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RESEARCH ARTICLE

Improved Gut Microbiota Escalates Muscle Function Rehabilitation and Ameliorates Oxidative Stress Following Mechanically Induced Peripheral Nerve Injury in Mice

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A B S T R A C T

Peripheral nerve injury (PNI) is among the leading health issues affecting the modern era. Currently, there are no effective therapeutic strategies to heal nerve damage and ensure fully functional recovery. Probiotics can serve as an appealing and effective option to close this gap via the gut microbiota. The purpose of the study was to evaluate the role of probiotics on functional recovery after nerve injury. For this purpose, sixty healthy BALB/c mice were divided into 04-groups. The control group was given a routine diet. In contrast, positive control, pre-injury probiotics and post-injury probiotics were administrated their respective treatments orally from the day of nerve injury to the end of the project. The sciatic functional index, grip strength, pinprick, and hot plate tests were used to analyse the retrieval of motor and sensory functions, and the results for the pre-injury probiotics group were highly significant. Additionally, the fiber count and surface area of the Tibialis anterior muscle were significantly improved in this group. When compared to the control and post-injury probiotics groups, this group's much lower total oxidant status and increased total antioxidant capacity indicate that probiotics have a strong potential to improve the restoration of muscle function when introduced before the injury. These results imply that probiotics are able to accelerate functional recovery following a peripheral nerve injury via the gut-brain axis. Nonetheless, future studies are warranted to identify the underlying mechanism of probiotics that boosts functional restoration.

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INTRODUCTION

The Peripheral Nervous System (PNS) encompasses a sophisticated and delicate network of nerves linking the brain and spinal tissue with the entire body for harmonizing the coordination in body functions. Any physical insult to the nerve triggers a series of pathological consequences that include oxidative stress, inflammation and initiation of phagocytosis that may result in demyelination. The eventual abrupt interruption in the conduction of nerve signals from CNS to the affected organ causes the nerve to degenerate. The damaged nerve may regenerate by itself over the time depending upon the type of injury (Lopes *et al.,* 2022; Golshadi *et al.,* 2023), but the pace of regeneration to reinnervate the target muscle is substantially slow and may takes months to years, during which sensorimotor loss in the denervated muscle leads to muscle dystrophy (Hussain *et al.,* 2020; Lopes *et al.,* 2022). The serious repercussions of physical dependency and lifelong disability linked with PNI drive researchers and clinicians to hunt for therapeutic approaches that can speed up the axonal regeneration to restore the loss of sensorimotor functions thereby preventing the atrophy of the target tissue and repairing such injuries to prevent inevitable lifelong impairment (Akram *et al.*, 2022). Traumatic nerve plexus avulsion is quite common among animals in veterinary medicine (Van Soens *et al.*, 2009). The evaluation of nerve regeneration by employing animal models in translational research provides future therapeutic opportunities for both humans and veterinary medicine (Maugeri *et al.,* 2021).

Probiotics are living and non-pathogenic microorganisms that are utilized for health benefits as dietary supplements. The use of probiotics is supposed to maintain balance in the intestinal microbial flora by inhabiting the host organ and avoiding settlement of

pathogenic strains (Markowiak and Śliżewska, 2018; Akram *et al.,* 2021). It has been demonstrated that anxiety and chronic psychological stress are prevented by regular probiotics intake. The *Lactobacillus* and *Bifidobacterium* have been reported to play important roles (Bravo *et al*., 2011, Ait-Belgnaoui *et al*., 2014) to reduce apoptosis in different functional regions of brain, and thereby to improve learning and enhance memory in experimental mice (Girard *et al*., 2009). Several studies have explored the important roles of probiotics in neuronal pathologies, describing their bidirectional interaction in the gut-brain axis (Grenham *et al*., 2011; Cryan and Dinan, 2012). The reported gut-brain axis indicates that gut microorganisms may influence both normal brain function and neurological diseases (Berer *et al.,* 2011; Kigerl *et al.,* 2018).

