Arch Biol Sci. 2025;77(3):225-236 https://doi.org/10.2298/ABS250531018M

Therapeutic effects of Mongolian medical warm acupuncture on neuropathic pain via gut microbiota and inflammatory pathway regulation in a chronic constriction injury rat model

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Received: May 31, 20255; **Revised**: June 23, 2025; **Accepted**: June 24, 2025; **Published online**: June 30, 2025

Abstract: The study was designed to determine whether Mongolian medical warm acupuncture (MMWA) can alleviate neuropathic pain through regulating gut microbiota and inflammation-related pathways in the chronic constriction injury (CCI) rat model. Rats were randomly divided into three groups: sham, model, and treatment. A bilateral chronic constriction injury (CCI) was induced to establish a rat model of neuropathic pain. Rats in the treatment group received MMWA at the Heyi and Shen acupoints once daily for seven consecutive days. Pain-related behaviors were evaluated using mechanical pain threshold and thermal withdrawal latency measurements. The mRNA levels of *c-fos* in spinal dorsal horns were determined using real-time PCR. Gut microbiota was analyzed by 16S ribosomal RNA sequencing. Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were evaluated by enzyme-linked immunosorbent assay (ELISA) analysis. MMWA could relieve behavioral symptoms ($P<0.01$). MMWA intervention significantly suppressed the expression of *c-fos* in CCI rats ($P<0.05$). The relative abundance of gut bacteria was disturbed in CCI rats, with the microbiota disorder ameliorated by MMWA. KEGG enrichment analysis revealed that changes in gut microbiota were associated with the pentose and glucuronate interconversion pathway and secondary bile acid biosynthesis. The concentrations of IL-1β, IL-6, and TNF-α in CCI rats were increased ($P<0.05$), an effect that was reversed by MMWA intervention ($P<0.05$). The current study indicated that the MMWA could effectively alleviate neuropathic pain by modulating gut microbiota and inhibiting inflammation in the CCI rat model, suggesting that gut microbiota could be a promising potential biological target for treating neuropathic pain.

Keywords: Mongolian medical warm acupuncture, neuropathic pain, chronic constriction injury, gut microbiota, inflammation

Abbreviations: CCI – chronic constriction injury; ELISA, Enzyme-linked immunosorbent assay; MMWA – Mongolian medical warm acupuncture; IL-1β – interleukin-1β; IL-6 – interleukin-6; TNF-α – tumor necrosis factor-α

INTRODUCTION

Neuropathic pain is defined as a painful condition caused by neurological lesions or pathological changes affecting the somatosensory system [1]. It has been reported that the prevalence of pain with neuropathic characteristics is 6.9-10% in the general population [2]. *c-fos*, an immediate-early gene, serves as a general marker of neuronal activity, allowing for more direct analysis of neuronal networks involved in neuropathic pain [3]. Despite advances, the underlying mechanism of neuropathic pain remains largely unknown. Current treatments for neuropathic pain are often ineffective and/or produce severe side effects [4]. Gabapentin, pregabalin, amitriptyline, and duloxetine are commonly used as first-line pharmacological treatments for neuropathic pain. The side effects of these agents include lethargy, vertigo, peripheral swelling, blurred vision, nausea, anticholinergic effects, or hypertension [5]. Their use is limited by their side effects. To develop more effective therapeutic strategies for neuropathic pain, it is essential to first understand the pathogenesis of neuropathic pain.

The human gut contains trillions of bacteria [6]. Recent evidence has shown that the gut microbiota is essential for maintaining the two-way interaction between the gut and the brain through neural, endocrine, and immune pathways [7]. It is noteworthy that the disorders of this gut-brain signaling contribute significantly to causing various nervous system diseases, such as Parkinson's disease, Alzheimer's disease, schizophrenia, multiple sclerosis, and stroke [8]. Accumulating evidence suggests that the gut microbiota and its metabolites play key roles in neuropathic pain [9-11]. They directly or indirectly influence the development of neuropathic pain through the regulation of immune, metabolic, endocrine, and neural signaling pathways. Preclinical studies investigating interventions related to the gut microbiome for the treatment of neuropathic pain demonstrate substantial potential [12]. Jing et al. reported that fecal microbiome transplantation from healthy mice into spinal cord injury animals exerts neuroprotective effects, possibly through the remodeling of the gut microbiome [13].

Traditional Mongolian medicine, an ancient practice, has demonstrated beneficial therapeutic effects on neuropathic pain [14]. Among its treatments, Mongolian medical warm acupuncture (MMWA) is a key traditional therapy. It has been widely used in the treatment of nervous system diseases through acupoint stimulation with warm needles and is considered a safe and feasible therapy [15,16]. MMWA has been reported to alleviate chronic exhaustive swimming-induced fatigue and enhance learning and memory by reducing levels of nitric oxide synthase and the proinflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α [17]. In a recent study, we found that MMWA regulated the signaling pathways associated with insomnia by modulating gut microbial dysbiosis in p-chlorophenyl alanine (PCPA)-induced insomnia rats [18]. Additionally, with the growing recognition of the gut-immune interaction's role in neuropathic pain [19], MMWA presents a promising treatment option. However, its effects on gut microbiota dysbiosis in neuropathic pain models remain unclear.

