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Bifidobacterium animalis subspecies lactic derived-vesicles alleviate cognitive impairment in mice *via* modulating gut microbiota metabolism and 5-HT/BDNF pathway

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ABSTRACT: Hippocampal neuroinflammation (**HNF**) causes gut microbial abnormalities to aggravate cognitive impairment. While *Bifidobacterium* has been shown to alleviate HNF and improve cognitive function, the role of *Bifidobacterium*-derived membrane vesicles (**MV**) in reshaping gut microbiota and their connection to cognitive impairment remains poorly understood. This study employed LPS-induced mice to determine the *Bifidobacterium animalis subsp. lactis* (**BL**) 99-MV on preventing cognitive impairment and the relevant mechanisms involved. Phenotypic results showed that BL99-MV supplementation inhibited IL-33, IL-1β and TNF-α level, increased brain-derived neurotrophic factor (**BDNF**) level, reduced Aβ, Tau, P-Tau (Ser404) and GSK-3β expression and improved cognitive impairment in mice. Metagenomic analysis revealed that BL99-MV supplementation restored gut microbiota dysbiosis, increased the relative abundance of *Clostridium* and *Oscillibacter*. Metagenomic analysis revealed that BL99-MV increased the hippocampal BDNF expression, which enhanced the conversion 5-hydroxytryptophan (**5-HTP**) to 5-hydroxytryptamine (**5-HT**) to improve learning. More importantly, the level of 5-HT exhibited a positive correlation with the abundances of *Clostridium* and *Oscillibacter*. Our findings illuminate that a novel and promising BL99-MV to improve LPS-induced HNF and accompanying gut disorder, and provide options for preventing gut-brain axis- related cognitive function.

Keywords: *Bifidobacterium animalis subsp. lactis*-derived vesicles; Hippocampal neuroinflammation; gut microbiota; 5-hydroxytryptamine; brain-derived neurotrophic factor

1. Introduction

The coronavirus disease 2019 (**COVID-19**) has been associated with the development of neurological sequelae, particularly a condition commonly referred to as 'brain fog'[76]. This condition is characterized by hippocampal neuroinflammation (**HNF**), which may contribute to its cognitive impairments, including memory dysfunction and cognitive decline[27]. HNF is primarily driven by the accumulation of amyloid-beta (**Aβ)** peptides and the formation of neurofibrillary tangles (**NFTs)**, which consist of hyperphosphorylated

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tau (**Tau**) protein[60; 88]. Furthermore, pro-inflammatory cytokines exacerbate HNF by contributing to neuronal injury and disrupting the integrity of the blood-brain barrier (**BBB**)[21; 38; 64].

When lipopolysaccharide (**LPS**) enters the central nervous system (**CNS**), it activates Toll-like receptor 4 (**TLR4**), triggering HNF and contributing to gut microbiota dysbiosis[89; 107], including quantitative and composition changes in the gut microbiome[31; 75]. Recent studies have highlighted the potential role of *Oscillibacter* and *Clostridium* in modulating HNF and its association with cognitive impairment[46; 55; 58; 83]. *Oscillibacter* partially ameliorates cognitive impairment by reducing HNF, activating EM CD4+ T cells and enhancing synaptic plasticity, thereby potentially mitigating cognitive decline[24; 37; 90; 100]. *Clostridium* alleviated cognitive impairment by attenuating deficits in hippocampal neurite outgrowth and synaptic ultrastructure, as well as improving hippocampal transcriptome profiles related to synaptic function[105]. Furthermore, *Clostridium* may exert neuroprotective effects under hypobaric hypoxia by increasing hippocampal levels of L-valine[108].

Following the activation of proinflammatory pathways, a significant increase in Interleukin (**IL**)-33 levels has been observed. This increase subsequently contributes to the accumulation of Aβ and enhances the release of tumor necrosis factor-α (**TNF-α**) and IL-1β.[20; 54]. The upregulation of IL-1β exacerbates tau protein pathology by enhancing the phosphorylation of Tau (**P-Tau**) and promoting NFT formation. This process is mediated through the activation of glycogen synthase kinase 3β (**GSK-3β)**, also known to as Tau kinase 1[43]. Notably, GSK-3β is highly expressed in the hippocampal region, where it mediates increased BBB permeability, ultimately resulting in cognitive dysfunction[9; 23; 29; 65]. In addition to these mechanisms, neuroprotective factors such as brain-derived neurotrophic factor (**BDNF**) and microtubule-associated protein 2 (**MAP2**) have been identified as crucial mediators of hippocampal neuronal survival and synaptic transmission[3; 22; 59; 72].

Accumulating research has increasingly highlighted the relationship between *Bifidobacterium* and cognitive function[18; 110]. *Bifidobacterium* enhances cognitive function by promoting the abundance of *Bifidobacterium* and *Akkermansia* abundance[109]. Interestingly, *Bifidobacterium* mitigates HNF by significantly elevating *Oscillibacter* abundance and concurrently enhancing BDNF and 5-hydroxytryptamine (**5-HT**) levels[86]. 5-HT is a crucial neurotransmitter that plays role in neuronal synaptogenesis and its involvement in learning and memory processes[1; 30]. This neurotransmitter is synthesized from its precursor, 5-hydroxytryptophan (**5-HTP**), through the catalytic action of tryptophan hydroxylase 1 (**TPH1**)[32]. Notably, *Bifidobacterium* supplementation attenuates HNF by significantly upregulating hippocampal TPH1 and 5-HTP levels[1; 15; 18; 34; 73; 74]. Furthermore, *Bifidobacterium* has been demonstrated to modulate fatty acid metabolism, particularly through the elevation of essential fatty acids including Linolenic Acid (**LA**), α-Linolenic Acid (**ALA**), Docosahexaenoic Acid (**DHA**), and Eicosapentaenoic Acid (**EPA**) [41; 61; 69]. The metabolic conversion of LA to ALA, and subsequently to EPA and DHA, establishes a biochemical pathway that enhances 5-HT and BDNF levels while stimulating synaptic plasticity, ultimately contributing to the alleviation of HNF[5; 14; 52].

Emerging research recognizes that probiotic-derived membrane vesicles (**MV**) as innovative bioactive carriers capable of modulating gut microbiota composition and function, thereby exerting therapeutic effects on cognitive impairment through the gut-brain axis[57]. For example, *Akkermansia muciniphila*- derived MV have been demonstrated to promote *Lactobacillaceae* abundance while concurrently upregulating BDNF and 5-HT expression, resulting in significant improvement of cognitive function[40]. Similarly, *Lactobacillus paracasei*-derived MV exhibit neuroprotective effects by enhancing BDNF expression and reducing Aβ accumulation, thereby attenuating HNF[13; 45]. Furthermore, *Bifidobacterium*-derived MV have shown promising potential in alleviating cognitive impairment through the upregulation of hippocampal BDNF expression[56]. *Bifidobacterium animalis subsp. lactis* (**BL**)-99 modulates gut microbiota composition by increasing the relative abundance of *Bifidobacterium*, *Akkermansia*, and *Lactic acid* bacteria, thereby ameliorating colitis symptoms[10; 47]. Furthermore, BL-99-derived MV attenuate HNF by targeting IL-33 to reduce Aβ accumulation[16]. Nevertheless, the precise mechanisms by which BL-99-derived MV modulate the gut microbiota and associated metabolites to ameliorate HNF remain unclear.

Therefore, this study aims to systematically evaluate the protective effects and underlying molecular mechanisms of BL99-MVs in HNF, with particular focus on their modulation of key gut microbiota and neural metabolic markers. We hypothesize that BL99-MVs ameliorate HNF-induced cognitive impairment by remodeling the gut microbiota, which in turn enhances hippocampal levels of 5-HT and LA, ultimately reducing Aβ deposition and mitigating neuroinflammation. The findings of this study may provide a theoretical foundation for further exploration of the health benefits and applications of probiotic-derived MV.

