

Compound Probiotics Improve Neuropathic Pain Prognosis in a Murine Model of Chronic Constriction Injury

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Purpose: Compound probiotics have been reported to ameliorate imbalances in the intestinal flora that may play a critical role in neuropathic pain. This study aimed to investigate the efficacy of compound probiotic treatment on neuropathic pain.

Methods: Thirty mice were randomly divided into three groups: 1) sham group, 2) mouse with chronic constrictive injury (CCI), and 3) probiotic gavage with CCI (CCI+Prob). The degree of pain and gait recovery was assessed by Mechanical withdrawal threshold (MWT), thermal withdrawal latency (TWL), and mouse footprints. The degree of atrophy of the gastrocnemius muscle was assessed by muscle weight, hematoxylin and eosin (H&E) staining. Gut microbiota were analyzed by 16S ribosomal RNA sequencing (16SrRNA).

Results: Four weeks after surgery, TWL and MWT assessment showed significant increases in the CCI+Prob group compared with the CCI group ($P < 0.01$). Gait analysis results as well as gastrocnemius muscle weight showed a significant improvement in the CCI+Prob group compared with the CCI group. Measurement of alpha diversity showed a significant increase in the CCI group compared with the sham group, but this increase was attenuated by probiotic intervention in the CCI+Prob group. Although the CCI group had significantly decreased levels of Akkermansia and significantly increased levels of Ruminococcaceae, probiotic treatment reversed these changes.

Conclusion: Compound probiotics treatment can improve the pain and muscle atrophy in mice with CCI-induced neuropathic pain. The improvement of symptoms is associated with changes in the composition of gut microbiota.

Keywords: probiotics, neuropathic pain, gut microbiota, muscle atrophy, Akkermansia, Ruminococcaceae

Introduction

Neuropathic pain can occur within the case of a lesion or disease of the somatosensory nervous system^{1,2} and has the key manifestations of hyperalgesia, spontaneous pain, and psychiatric comorbidities, which in turn enhance pain perception.³ Neuropathic pain may also directly and indirectly affect muscle function via activity restriction and immobility.⁴ This pain impairs physical, mental, and emotional function, which can significantly reduce quality of life. A multidisciplinary approach toward treatment combining pharmacological interventions, such as antidepressants, antiepileptics, or opioids, with physical or cognitive interventions is now advocated.^{5,6} Nevertheless, epidemiological surveys have shown that a significant number of patients do not receive appropriate treatment.^{7,8} The recurrent nature of refractory pain and the

adverse effects stemming from the prolonged use of opioids are well known. It is imperative to account for the potential risks associated with long-term opioid consumption and consider the use of other gentler and more effective strategies for treating intractable pain and related symptoms.

Humans harbor trillions of microorganisms, most of which reside in microbial communities within the gastrointestinal tract. Previous studies have verified the role of gut microbiota in maintaining host health by modulating intestinal permeability, digestion, metabolism, and immune response.^{9–12} However, most studies have focused on conditions such as cancer, malnutrition, and age-related diseases.^{13–15}

Gut microbial composition is dynamic and can be affected by the host's diet, lifestyle, hygiene, and use of antibiotics. The relationship between gut microbiota and pain has been described in the last years, specifically in alleviating abdominal pain.¹⁶ In recent years, growing evidence suggests that gut microbiota plays a role in both acute and chronic pain^{17–19} The findings have also shown the potential effectiveness of probiotics in the treatment of inflammatory diseases.^{20–22} It works primarily by modulating the immune system.^{23–25} We know that pain and inflammation are closely related. The inflammatory response plays a pivotal role in the transition of the pain process. A study using an in vitro model of sensitive neuron reported that paclitaxel (PTX)-induced neuropathic pain could be counteracted by administration of a high-concentration probiotic formulation.²¹ Despite the possibility of modulating gut flora–pain interactions with probiotics, no studies have evaluated the prognosis of compound probiotics in neuropathic pain. Therefore, in the present study, we described for the first time the effect of compound probiotics on pain threshold, degree of muscle atrophy, and gait using the CCI mouse model and conducted a preliminary exploration of possible mechanisms.

Material and Methods

Animals

Adult male C57BL/6J mice weighing 22–25 g were obtained from the Animal Experiment Center Institute of Regenerative Medicine, Shanghai East Hospital. All animals were raised in a 12:12 h light-to-dark cycle with ad libitum access to food and water. To ensure the reliability of the experiment two pain-hypersensitive mice and two hypalgesic mice were excluded from each group.

