

Neuroprotective Effects of Cerebroprotein Hydrolysate on MPTP-induced Parkinson's Mice by Increasing Neurotrophin and Regulating Intestinal Microbiota

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Abstract It is hypothesized that the neurotrophin loss and the intestinal tract could be a contributing factor to the neurodegenerative processes. Cerebroprotein hydrolysate-I (CH-I), a mixture of amino acids and low molecular peptides had been reported to show remarkable therapeutic effect on many neurological diseases. However, the reports about the effects of cerebroprotein hydrolysate on PD was little. In this study, we applied CH-I on MPTP-induced mice to detect the neuroprotective effects. The results showed that CH-I could enhance the expression of neurotrophin (NGF and BDNF) and improve the behavioral deficits in MPTP-induced Parkinson's mice. Furthermore, CH-I restored the level of tyrosine hydroxylase (TH), and inhibited the apoptosis induced by MPTP. In addition, CH-I made a contribution to reducing the abundance of pathogenic microbiota and increasing the relative abundance of beneficial bacteria. The data demonstrate that CH-I shows promise as a novel treatment of PD.

Keywords: Cerebroprotein hydrolysate-I, Parkinson's disease, intestinal microbiota, neurotrophins, mice

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1. Introduction

Parkinson's disease (PD), characterized by the accumulation of intracellular aggregates of misfolded alpha-synuclein called Lewy bodies, is regarded as the second most common neurodegenerative disorder next to Alzheimer's disease [1], leading to clinical symptoms of progressive dyskinesia, including, tremor, resting, rigidity and bradykinesia [2,3,4]. In addition, PD patients also show many of non-motor symptoms, mainly including anosmia, cognitive impairment, sleep disorder and gastrointestinal tract dysfunction [5], which may occur decades earlier than motor dysfunction, among which intestinal pathology such as increased intestinal permeability and flora dysbiosis is the most common non-motor symptom in the early stage of PD [6,7]. Now, some clinical and neuropathological evidence indicates PD originated in the gut: in the early stages of PD, abnormal aggregation of α -synuclein fibrils coupled with neurodegeneration in the enteric nervous system (ENS),

can be transmitted through the vagus nerve and ascends to the midbrain, supporting the "gut-to-brain" hypothesis [8,9].

The intestine that supports a dense, diverse and dynamic bacterial ecosystem is the body's largest digestive organ [10]. The intestinal microbiota depend on the human gut and supports the gut by digesting food, thus providing nutrients to the host, while maintaining the integrity of the natural barrier by promoting the maturation of the human immune system [11]. These floras normally maintain a dynamic balance with their hosts and play a vital role in the body, which the researchers call "forgetting organs" [12]. It is estimated that the resident flora of the human gut can support 1,000 distinct bacterial species, with the total number of bacteria approaching 1,000 cells and its total genome is about 3 million, 150 times the size of the human genome [11,13]. Studies have shown that intestinal flora can influence intestinal function, which in turn participates in the regulation of brain-intestinal axis function, resulting in effects on neurological disorders [14,15]. Interestingly, the researchers found that changes in the composition of intestinal microbiota and its metabolites may be in

connection with the pathogenesis and clinical phenotype of PD [16]. Hence, the regulation of intestinal microbiota may become a new strategy for PD treatment.

Holzer and colleagues reported that some neuropeptides such as calcitonin gene-related peptide, substance P and neuropeptide Y, played a role in bidirectional intestinal brain communication, affecting the activities of gastrointestinal microflora and its interaction with the intestinal brain axis [17,18]. Cerebroprotein hydrolysate, a mixture of amino acids and low molecular peptides obtained from animal brain [19], was reported to penetrate blood-brain barrier, promote protein synthesis, affect the respiratory chain, and provide precursors for neurotransmitters, peptide hormones and coenzymes [20,21,22]. Neveen et al found that cerebrolysin, a purified porcine brain-derived peptide preparation could restore the midbrain and striatum dopamine levels and improve the behavioral deficits in 6-OHDA rat model [23]. However, the neuroprotective mechanisms are unclear. Cerebroprotein hydrolysate-I (CH-I) is a polypeptide mixture extracted from pig brain protein by lysozyme without adding other components. Based on the theory of Holzer that peptides can regulate intestinal flora, we will apply CH-I on MPTP-induced mice to further explore the neuroprotective effect of CH-I.