2. Materials and methods

2.1 Imaging of BL99-MV In Mice

To monitor the hippocampus localization of BL99-MV in mice, BL99-MV were labelled with DiR (Cyanine 5 NHS ester, Lumiprobe, 43020) and administered by gavage. Briefly, BL99-MV (200μg/μl) was incubated with 1μl of DiR (5mg/ml) (cat. #D22070; Bridgen) at 37°C for 30 min. Mice were anesthetized and imaged over a 100 min period using IVIS Spectrum In Vivo Imaging System (Caliper, Shanghai, China). Afterward, the gastrointestinal (GI) tract and brain were collected and imaged using IVIS. The same amount of free DiR dyes in PBS was administered to mice as control.

2.2 BL99-MV Isolation

The preparation of BL-99 and subsequent isolation of BL99-MV were conducted according to established protocols[16]. Briefly, the BL-99 was prepared as a powder at 1.5×10^{11} CFU g^{-1} by Yili Co., Ltd. BL-99 was anaerobically cultured in an MRS medium with a supplement of 0.05% L-cysteine before being harvested at 37℃ overnight. The bacterial suspensions (2.24×10^{8} CFU/mL) were subjected to sequential centrifugation steps (3,600× g, 5,000× g and 11,000× g) to remove cellular debris. The resulting supernatant was concentrated 10-fold using 100-kDa molecular ultrafiltration tubes (Millipore, Burlington, NJ, USA) for

10 min (5,000× g) and ultracentrifuged for 90 min (120,000× g). The obtained BL99-MV were passed through 0.22-μm filter, resuspended in PBS, and stored at -80°C until further use.

2.3 Animal Experiments

All experiments were approved by the Animal Experimentation Ethics Committee of the China Agricultural University (AW02113202-5-1). 36 male Balb/c mice (6 weeks old, weighing 20-24 g, Beijing Huafukang Biotechnology) were randomly divided into four groups: control group (control), LPS model group (LPS), BL99-MV group (3 mg/kg per day per mice) and BL-99 (1×10^8 CFU per day per mice) group. The control and LPS group were gavaged a 0.9 % NaCl solution with 28 days. The BL99-MV group were gavaged BL99-MV (3 mg/kg) with 28 days. The BL-99 group were gavaged BL-99 (1×10^8 CFU) with 28 days. In 29 Days, except for the control the rest of the groups were intraperitoneally injected with 1mg/kg LPS (cat. #L2880-100MG; Sigma) for 6 hours (Figure 2A). The body weight of mice in each group was documented daily.

2.4 Behavioral Tests

Behavioral assessments were conducted using a double-blind experimental design to evaluate spatial memory and learning capabilities[62]. (1) Morris water maze (**MWM**): The MWM apparatus consisted of a circular pool (120 cm diameter × 40 cm height) maintained at 25 ± 1°C, containing an escape platform (10 cm diameter) submerged 2 cm below the water surface. The pool was conceptually divided into four equal quadrants. Following a training session focused on place navigation within a four-quadrant framework, data were collected on the mice regarding the frequency of platform crossings, the duration of time spent in the designated areas, and the distance swum within the targeted quadrants. (2) Novel object recognition (**NOR**): mice were allowed to explore two identical objects for 10 min. After a 24-hour retention interval, one familiar object was replaced with a novel object while maintaining identical spatial configuration. The exploration time of the mice was subsequently recorded for both the new and the old object over a period of 5 min. The recognition index was calculated according to the formula: New object / (New object + old object) × 100%[81].

2.5 Hematoxylin and Eosin Staining (HE) and Immunohistochemistry (IHC)

For HE, hippocampal tissues were fixed, processed, and embedded in paraffin according to standard protocols. Tissue sections (4 μm thickness) were prepared and mounted on slides[19]. For IHC, tissue sections underwent antigen retrieval and were subsequently incubated with primary antibodies against Aβ1-42 (cat. #25524-1-AP, Proteintech) and MAP2 (cat. #17490-1-AP, Proteintech). Following this, the sections were incubated with secondary antibodies (cat. #SE131, Solarbio), and coverslips were affixed using neutral balsam (cat. #G8590, Solarbio). Microscopic imaging was conducted to examine morphological alterations.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of cytokines in serum were determined using ELISA according to the manufacturer's instructions. In short, serum sample were centrifuged at 2,000 × g for 20 min at 4°C to remove cellular components, and collect the supernatant for subsequent analyses. Serum levels of 5-HT (cat.

#RXJ203223M, RUIXIN), IL-33 (cat. #RX203053M, RUIXIN), TNF- α (cat. #EK282, MULTI), IL-1β (cat. #RX203063M, RUIXIN) and IL-10 (cat. #EK210, MULTI) were determined using ELISA kits[111].

For hippocampal tissue analysis, hippocampus segments (1g) were homogenized in 9 ml of PBS using a tissue homogenizer. The homogenate was centrifuged at 5,000 × g for 15 min at 4°C to obtain a clear supernatant. Supernatants were collected to quantified 5-HT (cat. #MM-0443M2, MEIMIAN), 5-HTP (cat. #MM-45703M2, MEIMIAN) and LA (cat. #MM-928387O2, MEIMIAN) levels with ELISA kits[106].

2.7 Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA from hippocampal tissue was extracted using Trizol Reagent (cat. #15596026, Invitrogen)[24]. Quality of extracted RNA was determined using Nano Drop 2000(Thermo, Shanghai, China). The cDNA was synthesized using the Prime Script RT Reagent Kit (cat. #DRR037A, TaKaRa). The quantitative RT-PCR was conducted using TB Green® Premix Ex Taq™ (cat. #RR420A, Takara), following the manufacturer's instructions. Relative expression of genes was calculated using the $-2^{\Delta\Delta Ct}$ method. β-actin was used as a housekeeping gene. Primers used in this study are listed in Table S1.

2.8 Western Blot Analysis (WB)

As previously described[16], the hippocampal tissues protein were extracted using radioimmunoprecipitation assay (**RIPA**) buffer (cat. #R0020, Solarbio). Protein concentration were quantified using a BCA assay kit (cat. # PC0020, Solarbio). Protein lysates were resolved by sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) (cat. #CW0022S, CWBIO) and transferred into 0.45μm polyvinylidene fluoride (**PVDF**) (cat. #IPVH00010, Millipore) membranes for further analysis. The membranes were exposed to the primary antibodies for Aβ1-42 (cat. #25524-1-AP, proteintech), Tau (cat. #10274-1-AP, proteintech), P-Tau (Ser404) (cat. #81383-1-RR, proteintech), GSK-3β (cat. #22104-1-AP, proteintech), IL-33 (cat. #66235-1-Ig, proteintech), BDNF (cat. #25699-1-AP, proteintech), MAP2 (cat. #17490-1-AP, proteintech) and β-actin (cat. #66009-1-IG, proteintech) and incubated at 4 ℃ overnight, followed by incubation with secondary antibody (1:20000) for 1 h. Utilize the ECL (cat. #P0018FS, Beyotime) for spectral visualization. Following this, the grayscale intensity of the target strip was recorded and analyzed by image J2x 2.1.4.7 software (Rawak Software Inc., Stuttgart, Germany).

2.9 Fecal Metagenome Sequencing of Mice

Fecal samples were processed for metagenomic analysis at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Microbiome genomic DNA was extracted utilizing the FastPure Stool DNA Isolation Kit (YUHEALTH, China), with quality and quantity assessed by NanoDrop 2000 (Thermo, Shanghai, China) and agarose gel electrophoresis. High-throughput sequencing was performed on the Illumina NextSeq2000 platform (Illumina Inc., San Diego, USA). The subsequent processes encompassed sequence quality control, genome assembly, gene prediction, taxonomy, and functional annotation, were executed as outlined in prior studies[92]. Data analysis was carried out using the complimentary online platform provided by Majorbio (cloud.majorbio.com).