Probiotics are often regarded as a secure and affordable choice and based on recent research it can be considered a strong contender as a neuroprotective agent, protecting against microbiota dysbiosis (Hou *et al.,* 2022). Probiotics are beneficial for healthy brain and neuronal function and can be utilized to treat a variety of neurological illnesses including neuropathy and neuroinflammation-based brain disorders (Umbrello and Esposito, 2016; Pane *et al.,* 2022). Recent studies have demonstrated that there is an association between peripheral neuropathy and use of wide-spectrum oral antibiotics, which may perturbate gut microbiota and influences neuropathic pain (Morales *et al.,* 2019; Ding *et al.,* 2021). Interestingly, traumatic spinal cord injury (SCI) has been linked to gut dysbiosis, which hinders functional recovery and worsens intra-spinal inflammation and lesion pathology (Kigerl *et al.,* 2018). Given the context, there has been a scarcity of information about the ability of probiotics to regenerate damaged nerves. Therefore, we used a well-established sciatic nerve-injury induced mouse model to assess the role of probiotics in the process of nerve regeneration.

MATERIALS AND METHODS

Experimental animals: Adult BALB/C mice (8–10wks and 25–30g) used in the study. The animals were housed at the animal house at Government College University, Faisalabad-Pakistan. The animals were acclimatized to standard climatic conditions (25°C±2) and relative humidity of 50% with a 12-hour cycle of daylight and dark. They animals were fed with standard mouse fodder and water *ad-libitum*. The experiments of the study were conducted in accordance with the approval (Ref. No. GCUF/ERC/281) from Ethical Review Committee, Government College University, Faisalabad-Pakistan.

Experimental design: The experimental animals were randomly allocated into 04 groups (n = 15 per group): (i) Group-1: control, (ii) Group-2: positive control (Vitamin B12), (iii) Group-3: pre-injury probiotics and (iv) Group-4: post-injury probiotics group. Mice in group-3 were given probiotics by gavage (1.72 x 10^{10} CFU/kg) once daily for 7-days before the onset of sciatic nerve injury and probiotics administration continues after induction of nerve injury. Group-4 received probiotics only after nerve injury, the day of nerve injury being the first day of probiotics. The group-2 was treated with vitamin B12 (by gavage) (0.5mg/kg/day), once daily from the day of nerve injury till the final day of the experiment. The animals were sacrificed on the 13th day of experiment, and samples were taken for further evaluation and analysis using standard protocols.

Probiotic dosage and administration: A 23-strains probiotic blend (1.72 x 10^{10} CFU/capsule, Nexabiotic® Probiotics; ProVita Labs, Speedway, IN) was used in the study. Each mouse was gavaged with 200µL suspension in normal saline, corresponding to 10^{9} CFU bacteria (Tan *et al.,* 2016).

Sciatic nerve lesion: The experimental mice were anesthetized with intraperitoneal mixture of Ketamine (70 mg/kg) and Xylazine (5 mg/kg). After exposing the sciatic nerve, it was crushed mechanically with an identical set of forceps (Imran *et al.,* 2019; Razzaq *et al.,* 2020).

Motor function' analysis: The assessment of motor function is done by *Sciatic Functional Index* (SFI) and *Grip Strength Test* as described in earlier investigations (Imran *et al.,* 2019; Razzaq *et al.,* 2020). For SFI, non-hazardous blue ink was used to dye the hind feet, and the animal's walk pattern was taken on white paper placed on the wooden track box. The finest prints from every walk were considered to collect data for SFI for normal (N) and experimental (E) paws. SFI measurement was done by using the formula given:

$$SFI = \left(-38.3 \times -\frac{EPL - NPL}{NPL}\right) + \left(109.5 \times \frac{ETS - NTS}{NTS}\right) \times \left(13.3 \times \frac{EIT - NIT}{NIT}\right) - 8.8$$

In the equation, the Print Length (PL) and Toe Spread (TS) measurements were taken by standard methods.

The grip strength meter (Bioseb, Chaville, France) was used to measure the grasping force of each mouse's hind limbs (ipsilateral and contralateral to the lesion site). An average of three readings separated by a gap of 1-2 minutes was computed.

