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Effect of dendrobium mixture in alleviating diabetic cognitive impairment associated with regulating gut microbiota

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ABSTRACT

Dendrobium mixture (DM) is a patent Chinese herbal formulation consisting of Dendrobii Caulis, Astragali Radix, Rehmanniae Radix as the main ingredients. DM has been shown to alleviate diabetic related symptoms attributed to its anti-hyperglycaemic and anti-inflammatory activities. However, the effect on diabetic induced cognitive dysfunction has not been investigated. This study aims to investigate the effect of DM in improving diabetic cognitive impairment and associated mechanisms.

Our study confirmed the anti-hyperglycaemic effect of DM and showed its capacity to restore the cognitive and memory function in high fat/high glucose and streptozotocin-induced diabetic rats. The neuroprotective effect was manifested as improved learning and memory behaviours, restored blood-brain barrier tight junction, and enhanced expressions of neuronal survival related biomarkers. DM protected the colon tight junction, and effectively lowered the circulated proinflammatory mediators including tumour necrosis factor-α, interleukin-6 and lipopolysaccharides. In the gut microbiota, DM corrected the increase in the abundance of *Firmicutes*, the increase in the ratio of *Firmicutes/Bacteroidetes*, and the decrease in the abundance of *Bacteroidetes* in diabetic rats. It also reversed the abundance of *Lactobacillus*, *Ruminococcus* and *Allobaculum* genera. Short chain fatty acids, isobutyric acid and ethylmethylacetic acid, were negatively and significantly correlated to *Ruminococcus* and *Allobaculum*. Isovaleric acid was positively and significantly correlated with *Lactobacillus*, which all contributing to the improvement in glucose level, systemic inflammation and cognitive function in diabetic rats.

Our results demonstrated the potential of DM as a promising therapeutic agent in treating diabetic cognitive impairment and the underlying mechanism may be associated with regulating gut microbiota.

1. Introduction

Type 2 diabetes (T2D) is one of the most prevalent chronic diseases. There are 422 million T2D patients worldwide with 1.6 million deaths each year [1]. Diabetic cognitive impairment (DCI), a chronic and severe complication of T2D has serious consequences for public health and has markedly affected quality of life and wellbeing of many T2D patients [2]. DCI is manifested by impaired cognitive function and memory loss caused by hyperglycaemia-induced neuropathological abnormalities in the central nervous system [3,4]. The underlying mechanisms remain

Abbreviations: ANOVA, analysis of variance; BBB, blood brain barrier; BDNF, brain-derived neurotrophic factor; Con, control; DCI, diabetic cognitive impairment; DM, Dendrobium mixture; FBG, fasting blood glucose; HFD, high fat/high glucose diet; IL-6, interlukin-6; LPS, lipopolysaccharides; Mod, model; OGTT, oral glucose tolerance test; SD rats, Sprague-Dawley rats; SCFAs, short-chain fatty acids; STZ, streptozotocin; T2D, type 2 diabetes; TNF-α, tumour necrosis factor-α; MWM, Morris water maze; ZO-1, zonula occludens.

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unclear, however, pathological events including impaired insulin signalling, inflammation, and microbiota dysregulation were suggested to be involved in the development of DCI [5].

Most recent studies have shown that gut microbiota impacts on the cognitive function of patients with metabolic disorder through the pathway referred to as the microbiota-gut-brain axis [6]. The cognitive function is particularly related to the relative abundance, distribution and diversity of the microbiota, and *Firmicutes* and *Bacteroides* are two most important phyla which account for over three-quarters of the total microbiota [2]. In addition, peripheral inflammatory process was suggested to contribute to the neurochemical abnormalities and neuronal damage mediated by the release of proinflammatory mediators including tumour necrosis factor-α (TNF-α), interlukin-6 (IL-6) and lipopolysaccharides (LPS) from gut microbiota leading to impaired gut barrier and subsequent activation of macrophages [7]. The gut microbiota dysbiosis, with the predominance of LPS has been suggested to perpetuate the inflammation process, and the microbiota-derived inflammatory response impairs the blood-brain barrier (BBB) and contributes to neuroinflammation and neurodegeneration in the brain [8].

Dendrobium mixture (DM) is a patent Chinese herbal formula developed by The Second Affiliated Hospital of Fujian Traditional Chinese Medical University (patent. no. ZL201110408411.0). The herbal ingredients in DM include Shihu (Herba Dendrobii 15 g), Huangqi (Radix Astragali 20 g), Wuweizi (Fructus schisandrae chinensis 8 g), Gegen (Radix Puerariae 15 g), Danshen (Radix salviae miltiorrhizae 20 g), Dihuang (Radix rehmanniae 18 g), Zhimu (Rhizoma anemarrhenae 12 g), *etc.* [9]. Our previous studies have demonstrated that DM effectively reduced the fasting blood glucose (FBG), blood lipid and body weight in diabetic rats [9]. It also attenuated renal damage and regulated hepatic gluconeogenesis related to the phosphatidylinositol 3-kinase signalling pathways [9,10], respectively. Dendrobii Caulis (Dendrobium Officinale Kimura et Migo), the key herbal ingredient in DM, has been suggested to ameliorate insulin resistance in rats with diabetic nephropathy, which was related to the reduction of proinflammatory regulators including TNF-α and IL-6 [11]. Its combination with American ginseng was demonstrated to modulate the gut microbiota, especially five genera that were closely associated with diabetes namely Collinsella, Rothia, Howardella, Slackia and Intestinibacter [12].

Results from previous studies have demonstrated the capacity of DM to attenuate T2D related pathological events such as hyperglycaemic, inflammation and gut microbiota disorder which were also involved in DCI. However, the effect of DM on DCI has not been investigated. Thus, the present study aimed to determine the effect of DM in attenuating DCI and its underlying mechanisms of action related to the regulation of gut microbiota.

2. Material and methods

2.1. Animals and experimental design

40 male Sprague-Dawley rats (SD rats, 6 weeks, 200 ± 20 g) were purchased from Shanghai Silaike Experiment Animal Co., Ltd. (Shanghai, China) and maintained under the following environment: 25 °C, 80% humidity, 12-h light/dark cycle, food and water ad libitum. All of the animal experiments were approved from the Animal Ethics Committee at Fujian University of Traditional Chinese Medicines. The ARRIVE guidelines [13] and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) were followed throughout the study.

After 1-week adaptive regular diet, 8 SD Rats were randomly selected as the normal control group and were fed with basal diet supplied by the Animal House affiliated to Fujian University of Traditional Chinese Medicine. Week 0, the other 32 rats were fed with high fat/high glucose diet (HFD) consisted of 10% fat, 15% sucrose, 4% cholesterol, 10% yolk powder, 0.3% cholate and 60.7% regular chow based on previous published studies [9,14]. After six weeks on HFD, rats were administered with streptozotocin dissolved in 0.1 mol/L citrate buffer (pH 4.2) twice with 72 h interval (STZ, IP injection, 25 mg/kg). Five days after the second STZ injection, fast blood glucose (FBG) level was tested from the tail vein blood. SD rats (n = 24) with FBG higher than 11.1 mmol/L were randomly divided into 3 groups based on the blood glucose stratification including model group (n = 8), DM-treated group (n = 8) and metformin group (n = 8).

The DM (patent. no. ZL201110408411.0) was prepared and provided by the Third People's Hospital, Fujian University of Traditional Chinese Medicine. The composition and preparation of the formula were described in our previous published study [9]. Briefly, the mixture was decocted by stilled water, filtered, concentrated and stored at − 80 °C until further use. The final concentrated extract of DM was equivalent to 1.08 g/mL of the raw materials in the formula. The dosage for the administration of rats in the present study was calculated equivalent to the clinical dosage which was recommended as 108 g of the raw materials per day [9]. Prior to the experiments, the key chemical components including catalpol, harpaside, puerarin, timosaponin B II, astragaloside IV, tanshinone IIA and schisandrin were confirmed using a Xevo Triple Quadrupole Mass Spectrometry (Waters, Milford, MA, USA). The total ion chromatogram of DM formula and related MS data of analytes are shown in Supplementary Material Fig. 1 and Table 1. Metformin hydrochloride (Sino-American Shanghai Squibb Pharmaceuticals Ltd., Shanghai, China) was used as the positive control group which was prepared at 20 mg/mL before the administration.