Materials and Methods

Materials

Rabbit antibodies to TH (#ab112), NGF (#ab52918), BDNF (#ab108319), and GAPDH (#ab8245) were obtained from Abcam, Cambridge, USA. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahy

-dropyridine) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cerebroprotein Hydrolysate-I (CH-I) was provided by Hebei Zhitong Biopharma Pharmaceutical CO., LTD.

Experimental animals

Male C57BL/6 mice of 8-10 weeks were obtained from Model Animal Research Center of Nanjing University, were kept in a constant environment ($22 \pm 2^\circ\text{C}$, 60%-70% humidity) under a 12 h light / dark cycle with free access to food and filtered water.

No mice were excluded from statistical analysis. All animal procedures were approved by the Animal Ethics Committee of the Affiliated Hospital of Qingdao University (Permit No. QYFY WZLL26000) and followed the Principles of Laboratory Animal Care. The Laboratory animal permit was SYXK (Lu) 20150003.

Experimental design

All 48 mice were randomly assigned into four groups of 12 mice: (1) Control; (2) MPTP only; (3) MPTP + CH-I (10 mg/kg); (4) MPTP + CH-I (20 mg/kg); The mice in group (1) received saline hydrochloride intraperitoneally for 7 days, and the mice in groups (2-4) received the same volume of MPTP at a dose of 30 mg/kg·day. Thereafter, the groups (3-4) were treated intraperitoneally with CH-I

for another 14 consecutive days, while the mice in group (1-2) received the equivalent volume of saline.

Behavioral Evaluation

After CH-I treatment, the animals were subjected to behavioral evaluation, including pole test and traction test.

Pole Test

The pole test is commonly used to evaluate bradykinesia in mice PD [24]. The mice were placed head at the top of a rough pole (10 mm diameter, 500 mm length). Once the mice turned and climbed down the pole, the time that the mouse turn downward (T-turn) and the time that the hind paw touch down the ground (T-total) were measured. Three times in a single session was recorded.

Traction Test

Traction test is used to measure muscle strength and equilibrium [25]. Briefly, the mice forepaws were hung on a horizontal wire and its hind limb placements were scored. If no hind limb grasped the wire, the score was 1, if one hind limb grasped the wire, the score was 2, if both hind limbs grasped the wire, the score was 3.

Western blot analysis

Fresh sections of the midbrain striatum from five mice were collected (-80°C) for western blot measurements. The tissues were mixed with ice-cold lysis buffers and homogenized, then centrifuged at 12,000 rpm at 4°C for 5 min. After the protein concentration was measured, equal concentrations of protein were added with loading buffer, boiled, and centrifuged. The denatured proteins were separated using 10%-15% SDS-PAGE gels, then transferred to a PVDF membrane. The membranes were incubated with 5% skimmed milk or bovine serum albumin to block nonspecific reaction for 1 h and then probed with rabbit anti-TH (1:2,000), rabbit anti-NGF (1:1,000), rabbit anti-BDNF (1:2,000), and rabbit anti-GAPDH (1:5,000) in 4°C overnight. After rinsed with TBST three times for 10 min, the sample was incubated with horseradish peroxidase-conjugated IgG secondary antibody (1:2,000) for 2 h. Finally, the binding antibodies were developed with the chemiluminescence detection system.

Immunohistochemistry

After deeply anesthetized, the mice were transcardially perfused with saline (20 mL), 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (20mL) in sequence. Then the brains were immediately extracted and subsequently post-fixed in 4% PFA for 24 h. Brains were embedded in paraffin and coronal sections were conducted for immunohistochemical study. Then, the slices were subjected to a heat-mediated antigen retrieval using citrate buffer. After washed three times with 0.1 M PBST, the slices were pre-treated with 3% hydrogen peroxide for 10 min at room temperature. Then slides were incubated with

5% bovine serum albumin for 1 h at room temperature and incubated with primary antibodies (rabbit anti-mice monoclonal TH, 1:200) in 4 °C for overnight. After rinsing, the sections were incubated with secondary antibody for 1 h at 37 °C. Finally, the sections were visualized with 3,3'-diaminobenzidine for 5 min. The images were viewed on an Olympus AX-70 photomicroscope (Tokyo, Japan).

