $MPP^+$ induction for a further 6 h, after which the mRNA levels of TNFα and IL-1β were measured. We found that $MPP^+$ could induce inflammation in BV-2 cells as demonstrated by a 2–3-fold increase in mRNA levels of TNFα (Fig. 6A: t = 9.864, $p = 0.0006$; F=3.889, $p = 0.4091$ and Fig. 6 C: t = 7.980, $p = 0.0013$; F=2.240, $p = 0.6173$ and Fig. 6E: t = 6.276, $p = 0.0033$; F=7.750, $p = 0.2286$) and IL-1β (Fig. 6B: t = 15.07, $p = 0.0001$; F=10.00, $p = 0.1818$ and Fig. 6D: t = 10.56, $p = 0.0005$; F=7.516, $p = 0.2349$ and Fig. 6 F: t = 5.254, $p = 0.0328$ with Welch's correction; F=89.40, $p = 0.0221$). Pre-treatment with MA failed to influence TNFα or IL-1β mRNA levels at concentrations ranging from $10^{-8}$ M to $10^{-5}$ M,

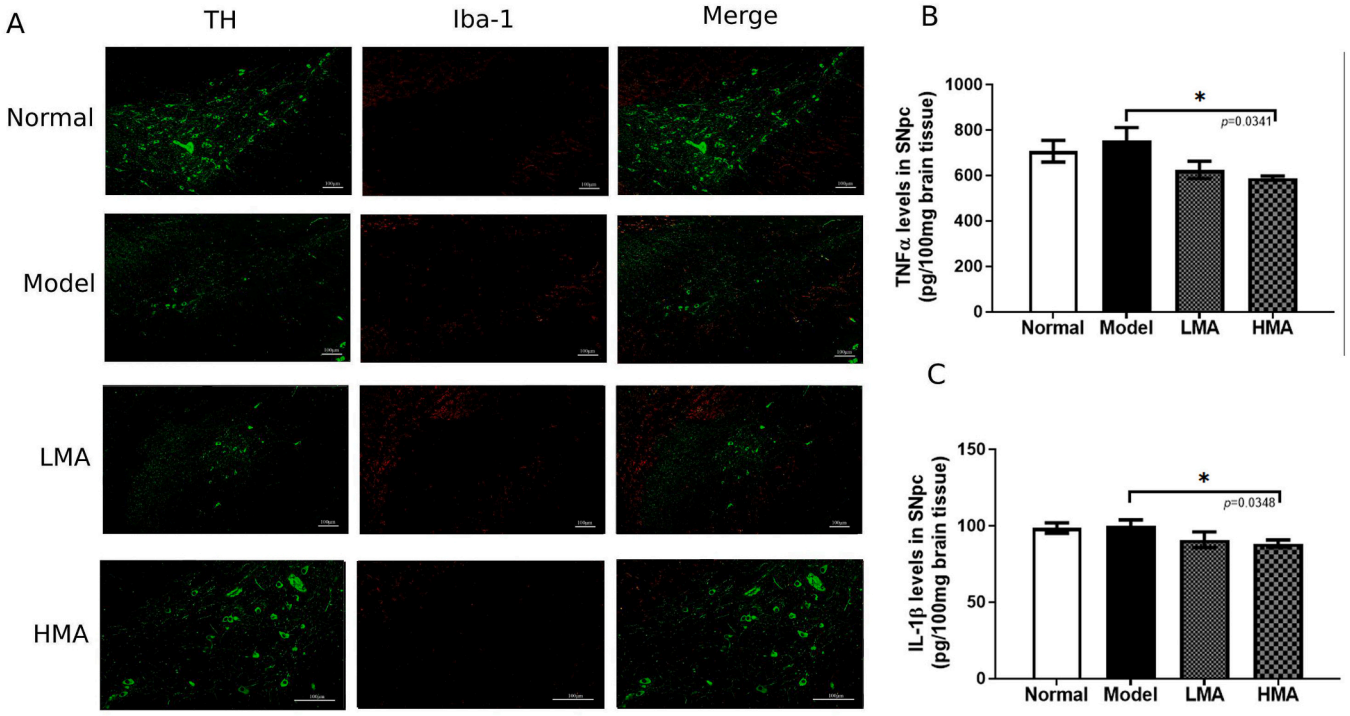



**Fig. 5.** Double IF staining of TH-IR neurons with microglia and TNFα and IL-1β levels in the SNpc of mice. (A) Double IF staining of TH-IR dopaminergic neurons (TH as marker; green) and microglia (Iba-1 as marker; red) (n = 2). One representative image from each group under 100 × magnification; scale bar represents 100 μm. (B) Protein levels of pro-inflammatory cytokines of TNFα and IL-1β were measured by ELISA kits and expressed as pg/100 mg brain tissues with n = 6. Data are expressed as mean ± SEM values. The unpaired t-test, with or without Welch's correction, was used to compare values between two groups. The exact t-test $p$ values are shown in the figures. *$p < 0.05$ vs. Model group. IF, immunofluorescence; TH-IR, tyrosine hydroxylase immunoreactive; TNFα, tumor necrosis factor alpha; IL-1β, interleukin 1β; SNpc, substantia nigra pars compacta.