Sensory function' analysis: *Hot plate Test* and *Pinprick Test* (Chen *et al.,* 2017) were used for the analysis of sensory function, as described in earlier investigations (Imran *et al.,* 2019; Razzaq *et al.,* 2020), before and after sciatic nerve injury on specified days mentioned in the figures. Briefly in the *Hot Plate Test*, mice were permitted to stand with their operated hind paw in contact with the hot plate's surface at a predetermined temperature of 56±2°C until they responded. Three readings were obtained for each mouse over the course of a 2-minute lap. In the pinprick test, the lateral section of the plantar area of the hind paw was hypothetically distributed into five parts and the animal was placed on a wire-meshed cage, and each location was gently pinpricked (with "Austerlitz insect pin" of size "000") and the rapid withdrawal of the paw was noted as a favorable reaction. The animal with positive reaction at all five locations received 5-score, which indicate a completely functional sensory recovery. Less than 5-score designates partial recovery, whereas a score of "0" indicates a full functional loss.

Total antioxidant capacity (TAC): The ability of the live body's defence mechanism to battle free radicals produced as a result of various pathophysiological processes occurring in the body is known as the antioxidant capacity. The total antioxidative capacity in blood from all experimental animals was assessed using the Erel, 2004 method, and the results were represented in mM of vitamin-C Eq/L (Erel, 2004; Imran *et al.,* 2019).

Total oxidant status (TOS): This term describes the overall amount of oxidants present in a living system. This test is used to evaluate the total level of oxidative stress. The principle of the assay relies on the conversion of the ferrous o-dianisidine into the ferric form, due to the action of oxidants present in the sample. The color intensity (which represents the sample's level of oxidation) was determined by spectrophotometer (Razzaq *et al.,* 2020).

Glycaemic level: A drop of blood was obtained from the mouse's tail to check glucose levels. The glycaemic level was determined using a glucometer (Accucheck) (Maqbool *et al.,* 2021).

Morphometric analysis: For morphometric analysis, muscles (tibialis muscles) from both of the hind limbs were removed at the end of the study, and processed using standard protocols (Maqbool *et al.,* 2021). The mounted sections were stained with H&E-stain using standard procedures, assessed at 40X, and digital camera was used to take pictures (Optica B1). Image-J is used to figure out the cross-sectional area of each muscle fibre. All fibres in the image were averaged out to compare between groups (Maqbool *et al.,* 2021).

Statistical analysis: All the findings were presented as mean ± SEM. The statistical analysis of the data was accomplished using GraphPad Prism (8.0.1). The means of the groups were compared using an ANOVA. Statistical significance was set at a value of $p < 0.05$.

RESULTS

Impact of probiotics on food intake and body mass: To examine the effect of *probiotics* on the pattern of food intake and the body mass of the studies animals, the daily consumption of food and animal's body weight were recorded during the experiment (both before and after inducing the injury to the nerve) in all the groups. The intake of the food was recorded daily at a specific time interval by subtracting the quantity of the food available at that specific time from the food quantity from yesterday. Tukey multiple comparisons test showed that there are no significant differences in the total food intake between any group (Fig. 1a). But the pattern of diet consumption significantly changed (P=0.001) across all groups. Furthermore, each animal's daily body weight was documented, and no statistically significant data was found (Fig. 1b). Therefore, we concluded that neither the body weight nor the pattern of food-intake was disturbed before and after the injury, which validates that the obtained results were exclusively attributed to probiotics.

Probiotics promote motor function recovery: The complete functional loss of motor neurons is the hallmark of the sciatic nerve injury. The development of functional reclamation is associated with the rate of nerve regeneration that is sluggish under normal conditions. The grip strength test (% of initial force) and SFI are utilized to evaluate the pattern of motor function retrieval after sciatic nerve crush in mice. We discovered that group-3 recovered their motor function more quickly. The motor function recovery as assessed by SFI at days 9, and 12 after injury (**p =.007, and *p =.029, respectively) and muscular grip strength at days 10, 11, and 12 after injury (**p =.002, *p=0.03, *p=0.01) in group-3 are shown in the accompanying graph. Our results indicate that probiotics promoted a rapid and statistically significant retrieval of motor functions (Fig. 2a, b).