Recent studies have explored the relationship between gut microbiota, inflammation, and neuropathic pain in chronic constriction injury (CCI) rat models. For example, gut microbiota has been associated with neuropathic pain, potentially affecting pro- and anti-inflammatory T-cell responses [11]. Also, changes in gut microbiota and metabolite profiles were observed in CCI rats, with significant correlations between microbial abundances and serum metabolite levels [9]. MMWA has demonstrated potential in alleviating insomnia by modulating gut microbiota and serum metabolites, suggesting similar mechanisms may apply to neuropathic pain [18]. Additionally, emodin, a natural compound extracted from *Rheum palmatum*, has been shown to have analgesic effects in CCI rats by altering gut microbiota community structure, inhibiting inflammatory responses, and increasing beneficial metabolites like S-adenosylmethionine and histamine in the spinal cord [20]. These findings highlight the importance of the gut-brain axis in neuropathic pain and potential therapeutic approaches. In the present study, we investigated the influence of MMWA on alterations in gut microbiota and inflammation in the CCI rat model. Our findings may assist in understanding the possible mechanism through which the MMWA exerts its therapeutic effect on neuropathic pain.

MATERIALS AND METHODS

Ethics statement

Procedures for the animal experiments followed the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain. This study was approved by the Animal Ethics Committee of Baotou Medical College (Ethics number: 2021039).

Animals and CCI rat model

Male Sprague-Dawley (SD) rats (6 weeks of age, weighing 160-180 g) provided by Vital River Laboratory Animal Technology (Beijing, China) were maintained in individual ventilated cages under controlled conditions of a 12-h day/night cycle and 24±2°C. All animals were allowed free access to food and water. Animals were included in the study only if they successfully underwent CCI surgery. Those that died prematurely, preventing the collection of behavioral and histological data, were excluded. In this study, a total of 24 rats were enrolled and randomly divided into the following three groups: sham operation group (sham), CCI surgery group (model), and CCI rats + MMWA

treatment group (treatment) (n=8 in each group). All animals were acclimated to the environment for 7 days before the experiments. Rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (20 mg/kg). After dissecting the skin and the musculature of the middle and back of the left thigh, the sciatic nerve was exposed. The nerves were then loosely ligated with 4-0 sutures, creating four ligation loops spaced 1 mm apart. Rats in the sham group underwent similar procedures, but the nerves were not subjected to ligation.

Acupuncture treatment

In this study, acupoints similar to human Heyi and Shen acupoints (Dazhui and Sanjiaoshu acupoints in traditional Chinese medicine, respectively) were selected as acupuncture sites (Supplemental Figure S1). Acupoint Heyi (GV 14) has been used to alleviate confusion, dizziness, palpitation, agitation, and delirium. Acupoint Shen (BL 22) is stimulated to treat pain, spermatorrhea, and cholera [15,21]. Acupuncture needles were heated to 40°C using an MY-I electrical heating needle warmer (Shanghai, China) and were inserted approximately 5 mm deep at the designated acupoints. The rats were treated with MMWA for 20 min, once daily, for seven consecutive days. During the MMWA stimulation, the rats were constrained in a specialized cloth bag to keep them awake and calm. The sham and model groups received identical catching-grasping stimuli without acupuncture.

Behavioral tests

Animals were tested at baseline, 7, and 14 days after surgery. Mechanical pain threshold was measured with von Frey hairs (YUYAN Instruments, Shanghai, China). The rats were placed in a testing cage and left to acclimate for 30 min. Then, a von Frey filament was applied to the left hind paw with increased pressure, as previously described [22]. If an ambiguous response occurred, mechanical stimulation was repeated 2 times with a 5-min interval between each test. The mean of 3 measurements was used as the paw withdrawal threshold. Thermal withdrawal latency was measured using a hot-plate apparatus (Taimeng, Chengdu, China). Rats were placed on the surface of the hot plate (52°C±0.2°C) to assess the thermal withdrawal latency of the left hind paw. The cut-off latency was 20 s to avoid tissue damage. Heat stimulation was repeated 3 times with a 5 min interval between stimuli, and then the average value of paw withdrawal latencies was calculated.

Sample collection

At the end of the experimental period, the rats underwent deep anesthesia with pentobarbital sodium (intraperitoneal injection at a dose of 40 mg/kg). Fecal contents were directly collected from the rat's cecum and stored at -80°C until analysis. Blood samples from the abdominal aorta were obtained. The serum samples were rapidly harvested from the blood for pro-inflammatory cytokines determination. The L4/5 section of the spinal dorsal horns was quickly removed for RNA extraction.

Real-time PCR

Total RNA was extracted using an RNAprep Pure Tissue Kit (TIANGEN, Beijing, China). Then, 1 μg of total RNA was converted into cDNA using the Prime Script RT Master Mix (TaKaRa, Beijing, China). The TB Green Premix Ex Taq II (TaKaRa, Beijing, China) was used for PCR reactions, which were run on the ABI PRISM 7500 system (Applied Biosystems, Foster City, USA). PCR amplification was performed as follows: initial denaturation at 95°C for 15 s, followed by 35 cycles at 95°C for 10 s, 60°C for 30 s, and 95°C for 15 s. The ratio of mRNA expression relative to the control was evaluated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Supplementary Table S1.