Nissl Staining

Nissl staining is commonly used to assess the degree of neuronal cell death for histopathological analysis [26]. In this study, we referred to the previous study to detect the effects of CH-I on the neurons in striatum [27]. After deparaffinised and rehydrated, 3 µm-thick slices were stained in 1% toluidine blue in a 60 °C incubator for 40 min. Then, the slides were washed 3 times with distilled water for 10 min and dehydrated in a graded ethanol series (70%, 95%, and 100%, for 1 min each). Finally, the slides were sealed with neutral resin and a light microscope was applied to count the numbers of necrotic cells.

Fecal bacterial genomic DNA extraction

Referred to the manufacturer's instructions, we used a MN NucleoSpin 96 kit to extract the total bacterial genomic DNA. Briefly, 300 mg fecal samples were lysed and homogenized in 1mL Inhibit EX buffer. After heated at 70 °C for 5 min and centrifuged, the supernatant was collected and mixed with 15 µL of proteinase K and 200 µL of Buffer AL for 15 sec. Then the supernatants were heated at 70 °C for 10 min, followed washed separately with 500 µL AW1 and AW2. At last, the DNA was eluted with 200 µL buffer ATE. And the DNA concentrations and purity were determined.

16S rDNA Sequencing

Referred to the protocol described in Caporaso et al [28], 16S rDNA sequencing was performed. Universal primers, 338F: 5'-ACTC CTA CGG GAG GCA GCA-3' and 806R: 5'-GGA CTA CHV GGG TWT CTA AT-3', were used. The PCR total reaction volume was: 10 × PCR ExTaq Buffer 2.0 µL, H₂O 13.25 µL, prime 1 (10 mM) 1.0 µL, ExTaq (5 U/mL) 0.25 µL, prime2 (10 mM) 1.0 µL, dNTP 2.0 µL, DNA template (100 ng/mL) 0.5 µL. The PCR cycle system was: 95 °C for 5 min, 30 cycles of incubations for 30 sec at 95 °C, 20 sec at 58 °C, and 6 sec at 72 °C, then 72 °C for 7 min. After the amplified products were purified and recovered, the library construction and sequencing steps were performed by Beijing Biomarker Technologies Co. Ltd.

Bioinformatics analysis

The bioinformatic analysis in this study was completed at the Biomarker biocloud platform (www.biocloud.org). In this study, paired-end reads were merged by FLASH (v1.2.7, <http://ccb.jhu.edu/software/FLASH/>) To obtain the raw tags. Then we filtered and clustered the raw tags. The merged tags were discarded by FASTX-Toolkit if the merged tags are with more than six

mismatches compared to the primers. Trimmomatic was applied to ensure the truncation of tags with an average quality score <20 in a 50 bp sliding window and the removal of tags shorter than 350 bp. We employed UCHIME to identify possible chimeras and clustered the denoised sequences using USEARCH. We defined tags with similarity ≥97% as a OUT. We searched against the Silva databases using the uclust within QIIME to assign taxonomy to all OTUs.

Statistical analysis

SPSS 17.0 software was applied in data analysis. One-way ANOVA was used in the multi-group comparison. The measurement data are shown as standard error of measurement (mean ± SEM). The values ($P \leq 0.05$, $P \leq 0.01$) were regarded to be statistically significant.

Results and Discussion

Characterization of CH-I

The content of peptides with molecular weight less than 10 kDa accounted for 25%-35% of the hydrolysate of brain protein, and the total nitrogen content was more than 120 mg/g. Total amino acids was 91% of total nitrogen. The free amino acid profiles of CH-I were shown in Table 1. As can be seen, CH-I was rich in Lys and Leu.