CCI Modeling

The CCI model was developed as described by Bennett in 1988.²⁶ Each mouse was anesthetized by intraperitoneal injection of 5% chloral hydrate (0.1 mL/10 g). The sciatic nerve was bluntly separated and surrounded with 4–0 sutures made with 2 light ligation loops in 1 mm intervals and tightened until the thigh muscles twitched. The mice were randomly assigned to three groups: CCI with normal saline gavage group (CCI group), CCI with probiotic gavage group (CCI+Prob group), and a sham group that underwent the same procedure without sciatic nerve ligation (sham group).

Compound Probiotics

Probiotics were purchased from Wuhan Senlan Biological Company, China, containing the following 15 strains: *Bifidobacterium animalis* ssp. Lactis HN019, *Bifidobacterium bifidum* Bb06, *Bifidobacterium animalis* ssp. lactis BB-12, *Bifidobacterium animalis* ssp. lactis Bi07, *Bifidobacterium longum* R175, *Bifidobacterium animalis* B94, *Lactobacillus rhamnosus* GG, *Lactobacillus casei* LC11, *Lactobacillus helveticus* R52, *Lactobacillus paracasei* Lpc37, *Lactobacillus plantarum* R1012, *Lactobacillus reuteri* HA188, *Lactobacillus rhamnosus* R11, *Lactobacillus acidophilus* NCFM, and *Streptococcus thermophilus* St21. Each gram contained 10 billion viable colony-forming units (CFUs). According to dose translation from animal to human,²⁷ each mouse in the CCI+Prob group was administered 2.5×10^7 CFU/g/day. To avoid loss of active cultures, the probiotic powder was stored at 4°C and kept away from heat and light. The probiotic mixture was added to saline to make a probiotic suspension, and administered through intragastric gavage.

Thermal Withdrawal Latency Test

A plantar algometer (Tes7370, Ugo Basile, Comerio, Italy) was used to measure the TWL of the injured limb by detection of infrared irradiation on the plantar surface of the mouse paw. Three independent measurements were performed for each mouse with 30-s intervals. Latency was defined as the average interval duration of three measurements before the mouse licked a hind paw or withdrew. A threshold time of 22 seconds was set to prevent tissue damage.

Mechanical Withdrawal Threshold Test

Before the assessment of the MWT, mice were placed on metal grids and acclimated for 30 min, avoiding the stress resulting from the test conditions. While the mice were quiet, Von Frey fibers were used to vertically stimulate the operative paws of the mice for 4–6 s with a 30-s interval. When foot withdrawal or foot licking was observed, it was regarded as a positive reaction and recorded as “X”, whereas no reaction was recorded as “O”. The order of stimulation intensity of fiber silk was 0.4, 0.6, 1, 2, 4, 6, 8, 10, and 15 g. Stimulation intensity was started at 2 g and decreased when a positive response occurred and increased if no response occurred. Measurements were applied six times, and the final mechanical pain threshold was recorded against the threshold table.

Measurement of Murine Footprints

The experimental design was based on a previous study by Wertman et al,²⁸ which took advantage of natural mouse behavior and affinity for small, dark places. Mice were placed on a tunnel 5 cm in width, 10 cm in height, and 100 cm in length. The soles of the mice were evenly dipped in ink before they walked across smooth white paper within a tunnel. Footsteps that were consistently clearly spaced and non-smudged were used for analysis.

Tissue Collection

After behavioral testing was conducted on day 28 post-surgery, the mice were sacrificed by an overdose of sevoflurane. The gastrocnemius muscle on the operated side of the mouse was removed and fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin wax, and serially sectioned at 6 mm for immunofluorescent and hematoxylin and eosin (H&E) staining.

Statistical Analysis

All data are presented as the mean \pm standard deviation. Data were analyzed by one-way analysis of variance followed by the Bonferroni multiple comparison post-hoc test using GraphPad Prism Software version 6.0 (Boston, MA, USA). A P value <0.05 was considered an indication of statistical significance.