Gut microbiota analysis

The MagPure Stool DNA KF kit B (Magen, China) was used to extract fecal microbial genomic DNA according to the manufacturer's instructions. The Qubit Fluorometer (Thermo Fisher Scientific, Inc., Waltham, MA, United States) and agarose gel electrophoresis were used to determine the concentration and integrity of the extracted DNA, respectively. The hypervariable V3-V4 16S rRNA region of bacterial DNA was chosen for amplification. Both forward and reverse primers were tagged with adapter, pad, and linker sequences. PCR cycling conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 30 sec, 56°C for 45

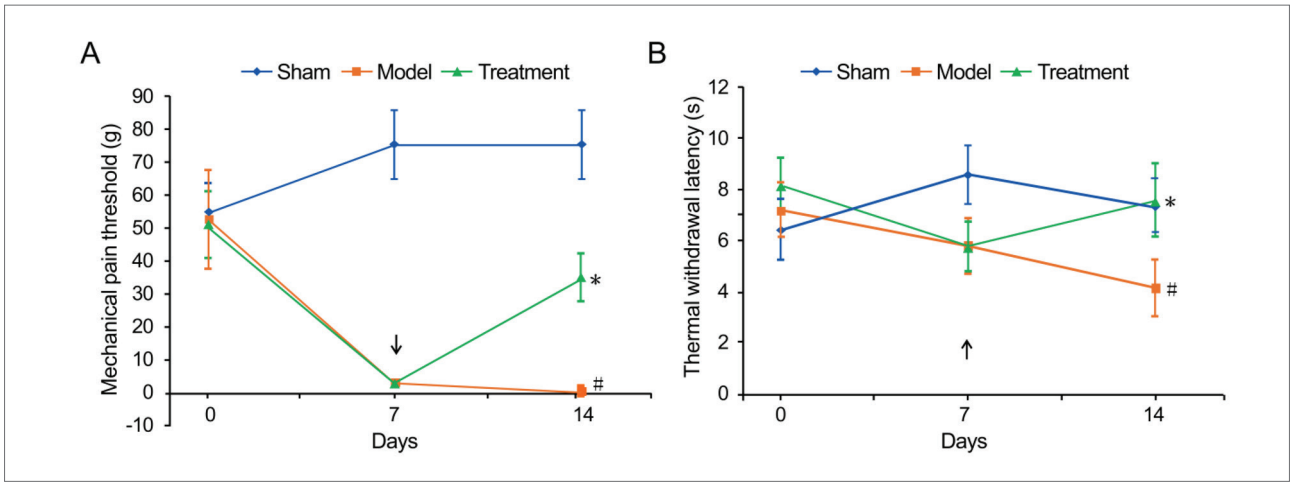

Fig. 1. Effects of MMWA on thermal and mechanical hypersensitivity in the CCI rat model. **A** – mechanical threshold; **B** – thermal threshold. #P<0.01, compared with the sham; *P<0.01, compared with the model.

sec, 72°C for 45 sec, and final extension for 10 min at 72°C. AmpureXP beads (Beckman Coulter, USA) were used to purify the PCR amplification products. Then libraries were constructed, and the quality was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). The library was sequenced on an Illumina MiSeq sequencing platform (BGI, China). A total of 556,519 reads and 18,898 operational units (OTUs) were obtained from the 24 samples. Bioinformatics analysis was then performed as previously described [18]. In brief, OTUs were clustered with 97% sequence similarity using UPARSE (version 7.0.1090). Alpha and beta diversity were calculated by the QIIME pipeline (version 1.8.0). Linear discriminant analysis (LDA) effect size (LEfSe) was performed using the LEfSe software. Only taxa that obtained an LDA score >2.0 were ultimately considered. Principal coordinate analysis (PCoA) was performed based on a distance matrix of weighted UniFrac among samples. The microbiota functions were further predicted using the annotated pathways based on a database search (KEGG, http://www.genome.jp/kegg/).

Enzyme-linked immunosorbent assay (ELISA) analysis

The serum samples were used for detecting the concentrations of IL-1β, IL-6, and TNF-α with a commercial ELISA kit (Jiangsu Zeyu Biological Technology Co., Ltd, Yancheng, China) according to the manufacturer's instructions.

Statistical analysis

SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. A one-way ANOVA test was performed for comparison between multiple groups, and an independent-sample t-test was performed for comparison between two groups. Values were presented as means±SEM. Differences were considered significant when P<0.05.

RESULTS

Behavioral changes

For each animal, four different investigators were involved as follows: the first investigator (HM) was responsible for the anesthetic procedure, the second (ZW) performed the surgical procedure, the third investigator (HY) performed the acupuncture treatment, and the fourth (SG) assessed the mechanical pain threshold and thermal withdrawal latency. We assessed the mechanical pain threshold and thermal withdrawal latency in CCI rats on days 0, 7, and 14 post-operation. On days 7 and 14 post-operation, the model group showed significantly lower mechanical pain thresholds and thermal withdrawal latencies compared to the sham group (P<0.01). Compared with those of the model group, the mechanical pain threshold (P<0.01) and thermal withdrawal latency (P<0.01) of the treatment group were significantly elevated on the 7th day post-treatment (Fig. 1).

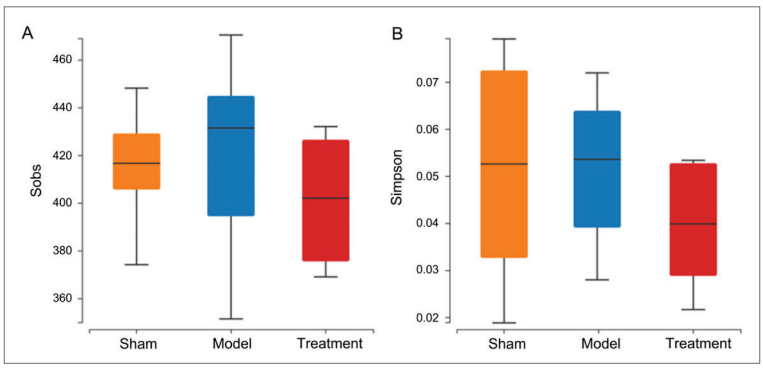

Fig. 2. Alpha diversity indices of gut microbiota. **A** – Sobs index; **B** – Simpson index.