Table 1. Free Amino Acid Composition (mg/ml) of CH-I

AA	Pro	Val	Met	Lys	Ile	Leu	Phe	Try
content	4.20	3.85	1.04	10.92	3.93	10.55	3.90	0.87

Effects of CH-I on the behavioral deficits in MPTP-induced Parkinson's mice

PD is a common neurodegenerative movement disorder. The study on PD in recent years has promoted the development of many PD models, and the behavioral tests of animals similar to PD symptoms have been used to preliminarily evaluate the degree of illness or the therapeutic effect of drugs [29]. In this study, we applied traction test and pole test to evaluate the effects of CH-I on the behavioral deficits in PD mice. As shown in pole test in Figure 1A, MPTP markedly encumbered the behavior of the mice, prolonging the time of turn and crawling, however CH-I markedly alleviated this behavioral disorder, especially CH-I at the level of 20 mg/kg significantly improved the athletic ability of PD mice. Figure 1B showed that MPTP group scored half as well as the control group, implying the physical coordination was impaired, however, CH-I significantly improved this impairment through enhancing the muscular strength in mice. Taken together, the results indicate that CH-I at both 10 mg/kg and 20 mg/kg potentiated sensorimotor function in PD mice. And there was no significant difference between the two concentration

groups. This data points to the applicability of CH-I to prevent behavioral deficits in PD mice.

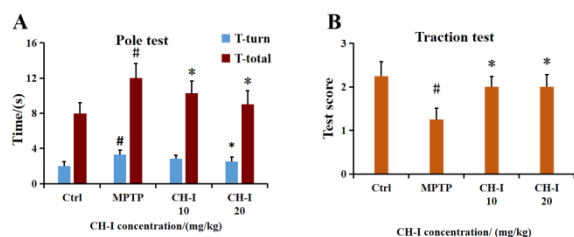
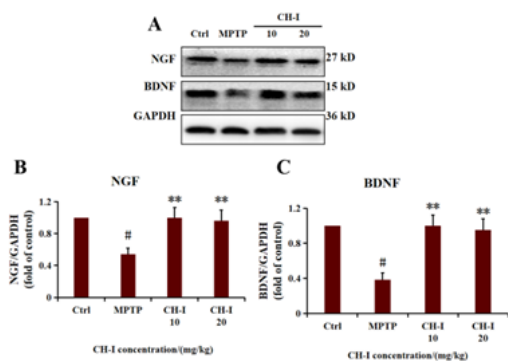


Figure 1. Effects of CH-I on behavioral deficits in PD mice. (A) Pole test. (B) Traction test. [#] $P < 0.05$ vs control group; ^{*} $P < 0.05$ vs MPTP group.

CH-I enhanced the expression of neurotrophin

Nerve growth factor (NGF) is a survival-promoting factor in regulating the growth and development of peripheral and central neurons [30]. In Figure 2A-B, MPTP decreased the level of NGF to 0.53-fold of the control group, however CH-I significantly improved NGF at both concentration of 10 mg/kg and 20 mg/kg.

Brain derived neurotrophic factor (BDNF), a protein widely distributed and synthesized in the central nervous system, makes a significant contribution to the survival, differentiation, growth and development of neurons [31]. In Figure 2C, the injection of MPTP attenuated the protein of BDNF, however CH-I increased BDNF expression, especially CH-I at the concentrations of 10 mg/kg improved BDNF to the level observed in control group. These results suggested that CH-I could enhance the expression of NGF and BDNF to provide nutritional factors for neurons.



[#] $P < 0.05$ vs the control group; ^{*} $P < 0.05$ or ^{**} $P < 0.01$ vs MPTP group.

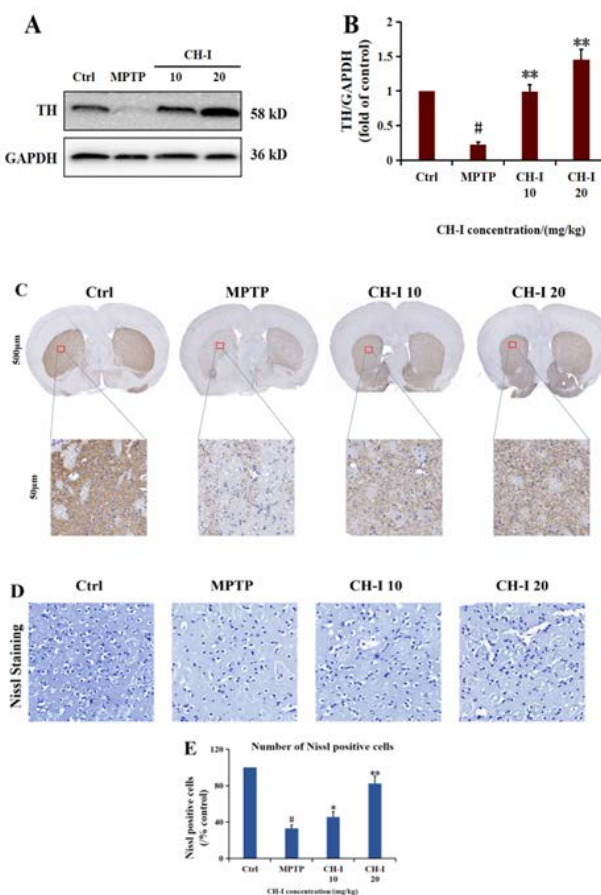
Figure 2. The levels of the expressions of NGF and BDNF. (A) The western blotting bands of NGF and BDNF. (B) Quantification of NGF. (C) Quantification of BDNF.