Results

Compound Probiotics Improve Pain and Gait in Neuropathic Murine Model

One week after surgery, compound probiotics or normal saline was administered by intragastric gavage for three consecutive weeks according to the requirements of the group. The MWT and TWL of each group were assessed before surgery and at 1, 2, 3, and 4 weeks after surgery. All the results showed that the MWT and TWL of the CCI and CCI+Prob groups had significantly decreased 1 week after CCI surgery ($P<0.05$). Compared with those of the CCI group, the MWT and TWL of the CCI+Prob Group had significantly increased after 3 weeks of gavage ($P<0.01$; [Figure 1A](#) and [B](#)). The weight of the surgery groups all showed a decrease, but the prob group tended to increase compared with the CCI group, although the difference between them did not reach statistical significance ([Figure 1C](#)).

Gait analysis of the right hind paw revealed that both the shape and clarity of the footprints of the CCI and CCI+Prob groups significantly differed from those of the sham group ([Figure 2A](#)). The clarity of the footprints of the CCI+Prob group was relatively improved compared with that of the CCI group, suggesting that the CCI+Prob group had a stronger grip on the ground. The analysis revealed no significant change in the toe length of the surgical paws of the three groups ($P > 0.05$; [Figure 2B](#)), The CCI+Prob group displayed a significantly increased toe spreading length ($P < 0.05$; [Figure 2C](#)) and stride length ($P < 0.05$; [Figure 2D](#)) compared with the CCI group.

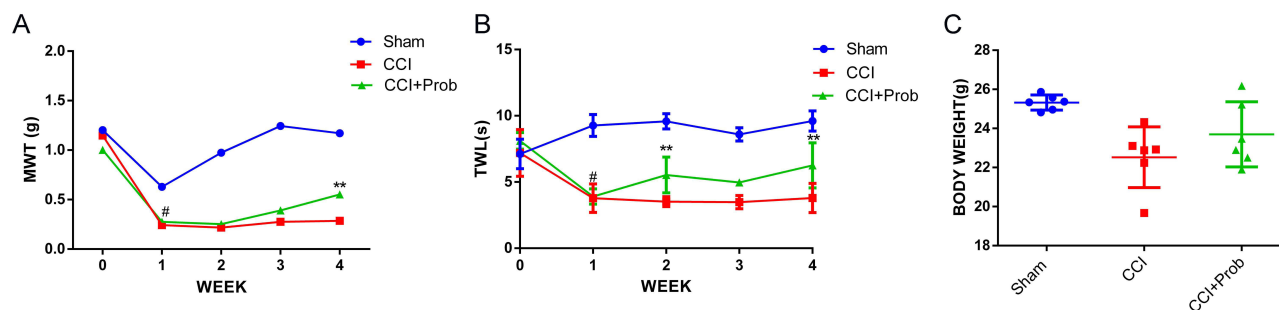


Figure 1 Comparison of mechanical withdrawal threshold (A), thermal withdrawal latency (B), and body weight (C) of sham, CCI, and CCI+Prob groups before and after surgery. Values were measured 0, 1, 2, 3, and 4 weeks after surgery. # $P < 0.05$ 0 vs 1 week for mechanical withdrawal threshold and thermal withdrawal latency; ** $P < 0.01$ CCI vs CCI+Prob group.