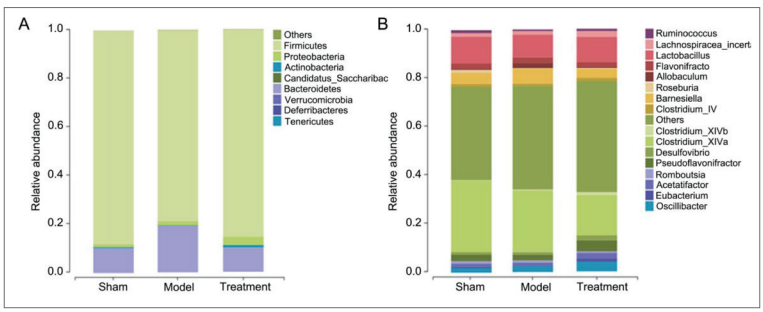

Fig. 3. Alterations in gut microbiota composition among the three groups. **A** – taxonomic composition at the phylum level; **B** – taxonomic composition at the genus level.

The expression of the *c-fos* gene

To confirm whether the MMWA was involved in the central sensitization mechanism, the mRNA levels of *c-fos* in spinal dorsal horns were determined using real-time PCR. As shown in Supplemental Fig. S2, compared with the sham group, there was a significant increase in *c-fos* expression in the model group ($P<0.05$). The MMWA intervention significantly inhibited *c-fos* gene expression in CCI rats ($P<0.05$).

Analysis of sequencing quality metrics

To study whether MMWA intervention affects the gut microbiota in a CCI-induced neuropathic pain rat model, the bacterial community composition was identified by bacterial 16S rRNA gene amplicon sequencing. The OTU rank curve represents the richness and distribution evenness of species in different samples from the three groups (Supplemental Fig. S3A). The species accumulation curve revealed that the sampling depth was adequate to evaluate the bacterial composition (Supplemental Fig. S3B). In the study, the rarefaction curves for Shannon diversity indices approached saturation (Supplemental Fig. S3C), suggesting that the majority of microorganisms present in the samples from the three groups were successfully captured. The Good's coverage for all samples exceeded 98% (Supplemental Fig. S3D), confirming the adequacy and completeness of the sampling.

Analysis of alpha diversity

Alpha-diversity indices of microbiota (Sobs and Simpson) were calculated. The Sobs index, indicating total sample richness, was 414.50 ±22.55, 419.00 ±38.28, and 401.13 ±25.68 in the three groups, respectively (Fig. 2A). Similar trends were also observed for the Simpson index, with values of 0.59±0.36, 0.60±0.43, and 0.45±0.23, respectively (Fig. 2B).

Comparison of microbial communities

To examine the microbial community composition and diversity across the three groups, dominant taxa were analyzed at both the phylum and genus levels. At the phylum level, eight distinct phyla were identified in the fecal samples (Fig. 3A). *Firmicutes* were the predominant bacterial group, accounting for 88.0%, 78.7%, and 85.5% of the sequences in the three groups, respectively. The relative abundance of *Firmicutes* in the sham and treatment groups was particularly high (>85%). *Bacteroidetes* were found in the three groups, making up 10.2%, 19.2%, and 9.76% of sequences, respectively. In contrast, the abundance of *Actinobacteria* and *Verrucomicrobia* was lower in the model group (0.18% and 0.0003%) than in the sham (0.41% and 0.0005%) and treatment (1.02% and 0.137%) groups.

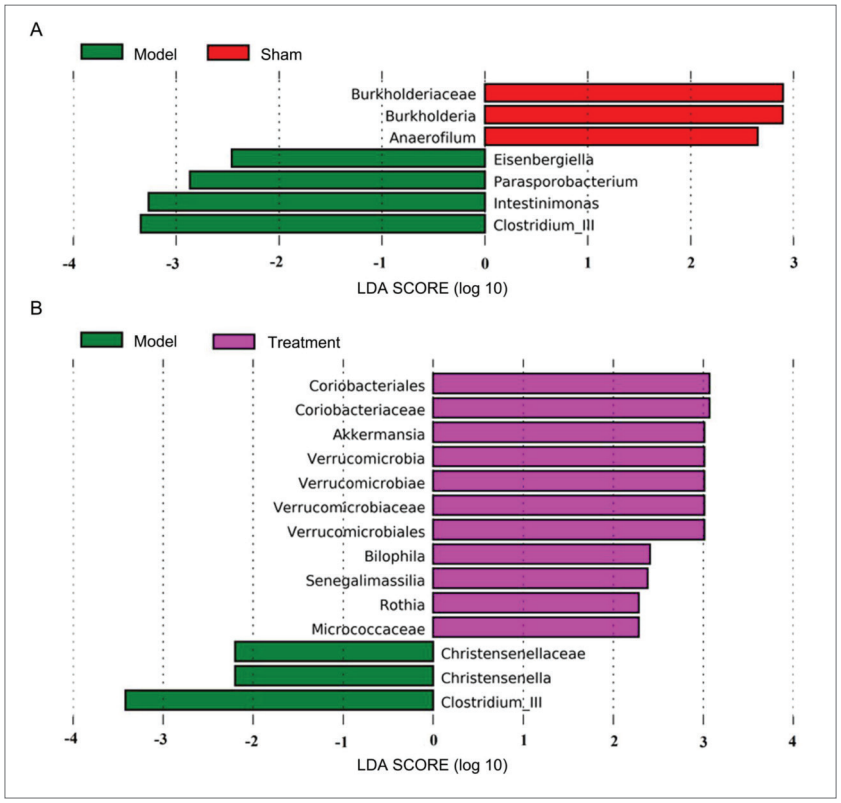

Fig. 4. Differences in intestinal flora among the three groups. **A** – LEfSe analysis of intestinal flora between the sham and model groups; **B** – LEfSe analysis of intestinal flora between the model and treatment groups.