CH-I enhanced the expression of TH in PD mice

Tyrosine hydroxylase (TH), as a marker for the integrity of dopaminergic neurons, is the rate-limiting enzyme in the synthesis of dopamine [32,33]. We applied western blot and immunostaining to analyse TH. In Figure 3A-B, compared with the control group, MPTP

significantly inhibited the expression of TH, however, CH-I restrained this trend, and especially CH-I at 20 mg/kg markedly improved the protein level of TH. In Figure 3C, we showed the representative microphotographs of TH immunostaining in the striatum. The striatal TH-immunoreactive fibers were easily detectable in the striatum of control mice, but a marked decrease of TH-immunopositive fibers was observed after MPTP treatment. In contrast, administration of CH-I increased the expression of TH, which was in accordance with the results observed in Western blot bands, implying that CH-I could improve the expression of TH in PD mice.

Neurons are the basic structural and functional unit of the nervous system. Nissl body distributed in the cell body and dendrites of neurons, is regarded as the characteristic structures, which main function is to synthesize proteins as needed for neural activity, such as the renewal of certain components in cells and the production of neurotransmitter related proteins and enzymes [27, 34]. As shown in Figure 3D, Nissl-stained striatum neuron stereological quantification confirmed a significant neuronal loss in MPTP administration mice in comparison to the control mice. However, mice treated with 10 mg/kg and 20 mg/kg CH-I had higher cell counts, prevented MPTP-induced neuronal loss in the striatum areas, implying that CH-I could inhibit the neurons loss induced by MPTP (Figure 3.E).



[#] $P < 0.05$ vs the control group; ^{*} $P < 0.05$ or ^{**} $P < 0.01$ vs MPTP group.

Figure 3. The levels of the expression of TH in PD mice. (A) The western blotting bands of TH in striatum. (B) Quantification of TH in Str. (C) TH immunostaining in striatum. Scale bar = 50 / 500 μ m. (D) Nissl Staining. Scale bar = 50 μ m.

The effects of CH-I on gut microbiota dysbiosis at different taxonomic levels

In the intestine, the microbial ecosystem is closely in connection with many aspects of human physiology, including vitamin production, dietary fiber breakdown, modulation of neurological function and regulation of metabolism [1]. It is reported that gastrointestinal dysfunction is a significant contributor to the pathogenesis of PD, especially the transit time of constipation and reduced colonic, and the gut microbiota alterations, which is considered a possible presymptomatic stage [35].

In order to detect the effects of CH-I on PD mice, the bacterial diversity presented in fecal microbiota was characterized. Double-terminal sequencing (Paired-End) method based on Illumina HiSeq sequencing platform was used in this study to construct small fragment library for sequencing of microbial diversity. The species composition of the samples can be revealed by Reads splicing filtration, OTUs clustering and abundance analysis. And Alpha Diversity, Beta Diversity and significant species difference analysis can excavate the differences between samples [36].

In this study, multiple sequencing of the V3-V4 hypervariable region covering the 16S rDNA gene was applied to detect the diversity of gut microbiota in PD mice. 35,566,526 sequences were obtained from 15 samples and 3,394,779 Clean tags with an average of 226,319 Clean tags per sample were gained through double Reads chipping and filtering. We applied Taxonomic assignment using the Green genes 99% OTU database. OTU is the classification operating unit, which is the same mark artificially set for a certain taxon in phylogenetic research or population genetics research for the convenience of analysis. In general, we defined the sequences as an OTU if the similarity is more than 97%, and each OTU corresponds to a representative sequence. Based on the Taxonomy database of Silva (bacteria) and UNITE (fungus), we use Usearch software to cluster Tags at 97% similarity level, obtain OTU, and conduct taxonomy annotation on OTU. Table 2 showed the sample coverage of bacteria among the different groups based on 97% consistency threshold.