One week after the 2nd STZ injection, SD rats in the control and model groups were supplied with 0.9% saline (10 mL/kg/day) only. DM and metformin groups were treated with DM (10.8 g/kg/day) and metformin (100 mg/kg/day) by intragastric administration, the administration volume was the same as control and model group for consecutive 8 weeks. At the end of the experiment, rats were fasted for 12 h before the rapid anaesthesia using 20% Ulatan (1 g/kg i.p). Then, blood was collected from the aorta artery. Plasma was obtained by blood centrifugation at 500g for 15 min, and the supernatant was frozen at − 80 °C until analysis. The brain and colon were removed and part of them were embedded in paraffin (4%) according to the methods described in our previous study [15], the rest were stored at − 80 °C until the Western blot analysis. The faecal samples were collected and store at − 80 °C till the gut microbiota sequencing and faecal short-chain fatty acids (SCFAs) analysis.

2.2. Fast blood glucose test

For the FBG test, the animals were fasted for 9 h before their tail blood was collected and tested on the blood glucose test strips measured by the glucometer (Roche, USA). The FBG was conducted on Week 0 (baseline), Week 7 (5 days after the 2nd STZ injection), Week 11 (mid of the DM treatment) and Week 14 (the end of the DM treatment). The blood glucose measurements at each time point were conducted three times for each rat, and the averaged value was recorded.

2.3. Behavioural testing by Morris water maze task

Spatial learning and memory were examined 8 weeks after the HFD and STZ-induced DCI (diabetic) modelling using the Morris water maze (MWM) [16]. The animals were fasted 4 h before the experiment. The maze was divided into four equal quadrants by two principal axis and the end of each line demarcates four cardinal points: North (N), South (S), East (E) and West (W). A hidden circular platform was located in the centre of the Southwest quadrant as the targeted quadrant. A tracking system was used to trace the movement of each rat and record their escape latency time to reach the targeted platform. The rats received trials starting from each of the four quadrants for a total of four trials per day for four consecutive days (Day 1–Day 4) with the platform located in the same position. Each trial was terminated as soon as the rat had

climbed onto the targeted platform or when 90 s had elapsed. Escape latency times in seconds were recorded for each rat in the four groups. On the fifth day, a 90 s probe trial was performed with the platform removed from the tank. Time spent in the target quadrant and platform crossing times in the probe trial were calculated.

2.4. Pathological staining

The pathological staining followed a previous protocol [17]. Briefly, SD rat brain and colon tissues were waxed, and sectioned 4 μm thick. After dewaxed, the tissues were perfused in xylene I and II for 30 min, respectively. Then, the tissues were embedded in paraffin 4% for 72 h, dehydrated by ascending ethanol, washed and dehydrated, followed by the staining with hematoxylin and eosin. The stained sections were sealed, imaged and analysed under the light microscopy.

2.5. Peripheral proinflammatory markers

Proinflammatory biomarkers in plasma samples including TNF-α and IL-6 were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D, USA) according to the manufacturer's protocol. The absorbance was measured at 410 nm using a microplate reader (TeBao, USA). The plasma LPS levels in each group were examined using a limulus amebocyte lysate kit (Xiamen Bioendo Technology Ltd, Co., Xiamen, China) according to the manufacturer's instruction. Briefly, 100 μL of diluted plasma was dispensed to each well in a 96-well plate. At the initial time point, 100 μL of the limulus amebocyte lysate reagent was added, respectively. The plate was incubated at 37 °C for 30 min. Then, 100 μL of the chromogenic substrate warmed to 37 °C was added to each well, and incubation was extended for an additional 5 min at 37 °C. Optical density at 545 nm was measured with a microplate reader (Tebao, USA).

2.6. Immunoassays

The protein levels of zonula occludens (ZO-1), occludin, brain-derived neurotrophic factor (BDNF) tubulin-associated unit (tau), phosphorylated tau (p-tau) in hippocampal tissue and ZO-1, occludin protein in colon tissue from each group were measured by Western blot analysis. Hippocampal and colon tissues were washed twice with ice-cold phosphate buffered saline (PBS), then whole cell protein lysates were prepared by a lysis buffer supplementing with protease inhibitor mixture (Solarbio, Beijing, China) and quantified by BCA kit (Shanghai Beyotime, Ltd., Co.). Equal amount of protein samples from each group was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes (Millipore Corporation, Billerica MA 01821, USA). After blocked with 5% Skim Milk at room temperature for 1.5 h, the membranes were incubated with the following primary antibodies overnight at 4 °C: β-actin (1:3000, catalogue number: 60008-1-Ig), ZO-1 (1:1000, catalogue number: 21773-1-AP), occludin (1:500, catalogue number: 13409-1-AP), BDNF (1:1000, catalogue number: 28205-1-AP) purchased from Proteintech (USA), tau (1:1000, catalogue number: ab254256), p-tau (1:1000, catalogue number: ab109390) purchased from Abcam (UK), β-actin was used as the internal control in this study. Then, the blots were washed in PBS with Tween 20 three times (three minutes at a time) and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (Proteintech, USA) for 1.5 h. The images of the targeted bands were taken by the ChemiDoc XRS plus imaging system (Bio-Rad, Hercules, CA), and intensity was quantified by ImageJ software. The quantitative data was presented as the ratio of intensity of targeted protein to that of β-actin.

2.7. Gut microbiota sequencing and data analysis

At the end of the treatment on week 14, the fecal samples were collected in 1.5 mL centrifuge tube on ice prior to the post mortem. DNA was extracted based on the methods described in a previous study [18], and the purity and concentration of the DNA were detected by agarose gel electrophoresis. The V3-V4 region of the bacterial 16 s ribosomal RNA gene was examined by Shenzhen Weike Fuke Technology Co., Ltd. The Quantitative Insights into Microbial Ecology II (QIIME) was used for data demultiplexing and filtering. The Amplicon Sequence Variants were generated by the open-source software R package DADA2. The quality control, edits, and removal of chimeric sequences were also conducted by DADA2 to generate the taxonomical classification of operational taxonomic units (OTUs). Then the clustering of OTUs was performed using QIIME2 feature-classifier and the similarity level was set at 99% against the curated Greengenes database (version 13.8). The distance metric was visualised by principal coordinate analysis and non-metric multidimensional scaling figures. The α diversity was analysed by Shannon index and Simpson index, and the β diversity was analysed by principal coordinates analysis. The difference between groups on the Shannon index and Simpson index was analysed by Wilcoxon signed-rank test. The relative abundance of gut microbial taxa at phylum and genus-level were analysed. Specific taxa comparisons between groups were analysed using One-way analysis of variance (ANOVA) or unpaired t test. Significance was determined by $p < 0.05$.

2.8. Determination of fecal short-chain fatty acids

The SCFAs from the fecal samples were determined by an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-TQ-MS) system (Acuity UPLC-XEVO TQS, Waters Cop., Milford, MA, USA). The preparation and purification of fecal samples followed the previous protocol with a slight modification [19]. QuanMET software (V2.0, Metbo-Profile, Shanghai, China) was used to process the original data files generated by UPLC-MS/MS, and peak integration, calibration and quantification were performed for each metabolite including acetic acid, propionic acid, isobutyric acid, butyric acid, ethymethylacetic acid, isovaleric acid, valeric acid and caproic acid (purchased from Sigma, China). Phenylalanine (Sigma, China) was used as an internal standard during sample preparation to improve the stability and accuracy of the test. Calibration curves were constructed for each SCFA with linear ranges. Regression equations, $y = ax + b$, were calculated, where x and y were the concentration of the reference samples and the peak area, respectively. The quantity of each analyte was obtained from the corresponding calibration curve.

2.9. Statistical analysis

Statistical analysis was conducted using the GraphPad Prism 8.0 Software. All the results were expressed as mean ± standard deviation and/or individual data collected from rats in each group. The normality of all data was assessed by GraphPad's Shapiro-Wilk test. The differences between groups were analysed using one-way or two-way ANOVA followed by Dunnett's multiple comparison test or unpaired t test, and p value < 0.05 was considered statistically significant. The ROUT test was performed for the outliers. Pearson correlation in the GraphPad Prism was conducted to elucidate the correlation and significance of different variants. R value < 1 was considered as negative correlation whereas r value> 1 presented positive correlation. $P < 0.05$ suggested significant correlation.