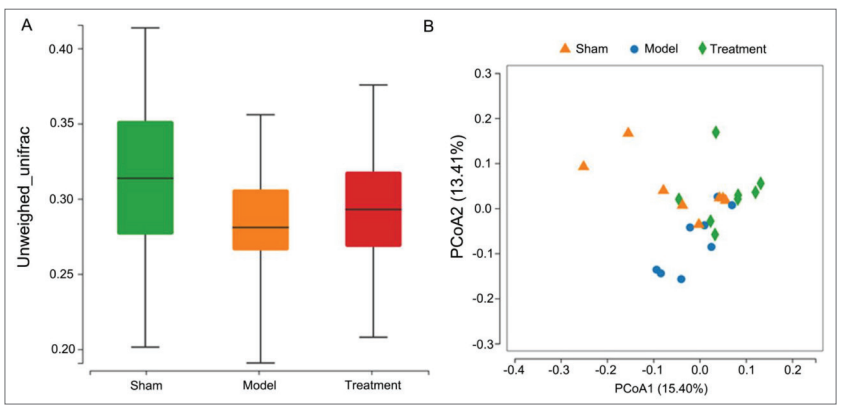

Fig. 5. Beta diversity indices of gut microbiota from the three groups. **A** – unweighted UniFrac distances; **B** – PCoA plots.

At the genus level, there were 16 genera tested in the samples (Fig. 3B). The model group showed a significant increase in *Barnesiella* and *Allobaculum* counts and a reduction in *Lactobacillus*, *Ruminococcus*, *Clostridium* cluster IV, and *Eubacterium* when compared with the sham group. Interestingly, MMWA intervention was found to significantly reverse the relative abundance of these genera.

To identify the specific taxa within each group, LDA effect size (LEfSe) analysis was applied to compare the gut microbiota between different groups (Fig. 4). As shown in Fig. 4A, LEfSe analysis identified seven bacterial clades with significant differences in the fecal microbiota between the sham group and the model group. In the sham group, 3 taxa were enriched, including one family (*Burkholderiaceae*), and two genera (*Anaerofilum* and *Burkholderia*). However, the bacterial taxa detected in the model rats were characterized by 4 genera, *Eisenbergiella*, *Intestinimonas*, *Parasporobacterium*, and *Clostridium* cluster III. Similarly, the LEfSe analysis revealed that 14 distinguishing components were statistically significant in the model group and the treatment group (Fig. 4B). In the model group, 3 taxa were enriched, including one family (*Christensenellaceae*), and 2 genera (*Christensenella* and *Clostridium* cluster III), whereas the bacterial taxa detected in the treatment group were characterized by 11 bacterial clades including one phylum (*Verrucomicrobia*), one class (*Verrucomicrobiae*), two orders (*Coriobacteriales* and *Verrucomicrobiales*), three families (*Verrucomicrobiaceae*, *Coriobacteriaceae*, and *Micrococcaceae*), and four genera (*Rothia*, *Senegalimassilia*, *Bilophila*, and *Akkermansia*). However, LEfSe analysis results confirmed that 18 bacterial clades showed significant differences in the fecal microbiota of the sham group and the treatment group (Supplementary Fig. S4). It is worth noting that the proportion of *Intestinimonas* in the MMWA-treated rats was closer to that in the model group (Supplementary Fig. S3), indicating that the warm acupuncture intervention did not regulate the structure of intestinal flora to reach a healthy state.

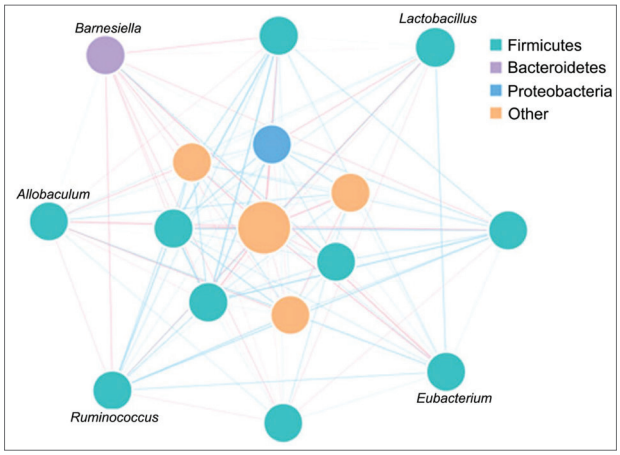

Fig. 6. Co-occurrence and co-exclusion network of the microbial community.

Analysis of beta diversity

As depicted in Fig. 5A, beta diversity based on the unweighted UniFrac showed that there was a difference in intestinal microbiota among the three groups (P=0.049). Beta diversity results of PCoA based on unweighted UniFrac showed the data distribution of samples (Fig. 5B). Our results indicated that the microbial community structure of the three groups showed big differences.

Interactions of the microbial community in fecal samples

The microbial community network constructed from fecal samples consisted of 15 nodes and 105 edges (Fig. 6). *Lactobacillus* (*Firmicutes*) had a negative relationship with *Barnesiella* (*Bacteroidetes*) and had a positive one with *Ruminococcus* (*Firmicutes*) and *Eubacterium* (*Firmicutes*). *Barnesiella* had a positive relationship with *Allobaculum* (*Firmicutes*) and a negative one with *Lactobacillus*, *Ruminococcus*, and *Eubacterium*.