Probiotics accelerate the restoration of sensory functions: The restoration of sensory function following induction of nerve damage was evaluated using the hot plate test and pinprick test. The paw withdrawal latency was measured on the experimental hind paw by exposing it to a hot-plate and the amount of time it took to react was noted. In the pinprick test, the animal was placed on a wire-meshed cage, and the specified location was gently pin-pricked and the withdrawal reaction was observed. The hotplate and pinprick tests were applied on different days to evaluate the recovery of sensory function. A significant reduction in paw withdrawal latency (P=0.006) and improved pinprick score (P<.001) was noticed in group-3. These results point to the treated group's quicker restoration of sensory function, which is thought to be due to probiotics' potential to encourage axonal regeneration leading to a quick functional recovery (Fig. 3a, b).

Effects of probiotics on oxidative stress and glycaemic level: It is established fact that normal oxidative levels and glucose metabolism is disturbed at the location of nerve damage. The degree of oxidative stress in each group was evaluated as TOS and TAC at the end of the study. To evaluate the therapeutic potential of the probiotics in glucose metabolism, the blood sugar levels were monitored both before and after the injury. Group-3 demonstrated a significant rise in TAC values (***P<0.001) and a reduction in TOS (***P<0.001), confirming probiotics' antioxidative capacity (Fig. 4a, b). The blood glucose level was monitored at different time intervals before and after the induced nerve injury. In contrast to group-2, a substantial decrease in glucose level was seen in group-3 (P<.001) (Fig. 4c). These results suggest that probiotics have hypoglycaemic properties.

Effect of probiotics on the morphology of muscle fibre: The sciatic nerve directly innervates the gastrocnemius and tibialis anterior muscles, which are used for walking and running by inward and outward flexion of the foot. As the sciatic nerve is squeezed, these muscles get denervated, and muscle mass and number of fibres continues to lose. So, to check the recovery status, the muscle mass is evaluated using a small fibre cross-sectional area with irregular shape and their fibre count. The cross-sectional area and fibre count of the muscles in the treatment group particularly

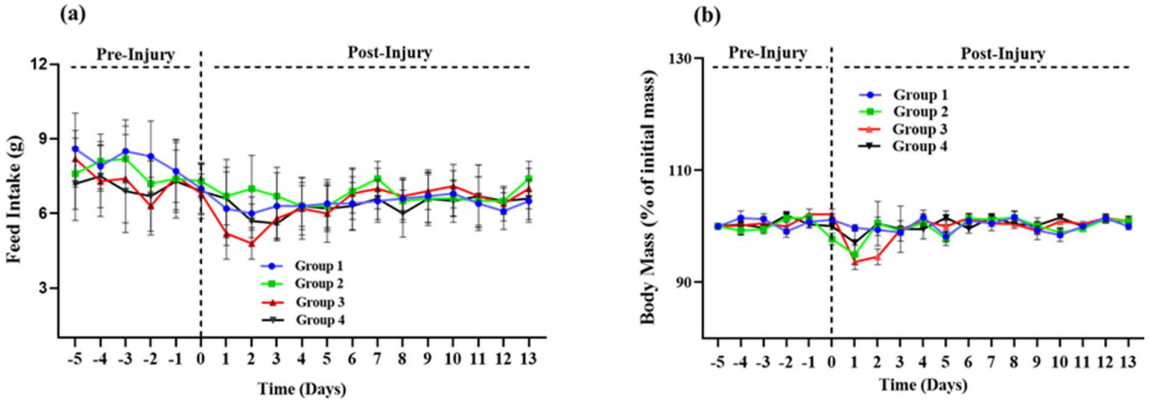

Fig. 1: Probiotics significantly affect the intake of the feeding pattern, but this change in the feeding pattern has no significant effects on the overall food total food intake as well as on the body weight of the studied animals: Group-1 (control), (ii) Group-2 (positive control), (iii) Group-3 (pre-injury probiotics), and (iv) Group-4 (post-injury probiotics) Results are represented as mean ± SEM (n= 15). (a) The effect of probiotics on the pattern of food intake was measured on daily basis, and significant (P=0.001) differences between the two groups at the times under study were found by two-way ANOVA analysis, showing change in the feeding pattern. But non-significant effect of overall diet intake was found (P=0.694) (b) The body-mass measurements were taken throughout the study. Each animal's body-mass is shown as a daily %age of the original mass. There were no differences between the two groups at any time intervals, according to a two-way ANOVA analysis (P=0.140).