2.10 Serum Metabolomics

Serum samples were processed for metabolomic analysis at Majorbio Bio-Pharm Technology (Shanghai, China). Metabolite extraction was performed by mixing 100 μL of serum with 400 μL of extraction solution (acetonitrile: methanol = 1:1, v/v) containing 0.02 mg/mL L-2-chlorophenylalanine as an internal standard. For analysis, the supernatant was concentrated, dried, and re-solubilize in solution (acetonitrile: water = 1:1). The Thermo UHPLC-Exploris240 system coupled with ACQUITY HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, USA) was used for supernatant analysis. A gradient elution program ran with mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). Positive mode separation gradient: 0-3 min, 20% B; 3-4.5 min, 35%B; 4.5-6,3 min, 100%B; 6.3-8 min, 0% B. Negative mode separation gradient: 0-1.5 min, 5% B; 1.5-2 min, 10% B; 2-4.5 min, 30% B; 4.5-6.3 min, 100% B; 6.3-8 min, 0% B. The flow rate was 0.40 mL/min and the column temperature was 40℃. All samples were collected in positive (ESI+) and negative ion (ESI−) switching full scan modes. The data were analyzed through the free online platform of majorbio choud platform (cloud.majorbio.com).

2.11 Statistical analysis

All data are presented as mean ± standard error of the mean (**SEM**) and the statistical tests were analyzed by one-way ANOVA followed by Tukey's test with GraphPad Prism version 9.2 (San Diego, California, USA). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ were considered to be statistically significant.

3. Results

3.1 Biodistribution of BL99-MV In Mice

Our results showed that BL99-MV could be detected the GI tract and brain in mice 10 min, and the fluorescence signal gradually decreased with the extension of time (Figure 1A). Since the fluorescence distribution of the brain could not be photographed in supine, we performed mice experiment in vitro (Figure 1B).

We have shown that BL99-MV reach the GI tract and brain approximately at 20 min following gavage in mice, the signal was mainly observed in GI tract (2.58×10^{11} p/s/cm^2/sr), while minor but significant amount of fluorescence was detected in brain (1.551×10^{8} p/s/cm^2/sr). Importantly, the accumulation of fluorescence signals was maximum in the brain, reaching a peak value of 3.78×10^{8} p/s/cm^2/sr at 80 min. The accumulation of fluorescence signals in the GI tract (1.054×10^{11} p/s/cm^2/sr) gradually decreased. After 100 min, the fluorescence signal was minimized in the GI tract and brain (Figure 1C-D).

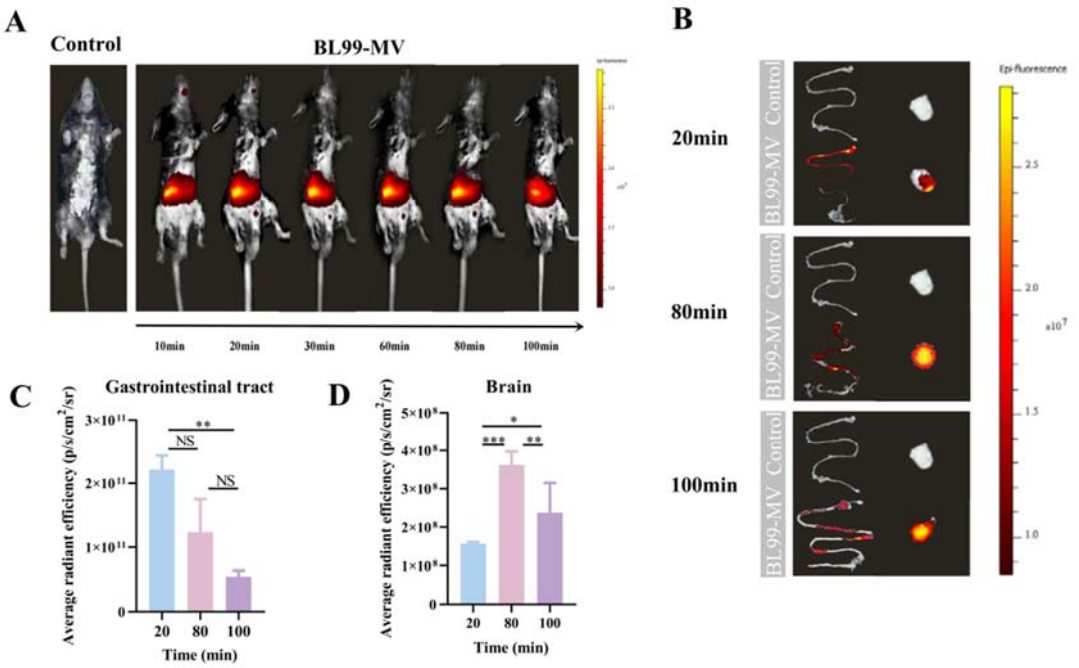

Figure 1. Biodistribution of BL99-MV in *vivo* and *vitro*. (A) Biodistribution of DiR-labelled BL99-MV in *vivo.* (B) Biodistribution of DiR-labelled BL99-MV in *vitro.* (C) Fluorescence signal quantification of DiR-labelled BL99-MV in GI tract in *vitro*. (D) Fluorescence signal quantification of DiR-labelled BL99-MV in brain in *vitro*. Data presented as means ± SEM (N=3). Statistically significant differences were indicated: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS *P* > 0.05.

3.2 BL99-MV Supplementation Improved LPS-Induced Cognitive Impairment

To investigate the impact of BL99-MV on mitigating memory and cognitive deficits, mice were respectively administrated with BL99-MV and BL-99 for 28 days with LPS treatment for 6 hours (Figure 2A). Compared to the LPS group, BL99-MV and control group exhibited a significant increase in weight for 6h ($P < 0.05$) (Figure 2B). In behavioral analyses, we employed the MWM and NOR to assess spatial memory capabilities. In the LPS group, mice reduce locate hidden platforms, crossing platform times and movement trajectories in quadrant platform lines. Compared to LPS group, BL99-MV group significantly increasing crossing platform number ($P < 0.05$), activity trajectories in the quadrant platform ($P < 0.01$), target quadrant time and distance ($P < 0.05$) (Figure 2C-G). Additionally, LPS group spent remarkably less time and entries in new object during NOR test ($P < 0.001$). Compared to the LPS group, BL99-MV ($P < 0.001$), BL-99 ($P < 0.01$) and control ($P < 0.001$) groups investigated new objects considerably more frequently (Figure 2H-I). Therefore, BL99-MV and BL-99 have the potential to enhance cognitive function in mice.

Each group mice hippocampus pathological morphology is shown in Figure 1J. In the LPS group, the intercellular space increased, and neurons within the hippocampal CA1 region were observed to be arranged in a more dispersed manner. BL99-MV and BL-99 group exhibited a closely organized structure characterized by distinct nucleoli. Compared to the LPS group, BL99-MV group significantly increasing the neurous numbers ($P < 0.01$) (Figure 2J-K).