KEGG pathway enrichment

The results of the KEGG pathway analysis revealed that the differentially expressed genes were mainly enriched in 118 pathways. The top 23 pathways are listed in Supplemental Fig. S5. Among them, the enrichment values for the pentose and glucuronate interconversions pathway were 0.00738, 0.00822, and 0.00709 in the three groups, respectively. In addition, the enrichment values for the secondary bile acid biosynthesis pathway were 0.0159, 0.0178, and 0.0154 in the three groups, respectively. Both pathways were significantly enriched and are considered particularly important in the development of neuropathic pain.

Analysis of IL-1β, IL-6, and TNF-α levels

The levels of inflammatory factors IL-1β, IL-6, and TNF-α in the serum were analyzed using the ELISA assay. As shown in Fig. 7, the rats in the model group showed higher levels of IL-1β, IL-6, and TNF-α compared to the controls (P<0.05). MMWA intervention contributed to a reduction in inflammatory factors levels (P<0.05).

DISCUSSION

Mongolian medical warm acupuncture (MMWA) could be a potential therapeutic strategy for the treatment of neuropathic pain [23]. In the present study, a CCI rat model of neuropathic pain was successfully established. The validity of the established model was proven by both heat and mechanical hypersensitivity following surgery. This was similar to a previous study [24]. We found that MMWA attenuated the decrease

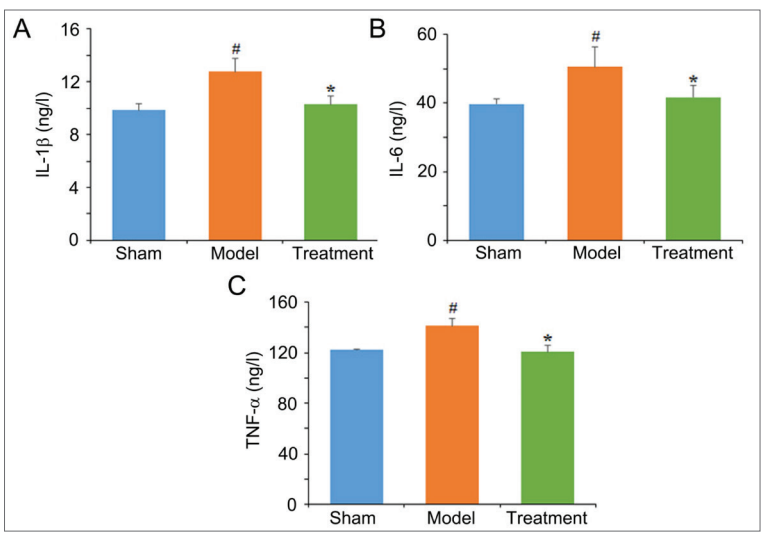

Fig. 7. The effects of MMWA on inflammatory factor lelevs. **A** – IL-1β; **B** –IL-6; **C** – TNF-α. #P<0.05, compared with the sham; *P<0.05, compared with the model.

in mechanical pain threshold and thermal withdrawal latency following CCI surgery. *c-fos* mRNA expression results showed that MMWA intervention directly affects neuron sensitization in CCI rats. Altogether, our findings underscore the critical roles of MMWA intervention in the treatment of neuropathic pain.

Growing evidence suggests that intestinal flora plays a key role in the pathogenesis of neuropathic pain [25,26], which prompted us to investigate whether MMWA exerts its regulatory effects on neuropathic pain through modulation of the gut microbiota. Firstly, the effects of MMWA on the composition and diversity of gut microbiota were determined. Consistent with a previous report [27], we observed that the dominant bacterial phyla of CCI rats were *Firmicutes* and *Bacteroidetes*. However, MMWA intervention resulted in increased levels of *Firmicutes* and a decreased relative abundance of *Bacteroidetes*. This finding is consistent with the results of [28], which reported that drinking hydrogen-rich water regulates the gut microbiota of mice with neuropathic pain. We further analyzed the quantitative changes in the microbiota at the genus level. Compared with the sham group, a significantly increased abundance of *Barnesiella* and *Allobaculum* was observed in CCI rats. In contrast, *Lactobacillus*, *Ruminococcus*, *Clostridium* cluster IV, and *Eubacterium* were significantly decreased. It has been reported that *Allobaculum* was associated with inflammation levels [29], while *Barnesiella* is a dominant genus in patients with depression [30]. Additionally, *Lactobacillus* has been reported to be associated with neuropathic pain [31]. Also, the abundances of *Ruminococcus*, *Clostridium* cluster IV, and *Eubacterium*, all short-chain fatty acid (SCFA)-producing bacteria [32-34], may help prevent inflammation in both the central and enteric nervous systems [35]. LEfSe analysis revealed that the relative abundance of *Clostridium* cluster III differed significantly between the model and sham groups. Therefore, these bacteria in CCI rat models may play a role in the mechanisms underlying neuropathic pain. Interestingly, MMWA intervention partially restored the dysbiosis of these bacteria, suggesting its modulatory effects on the gut microbiota. KEGG pathway enrichment analysis was performed, and the results revealed that neuropathic pain is related to several signaling pathways. Among these, two pathways, pentose and glucuronate interconversions and secondary bile acid biosynthesis, were considered to be especially important. It has been reported that the pentose and glucuronate interconversions pathway mediates anti-inflammatory and analgesic effects [36,37], and the secondary bile acid biosynthesis pathway contributes to the activation of colonic extrinsic afferent nerves and their neuronal cell bodies [38]. Our findings are consistent with these reports. It is reasonable to surmise that gut microbiota contributes to the regulation of neuropathic pain in response to MMWA intervention.