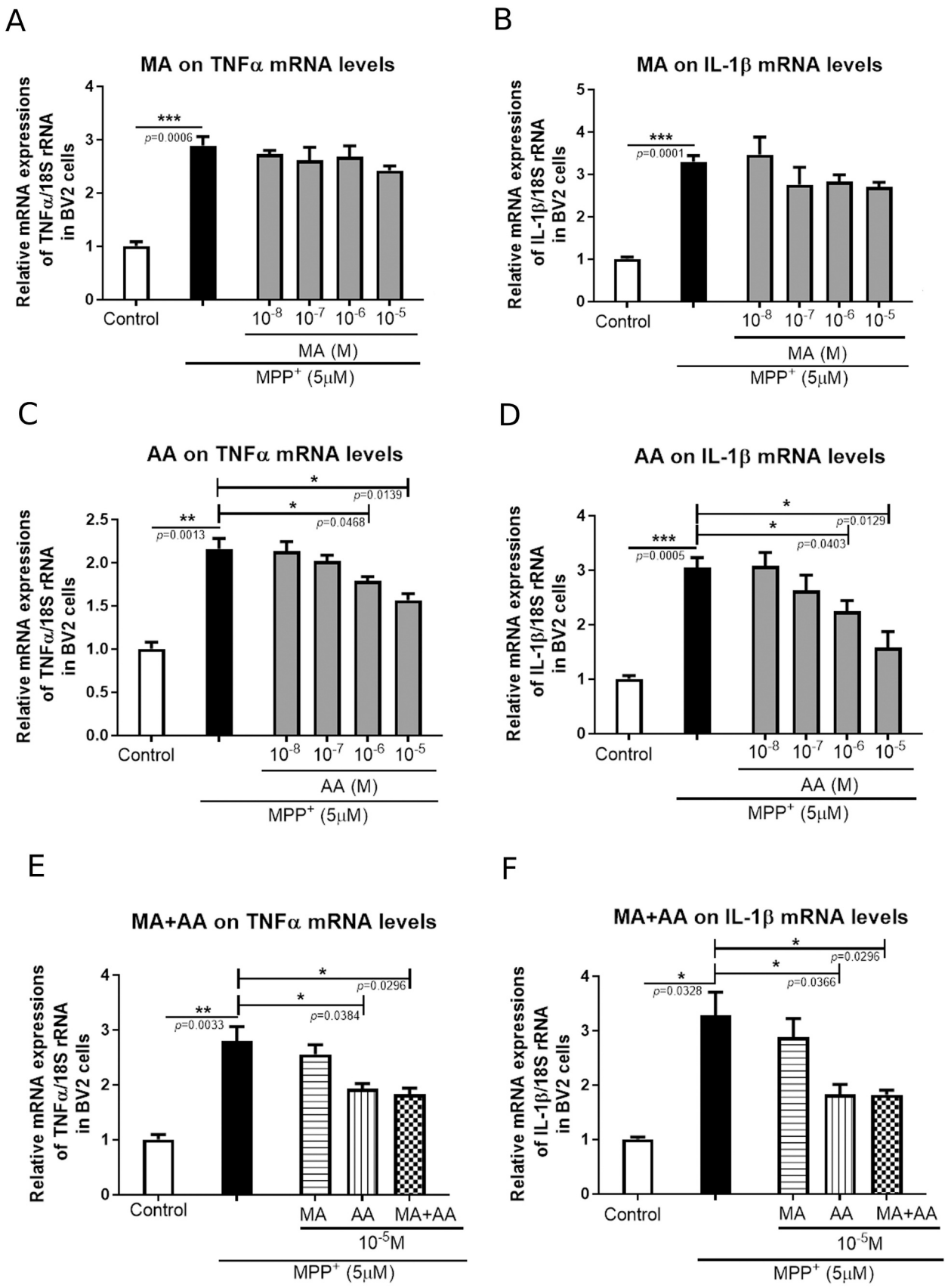



**Fig. 6.** TNFα and IL-1β mRNA levels in microglial BV-2 cells. mRNA expressions (ratio of target gene to 18 S rRNA gene and relative to group mean of Control group) of TNFα and IL-1β in response to different concentrations ($10^{-8}$ M to $10^{-5}$ M) of MA (A, B) or AA (C, D) alone or their combination (MA+AA, at the concentrations of $10^{-5}$ M) (E, F) in BV-2 cells. Data are expressed as mean ± SEM values, n = 6. The unpaired t-test, with or without Welch's correction, was used to compare values between two groups. The exact t-test *p* values are shown in the figures. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. negative control induced by $MPP^+$.

but pre-treatment with AA at a concentration of $10^{-6}$ M (TNFα: t = 2.841, $p = 0.0468$; F=5.618, $p = 0.3022$ and IL-1β: t = 2.990, $p = 0.0403$; F=1.196, $p = 0.9108$) or $10^{-5}$ M (TNFα: t = 4.178, $p = 0.0139$; F=2.589, $p = 0.5573$ and IL-1β: t = 4.279, $p = 0.0129$; F=2.538, $p = 0.5652$) significantly suppressed the elevated TNFα (Fig. 6C) and IL-1β (Fig. 6D) mRNA levels in $MPP^+$-induced BV-2 cells. When MA ($10^{-5}$ M), AA ($10^{-5}$ M), or their combination was pre-incubated with $MPP^+$-induced BV-2 cells, only AA (TNFα: t = 3.040, $p = 0.0384$; F=8.407, $p = 0.2126$ and IL-1β: t = 3.090, $p = 0.0366$; F=5.342, $p = 0.3153$) and MA+AA (TNFα: t = 3.311, $p = 0.0296$; F=5.5653, $p = 0.3006$ and IL-1β: t = 3.312, $p = 0.0296$; F=27.75, $p = 0.0696$) significantly downregulated the increased TNFα (Fig. 6E) and IL-1β (Fig. 6F) mRNA levels; moreover, the MA+AA combination was not observed to have any obvious synergistic actions.

## 4. Discussion

The major pathological features of PD are progressive loss of dopaminergic neurons in the midbrain, especially the SNpc, leading to markedly decreased levels of DA and its metabolites in the striatum [29]. A PD mouse model established by i.p. injection of the neurotoxin MPTP is a widely accepted classical model of PD [30], although there are many different protocols for MPTP