3. Results

3.1. DM improved blood glucose concentrations and attenuated cognitive deficit in HFD and STZ-induced DCI (diabetic) rats

Starting from week 7 (STZ injections) to week 14 (end of observations), we noticed that the rats in the HFD and STZ-induced DCI (diabetic) group presented the following clinical alterations: frequent

urination (manifested as wet cage), reduced spontaneous motor activity, and increased food consumption. Both DM and metformin showed improvement of symptoms compared to that of the model group, but rats in the DM group showed more active spontaneous motor activity than that of metformin.

FBG test was conducted to monitor the blood glucose level on week 0, week 7, week 11 and week 14. As shown in Fig. 1A, the blood glucose concentrations were comparable at the baseline (Week 0) among the three groups ($p > 0.05$). The model group showed significantly higher blood glucose concentration at week 7 (24.6 ± 5.9 mmol/L, $p < 0.0001$), week 11 (25.0 ± 7.5 mmol/L, $p < 0.001$) and week 14 (24.53 ± 5.81 mmol/L, $p < 0.0001$) compared with the control group at the same time points, suggesting that 6-week treatment of HFD and STZ injection induced a marked increase in blood glucose level. In contrast, DM treatment significantly reduced high blood glucose level from 25.0 ± 6.0 mmol/L at week 7 to 16.8 ± 5.9 mmol/L at week 11, and 14.3 ± 2.6 mmol/L at week 14 which was significantly lower than that of the model group ($p < 0.01$), suggesting that DM treatment successfully alleviated the HFD and STZ-induced hyperglycaemia in a time-dependent manner. In addition, the effect of DM was almost comparable to that of metformin which also time-dependently reduced FBG at week 11 (15.2 ± 3.5 mmol/L, $p < 0.05$) and week 14 (11.8 ± 3.9 mmol/L, $p < 0.001$).

The effects of DM and metformin on spatial learning and memory function were tested in the HFD and STZ-induced diabetic rats which primarily depends on the hippocampus function. As shown in Fig. 1B, significantly greater escaping latency time were constantly detected in the model group from day 1 (80.7 ± 11.5 s) to day 4 (53.7 ± 17.5 s) compared to that of the control group (day 1: 48.0 ± 18.3, $p < 0.01$; day 4: 18.7 ± 6.5 s, $p < 0.001$) during the training phase, suggesting an impaired cognitive function in the diabetic animals. Metformin did not show a significant effect in reducing the escape latency time during the training phase which were comparable to that of the model group. In contrast, DM treatment significantly reduced the latency time (day 1: 66.7 ± 23.2 s; day 4: 27.6 ± 15.3 s, $p < 0.05$) compared to the model group. The representative swim pattern of animals (track plot records) during the training trails are shown in supplementary material Fig. 2. It showed that the animals in the control group were able to reach the target platform within relative short trajectory tracks, whereas animals in the model group were not able to find the target platform from day 1 and day 3 and showed very long latency and distance to reach the target platforming in day 4. Animals in the DM groups showed a remarkable improvement along the training phase, and there was no obvious improvement in the metformin group.

The probe test results (day 5) of the spatial learning capacity test are shown as a trajectory chart in Fig. 1C which represents the dwell time in each quadrant of the pool collapsed across animals in each group. The rats in the control group organised their movements around all four quadrants and spent higher amount of time near the targeted (South-west) quadrant. The rats in the model and metformin groups presented less spatial learning capacity evidenced by less trajectory tracks and higher dwelling time in the edge rather than the targeted quadrant. The DM-treated rats showed more movements in each region, and their dwelling time close to the targeted quadrant increased compared to that of the model group. The statistical comparison in Fig. 1D showed that the DM group spent significantly longer time in the targeted quadrant (25.6 ± 7.3 s) and platform crossing times (3.5 ± 1.2) compared to the model group (18.1 ± 5.5 s and 1.8 ± 1.4, both $p < 0.05$), whereas metformin (19.0 ± 6.5 s and 2.8 ± 1.7) did not shown any significant difference compared to the model group. In addition, the total distance travelled by animals within 90 s in the probe test (day 5) in the model group was significantly lower than that of the control group ($p < 0.0001$), whereas DM group showed significantly longer distance

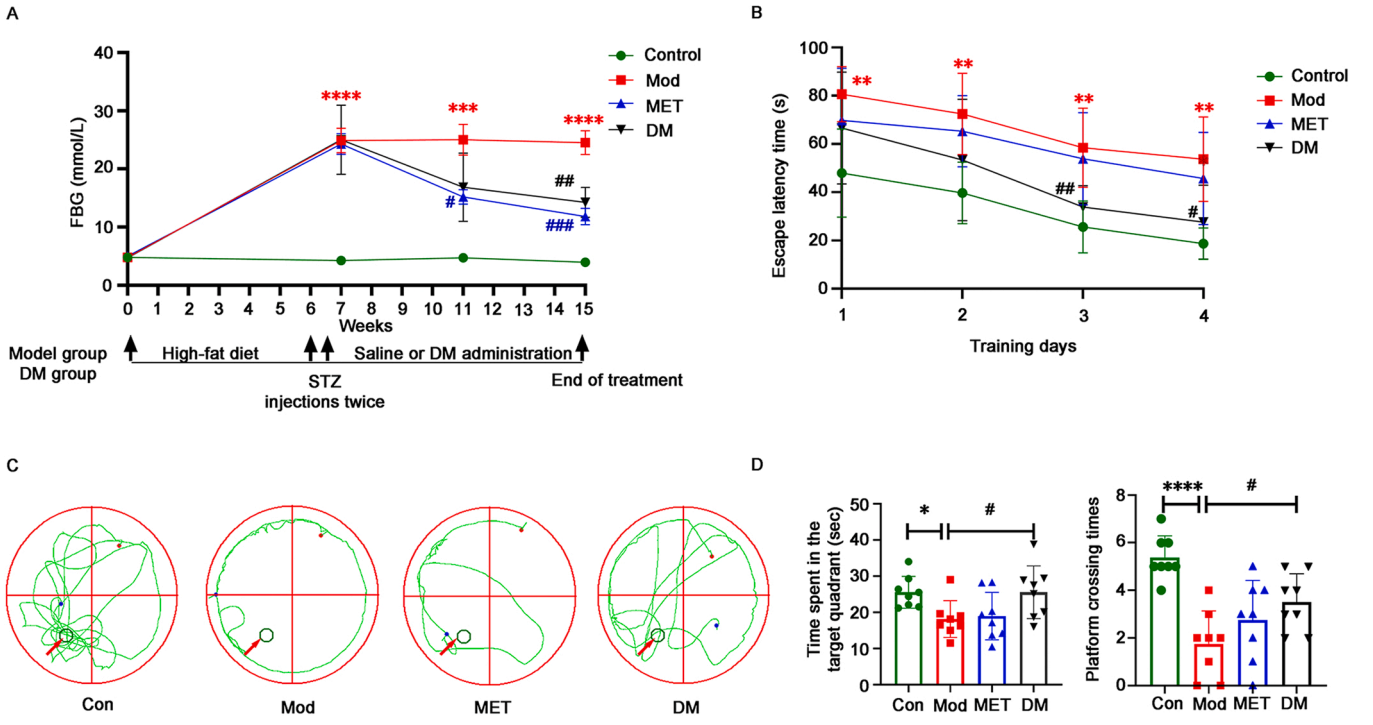

Fig. 1. The changes in blood glucose level and cognitive function in response to DM treatment. Con: Control group; Mod: Model group; DM: DM treatment group. (A). DM time-dependently reversed the STZ-induced high blood glucose level (mmol/L) in diabetic rats (n = 8 per group). (B) DM treatment exhibited time-dependent effect in reducing escape latency time in the training phase (from Day 1 to Day 4) and the probe test (Day 5). The escape latency time was compared among groups at the same time point as analysed by the two-way ANNOVA test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ *vs.* Con group. #$p < 0.05$, ##$p < 0.05$ *vs.* Mod group. (C) Representative trajectory chart (green colour) represents occupancy across the entire 90 s trial in the probe test (Day 5) with n = 8 per group. The black circles refer to the target platform. (D) Statistical analysis by one-way ANOVA showed that DM treatment increased the time spent in the target quadrant and platform crossing times compared to the Mod group. Results were shown as the mean ± SD. * $p < 0.05$ and **** $p < 0.0001$ *vs.* Con group, and #$p < 0.05$ *vs.* Mod group.

than that of the model group (both $p < 0.01$). Metformin group showed no significant difference to that of the model group. These data suggested that DM treatment appears to restore the impaired spatial memory function in the HFD and STZ-induced DCI (diabetic) rats.