Dysbiosis of gut microbiota after CCI surgery is known to lead to inflammation [39]. In the present study, SCFA-producing bacteria, such as genera *Lactobacillus*, *Ruminococcus*, *Clostridium* cluster IV, and *Eubacterium*, were found to be decreased in CCI rats. It has been well documented that SCFAs, particularly butyrate, acetate, and propionate, mediate inflammation [35]. Therefore, we speculated that MMWA might inhibit pro-inflammatory cytokine secretion in CCI rats via gut microbiota regulation. To test this hypothesis, IL-6 and TNF-α levels were measured in CCI rats. The results showed that both IL-6 and TNF-α were significantly elevated in the model group compared to the sham group. However, MMWA treatment suppressed the production of IL-6 and TNF-α. These results confirm that MMWA alleviates neuropathic pain in CCI rats, at least in part, by modulating inflammation-related pathways via the gut microbiota as described above, consistent with findings from another study [40].

This study suggests that MMWA intervention may modulate the gut microbiota to alleviate neuropathic pain in CCI rats. How MMWA might influence the gut microbiota in patients with neuropathic pain remains a key question for future research.

CONCLUSIONS

The MMWA intervention was shown to alter the diversity and composition of the gut microbiota in CCI rats, thereby enhancing multiple pathways and restoring balance to pro-inflammatory cytokine levels. These results may enhance our understanding of the pathogenesis of neuropathic pain and the therapeutic mechanisms of MMWA intervention. Furthermore, this study suggests that MMWA could become a promising therapeutic option for patients suffering from neuropathic pain worldwide in the future.

Funding: This study was supported by the National Natural Science Foundation of China (no. 82460987, 82074577, and 82260092), and the Natural Science Foundation of Inner Mongolia Autonomous Region of China (2023ZD01 and 2023QN08065).

Author contributions: Conceptualization, GA, and ZW; methodology, HM, SG, and HY; validation, HM, SG, and HY; investigation, HM; resources, GA, and ZW; data curation, GA; writing—original draft preparation, HM; writing—review and editing, ZW; visualization, SG; supervision, GA; project administration, GA; funding acquisition, HY, and GA. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: 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.

Data availability: The data presented in this study are openly available in: [CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb), https://doi.org/10.26036/CNP0003536.

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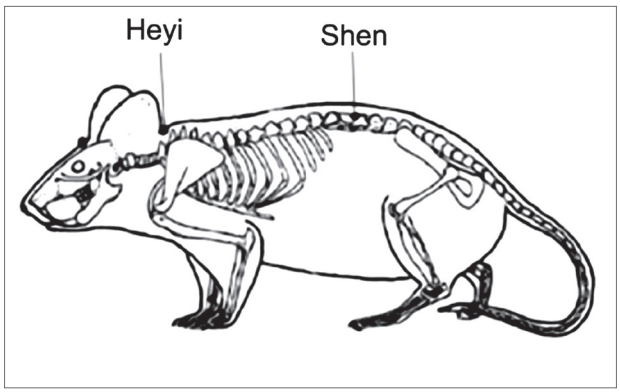
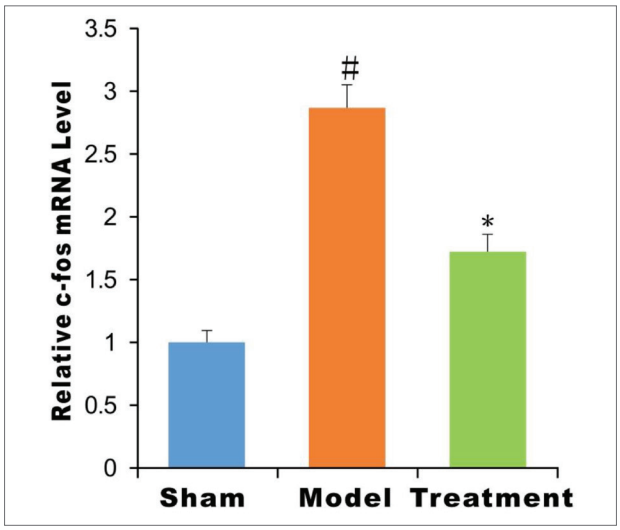
SUPPLEMENTARY MATERIAL

Supplementary Table S1. Specific primer sequences used for real-time PCR.

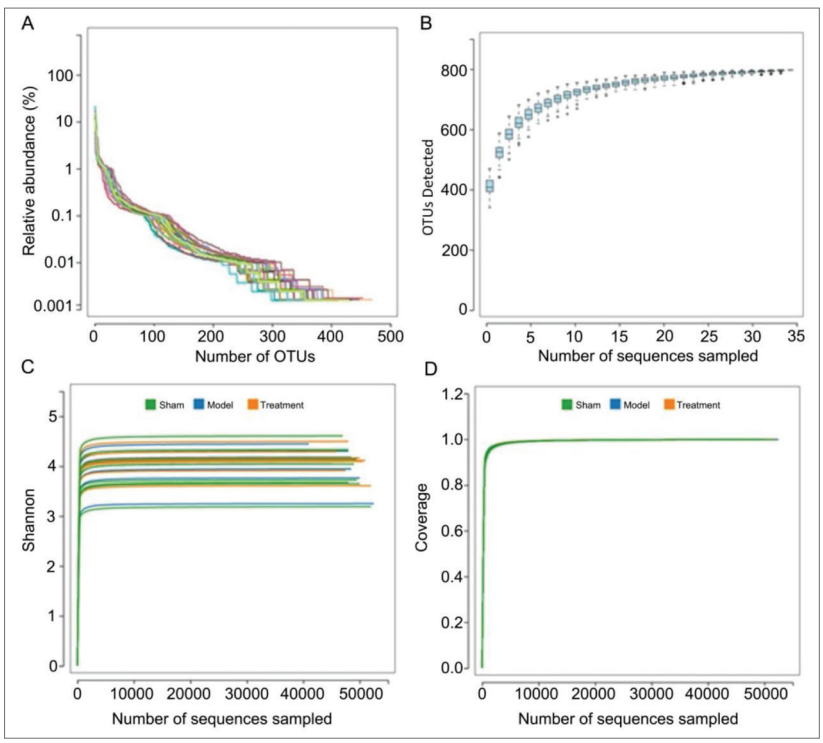
Primer name	Forward (5–3′)	Reverse (3–5′)
c-FOS	TGTGACCTCCCTGGACTTG	CACTGGGCCTAGATGATGC
GAPDH	GCAAGTTCAACGGCACAG	GCCAGTAGACTCCACGACAT