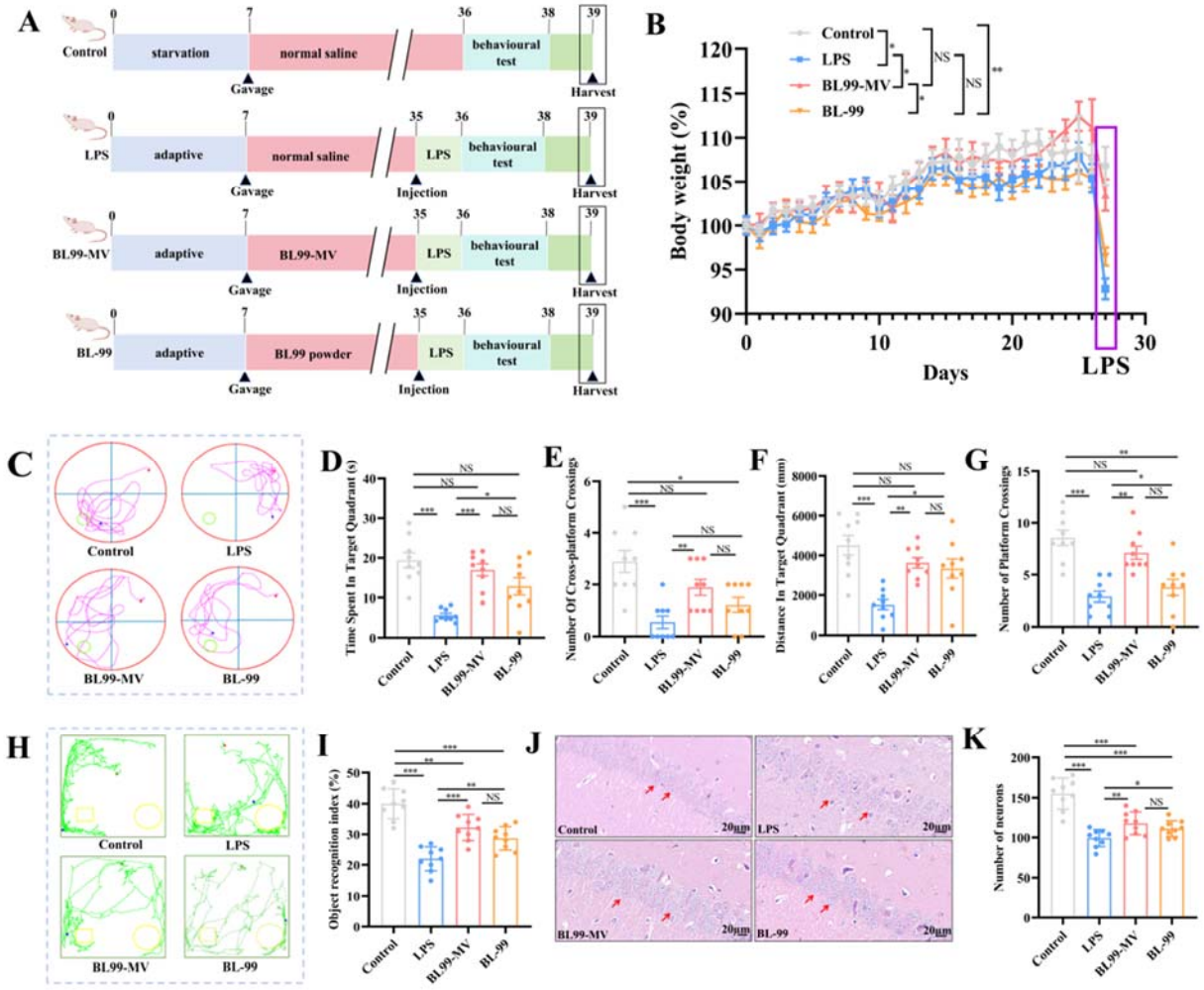

Figure 2. BL99-MV and BL-99 supplementation improves the Cognitive Impairment. (A) Experimental approach for LPS-induced HNF and BL99-MV administration. (B) Daily changes in body weight of the BL99-MV group during 28 days. (C) The mice track plots with representative image in the MWM. (D) The number of cross-platform crossings. (E) The number of cross-platform crossings. (F) Distance in target quadrant. (G) The number of platform crossings. (H) The mice track plots with representative image in the NOR. (I) The object recognition index. (J) HE staining of hippocampal morphology (60×). (K) Number of neurons. Data presented as means ± SEM (N = 9). Statistically significant differences were indicated: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS $P > 0.05$.

3.3 BL99-MV Supplementation Alleviates LPS-Induced Aβ1-42 Protein Expression

To further examine the impact of BL99-MV on Aβ1−42 protein expression in the hippocampus, IHC and WB were employed. The IHC analysis was performed to evaluate the expression of Aβ1−42 protein in the CA1 region of the hippocampus among different groups. In the LPS group，Aβ1−42 protein high expression was a brown-yellow sediment, which was widely distributed in the hippocampus. Compared with the LPS group, BL99-MV and BL-99 effectively decreased the brown-yellow sediment average optical density value of Aβ1−42 protein (Figure 3A-B). In alignment with the IHC results, BL99-MV and BL-99 supplementation significantly decreased Aβ1−42 expression by WB ($P < 0.01$) (Figure 3C-D).

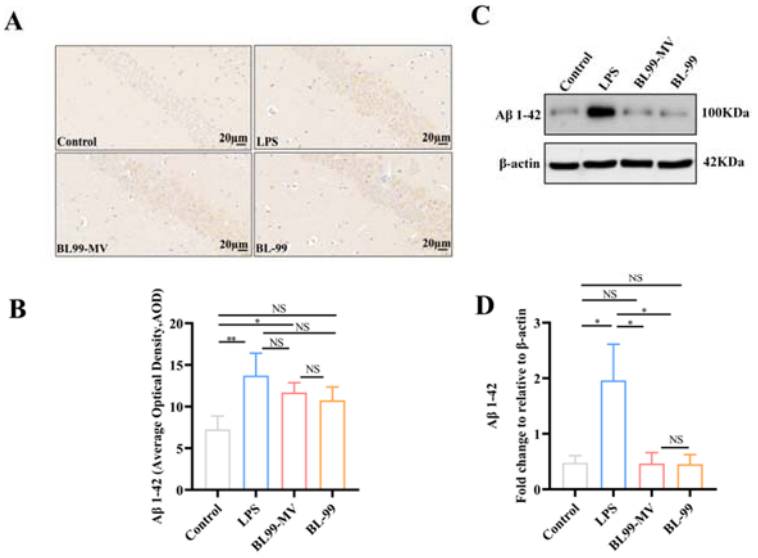

Figure 3. BL99-MV and BL-99 supplementation alters the expression of Aβ1-42 protein in the hippocampus of LPS-induced mice. (A) The Aβ1-42 protein with representative image by IHC (60×). (B) Quantitative analysis of IHC. The AOD of Aβ1-42 was measured with image J. (C) The Aβ1-42 protein with representative image by WB. (D) Quantitative analysis of WB. The bands of Aβ1-42 was measured with image J and normalised with β-actin. Data presented as means ± SEM (N = 3). Statistically significant differences were indicated: **P* < 0.05; ***P* < 0.01; NS *P* > 0.05.

3.4 BL99-MV Supplementation Alleviates LPS-Induced Tau and GSK-3β Protein Expression

The WB analysis was performed to evaluate the expression of Tau, P-Tau (Ser404), and GSK-3β protein in the hippocampus among different groups (Figure 4A). Compared with the LPS group, BL99-MV and BL-99 decreased Tau expression significantly ($P < 0.001$). BL99-MV induced a more significant decrease in P-Tau (Ser404) ($P < 0.05$) and GSK-3β ($P < 0.01$) protein expression, compared to the bacterium itself (Figure 4B-D).