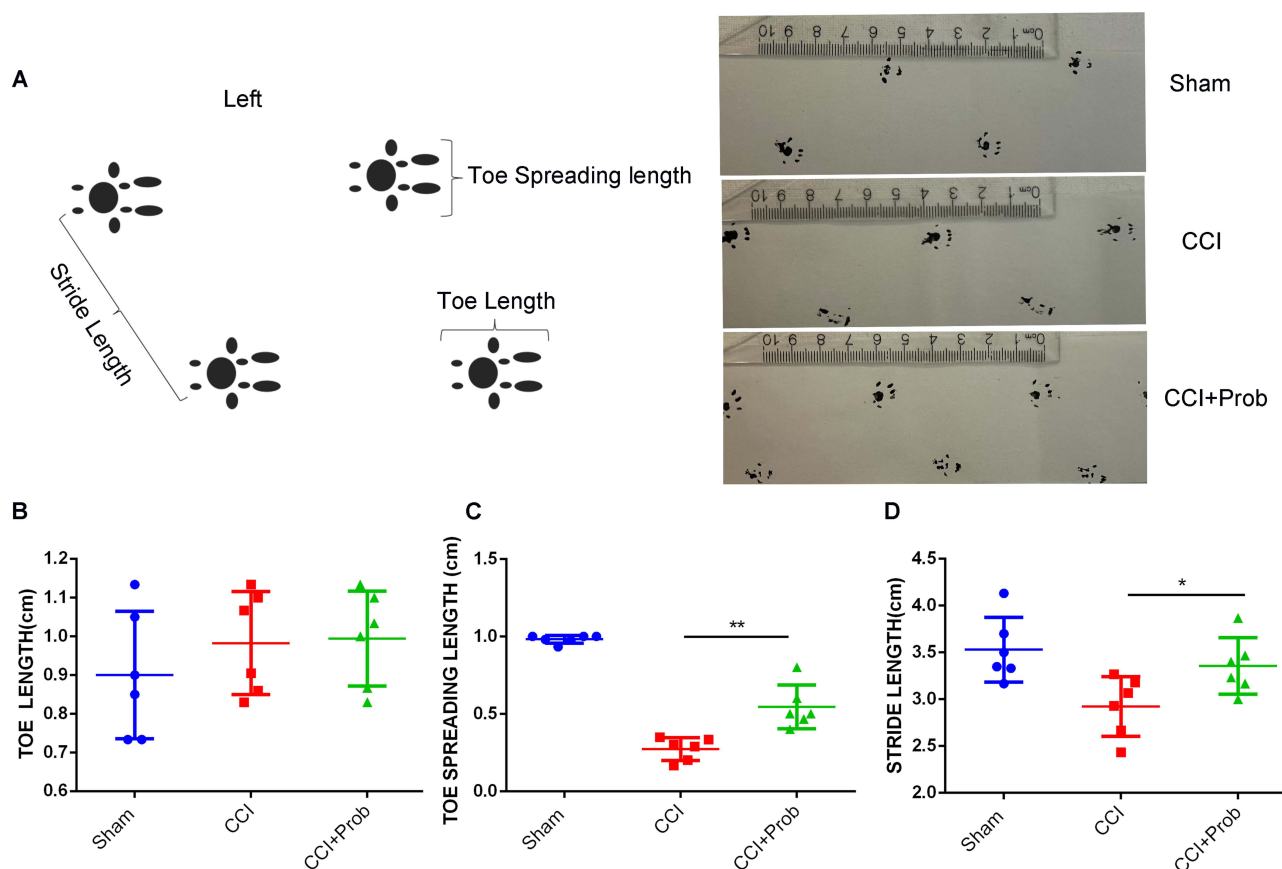


Figure 2 (A) Gait analysis results. (B) No changes were detected in the toe-spreading length of the surgical paw in the sham, CCI, or CCI+Prob group. (C and D) CCI+Prob group displayed a significantly increased toe-spreading and stride length compared with the CCI group. * $P < 0.05$ CCI vs CCI+Prob group; ** $P < 0.01$ CCI vs CCI+Prob group.

Compound Probiotic Treatment Improves CCI-Induced Neuromuscular Atrophy

The gastrocnemius muscle is one of the main muscles innervated by the sciatic nerve that showed varying degrees of atrophy after sciatic nerve ligation. The atrophy of the gastrocnemius muscle on the surgical side of the mice was significantly improved in the CCI+Prob group ($P < 0.05$; Figure 3A). Comparison of H&E-stained transverse sections of the gastrocnemius muscle revealed a high number of muscle fibers with centrally located nuclei in the CCI group, a primary pathological sign of muscular dystrophy. The muscle fibers of CCI mice were smaller compared with those of the other two groups, and smaller muscle fibers were more commonly distributed in the muscles of CCI mice. Such