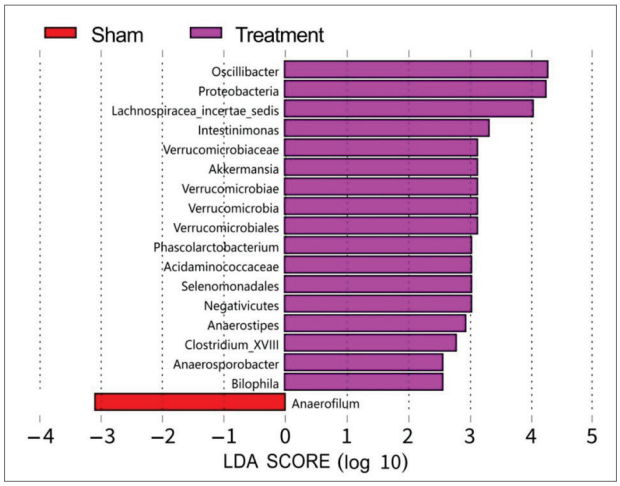
Supplementary Fig. S1. Acupoint locations.



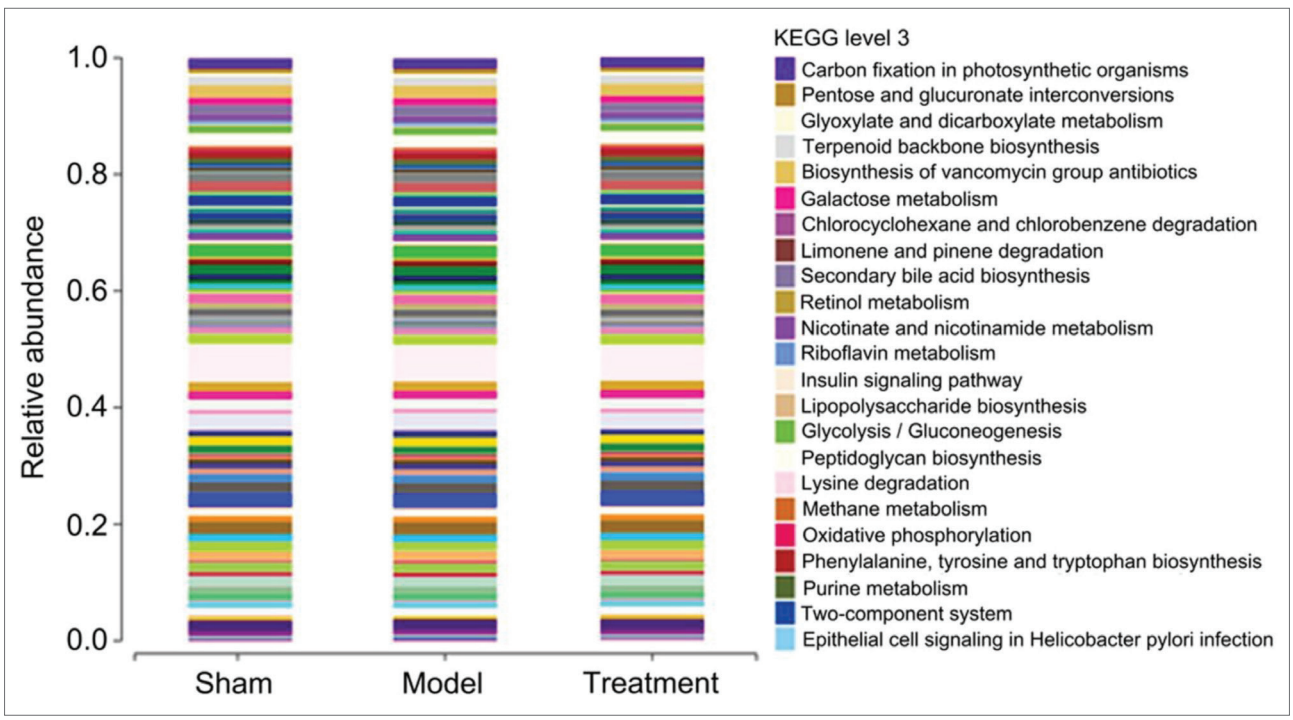
Supplementary Fig. S2. Effect of MMWA on the expression of the *c-fos* gene in the CCI rat model. #P<0.05, compared with the sham; *P<0.05, compared with the model.



Supplementary Fig. S3. Quality metrics of high-through sequencing analysis. **A** – Rank-abundance curves; **B** – species accumulation curves; **C** – Shannon index curves; **D** – Good's coverage index curves.



Supplementary Fig. S4. LEfSe analysis of intestinal flora between the sham and treatment groups.



Supplementary Fig. S5. KEGG pathway enrichment analysis of the gut microbiota among the three groups.