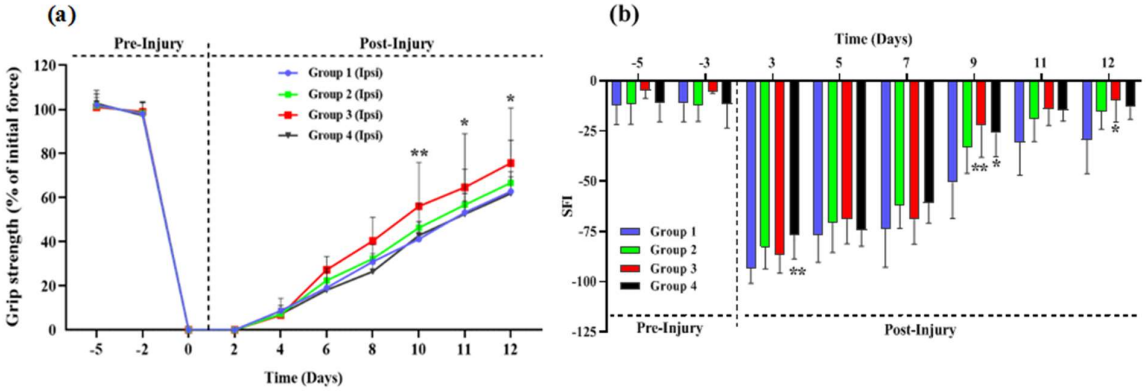

Fig. 2: Probiotics accelerates functional recovery of motor nerves after nerve injury. Group-1 (control), (ii) Group-2 (+ve control), (iii) Group-3 (pre-injury probiotics), and (iv) Group-4 (post-injury probiotics) Results are represented as mean ± SEM (n=15). (a) Data were acquired from the hind limbs ipsilateral to the injury (data in solid lines). The 3rd group improved muscle grip strength at days 10, 11, and 12 after injury (**p =0.002, *p=0.03, *p=0.01) as compared to the 1st group according to Tukey's multiple comparison test. (b) The record of functional index (SFI) of animals at mentioned time-points. Group 3 significantly improved SFI on days 9 and 12 (**p =.007, and *p =0.029, respectively) and Group 4 significantly improved SFI on days 3 and 9 (**p<0.005, and *p =0.031, respectively) on employing Tukey's multiple comparison test.

group 3 show a statistically significant change, indicating that the probiotics have improved the restoration of muscle fibre health (Fig. 5a, b).

DISCUSSION

Peripheral Nerve Injury (PNI) is a neurological condition that leads to serious and long-term functional and physiological disabilities in both human and animal. PNI is among the prevalent and life-threatening medical conditions affecting a huge number of people globally. PNI is also prevalent in veterinary practice as animals with the history of trauma are often seen with nerve plexus avulsion (Van Soens *et al.*, 2009; Lopes *et al.,* 2022). Following an event of a PNI, a cascade of immunological processes begins to eliminate the damaged tissues, and to initiate the intricate nerve-repairing mechanisms. The process of repair is pretty slow that may take months to years to restore functional status. Consequently, the impending muscle atrophy embarks an additional delay in functional retrieval and overall recovery process (Razzaq *et al*., 2020; Anwar *et al.,* 2021; Lopes *et al.,* 2022). Accordingly, the accelerated rate of nerve regeneration leading to its functional recovery before the start of the atrophy of muscle might be helpful. The probiotics have shown to induce anti-cancer, antioxidant, anti-inflammatory and neuroprotective potentials. Interestingly, it has been shown that the impaired functional recovery and exacerbations in intraspinal inflammation occurs in traumatic spinal cord injury (SCI) that has been linked to gut dysbiosis (Kigerl *et al.,* 2018). Importantly, the probiotics and gut microbiota regulate and improve various motor and non-motor symptoms in the experimental animal models (Lee *et al.,* 2023). The animal models entertain the researchers with the advantage of multimodal methodologies and their diverse combinations for the study of axonal regeneration (Maugeri *et al.,* 2021).