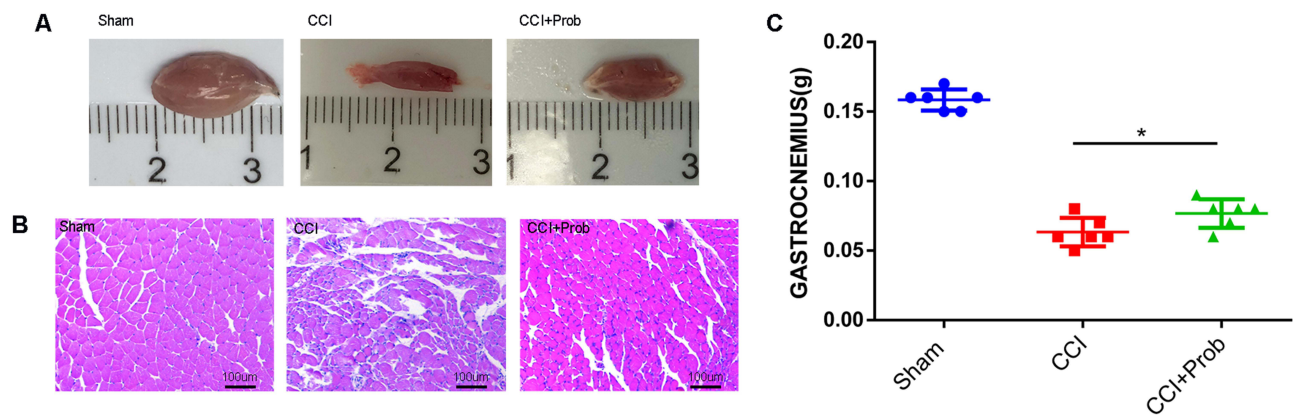


Figure 3 (A) Gross morphology of gastrocnemius muscle. (B) Gastrocnemius muscle cross section from hematoxylin and eosin stain. (C) CCI+Prob group displays a significant increase in gastrocnemius muscle weight. * $P < 0.05$ CCI vs CCI+Prob group.

phenomenon improved in the CCI+Prob group (Figure 3B). We observed that the weight of the gastrocnemius muscle was reduced in both the CCI group and the CCI+Prob group compared to the sham group. However, the weight of the gastrocnemius muscle was significantly increased in the CCI+Prob group than in the CCI group. ($P < 0.05$; Figure 3C).

Intestinal Flora Diversity is Altered with CCI-Induced Neuropathic Pain

Using 16S rRNA gene sequencing to measure microbial populations, we detected structural differences in the microbial composition of the feces of the three groups (Figure 4A). The Linear Discriminant Analysis Effect Size (LEfSe) tool was used to analyze the data, which shows a range of discriminating biomarkers across all taxonomic levels. We can identify those taxa of different levels were differentially abundant in the three groups (Figure 4B). Using the Shannon index to analyze the alpha diversity, which refers to the diversity of bacteria or species, we observed a significant increase in the CCI group compared with the sham group (Figure 4C). Furthermore, we found a clear downward trend in the diversity of the CCI+Prob group despite detecting no significant differences between the CCI and CCI+Prob groups.

Using the DESeq2 method to further analyze the results of pairwise comparisons, we found that the level of phylum Verrucomicrobia significantly decreased while the level of family Ruminococcaceae significantly increased in the CCI group. However, probiotic treatment reversed the CCI-induced changes in the relative abundance of Verrucomicrobia and Ruminococcaceae in the CCI+Probiotics group (Table 1). We also observed this phenomenon in the relative abundance of Akkermansia, which is the only genus of bacteria in the phylum Verrucomicrobiota known to be in the gut.

Discussion

Patients with neuropathic pain accompanied by muscle atrophy experience a decline in muscle strength, gait speed, and athletic ability, negatively impacting their quality of life. The results of our comparison of footprint clarity, stride length and toe-spreading length in three groups of mice indicate that compound probiotic treatment can attenuate nociceptive hypersensitivity in a CCI-induced neuropathic pain model. We found that the gait of the mice who had undergone probiotic treatment was closer to that of healthy mice than the mice that had not undergone probiotic treatment, suggesting that the probiotic treatment had improved lameness in the CCI group. These findings are consistent with previous findings that muscle wasting and dysfunction can be delayed by intervening in the microbe–muscle axis with probiotic treatment.^{29–31}

To compare the changes in gut microbiota in the three experimental groups, we measured the alpha diversity, an indicator of bacterial diversity, using the Shannon index. We found that the relative abundance of microbiota in the CCI group was higher than that of the sham group, as well as that the improvement of symptoms in the CCI+Prob group was accompanied by a reduction in changes in the gut microbiota. Interestingly, we also observed that probiotic treatment reversed the reduction in levels of Akkermansia, bacteria associated with inflammatory diseases and metabolic disorders that can convert mucin to short-chain fatty acids (SCFAs) that may mediate immunoregulatory effects.^{32–34} Akkermansia adheres to the mucus layer to strengthen intestinal barrier function to protect epithelial cells from microbial attacks.³⁵