Table 2. Sequencing Data Quality Control and Phylotype Coverage of Each Sample

Sample ID	PE Reads	Raw Tags	Clean Tags	Effective Tags	GC(%)	Q20(%)	Q30(%)	Effective(%)
Ctrl	159361	152382	151162	148637	54.71	96.82	90.88	93.23
MPTP	155587	148559	147331	144666	54.65	96.79	90.81	92.96
CH-I	153872	147045	145797	143111	54.63	96.83	90.88	92.98

Date are expressed based on 16S rDNA V3-V4 region data. (n = 5)

Rarefaction Curve, which verifies whether the amount of sequencing data is sufficient to reflect species diversity and indirectly reflect species richness in the sample, displayed the diversity of the bacterial communities in Figure 4A. The common and unique OTUs were shown in Venn diagram in different samples (Figure 4B). 469 OTUs were identified in all samples, the percentages of OTUs

unique to the Control, MPTP, and CH-I groups were 0.6%, 0.2% and 0%. We identified 421 OTUs shared by three groups, accounting for 89.76% of the total OTUs, which suggested the similarity in the bacteria structure among the treatment groups.

Non-Metric Multi-Dimensional Scaling (NMDS) is a sort method suitable for ecological research, whose main purpose is to simplify the research object of multidimensional space to the low-dimensional space for localization, analysis and classification under the condition of preserving the data analysis method of primitive relations among objects [37, 38]. In Figure 4C, the relatively tight clustering suggested the differences in the composition of the bacteria were observed between the three groups.

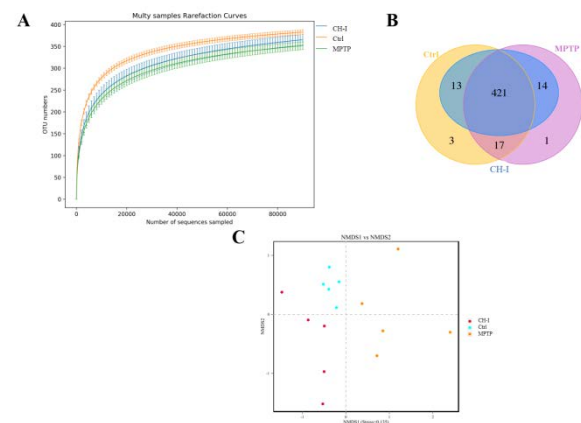
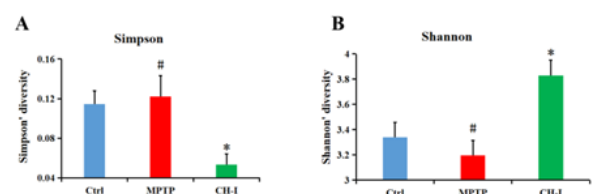


Figure 4. Diversities of bacterial community and the structures of the mice intestinal microbiota in different treatment groups. (A) Rarefaction curves. (B) Venn diagrams. (C) T NMDS analysis.

Effects of CH-I on Alpha-diversity index of gut microbiota

Alpha diversity reflects individual sample species diversity (diversity) and species abundance (richness), including: Chao1, Ace, Shannon, Simpson. Shannon and Simpson, which are affected by species abundance and species evenness in the sample community, are important to measure species diversity, [39]. The species diversity is positively correlated with the evenness of species in the community, based on the same species abundance [40]. As seen in Figure 5A and Figure 5B, comparing to control group, MPTP reduced the Shannon diversity significantly ($P < 0.01$), while the Simpson diversity was increased in CH-I group. The data indicated that MPTP-treatment reduced the diversity of gut microbiota, while CH-I treatment could improve the diversity.



$P < 0.05$ vs the control group; * $P < 0.05$ or ** $P < 0.01$ vs MPTP group

Figure 5. Alpha-diversity comparison of the microbiota of the cecum based on (A) Simpson's diversity and (B) Shannon's diversity and index.