3.2. DM treatment reduced the pathological changes in hippocampus CA1, protected blood brain barrier and increased neuronal survival in HFD and STZ-induced DCI (diabetic) rats

Representative H&E organization structure diagrams of the CA1 area for the hippocampal pyramidal neurons are shown in Fig. 2A. In the control group, numerous pyramidal neurons were found in the CA1 area which were closely and regularly packed with a normal morphology. However, in the model group, there was a loss of pyramidal neuronal cells, that demonstrated morphology of nuclear fragmentation (karyorrhexis) and cell shrinkage with condensed nuclei (pyknosis). DM treatment was associated with an increase in neurons number and the cells were closely packed similar to that of the control group indicating the treatment alleviated the HFD and STZ-induced pathologic changes. Our quantification results showed that the population of neurons n CA1 area per view in the model group was significantly lower than that of the control group ($p < 0.05$), whereas DM group significantly restored the neuron populations ($p < 0.05$).

The brain tight junction proteins, ZO-1 and occludin were also measured to evaluate integrity of BBB and the results were shown in Fig. 2B. ZO-1 and occludin expressions were significantly reduced in STZ-induced diabetic rats when compared with the control group ($p < 0.001$ and $p < 0.01$, respectively, Fig. 2C) suggesting impaired tight junction function of BBB in the model group. However, DM treatment was associated with a significant increase in ZO-1 ($p < 0.01$) and occludin ($p < 0.05$) expressions compared to the model group, suggesting a BBB protective effect.

The changes of BDNF protein expression, a biomarker for neuronal survival and growth were shown in Fig. 2B and C. A significant lower BDNF level was detected in the model group compared with control ($p < 0.05$), suggesting decreased neuronal activity. DM treatment group significantly increased the BDNF level ($p < 0.05$) compared with the model group. Moreover, DM treatment group significantly lowered the p-tau/tau level as compared to that of the model group ($p < 0.05$), highlighting the potential of DM in ameliorating HFD and STZ induced tau pathology. Taken together, these data suggested that the DM treatment attenuated the hyperglycaemia-induced hippocampal BBB impairment and neuron deficit.

3.3. DM restructured gut microbiota and microbial metabolites in diabetic rats

3.3.1. DM protected colon tight junction in diabetic rats

The effect of DM on the colon tight junction was evaluated by H&E staining as well as relevant tight junction protein expressions including ZO-1 and occludin. As shown in Fig. 3A, the model group showed marked pathological changes in colon sections including impaired epithelial barrier integrity and villi, damaged structure of glandular epithelium, increased lymphocytes infiltration, and goblet cell loss. Significant reductions in ZO-1 and occludin expressions were also observed in the model group (Fig. 3B, $p < 0.0001$ and $p < 0.01$, respectively). In the DM group, the colon epithelial cells demonstrated similar morphological characteristics to that of the control. Additionally, DM treatment restored protein expression of ZO-1 ($p < 0.05$) and occludin ($p < 0.05$) compared to that of the model group suggesting that DM attenuated hyperglycaemia-induced intestinal barrier impairment.

3.3.2. DM alleviated systemic inflammation in HFD and STZ-induced DCI (diabetic) rats

Peripheral inflammation was suggested to contribute to diabetes-induced neurochemical abnormalities and neuronal damage, and was

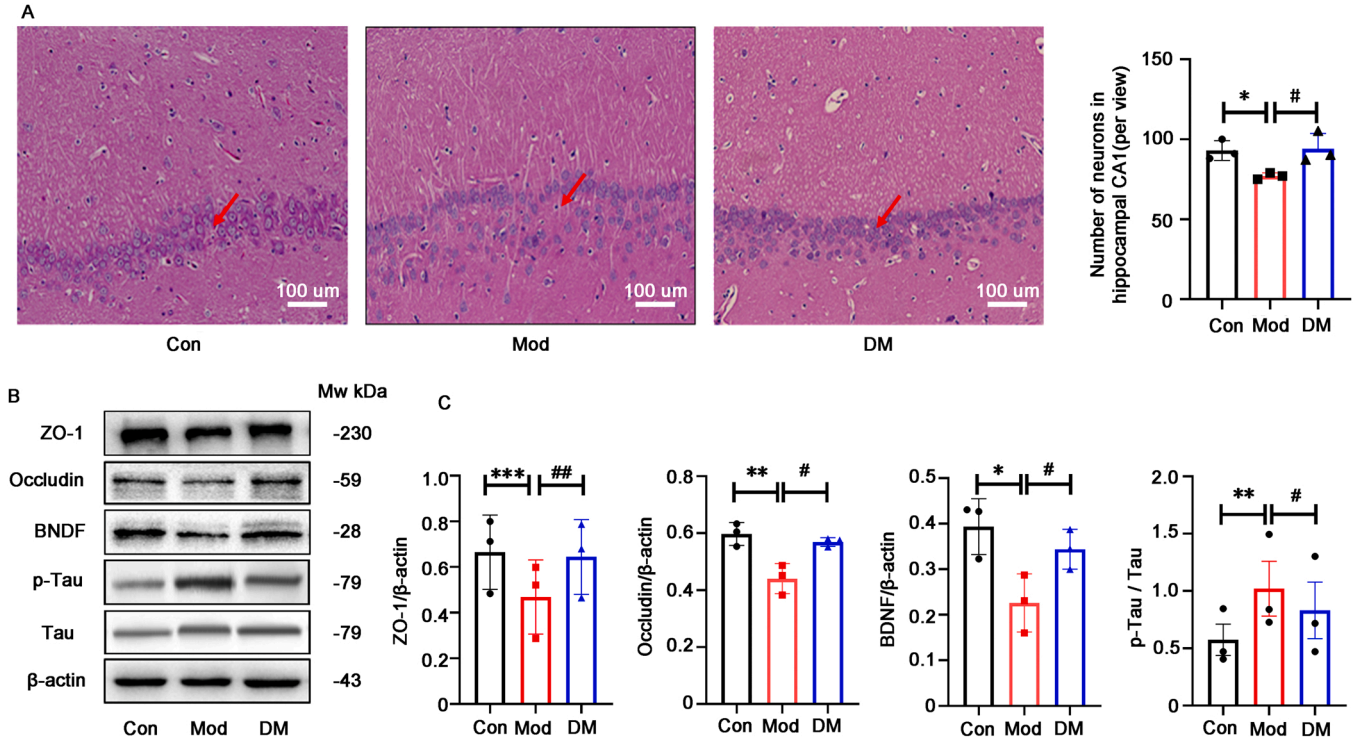

Fig. 2. Effect of DM on histopathologic changes in the rat hippocampal CA1 region. (A). Representative sections of H&E staining (magnification 200 ×). Red arrow points to the package of neurons. Statistical analysis of number of neurons in hippocampal CA1 area (per view). * $p < 0.05$ *vs.* Con group, # $p < 0.05$ *vs.* Mod group. (B). Representative Western blot images from three individual experiments and (C) their statistical analysis of protein expressions of ZO-1, occludin, BDNF, p-tau/tau in hippocampus (n ≥ 3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* Con group, # $p < 0.05$ *vs.* Mod group.