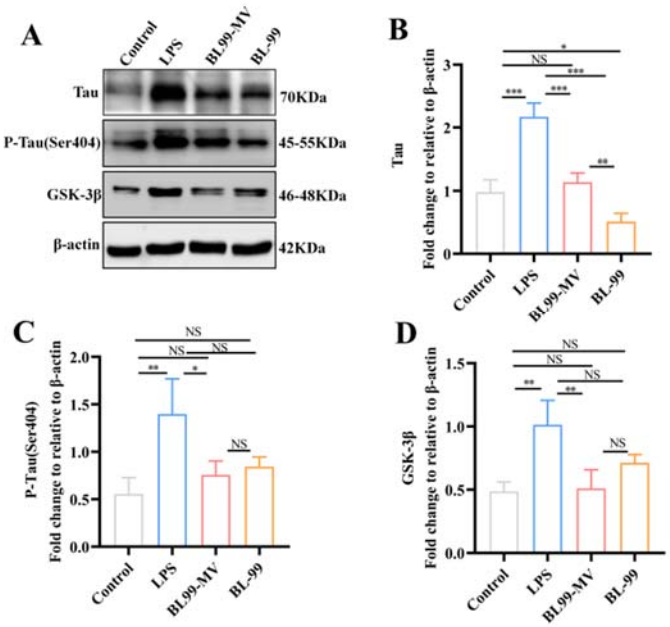

Figure 4. BL99-MV and BL-99 supplementation alters the expression of Tau, P-Tau (Ser404), and GSK-3β protein in the hippocampus of LPS-induced mice. (A) Tau, P-Tau (Ser404), and GSK-3β protein with representative plots by WB. (B-D) Quantitative analysis of WB. The bands of Tau, P-Tau (ser404), and GSK-3β were measured with image J and normalised with β-actin. Data presented as means ± SEM (N = 3). Statistically significant differences were indicated: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS *P* > 0.05.

3.5 BL99-MV enhancing BDNF Expression and Synaptic Plasticity

The IHC analysis was conducted to assess the expression of MAP2 protein in the CA1 region of the hippocampus among different groups (Figure 5E). In the control group, MAP2 high expression was a brown-yellow sediment, which was widely distributed in the hippocampus. Compared with LPS group, BL99-MV ($P < 0.001$) and BL-99 ($P < 0.01$) significantly increased the brown-yellow sediment average optical density of MAP2 (Figure 5F).

The WB analysis to evaluate IL-33, BDNF and MAP2 expression in the hippocampus among different groups (Figure 5A). Compared with the LPS group, BL99-MV decreased IL-33 expression ($P < 0.05$), increased BDNF ($P < 0.05$) and MAP2 ($P < 0.01$) expression significantly. Compared with the LPS group, BL-99 effectively decreased IL-33 ($P >0.05$) expression, increased BDNF ($P < 0.05$) and MAP2 ($P < 0.001$) expression significantly (Figure 5B-D). Taken together, the IHC results and WB results suggest that BL99-MV and BL-99 could potentially contribute to maintain synaptic plasticity *via* suppressing IL-33 expression.

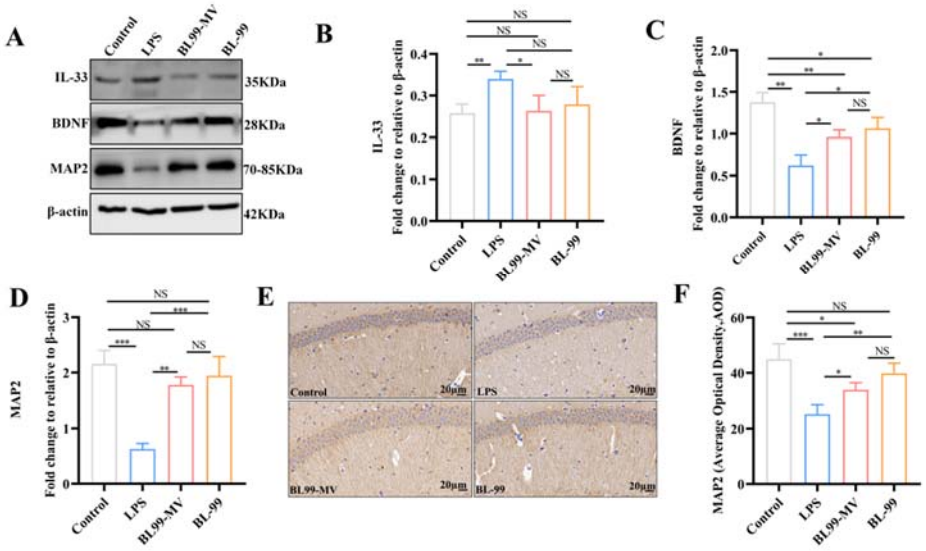

Figure 5. BL99-MV and BL-99 supplementation alters the expression of IL-33, BDNF and MAP2 in the hippocampus of LPS-induced mice. (A) Representative plots of IL-33, BDNF and MAP2 protein by WB. (B-D) Quantitative analysis of WB. The bands of IL-33, BDNF and MAP2 were measured with image J and normalised with β-actin. (E) Representative image of MAP2 protein by IHC (60×). (F) Quantitative analysis of IHC. The AOD of MAP2 was measured with image J. Data presented as means ± SEM (N = 3). Statistically significant differences were indicated: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS $P > 0.05$.

3.6 BL99-MV Supplementation Alleviates LPS-Induced Inflammatory Responses

To better understand BL99-MV and BL-99 effects on serum inflammatory response, we analyzed IL-33, IL-1β, TNF-α, and IL-10 levels. Compared with LPS group, BL99-MV significantly decreased IL-33($P < 0.001$) and TNF-α ($P < 0.01$) level, effectively decreased IL-1β ($P > 0.05$) level, while significantly increased IL-10 levels ($P < 0.05$). BL-99 group had same trend (Figure 6A-D).

Meanwhile, we measured the expression of inflammatory markers in the hippocampal lysates by RT-PCR. Our results showed that compared with control group, LPS group significantly increased IL-33 ($P < 0.01$), IL-1β ($P < 0.001$) and TNF-α ($P < 0.001$) expression, while significantly inhibiting IL-10 ($P< 0.01$)

expression in mice. However, compared with LPS group, BL99-MV significantly reduced IL-33 ($P < 0.05$), IL-1β ($P < 0.01$) and TNF-α ($P < 0.05$) expression, and significantly increased IL-10 ($P < 0.05$) expression (Figure 6D-H). These results indicate that BL99-MV possess anti-inflammatory properties.

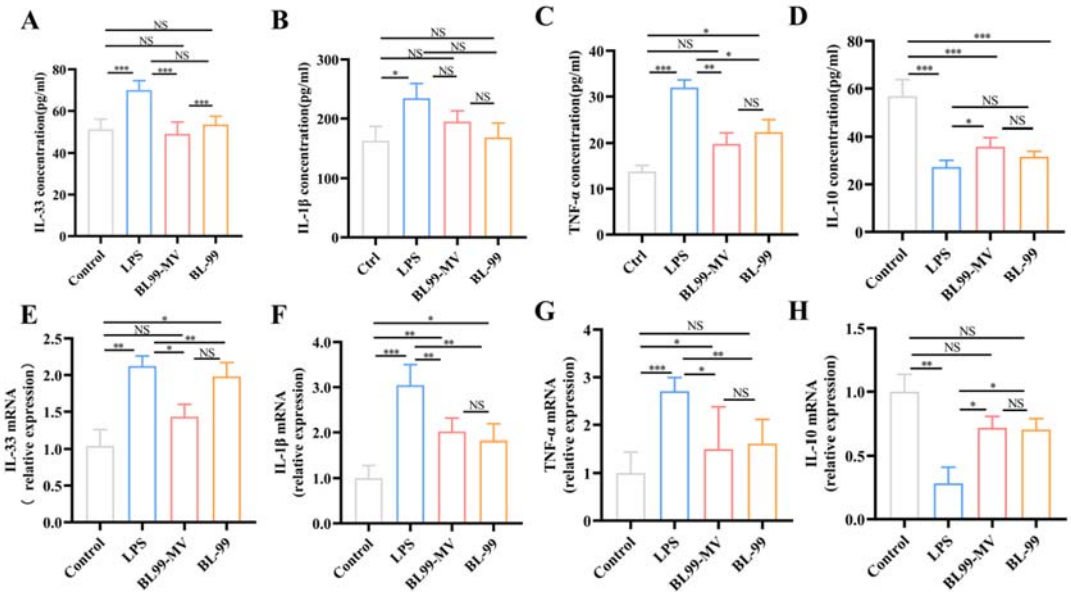

Figure 6. Effect of BL99-MV and BL-99 on proinflammatory and anti-inflammatory cytokine level. (A-D) IL-33, IL-1β, TNF-α and IL-10 level in serum by ELISA. (E-H) IL-33, IL-1β, TNF-α and IL-10 mRNA expression in hippocampus by RT-PCR. Data presented as means ± SEM (N = 5). Statistically significant differences were indicated: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS $P > 0.05$.