In general, any drug administered to an animal may result in altered feeding habits and weight change. The body mass and the feed consumption of animals were measured daily throughout the study to check the interference of feeding pattern. It has been observed that

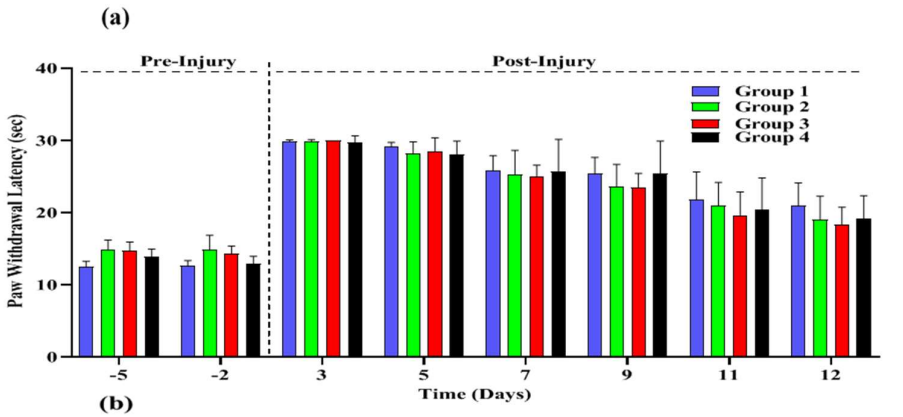

Fig. 3: Probiotics enhance the sensory threshold retrieval after induced nerve injury: Group-1 (control), (ii) Group-2 (+ve control), (iii) Group-3 (pre-injury probiotics), and (iv) Group-4 (post-injury probiotics). The data is denoted as mean ± SEM (n=15). (a) The record of the paw-withdrawal latency in the animals when contact with the thermal stimulus at the mentioned time point. After injury, there was a very significant difference (*P<0.05, **P<0.005) across all groups. (b) Measurement of Paw-withdrawal score in reaction to pinprick stimulation-test. The Two-way ANOVA showed a significant difference (*P<0.05, **P<0.005) in all groups.