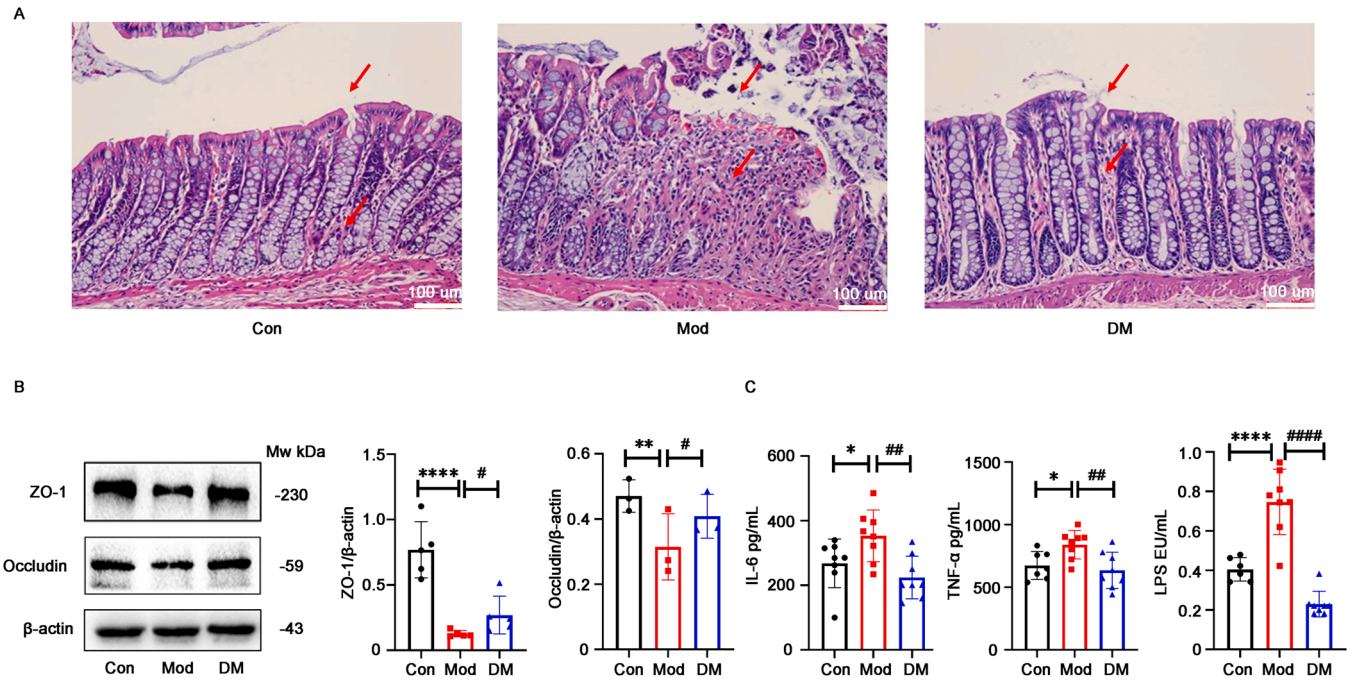

Fig. 3. Effect of DM on histopathologic changes in the rat colon sections. (A) Representative sections of H&E staining (magnification 200 ×). Red arrow represents the damaged villi. (B). Representative Western blot images and their statistical analysis of protein expressions of ZO-1, occludin in colon (at least three independent experiments), ** $p < 0.01$, **** $p < 0.00001$ *vs.* Con group, # $p < 0.05$ *vs.* Mod group. (C) DM treatment reduced plasma levels of IL-6, TNF-α and LPS in the STZ-induced diabetic rats, n = 8 in each group. * $p < 0.05$, **** $p < 0.0001$ *vs.* Con group. ##$p < 0.01$, #### $p < 0.0001$ *vs.* Mod group.

related to higher permeability of intestinal barrier. In this study the key inflammatory biomarkers in the plasma samples were evaluated. As shown in Fig. 3C, plasma IL-6, TNF-α and LPS levels increased significantly in the model group in comparison to the control group ($p < 0.05$, $p < 0.05$, $p < 0.0001$, respectively). However, the DM intervention markedly reduced the elevated levels of IL-6 ($p < 0.01$), TNF-α ($p < 0.01$) and LPS ($p < 0.0001$) compared to that of the model group. Interestingly, the LPS level in the DM treatment group was even slightly lower than that of the control. Taken together, DM treatment has markedly lowered the three key inflammatory levels in the plasma against the HFD and STZ-induced diabetes.

3.3.3. *DM regulated gut microbiota at phylum and genus levels in diabetic rats*

The gut-brain axis plays a role in regulating brain functions including learning and memory function. In this study, we have demonstrated the DM treatment restored intestinal barrier and reduced LPS in the circulation. We then examined if this effect was associated with the regulation of the gut microbiota.

Firstly, the high-quality sequences were represented into Operational Taxonomic Units (OTUs) based on 97% similarity. The species rarefaction curves tended to be flat in all three groups, indicating that the amount and depth of sequence were reasonable. We then compared the Shannon and Simpson indices which represent the overall number of bacteria in the microbial community among the three groups. DM treatment group showed a similar pattern as the control group in both Shannon and Simpson indices as opposed to that of the model group. These results indicated that the overall diversity of gut microbiota was impaired by the HFD and STZ-induced diabetes and this impairment was alleviated by the DM treatment which appeared to improve the diversity of gut microbiota.

The Unweighted Unifrac represents β-diversity. The results showed that the distribution of the dots in the model group were distinguished from that in the control group. In contrast, most dots in the DM treated group were distributed similar to that in the control group, indicating DM group shared the similar dominant species with the control group other than with the model group.

We have also compared the abundance of dominant phyla in three groups. As shown in Fig. 4A, the top five dominant phyla were *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Spirochaetes* constituting over 90% of the overall detected abundance of the gut bacteria in all groups. Heatmap in Fig. 4B shows the top 14 differentiated taxa with the highest phylum level. The results demonstrated a different clustering of the composition of microbiota community between the model and control groups, whereas the microbiota community composition in most of the DM treatment subjects were closer to that of the control group. We then conducted the statistical comparison of each of the five dominant phyla among groups as shown in Fig. 4C. *Firmicutes* ($p < 0.05$) and *Actinobacteria* ($p < 0.01$) were found to significantly increase, whereas *Bacteroidetes* decreased ($p < 0.01$) in the model group. DM treatment markedly reduced the levels of *Firmicutes* ($p < 0.001$) and *Actinobacteria* ($p < 0.05$), and restored the level of *Bacteroidetes* ($p < 0.01$). Additionally, HFD and STZ-induced diabetes significantly increased the ratio of *Firmicutes* and *Bacteroidetes* ($p < 0.01$), and such a change was significantly reversed by the DM treatment ($p < 0.001$) (Fig. 4C).

3.3.4. *DM regulated gut microbiota at genus level in diabetic rats*

The changes of gut microbiota were also investigated at the genus level. *Lactobacillus murinus*, *Prevotella* (family: Prevotellaceae), [*Prevotella*] (family: Paraprevotellaceae), *Bifidobacterium*, *Ruminococcus*, *Allobaculum*, *Oscillospira*, *Blautia* and *Streptococcus* were the dominant genus in all groups which constituted 50% of the total genus. The changes in the relative abundance of dominant microbial genus were illustrated in Fig. 5A. The relative abundance of *Lactobacillus*, *Blautia* and *Oscillospira* were significant reduced in the model group when compared to the control ($p < 0.01$, $p < 0.01$ and $p < 0.0001$, respectively) (Fig. 5B). The abundance of *Lactobacillus* was significantly restored in the DM group ($p < 0.01$), whereas there was a trend towards an increase in *Blautia* and *Oscillospira*. Increased abundance of genera in the model group was shown in *Prevotella*, *Ruminococcus*, [*Prevotella*], and *Allobaculum* ($p < 0.01$, $p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively).