3.7 BL99-MV Supplementation Reverse Gut Microbial Dysbiosis in LPS-Induced Mice

To identify the BL99-MV reverse gut microbial dysbiosis, we employed metagenomic sequencing to analyze gut microbial alterations in mice. To evaluate the β-diversity of the gut microbiota, we performed PCA utilizing the Bray-Curtis distance. Our results revealed notable variations in distance distributions among the various groups. The PCA visualization indicated that PC1 and PC2 variation percentages were 24.77% and 12.71%, respectively (Figure 7A). Subsequently, we analyzed the relative abundances of species at the genus level. In general, BL99-MV supplementation increased species abundance and diversity compared to the LPS group. We obtained that the dominant gut microbiota of mice in LPS group were *Enterococcus* and *Escherichia,* while in BL99-MV group were *Clostridium* and *Oscillibacter, Pseufoflavonifractor*, *Lachnoclostridium* and *Lactobacillus* (Figure 7B).

The Wilcoxon rank-sum test was employed to assess the interspecific differences between the groups. Compared with the LPS group, we observed that BL99-MV supplementation significantly increased the abundance of *Clostridium, Oscillibacter, Lachnoclostridium* and *Lactobacillus* ($P < 0.05$) (Figure 7C-D). These results proved that BL99-MV supplementation renovated LPS-induced gut microbiota disturbances.

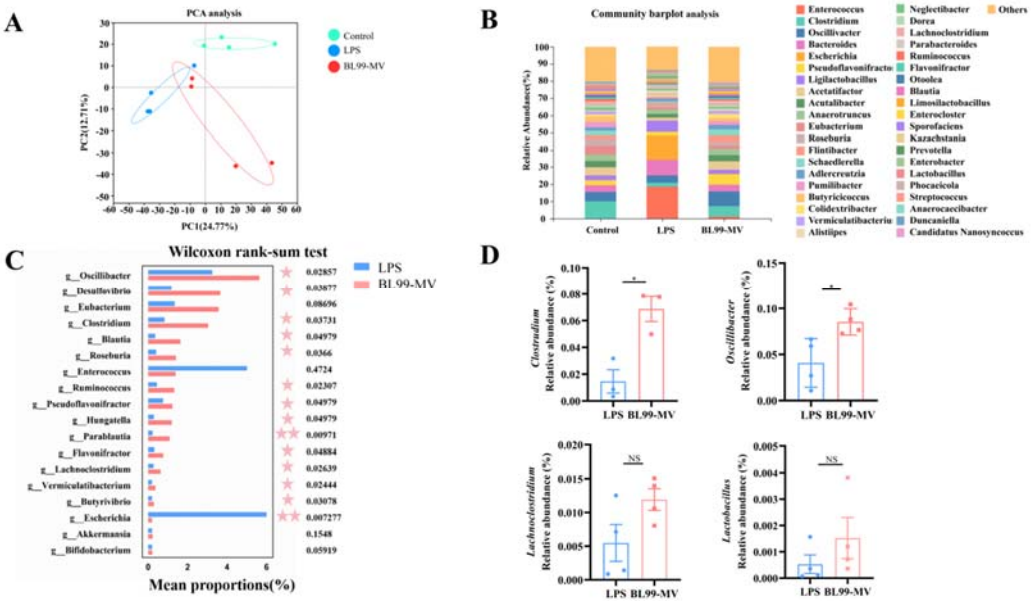

Figure 7. Alterations in the taxonomic composition of the gut microbiome following the supplementation of BL99-MV (A) PCA. (B) Species relative abundance at the genus level in different groups. (C) Species different abundances between BL99-MV and LPS groups by Wilcoxon−Mann-Whitney U test. (D) Characteristic species. Statistically significant differences were indicated: *$P < 0.05$; **$P < 0.01$; NS $P > 0.05$.

3.8 BL99-MV Supplementation Regulates Serum Metabolites in LPS-Induced Mice

To explore the metabolic alternations associated with the HNF process and the potential roles of BL99-MV in ameliorating cognitive impairment, we employed an untargeted metabolomics approach to characterize the serum metabolic profiles of mice. The partial least squares-discriminate analysis (**PLS-DA**) method was utilized to illustrate the metabolic differences among all groups in mice. Each point on the PLS-DA score plot represented an individual sample, with its position determined by the type and concentration of metabolites present. Samples exhibiting similar metabolic compositions were located in close proximity on the plot. The analysis revealed a distinct separation between the LPS and control groups, indicating significant changes in endogenous metabolites. This finding suggests that the LPS group was successfully established, demonstrating strong predictive capability and reliability (Figure 8A). The BL99-MV identified and quantified differential metabolites in comparison to the LPS group by volcano plot, which identified 420 differential metabolites (319 up- and 101 downregulate) between BL99-MV and LPS treatment mice (Figure 8B). Compared to the LPS group, BL99-MV significantly increased the level of 13-Oxoode, LA, Stearidonic Acid (**SDA**), Kynurenine Acid, 9S-Hpode, N-Formylanthranilx Acid, Tryptophan, 5-HT and Xanthurenic Acid (Figure 8C). With a significance threshold of $P < 0.05$ and VIP >1, the differentially expressed genes were primarily enriched in 20 signaling pathways. We observed that LA metabolism pathways and Tryptophan metabolism pathways were potential BL99-MV targets in treating LPS mice (Figure 8D). Additionally, we measured the 5-HT, 5-HTP and LA concentrations by ELISA. Compared with LPS group, BL99-MV increasing 5-HT ($P < 0.05$), 5-HTP ($P < 0.001$) and LA ($P < 0.05$) concentrations in the hippocampus, meanwhile increasing 5-HT ($P < 0.05$) concentration in serum significantly (Figure 8E-H).