injection. Chronic injection (twice a week for a total of 5 weeks), which has been tested by our research group to induce the major pathological features of PD in mice [27,28], is one of the most commonly used protocols. In this study, our results clearly demonstrated that both low and high dose MA treatments via oral gavage significantly prevented dopaminergic neuronal loss by ameliorating motor functions and improving TH expressions in the SNpc and increasing levels of DA and its metabolite of HVA in the striatum of classical chronic PD mice model. This is the first study to report that oral MA treatment could provide neuroprotection against PD in an animal model. MA has been reported to protect against oxygen-glucose deprivation-induced brain injury [6], promote synaptogenesis and axon growth in a cerebral ischemia model [7], and ameliorate schizophrenia-like behaviors in mice [8], but there was no report of its effects against PD until now. Thus, our findings add new evidence regarding the neuroprotective effects of MA.

The effects of MA in this PD mouse model were not dose-responsive, as similar beneficial effects of both low and high dose MA treatments were observed. Subsequent analyses revealed that low and high dose MA treatments might confer protection against PD via distinct mechanisms. Our results showed that administration of MA at both low and high doses could increase striatal levels of DA and HVA, but that only low dose MA treatment enhanced striatal levels of GABA and 5-HT and its metabolite 5-HIAA in the PD mice. Low dose MA treatment also induced significant changes in GM composition, but high dose MA treatment failed to influence the GM of PD mice. Moreover, low dose MA treatment downregulated fecal PA levels, and conversely, high dose MA treatment upregulated fecal AA levels in PD mice. Finally, only high dose MA treatment greatly inhibited neuroinflammation in PD mice. These contrasting effects of different doses of MA indicate that dose is an important factor determining the effects of MA in PD model mice.