Effects of CH-I on gut microbiota dysbiosis at different taxonomic levels

Figure 6A/C showed the faecal microbiota community structure composition at within-phylum/family. Figure 6B/D presented the pie chart based on the differential bacterial communities. The bacterial phylum with the top ten relative abundances: *Cyanobacteria*, *Tenericutes*, *Deferribactera*, *Patescibacteria*, *Actinobacteria*, *Verrucomicro*, *Proteobacteria*, *Epsilonbacteria*, *Firmicutes*, *Bacteroida*. The greatest relative abundance of the bacterial at phylum-level was observed at *Bacteroida*. As shown in Figure 6B, MPTP significantly changed the relative abundance of *Proteobacteria*, *Epsilonbacteria*, *Verrucomicro*, *Cyanobacteria*, *Deferribactera*. However, CH-I inhibited the disorder of these bacterial.

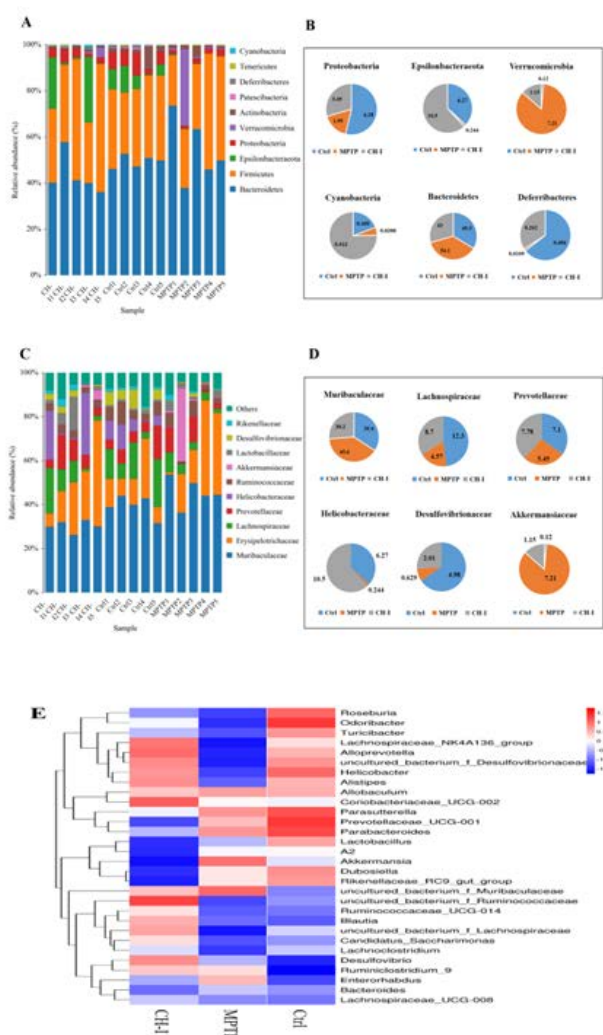


Figure 6. The relative abundance of all identified bacterial phylum (A-B), families (C-D) and Bata-diversity (E) among the control, MPTP and CH-I.

We selected the bacteria ranked at the top 40 with relative abundance at genus-level and generate heat maps through clustering bacterial taxa and samples (Figure 6E). Compared to the control group, a significant increase in the relative abundance of *Akkermansia* and *Muribaculaceae* and a significant reduce in the abundance of *Lachnospiraceae*, *Roseburia*, *Odoribacter*, *Turicibacter*, *Desulfovibrionaceae* and *Helicobacter*

group were found in the MPTP group. However, these changes were significantly regained after CH-I treatment. The data suggested that CH-I could enhance the protection of the intestinal health and reduce the intestinal infection by pathogens through regulating the composition of the intestinal bacteria.

Conclusion

In summary, our study demonstrated that the CH-I showed a promising effects on PD model. The study displayed that CH-I could improve the behavioral deficits and enhanced the expression of neurotrophic factors (NGF and BDNF) in MPTP-induced Parkinson's mouse. More importantly, CH-I restored the level of TH, the rate-limiting enzyme in the synthesis of DA, and inhibited the apoptosis induced by MPTP. In addition, CH-I makes a contribution to the regulation of intestinal microbiota by reducing the abundance of pathogenic microbiota and increasing the relative abundance of beneficial bacteria.

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Conflict of interest

The authors declare no any competing financial interest.

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