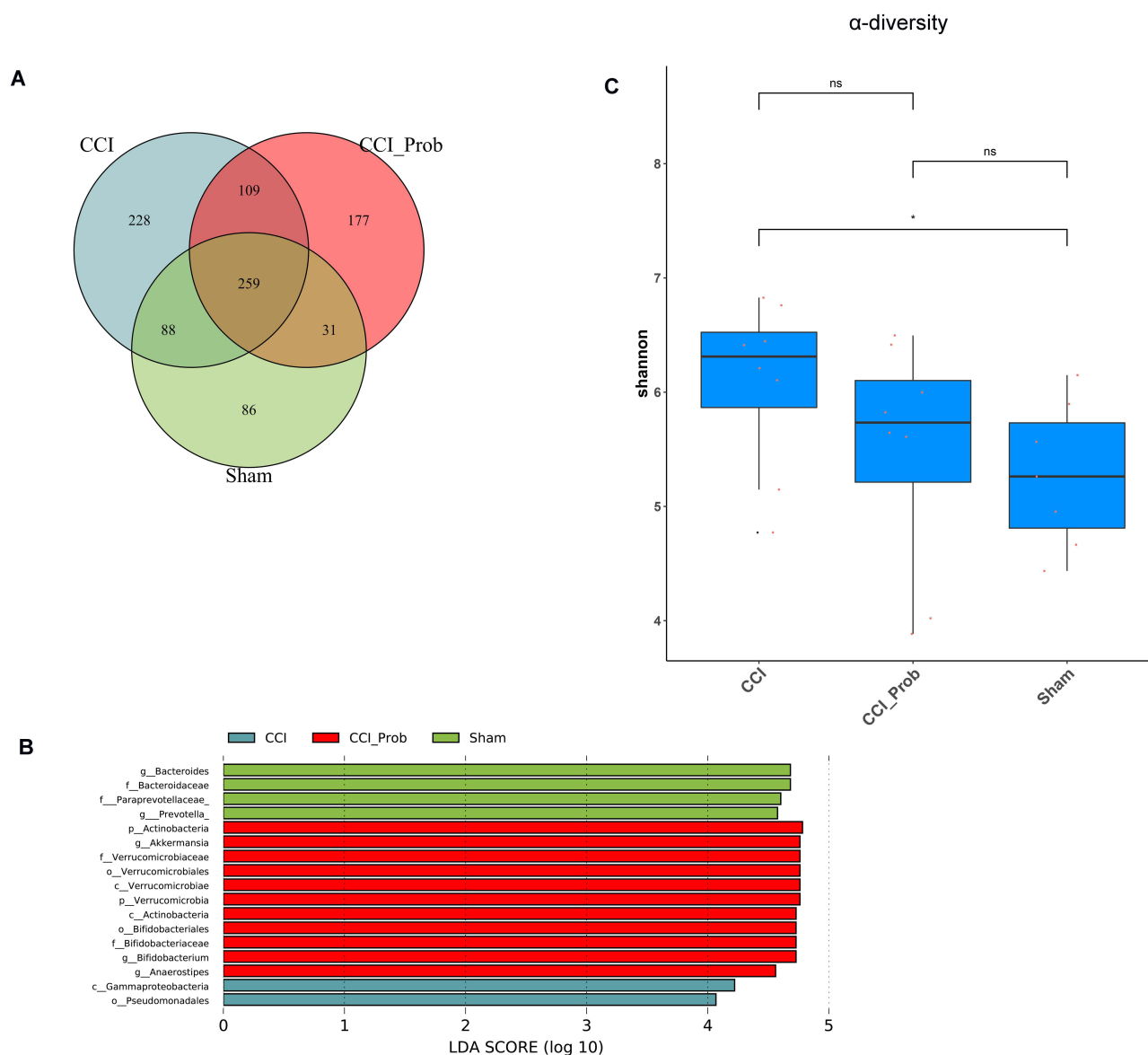


Figure 4 Differential profiles of gut microbiota among three study groups. **(A)** Taxonomic composition of gut microbiota. **(B)** Taxa identified by LEfSe as biomarkers of samples from the Sham group, CCI group, and the Prob group (cutoffs were LDA \geq 4 and p-value \leq 0.05) **(C)** Shannon index, an α -diversity indicator, of gut microbiota.