As mentioned in our previous study [19], the neurotransmitters DA, 5-HT and GABA interact in the regulation of motor neurons and are involved in PD pathogenesis. 5-HT and its metabolite 5-HIAA has also been reported to decline in patients with PD [31]. Besides DA, declining 5-HT and GABA levels in the striatum accompanied by motor deficits and degeneration of the SNpc, have been reported in many PD animal models [32,33]. Hence, upregulation of these neurotransmitters in the midbrain is expected to benefit dopaminergic neurons and motor functions in PD models. Our present results also showed that striatal levels of DA, 5-HT and GABA declined in PD mice and that oral administration of low dose MA improved not only striatal DA levels but also striatal 5-HT, 5-HIAA, and GABA levels, likely contributing to its beneficial actions against PD.

GM is now being considered as one of the pathogenic factors in PD, and oral agents targeting GM might be potential therapeutic options, as indicated in our previously published paper [19,28]. Pharmacokinetic studies of MA in rodents have demonstrated that MA has a low bioavailability of 5.13 % [9], but can distribute to most organs in mice, including the brain, after oral intake [10]. Another study found that orally ingested MA was detected in all intestinal segments, with higher concentrations in the distal part of the intestine, and that some MA in the intestine only undergoes phase I reactions, resulting in mostly monohydroxylated metabolites [34]. Based on this, the contrasting effects of low and high dose MA on striatal neurotransmitters in PD mice is hypothesized to be associated with their differential interactions with GM. Our results showed that significant changes in GM composition occurred in PD mice treated with low dose MA, while high dose MA did not show any effect on GM. Although both low and high dose MA treatments led to considerable changes in alpha diversity, low dose MA administration aggravated major GM changes in PD mice by further decreasing phyla *Bacteroidetes*. Phyla *Firmicutes* and *Bacteroidetes* are the two most common GM components, and account for over 90 % of the GM in mammals [35]. It has been proposed that the *Firmicutes/Bacteroidetes* (F/B) ratio can be a biomarker for metabolic diseases such as diabetes, obesity, and inflammatory bowel disease, because higher F/B ratios have been found in individuals with metabolic diseases than healthy controls in some human studies [36]. However, inconsistent F/B ratios in PD patients have been reported in different clinical trials [37,38], making it an inappropriate biomarker for PD. However, our study found that the abundance of *Bacilli* (class) under *Firmicutes* (phylum), and its subordinate *Lactobacillales* (order), *Lactobacillaceae* (family), *Lactobacillus* (genus) ($\log_{10}$ LDA scores > 3.0 in pairwise comparison and relative abundance > 1 %) increased greatly in response to low dose MA treatment. Many studies [39,40], including our previous work [28], have reported the neuroprotection provided by *Lactobacillus* against PD in animal models. *Lactobacillus* is also the major strain that produces 5-HT and GABA in the intestine [41], which are directly transported from the enteric nervous system (ENS) to the central nervous system (CNS) via neural processes [28]. This could explain why low dose, but not high dose MA treatment led to increased striatal levels of 5-HT and GABA in PD mice.

As we have reported previously [19,27,28], fecal SCFAs (mainly AA, PA, and BA) as the main GM metabolites and neuromodulators have been proven to provide neuroprotection but their levels decline considerably in patients with PD and in MPTP induced mouse models. The results of the present study also showed a considerable decline in SCFA levels, especially those of AA and PA, in the feces of PD mice. AA and PA are mainly produced by *Bacteroidetes* [42], and the significant decrease in *Bacteroidetes* abundance is expected to have lower fecal AA and PA levels in PD mice (vs. Normal group) as stated in our previous studies [27,28]. Hereby, the further decrease in fecal PA levels in low dose MA-treated PD mice could be attributed, at least in part, to the further decline of *Bacteroidetes* abundance in response to low dose MA (vs. Model group). In contrast, high dose MA treatment did not influence the GM much, but it significantly increased fecal AA levels in PD mice. This result indicated that the production of fecal SCFAs is not solely dependent on GM composition, and involves many other factors, such as dietary component, especially fibres, gut transit time and others, as described in our previous work [19].