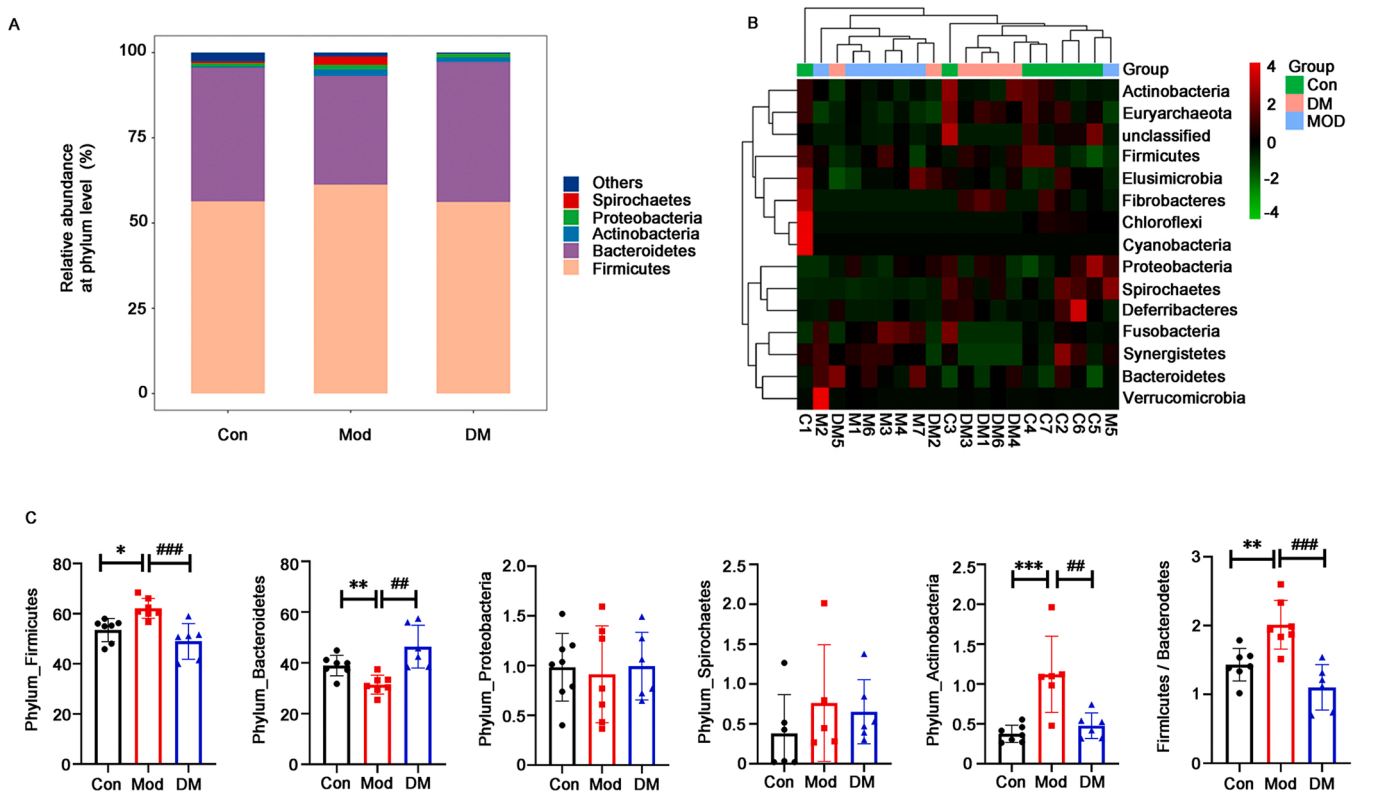

Fig. 4. DM affected the relative abundance of gut microbiota at the phylum level (n ≥ 5 rats in each group). (A) The relative abundance of the 5 top-ranked phyla. (B) Hierarchical clustering heatmap of the top differentiated taxa at the phylum level. (C) The relative abundance of *Firmicutes*, *Bacteroidetes, Actinobacteria*, *Proteobacteria* and *Spirochaetes* were compared among groups. The ratio of *Firmicutes/Bacteroidetes* was compared among three groups. The difference between groups was assessed by unpaired t-test. * $p < 0.05$, ** $p < 0.05$, *** $p < 0.001$ *vs.* Con group. ##$p < 0.01$, ###$p < 0.001$ *vs.* Mod group.

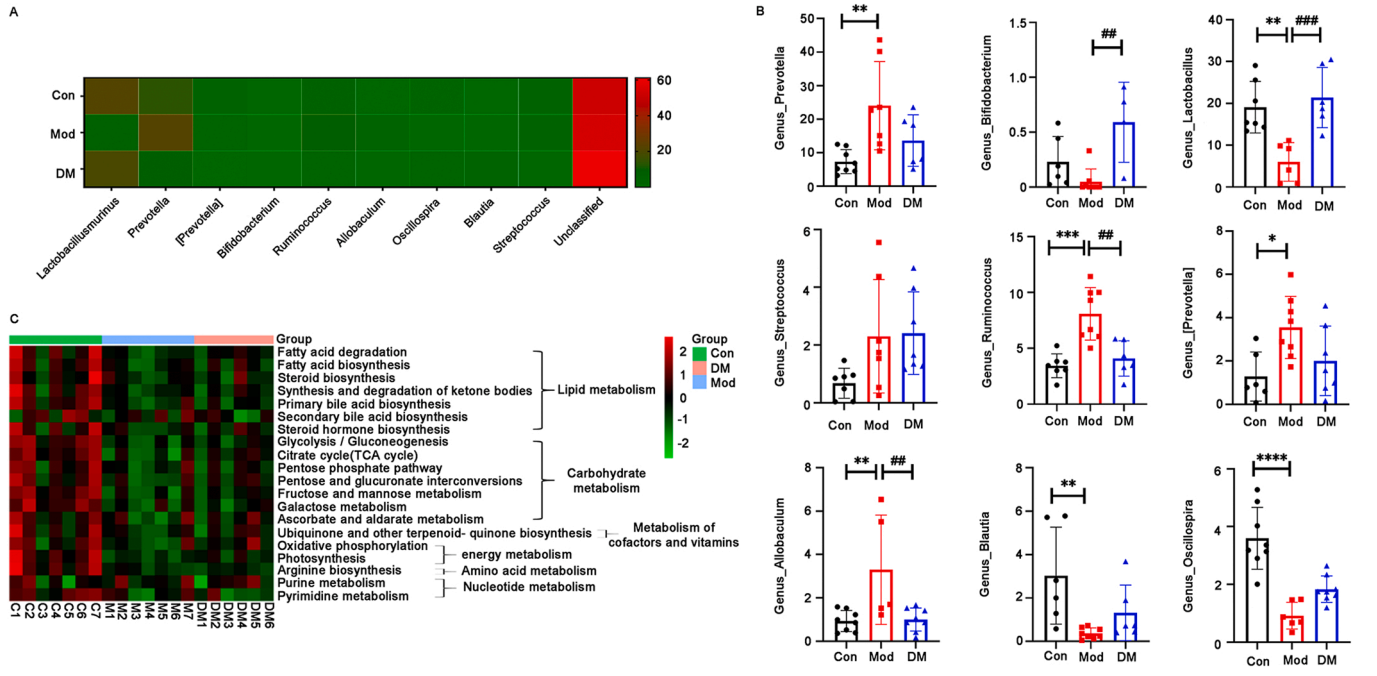

Fig. 5. DM corrected the relative abundance of gut microbiota at the genus level (n ≥ 5 rats in each group). (A) Heat map of the relative abundance of dominant microbial species altered by DM treatment. Red colour indicates high values, whereas green colour means low values. (B) The comparison of relative abundance of *Lactobacillus*, *Prevotella, [Prevotella], Ruminococcus*, *Allobaculum*, *Blautia*, *Oscillospira*, *Streptococcus*, *Bifidobacterium* in the three groups (n ≥ 6 rats per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ *vs.* Con group, ##$p < 0.01$, ###$p < 0.001$ *vs.* Mod group. Differences were examined by One-way ANOVA test. *Prevotella* was under the family *Prevotellaceae,* and *[Prevotella]* was under the family *Paraprevotellaceae.* (C) KEGG metabolic pathway prediction by PICRUSt analysis.

The DM treatment significantly reduced the abundance of *Ruminococcus* and *Allobaculum* compared to the model group ($p < 0.01$ and $p < 0.05$, respectively), and their levels were similar to the control group. Taken together, these finding suggested that the derivatives of *Lactobacillus*, *Ruminococcus* and *Allobaculum* were the key distinguishing alterations for group separation.

In addition, 20 altered functional characteristic pathways of Kyoto Encyclopedia of Genes and Genomes (KEGG) were predicted from the 16S rRNA gene profile through Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis, namely, 7 lipid metabolism, 7 carbohydrate metabolism, 1 metabolism of cofactors and vitamins, 2 energy metabolism, 1 amino acid metabolism and 2 nucleotide metabolism pathways (Fig. 5C). Thus, these findings indicated lipid metabolism maybe involved in the regulated gut microbiota as the main altered metabolic pathway by DM in diabetic rats.