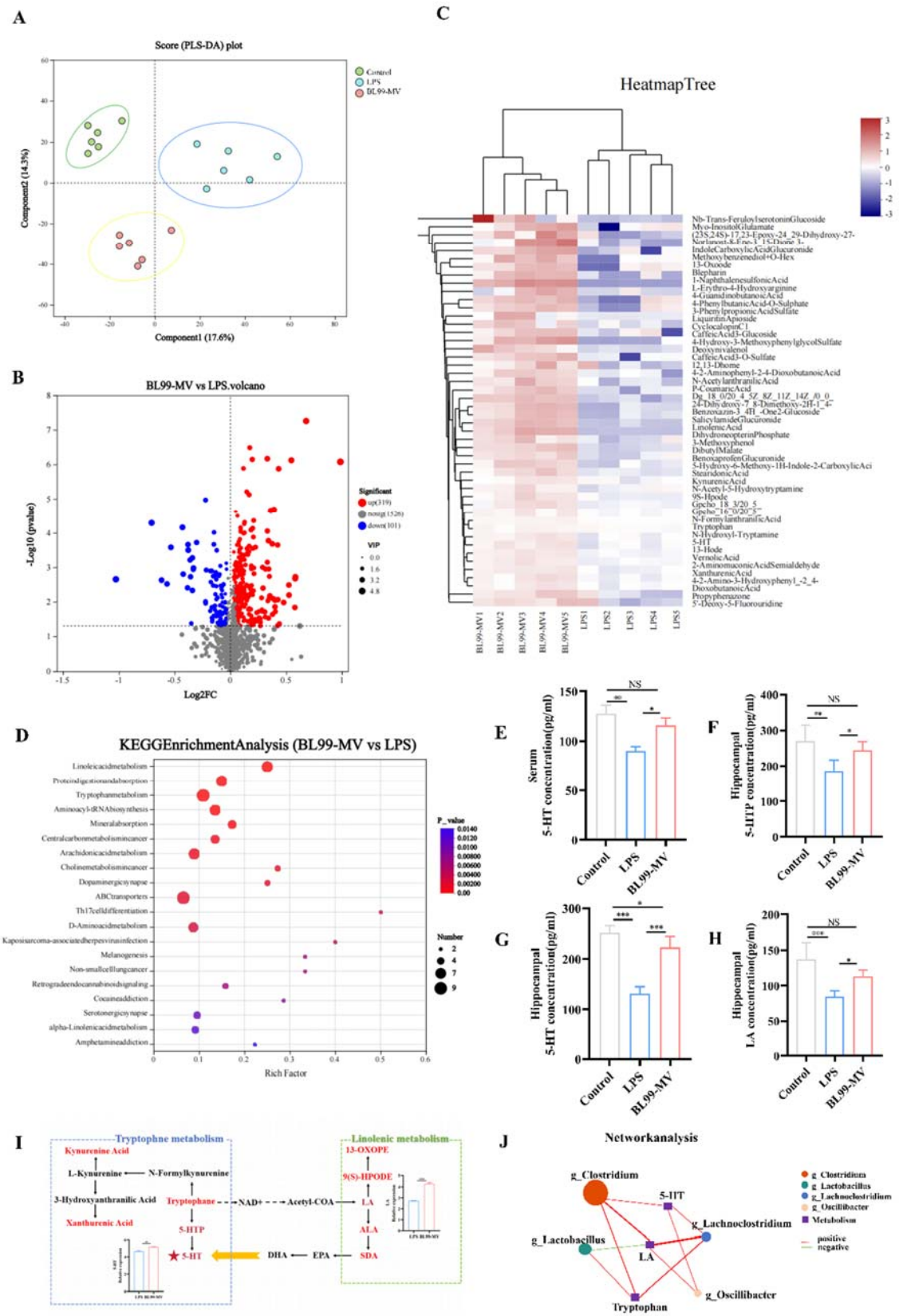

Figure 8. BL99-MV effects on host circulating serum metabolism. (A) Score plot. (B) The volcano plots between BL99-MV and LPS group. Each point in a volcano plot represents a metabolite. (C) Hierarchical cluster analysis of differential metabolites between BL99-MV and the LPS group. (D) Potential target metabolic pathway of BL99-MV group. (E) 5-HT concentrations in serum. (F) 5-HTP concentrations in the hippocampus. (G) 5-HT concentrations in the hippocampus. (H) LA concentrations in the hippocampus. (I) A schematic map depicting the enriched key pathways and the targeted compounds regulated by BL99-MV in mice. (J) Networkanalysis reveals that the microbiome is associated with changes in metabolites. Red edges indicate a positive correlation and green edges indicate a negative correlation.

To understand the relationships between genus-level gut microorganisms and the metabolites of serum, Pearson correlations were used to generate a Network. In the network, we focus on the interactions of specific metabolities and microbiotics. Our results showed that 5-HT positively correlated with *Clostridium, Oscillibacter, Alistipes, Desulfovibrio, Acetatifactor and Eubacterium* level, negatively correlated with *Enterococcus*, *Prevotella*, *Ligilactobacillus*, *Escherichia*, *Parabacteroides* and *Bacteroides* levels. LA positively correlated with *Clostridium, Oscillibacter, Desulfovibrio, Acetatifactor and Eubacterium* levels, negatively correlated with *Muribaculum*, *Paramuribaculul*, *Enterococcus, Prevotella*, *Ligilactobacillus*, *Escherichia*, *Parabacteroides, Alistipes* and *Bacteroides* levels. Tryptophan positively correlated with *Clostridium, Oscillibacter, Desulfovibrio, Acetatifactor and Eubacterium* levels, negatively correlated with *Muribaculum*, *Paramuribaculul*, *Enterococcus, Prevotella*, *Ligilactobacillus*, *Escherichia*, *Parabacteroides, Alistipes* and *Bacteroides* levels (Figure S1). Interestingly, we observed that 5-HT positively strong correlated with *Clostridium, Oscillibacter* and *Lachnoclostridium* levels. LA positively strong correlated with *Clostridium*, *Lachnoclostridium* and *Oscillibacter* levels, negatively correlated with *Lactobacillus* levels. Tryptophan positively strong correlated with *Lactobacillus* and *Lachnoclostridium* levels (Figure 8J).

4. Discussion

The probiotic-derived MV play a significant role in regulating host neuronal protection[11]. Interestingly, probiotic-derived MV modulates the gut microbiota via gut-brain axis, further reducing HNF, improving cognitive impairment[36; 53; 66; 99]. Recent studies have shown that *L. plantarum*-derived MV enhanced BDNF expression to rescue depressive-like behaviors in HT22 cells[13]. *Akkermansia muciniphila*-derived MV enhancing gut and BBB integrity to improve cognitive impairment in mice[25]. This study indicates that neuroprotective effects of BL99-MV were achieved by enhancing learning and memory functions, decreasing neuronal loss in the hippocampus, inhibiting Aβ1−42 deposition, reducing NFTs of tau protein, increasing BDNF expression and enhancing the levels of 5-HT, all of which effects were achieved by down-regulation of IL-33. These findings imply that BL99-MV might contribute to the stability of the neuronal cytoskeleton and be used in the nutritional intervention of HNF.

The findings of this study revealed notable disparities in cognitive function between control and the group. LPS group showed a significant increase in escape latency and preference for novel object (Figure 2C-I), thereby indicating that administration of LPS adversely affects cognitive ability in mice, which is consistent with the previous study[67; 78]. Compared with LPS group, BL99-MV and BL-99 supplementation demonstrated the ability to ameliorate behavioral deficits. BL99-MV supplementation more significant effective improve cognitive function, compared to the bacterium itself. The current research indicates that probiotic-derived MV could alleviate cognitive impairment. *Lactobacillus pentosus* derived MV had short latencies to find the hidden platform[79]. Our study also can prove this point.

The structure and function of the hippocampal CA1 region is critical for cognitive processes and is associated with spatial learning and memory[51; 63]. Our HE results showed that compared with LPS group, BL99-MV and BL-99 could effectively increase the number of neurons cells, while BL99-MV has

significant effect ($P < 0.01$) (Figure 2J-K). Our ELISA results showed that BL99-MV increasing 5-HT concentrations in serum ($P < 0.05$) and the hippocampus ($P < 0.001$), and also increasing 5-HTP in the hippocampus ($P < 0.05$) significantly (Figure 8E-G). Studies showed that the highly expression of 5-HT in hippocampus which could enhance the excitatory transmission of synapses and improves spatial memory thereby improves learning and memory abilities[84; 87]. Our results showed that BL99-MV could enhance the level of 5-HT to improve cognitive impairment in hippocampus.