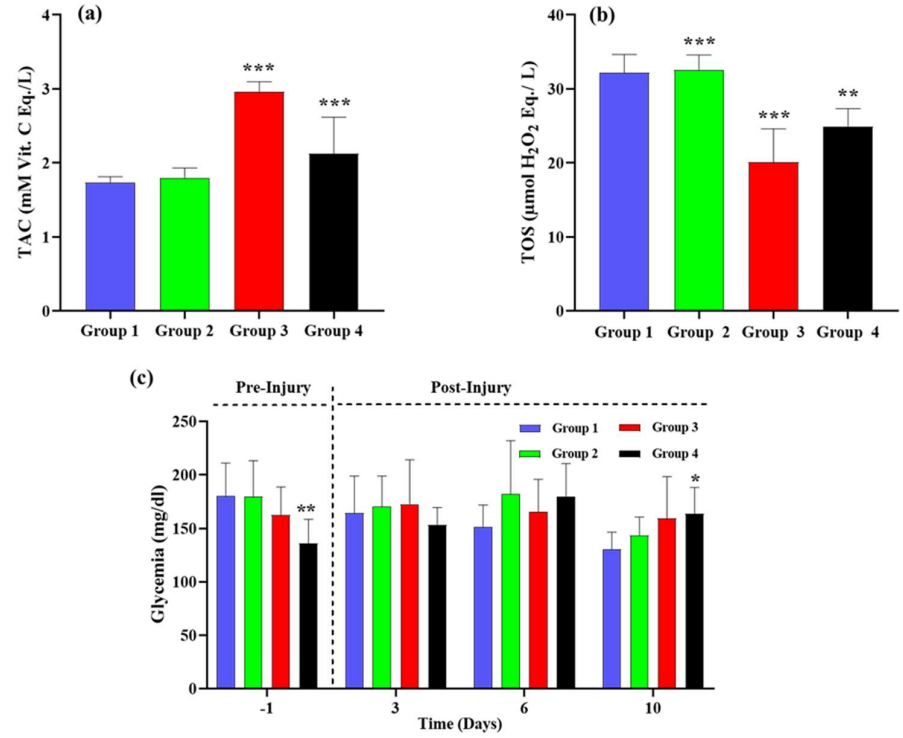

Fig. 4: Probiotics ameliorates the oxidative stress and glycaemic level: Group-1 (control), (ii) Group-2 (positive control), (iii) Group-3 (pre-injury probiotics), and (iv) Group-4 (post-injury probiotics). The results are denoted as a mean ± SEM (n=15). (a) Measurement of Total Antioxidant Capacity (TAC) in all the studied groups mentioned. One-way ANOVA revealed significant changes (***P<0.0001) among the groups. (b) Measurement of Total oxidant status (TOS) in all groups. One-way ANOVA revealed significant differences showed a significant difference (**P<0.005, ***P<0.0001) between all groups. (c) Measurement of the glycaemic level before and after injury. A significant difference was observed between pre- and post-injury glycaemia values for all groups (*P<0.05, **P<0.005).

the addition of probiotics affected feed intake but did not affect body mass. The recovery of the motor function is determined by the rate of nerve regeneration and the number of motor units that are providing the innervation to the target muscle, that may result in a change in muscle grip strength and SFI value. Accelerated motor function recovery was observed in Group-3 and Group-4. The current study revealed that the Group-3 showed improved grip strength (> 50% on day 10, >60 % on day 11, and >70% on day 12 post-injury) and improved SFI values on days 9, 11, and 12 post-injuries. Similarly, the improved sensory function was obvious in the Group-3. Probiotics improve the symptoms by modulating several molecular and cellular processes that control oxidative stress, the inflammatory, anti-inflammatory and immune-mediated pathways and apoptosis (Mirzaei *et al.,* 2022). According to a meta-analysis, taking probiotics may enhance carbohydrate metabolism to a small extent, but with a possibly better benefits when the period of intervention lasts for 8-weeks or when different strains of probiotics are

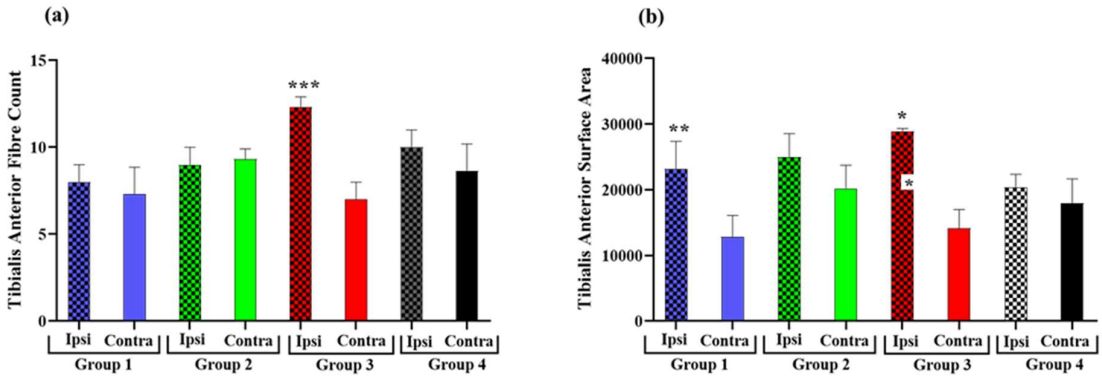

Fig. 5: Probiotics increase the morphological features (i.e., surface area and number of fibres) after nerve injury: Group-1 (Control), (ii) Group-2 (positive control), (iii) Group-3 (pre-injury probiotics) and (iv) Group-4 (post-injury probiotics). The results are shown as mean ± SEM (n= 15). (a) The cross-sectional area of the Tibialis Anterior was examined using a compound microscope; the H&E-stained pictures were captured using a 40x lens. The Ipsi- and Contra-muscles of the control and treatment groups showed significant differences (***P<0.0001) in the ordinary one-way ANOVA. (b) Probiotics' effect on muscle cross-sectional area: The ordinary one-way ANOVA displayed a significant change (*P<0.05, **P<0.005) between the ipsi and contra muscles of the control and treatment groups.