3.3.5. *DM modulated fecal short chain fatty acids in diabetic rats*

Since the lipid metabolism pathways have been shown as the leading metabolic pathways corresponding to the regulation of microbiota by DM, we have then examined the key SCFAs levels in the fecal samples as part of the lipid metabolism among the three groups. As shown in Fig. 6A, HFD and STZ-induced diabetes has significantly reduced the amount of isobutyric acid ($p < 0.01$), butyric acid ($p < 0.05$), ethylmethylacetic acid($p < 0.01$) and isovaleric acid ($p < 0.01$). The DM treatment significantly reversed the reduced isobutyric acid, ethylmethylacetic acid and isovaleric acid ($p < 0.01$). The DM treatment was also associated with an increase in butyric acid, however, the change did not reach a statistical significance.

The correlation between the key SCFAs and the biomarkers of DCI including MWM (escape latency time), LPS, TNF-α, IL-6 and FBG were investigated. As shown in Fig. 6B, genera that were upregulated by the DM treatment including *Lactobacillus, Bifidobacterium, Oscillospira* and *Blautia* were all negatively correlated with FBG, MWM, LPS, TNF-α and IL-6 ($r < 0$). The negative correlation between *Lactobacillus* and TNF-α reached statistical significance ($p < 0.05$). Genera that were reduced by DM treatment including *Prevotella, [Prevotella], Ruminococcus, Allobaculum* and *Streptococcus* were all positively correlated with FBG, MWM, LPS, TNF-α and IL-6 ($r > 0$). The significance was seen in the correlation of *Allobaculum* and MWM ($p < 0.05$). Additionally, we have noticed that isobutyric acid was negatively and significantly correlated with *Rumunocuccos* ($r = -0.998$, $p = 0.044$) and *Allobaculum* ($r = -1$, $p = 0.003$) in the fecal samples. Ethylmethylacetic acid was also negatively and significantly correlated with *Rumunocuccos* ($r = -1$, $p = 0.005$), and *Allobaculum* ($r = -0.998$, $p = 0.036$). Isovaleric acid was found to be positively and significantly correlated to *Lactobacillus* ($r = 0.943$, $p = 0.049$).

4. Discussion

We have previously reported that the DM treatment effectively reduced the high blood glucose level in diabetic rats [9]. In the current study, we investigated the effects of DM in attenuating DCI in HFD and STZ-induced diabetic rats and the results have shown DM improved the MWM test behaviour, and restored the levels of neuronal survival-related biomarkers. Moreover, our results demonstrated that the DM treatment alleviated the HFD and STZ-induced impairments in intestinal tight junction barrier, plasma proinflammatory marker levels, gut microbiota composition and their metabolites of SCFAs. The study has for the first time shown that the mechanism underlying DM's effects in restoring cognitive and memory function maybe associated with its capacity to regulate gut microbiota, particularly phyla of *Firmicutes*, *Bacteroidetes*, genera of *Lactobacillus*, *Ruminococcus* and *Allobaculum* and their metabolites, SCFAs including isobutyric acid, isovaleric acid and ethylmethylacetic acid.

The DM mixture, especially its principal herb Dendrobii Caulis has been traditionally used for the treatment of diabetic related syndrome [20–22]. Our current study showed that the blood glucose level was significantly reduced after 8-week treatment of DM compared to the model group and this is consistent with that of previous studies in high-fat/high-glucose rats [9]. Additionally, our results suggested that DM alleviated the impaired cognitive and memory function evidenced by the reduced escape latency time, prolonged time spent in the target quadrant and repaired the pathological changes in the hippocampus CA1 area. However, the treatment of metformin did not show a significant effect. These results provide new evidence to support the use of DM in DCI. BBB integrity is critical in neuroprotection and maintaining homoeostasis in the brain [23]. Our study demonstrated that DM protected BBB integrity by restoring tight junction proteins including ZO-1 and occludin leading to neuroprotective effects. In addition, the pathological mechanism underlying DCI involves direct neuronal damage and apoptosis caused by the lack of intracellular glucose. Our study

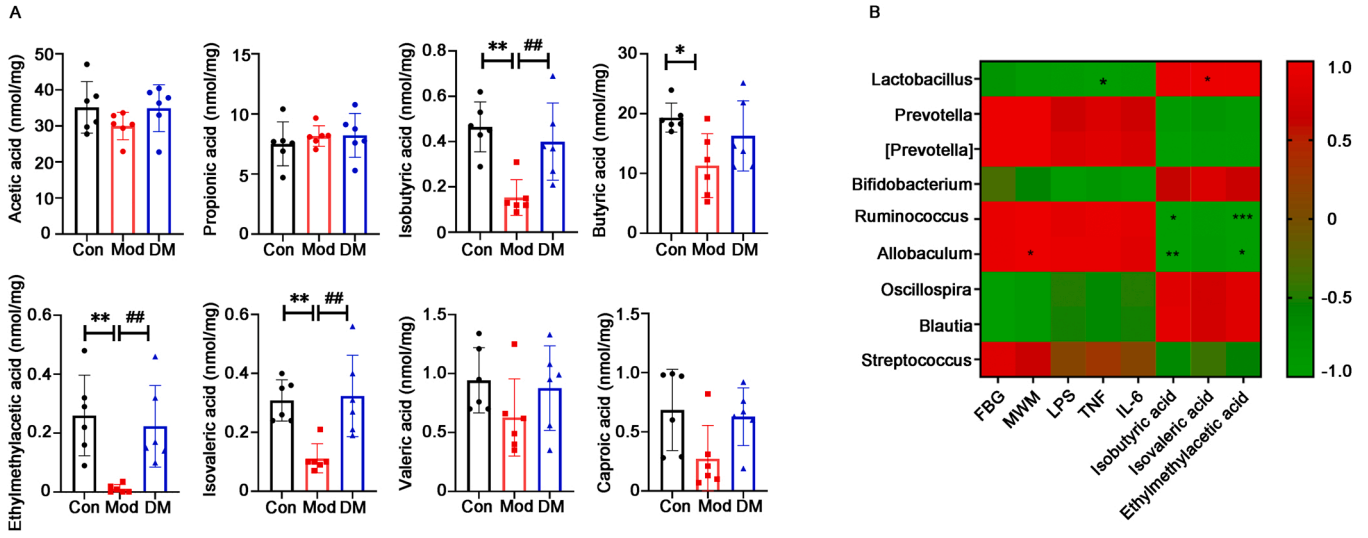

Fig. 6. DM restored the fecal SCFAs in diabetic rats, and the correlation between the changed genera responding to DM and diabetic-related index (n = 6 rats in each group). (A) Comparison of identified fecal short chain fatty acids in the three groups analysed by one-way ANNOVA test. * $p < 0.05$, ** $p < 0.01$ *vs.* Con group; ##$p < 0.01$ *vs.* Mod group. (B) Correlation heatmap of changed genera responding to DM and diabetic related index including FBG, MWM, LPS, TNF-α, IL-6, isobutyric acid, isovaleric acid and ethylmethylacetic acid. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for the correlation.

examined several key biomarkers related to neuronal survival to elucidate the mechanism of DM for neuroprotection. Activated BDNF has been suggested to exhibit a positive effect to promote neural regeneration and rehabilitation of cognition and memory, and to reduce inflammation, neurotoxicity and apoptosis [24]. In addition, the abnormally hyperphosphorylated tau is linked with diabetes-associated cognitive deficits and presently considered as a characteristic neuropathological hallmarker for tauopathies, of which Alzheimer disease (AD) is the most common form [25]. Our results demonstrated that DM treatment significantly reversed the elevated p-tau expression which suggested a potential of DM in preventing high glucose triggered tauopathies and consequent development of DCI.