Moreover, it acts as a nutrient providing energy for microorganism growth. Experiments on hamsters provided evidence that Akkermansia provides a competitive advantage during stressful events, such as fasting and malnutrition.³⁶ A similar significant trend regarding an increase in levels of Akkermansia has been reported in the context of immune-based diseases.^{37,38}

The family Ruminococcaceae acts as a key component of the microbiota in humans by maintaining homeostasis of the gut microenvironment. Although we found that levels of Ruminococcaceae significantly increased in the CCI group, this increase was reversed by probiotic treatment. Most studies have found higher levels of Ruminococcaceae in healthy controls, and only some studies reported mixed results, such as that increased levels of Ruminococcaceae correlate with longer Parkinson's disease duration.³⁹ A case study reported that Ruminococcaceae levels increased significantly and progressively with improvement in bipolar depression symptomology.⁴⁰ Nevertheless, few studies have examined the relationship between disease and Ruminococcaceae levels despite their fluctuations possibly being related to specific diseases. As the development and progression of CCI-induced neuropathic diseases could be associated with levels of

Table I Changes at Different Levels in Gut Microbiota

| OUT | Group | log2FoldChange | lfcSE | stat | p value ^a |
|-----------------|-----------------|----------------|-------|-------|----------------------|
| Verrucomicrobia | Sham VS CCI | -5.84 | 1.04 | -5.63 | <0.001 |
| Verrucomicrobia | CCI VS CCI-Prob | -8.54 | 1.01 | -8.50 | <0.01 |
| Ruminococcaceae | Sham VS CCI | 1.37 | 0.49 | 2.79 | 0.005 |
| Ruminococcaceae | CCI VS CCI-Prob | 1.40 | 0.48 | 2.93 | 0.003 |
| Akkermansia | Sham VS CCI | -6.90 | 1.12 | -6.16 | <0.001 |
| Akkermansia | CCI VS CCI-Prob | -8.18 | 1.08 | -7.55 | <0.001 |

Notes: P < 0.05 was considered statistically significant. ^aStatistical significance was determined using the DESeq2 test.

Abbreviations: OUT, Operational Taxonomic Unit; lfcSE, Standardized Error for log2FoldChange; stat, test statistic.

Akkermansia and Ruminococcaceae, they could serve as the pathological foundations of muscle atrophy and nociceptive hypersensitivity in disease progression and may even serve as therapeutic targets or markers.

Probiotics appear to act as antioxidants, namely the production of antioxidant enzymes and metabolites like lactate.^{41,42} In addition, probiotics inhibit the TLR4/NF- κ B pathway activated by gut-derived lipopolysaccharide and reduce the production of inflammatory factors.⁴³ Studies have shown that reactive oxygen species as well as inflammatory responses play an important role in neuropathic pain and are involved in central sensitization.^{44,45} Therefore, we speculate that the antioxidant properties of probiotics and the reduction of inflammatory factor production may also be one of the mechanisms in alleviating symptoms of neuropathic pain.

In our CCI-induced neuropathic pain murine model, we observed changes in gut microbiota that we attempted to modify with compound probiotics to treat the neuropathic pain. The most significant limitation of our study was the limited period of observation. Despite this limitation, our findings strongly suggest that targeting the gut microbiota might be a novel treatment for neuropathic pain or even a means of alleviating skeletal muscle atrophy. The potential role of compound probiotics should be examined within the context of the interaction of significant factors in pain development and management. Further studies are needed to explore how changes in gut microbiota can be used to improve disease symptoms in neuropathic pain.

Conclusion

Our findings provide evidence that compound probiotic treatment provides some degree of relief from neuropathic pain and its associated symptoms, supporting previous observations that probiotic treatment could be a means of managing inflammation-related pain symptoms. We also provide evidence that changes in the composition of gut microflora are associated with the development of neuropathic pain. Among the microflora for which we detected changes in levels, we observed that Akkermansia and Ruminococcaceae were related to disease progression, suggesting that they could be utilized as targets for treatment or as markers for disease progression.

Abbreviations

CCI, chronic constrictive injury; Prob, probiotics; TWL, thermal withdrawal latency; MWT, mechanical withdrawal threshold; H&E, hematoxylin and eosin; 16SrRNA, 16S ribosomal RNA sequencing; CFUs, colony-forming units; LEfSe, Linear Discriminant Analysis Effect Size.

Data Sharing Statement

Data supporting the findings of this study are available from the corresponding author on request.

Ethics Approval

All ethical principles are considered in this commentary. All ethical principles are considered in all experimental procedures. All procedures were conducted in compliance with the Animal Experiment Regulations of Tongji University Experimental Animal Center, the Animal Experiment Committee Regulations, and the Animal Experiment Approval Provisions (Approval Nos: TJBB03124102).

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Disclosure

The authors declare that they have no competing interests.

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