It has been hypothesized that SCFAs (including AA, PA, and BA) produced in the colon inhibit neuroinflammation after crossing the blood brain barrier (BBB) to enter the brain [19,43]. A recent study discovered that, among the various SCFAs, only microbially-derived AA showed significant inhibitory effects on microglial maturation and metabolic state in a mouse model of Alzheimer's disease [44]. Our present results showed that high dose MA treatment could significantly inhibit neuroinflammation by downregulating the levels of pro-inflammatory cytokines of TNFα and IL-1β in the SNpc of PD mice. This is speculated to be associated with higher fecal AA production in

the high dose MA treatment group. In order to decipher whether the effects of MA on neuroinflammation are direct or AA-mediated, we designed an in vitro experiment, wherein MA and AA alone or in combination were administered to $MPP^+$-induced microglial BV-2 cells for an indicated time. The results demonstrated that only AA showed significant inhibitory effects against $MPP^+$-induced elevation of TNFα and IL-1β expression, and that MA did not have any significant impact. As previously reported [9,10], MA can cross the BBB, but its bioavailability is low, and the present results strongly suggest that the suppressive effects of high dose MA treatment on neuroinflammation in vivo might not be direct, but mainly mediated by microbially-derived AA.

In summary, our results demonstrated that both low and high dose MA treatments via oral gavage significantly prevented dopaminergic neuronal loss by ameliorating motor functions and improving TH expressions in the SNpc and increasing striatal levels of DA and its metabolite HVA in a classical chronic PD mouse model. However, the effects of MA in this PD mouse model were not dose-responsive, as similar beneficial effects of the low and high dose MA treatments were observed. Further mechanism-based studies indicated that low dose MA administration to PD mice favored probiotic bacterial growth (from *Bacilli* class to *Lactobacillus* genus), which helped to increase the levels of 5-HT, 5-HIAA and GABA in the striatum, and contributed to its neuroprotective effects. High dose MA administration to PD mice did not influence GM composition, but did significantly inhibit neuroinflammation in the SNpc, and these effects were mainly mediated by microbial AA production in the colon. Overall, our results indicate that the neuroprotective effects observed can be attributed to increase in striatal neurotransmitters levels by low dose MA and inhibition of neuroinflammation by high dose MA treatment, and that these effects were all mediated by GM in PD model mice. Thus, oral MA at different doses protects against PD via distinct GM-related mechanisms. Our novel findings provide new evidence for the neuroprotective effects of MA and reveals the importance of dose and GM in determining the effects of MA as an oral agent against PD. Therefore, our study provides valuable scientific evidence to medical professionals in the context of the development of MA into an ideal oral agent for preventing or treating PD.

Nevertheless, the lack of further mechanism-related investigations on the interactions of MA and GM in this PD model is a limitation of this study. Future studies will be designed to further delineate the signaling pathways involved in the interactive actions between different doss of MA and GM.

## CRediT authorship contribution statement

Conceptualization: Xiao-Li Dong and Ka-Hing Wong; Data curation: Xu Cao; Funding acquisition: Xu Cao, Xiao-Li Dong, Ka-Hing Wong, Ping-Yi Xu; Investigation: Xu Cao, Zhong-Rui Du, Xin Liu, Xiong Wang, Chong Li, Sai-Nan Zhou, Jia-Rui Liu; Methodology: Xu Cao, Zhong-Rui Du, Xin Liu, Xiong Wang, Chong Li, Sai-Nan Zhou, Jia-Rui Liu, Fang Zhao; Project administration: Xiao-Li Dong; Resources: Ka-Hing Wong; Supervision: Xiao-Li Dong and Ka-Hing Wong; Writing-original draft: Xiao-Li Dong; Writing-review and editing: Ping-Yi Xu, Jun-Li Ye, Qing Zhao. All authors have read and agreed to the published version of the manuscript.

## Declaration of Competing Interest

None.

## Data Availability

Data will be made available on request.

## Acknowledgements

This research was funded by the Natural Science Foundation of Shenzhen University General Hospital, China (grant number: SUGH2018QD005); the Health and Medical Research Fund (HMRF) grant of Hong Kong, Hong Kong [grant number: 06173176]; Shenzhen Fundamental Research Program, China [grant number: JCYJ20200109142019319]; Basic and Applied Basic Research Foundation of Guangdong Province, China [grant number: 2022B1515230004].

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115100.

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