ingested (Zhang *et al.,* 2016). Recent results also indicate that gut microbiota are crucial for normal muscle function in mice by influencing the glucose regulation (Nay *et al.,* 2019). Like these findings, our data indicate that probiotics have the potential to lower blood glucose levels, which may be due to supply of glucose and nutrients to muscles, representing the compelling mechanism that may contribute to the gut microbiota-muscle axis. Oxidative stress performs a significant role in promoting the pathologic activities occurring at the site of injury (Wang *et al.,* 2015). The cascade of processes such as mitochondrial dysfunctions, neuro-inflammation, apoptosis and demyelination (Hussain *et al*., 2020; Lopes *et al.,* 2022) occur after nerve injury, that results in the formation of toxic oxidants further aggravating the injury and thus delay the curative and remedial processes (Pane *et al.,* 2022). Probiotics exhibits antioxidant activity as shown by *in vitro* and *in vivo* research. Consumption of probiotics alone or probiotic-enriched meals may lower oxidative damage, free radical scavenging rate, and changes in the activity of antioxidative enzymes (Mishra *et al.*, 2015). It has been demonstrated that alterations in gut microbiota leads to inflammation along with oxidative stress due to mounting effects of gut-derived toxins in renal disease (Mafra *et al.,* 2014). Similarly, in our experiments, the probiotics in mice significantly increase TAC and decrease TOS proving its potential antioxidant activity.

The probiotics efficiently improve muscle mass and function in different animal models in different metabolic situations. The meta-analysis of randomized controlled trials showed that probiotic supplement improves both muscle mass and global muscle strength in humans (Prokopidis *et al.,* 2023). Interestingly, it is demonstrated that probiotics control intestinal metabolites and regulate muscle fiber mass (Liu *et al.,* 2023). Our data showed that the treatment with probiotics lowers muscular dystrophy while improving the normal form and muscle area during the period of the nerve re-generation after the injury. The histopathological evaluation of the tibialis anterior muscle from the experimental and the control sides in each group supports the findings regarding sensorimotor regain after nerve injury. Different studies suggest existence of the gut-muscle axis contributing to skeletal-muscle mass and function. The mice devoid of gut-microbiota exhibited decreased skeletal muscle mass, and subsequently improvement in the gut microbiota leads to increased skeletal muscle mass with reduced markers of muscle atrophy and enhanced oxidative metabolic capability of muscle (Lahiri *et al.,* 2019). Based on these findings, it has been revealed that probiotics might be an effective therapeutic choice for combating the problem of nervous system injuries and nerve regeneration, but further studies are required to find exact metabolites or products produced by microbiota influencing nerve regeneration, skeletal muscle growth and function.

Conclusions: The current study's findings suggest that probiotics can accelerate nerve function recovery following a mechanically induced injury to a peripheral nerve. These intriguing early findings call for further research into the mechanism underlying the gut-brain axis-mediated neuromodulation and neuroprotection of probiotics. Additionally, it would be interesting to investigate all potential molecular processes that underlie this accelerated axonal regeneration and would be impacted by this treatment. Future extensive and detailed studies may be able to prove whether the probiotics are good candidates for nerve regeneration or not.

Conflicts of Interest: The authors declare no conflict of interest.

Authors contribution: SAB designed the project. SAB and GH supervised the project. JZ and GH performed the experiments. JZ, SAM, GH and SK analysed the data. SAB, GH and SK contributed reagents/materials/analysis tools. ZJ wrote original draft. SAB, SAM and GH revised the paper and critically reviewed it. All the authors read and approved the final version of the manuscript.

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