When HNF occurred, it would lead to Aβ1-42 aggregation and P-Tau NFT in the hippocampus[102]. Suppress the processes of Tau NFT accumulation and is important for maintaining the stability of the neuronal cytoskeleton[39; 71]. Our studies have demonstrated that Aβ1-42 accumulation and Tau NFT abnormalities in the LPS group. Compared with LPS group, BL99-MV and BL-99 exert a significant influence on reducing the level of the Aβ1-42, total Tau protein and P-Tau (Ser404) (Figure 3A-D, Figure 4A-D), which is consistent with previous studies[96]. GSK-3β, as a Tau kinase, highly expression leading to over-phosphorylation of Tau, further initiating HNF [24],[103]. Our results showed that GSK-3β protein level was dramatically improved in the LPS group ($P < 0.01$). Compared to the LPS group, BL99-MV significantly reduced the level of GSK-3β ($P < 0.01$). These findings suggest that BL99-MV and BL-99 have the ability to reducing GSK-3β and P-Tau (Ser404) level, thereby further improving HNF, which consistent with previous[79].

Furthermore, there were accumulating evidence that cognitive impairment is positive correlation with the secretion of proinflammatory cytokines[6; 80]. Our results showed that LPS mice following HNF, several proinflammatory biomarkers, including IL-33, IL-1β, and TNF-α, exhibit an increase in plasma levels within a mere 6 h timeframe. When HNF occurs, IL-33 is consistently highly expressed in the hippocampus, which upregulates Aβ1-42 expression. It has been shown that high expression of IL-33 decreases the expression of BDNF, which further down-regulates the expression of the synaptic plasticity marker protein MAP2[2; 50; 91; 93; 94; 104; 111]. IL-1β, as member of IL-1 family, its high expression could exacerbate tau phosphorylation[50]. Compared with the LPS group, BL99-MV and BL-99 suppressed inflammatory cytokine expression (IL-33, IL-1β, and TNF-α) and increased anti-inflammatory cytokine IL-10 expression in serum and hippocampus (Figure 6A-F), consistent with previous study. Our results revealed that BL99-MV and BL-99 improved neuroprotection and enhanced synaptic plasticity by inhibiting IL-33 expression and promoting BDNF expression, thus achieving the effect of improving HNF (Figure 5A-H).

Some research were established a close connection between gut microbiota and HNF, commonly referred to as the "gut-brain axis"[110]. In our study, when HNF occurs, gut microbiota richness and diversity was decreased, BL99-MV supplementation restoring the gut microbiota richness and diversity. At the genus level, compared with LPS, BL99-MV supplementation enhancing the abundance of *Oscillibacter*, *Clostridium*, *Pseudoflavonifractor*, *Lactobacillus*, *Lachnoclostridium*, *Ruminococcaceae*, *Akkermansia* and *Bifidobacterium*, while decreased *Bacteroides, Escherichia* and *Enterococcus* (Figure 7B). Compared with LPS group, *Oscillibacter* and *Pseudoflavonifractor* abundance were increased to improve cognitive deficits

in curcumin supplementation group[46]. Compared with LPS group, *Clostridium* and *Lactobacillus* abundance were increased to decreased the pro-inflammation levels in Jasmine tea supplementation group[101]. Compared with LPS group, *Lactobacillus*, *Lachnospiraceae*, and *Ruminococcaceae* abundance were increased to improve the neurotransmitter concentration (5-HT) in Tianyi Liquid supplementation group[12]. Ellagic acid significantly improved cognitive impairment and hippocampal damages in mice by enriching *Akkermansia muciniphila*, *Bifidobacterium*, *Clostridium* and *Lactobacillus*[95]. These microbiomes were associated with cognitive function, as shown in previous studies. In this study, compared with the LPS group, BL99-MV groups exhibited a progressive trajectory to the control group in the microbial composition. Notably, compared with LPS, BL99-MV supplementation significantly increased the abundance of *Oscillibacter* and *Clostridium* to improve cognitive impairment ($P < 0.05$) (Figure 7C-D), consistent with recent studies that increasing *Oscillibacter* and *Clostridium* expression to regulates gut homeostasis[24; 95]. While our integrated analysis establishes a compelling association between BL99-MV-induced gut microbiota restructuring and the alleviation of neuroinflammation. However, the correlative nature of this evidence constitutes a key limitation. Future studies employing fecal microbiota transplantation or antibiotic perturbation experiments to test whether gut microbiota remodeling is indispensable for BL99-MV-mediated neuroprotection.[7; 8]. Consequently, while our data support the proposed model of gut microbiota-mediated neuroprotection, this mechanism should be viewed as a well-supported but provisional hypothesis.

An important link between the gut microbiota and HNF was metabolites[35; 49]. Recent studies have highlighted the role of metabolites in HNF[33; 82]. Compared with LPS group, BL99-MV supplementation increasing the level of LA, SDA, Kynurenic Acid, N-Formylanthranilx Acid, Tryptophan, 5-HT and Xanthurenic Acid (Figure 8C). An enrichment of LA, SDA, Tryptophan and 5-HT could amelioratie cognitive dysfunction[42; 68; 97]. Compared with LPS group, BL99-MV supplementation significantly increasing the level of LA, Tryptophan and 5-HT to relieve HNF, further improve cognitive function (Figure 8I). 5-HT is synthesized from tryptophan through a series of enzymatic steps, which play a significant role in cognitive functions[44; 85]. It contributes to neuronal maturation and synaptic plasticity in the hippocampus[26]. The lack of 5-HT in the hippocampus affected the proper wiring of the brain in a manner that may trigger the emergence of cognitive disorders[17]. High level of LA and ALA were associated with cognitive performance[80]. In our study, when HNF occurs, BL99-MV promotes the conversion of LA to ALA. LA was significantly suppressed pro-inflammatory cytokines to alleviate memory loss in the hippocampus[4; 98]. Our ELISA results showed that BL99-MV increasing LA concentrations in the hippocampus ($P < 0.05$), which consistent with previous[106]. ALA supplementation significantly promoting Aβ1-42 degradation to prevent learning and memory deficits in the hippocampus[48]. Interestingly, ALA and SDA are precursor fatty acids for the endogenous synthesis of EPA and DHA [77]. The EPA and the DHA are prevalent in the hippocampus, altering the regulation of 5-HT to improve cognitive impairment[28; 70].

Because of the impact of the gut microbiome on cognitive impairments triggered by HNF, we hypothesized that microbial metabolites could potentially exert an influence on cognition. Furthermore, the

metabolomic results showed that BL99-MV reconstructed the metabolic disturbance by regulating the level of 5-HT and LA, and correlation analysis showed that there was a strong correlation between 5-HT and LA with ***Clostridium***, which is consistent with previous literature. Interestingly, we found an association between the gut microbiota (***Oscillibacter***, ***Clostridium***, *Lachnoclostridium* and *Lactobacillus*) and Tryptophan metabolism. BL99-MV significantly contribute to metabolome differentiation (Figure 8F). These differentially altered metabolites were enriched in Tryptophan metabolism and Linolenic metabolism.

In conclusion, our results indicate that BL99-MV supplementation influences the composition of gut microbiota, increases the production of 5-HT and BDNF in mice serum and hippocampus, thereby rescues cognitive dysfunction, and reduces Aβ1-42 deposition through IL-33 expression suppression under HNF conditions. Importantly, 5-HT modulation is a key mechanism driving the probiotic-derived MV benefits for both the brain and gut. Our study highlights the important signaling role of these metabolites, which ultimately contribute to an enhanced comprehension of metabolism integration, enhance cognitive abilities, and slow the progression of HNF. These findings suggest a novel and promising preventive measure for managing cognitive dysfunction or other gut-brain axis-related disorders.

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Informed Consent Statement

Not applicable.

Data Availability Statement

 Sequencing data generated in this study were deposited in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/, accessed on xxxx 2024) with the BioProject ID:xxxxxxx.

Notes

The authors declare no competing financial interest.

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