Gut microbiota plays an essential role in the cognitive function which involves a series of dynamic changes in the body through the microbiota-gut-brain axis [26]. The disordered gut microbiota composition is manifested as intestinal inflammation and impaired intestinal tight junction with higher permeability, which in turn causes peripheral inflammation, and ultimately promote the development of diabetes and DCI [27]. In this study, we examined the morphological changes of epithelial tight junction in the colon, and observed that DM repaired the damaged epithelial tight junction structure and restored the protein expressions of ZO-1 and occludin. A clinical study in T2D patients suggested that the loss of ZO-1 drives translocation of LPS and other bacteria from guts to the circulation. The circulated LPS activates microphages and thus increased the proinflammatory cytokines such as IL-6 and TNF-α *via* TLR4-NFκB pathway [28]. Our results showed that DM significantly lowered LPS, TNF-α and IL-6. The lowered LPS level in the plasma was likely to be associated with repaired colon tight junction leading to reduced LPS translocation. In addition, reduced TNF-α and IL-6 levels were suggested to contribute to the alleviated neurochemical and structural abnormalities, neuronal damage mediated by macrophages activation and release of proinflammatory mediators [7].

Many studies have linked the intestinal mucosal barrier to the regulation of intestinal flora, especially restored probiotics as an essential antidiabetic mechanism [29–31]. In the current study we observed a powerful regulative effect of DM on the overall diversity and dominant species of gut microbiota. Additionally, we examined the changes of dominant phyla and genera to determine the bacteria responsible for the regulatory effect of DM. At the phylum level, *Firmicutes* and *Bacteroidetes* are two most important phyla in the gastrointestinal tract that are relevant to energy metabolism homoeostasis [32], and the increased ratio of F/B is usually observed with obesity [33]. DM corrected the F/B ratio which may link to the anti-hyperglycaemic and anti-hyperlipidaemia properties of Dendrobii Caulis [34]. Many studies have suggested that probiotics contributed to the restoration of dysbiotic microbiota and may modify the F/B ratio. The most commonly tested probiotics that modify the F/B ratio were from the genus *Lactobacillus*. For example, a randomised, double-blind, placebo-controlled trial showed that administration of *Lactobacillus gasseri* showed beneficial influence on metabolic disorder in adults with obese tendencies [35]. Another randomised, double-blind controlled trial showed that probiotic yogurt contained *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 improved total cholesterol and low density lipoprotein cholesterol level in T2D people [36]. At the genus level, our results showed that DM significantly reversed the diabetes-induced reduction of *Lactobacillus* and *Bifidobacterium*, suggesting that the two genera restored the homoeostasis of gut microbiota. Numerous studies have demonstrated that the species from these two genera maybe linked with improved cognitive and memory ability. Their actions on cognitive function maybe linked with gut-brain axis through attenuated circulated inflammation [37] and neuro-inflammation as well as modulating regional brain metabolites [38]. As demonstrated in Ni (2019), the administration of *Lactobacillus casei* LC122 or *Bifidobacterium longum* BL986 ameliorated oxidative stress and inflammation in peripheral tissues, and improved gut barrier function. Moreover, they have altered the diversity and composition of gut microbiota leading to the regulated metabolism functions which may be linked with improved cognitive function [39]. The oral gavage of *Lactobacillus plantarum* NK151 and *Bifidobacterium longum* NK173 were shown to modified the microbiome and significantly reduced inflammation in hippocampus leading to alleviated cognitive impairment [40]. In particular, a study from Lee HJ et al. (2019) suggested that the mechanistic action of *Bifidobacterium longum* in mitigating cognitive decline through the gut-brain axis was associated with the suppression of gut microbiota endotoxins such as LPS induced hippocampal inflammation *via* the NF-κB activation which led to reduced hippocampal Aβ plaque accumulation [41]. The knowledge sheds light on the mechanistic study of DM on cognitive function through the influence of *Bifidobacterium and Lactobacillus* species and attenuated neuroinflammation induced by gut microbiota endotoxins such as LPS [41].

DM was also found to regulate some other excessively expressed genera including *Ruminococcus* and *Allobaculum*. A study by Wan et al. [42] suggested that *Ruminococcus* was typically responded to the treatment of Chlorella pyrenoidosa in rats, and thus it may play a vital role in the treatment of diabetes. A study from Zhou et al. [43] suggested that random blood glucose level was positively correlated to *Ruminococcus* and *Allobaculum* in Zucker diabetic fatty rats. Moreover, the actions of these two genera on gut-brain axis were also associated with inhibited level of LPS and TNF-α [44]. Thus, the restoration of these genera by DM may all contribute to its overall regulation of gut microbiota leading to the alleviation of diabetes and DCI.

As an important component of the brain, lipids play a crucial role in cell signalling and various physiological processes [45]. Our predicted KEGG results suggested that lipid metabolism pathways were one of the main alterations in the gut microbiota. As a member of the lipids family, SCFAs are the main metabolites produced in the colon by the intestinal microbiota which are closely associated with brain function and neurobiological effects in systemic circulation [46,47]. For example, lower levels of microbial SCFAs are associated with increased systemic inflammation in diabetic individuals which were linked to gut dysbiosis and cognitive impairment [48]. Our study has shown that HFD and STZ-induced diabetes reduced the levels of isobutyric acid, isovaleric acid and ethylmethylacetic acid. Similarly, previous study in rodents fed with saturated fat and added sugars (Western style diet) demonstrated that the reduced SCFAs were a putative contributor to the subsequent cognitive dysfunction. Our results showed that DM reversed the diminished fecal SCFAs, indicating that enhanced fecal SCFAs modulated by DM may play a role in the improved learning and long-term function against diabetes through the gut-brain axis. Isovaleric acid and isobutryic acid were hypothesised to serve as substrates for energy metabolism, providing an alternative energy source to rectify brain hypo-metabolism that contributes to neuronal dysfunction [49,50]. To elucidate the link of SCFAs to cognitive function related factors (FBG, MWM test and systemic inflammation) and the changes in genera responded to DM, we have conducted a Pearson correlation analysis. Our results revealed that isobutyric acid and ethylmethylacetic acid were principal SCFAs that may be involved in the negative regulation of *Ruminococcus* and *Allobaculum* which were both positively correlated with high FBG, systemic inflammation and impaired cognitive function. On the other hand, isovaleric acid was the key SCFA that was positively correlated with *Lactobacillus* which contributed to attenuate FBG, inflammation and cognitive dysfunction. However, further studies are warranted to confirm the specific genera that produced SCFAs metabolites contributing to the improved DCI.

5. Conclusion

Eight-week oral administration of DM improved blood glucose and cognitive function in HFD and STZ-induced DCI rats. The DM treatment restored the intestinal barrier integrity and reduced the circulated proinflammatory mediators including TNF-α, IL-6 and LPS. The reduced systemic inflammation was likely to be associated with gut microbiota

regulation. DM improved the overall diversity, and corrected relative abundances of *Firmicutes* and *Bacteroidetes* and their ratio at the phylum level, and *Lactobacillus, Ruminococcus* and *Allobaculum* at the genus level. Moreover, DM was found to reverse the reduced levels of several SCFAs including isobutyric acid, isovaleric acid and ethylmethylacetic acid. In particular, isobutyric acid and ethylmethylacetic acid were linked to reduced excessive abundances of *Ruminococcus* and *Allobaculum*, and isovaleric acid was correlated with the increased level of *Lactobacillus*, which all contributed to reduced blood glucose, improved cognitive function, and suppressed systemic inflammation.

Taken together, our findings provide new and comprehensive evidence to support the use of DM as a promising standardised herbal preparation for the treatment of diabetic cognitive dysfunction. Moreover, our study revealed that the mechanisms underlying the neuroprotective effect of DM were likely associated with the regulation of gut microbiota and metabolic short-chain fatty acids through the gut-brain axis.

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CRediT authorship contribution statement

YFZ, XZ, JPZ and **HS** participated in the study design. **JLZ, STZ, CXW, YC, WX, XNW, CGH, WL** and **XHL** performed the experiments. **XZ, JYC, YBS, LHN, LD** and **MQH** performed the data analysis. YFZ and HS confirm the authenticity of all the raw data. **XZ, YFZ** and **DC** drafted and reviewed the manuscript. All authors have read and approved the final manuscript.

Declaration of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. This manuscript is not under consideration for publication elsewhere. All authors agreed to submit the manuscript to Biomedicine&Pharmacotherapy. If accepted, it will not be published elsewhere in the same form. As a medical research institute, NICM Health Research Institute receives research grants and donations from foundations, universities, government agencies, individuals and industry. Sponsors and donors also provide untied funding for work to advance the vision and mission of the Institute.

Data availability

Data will be made available on request.

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Data Availability Statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author/s.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112891.

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