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Original Research

A Probiotic Mixture Decreases Neuropathy and Oxidative Stress Markers in Diabetic Rats

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Abstract

Diabetic neuropathy (DN) is a type of nerve damage caused by long-term hyperglycemia in diabetes mellitus (DM). The gut microbiota alters in DM. Therefore, improvement of the gut flora may affect neuropathic pain and oxidative biomarkers' responsiveness to the probiotic treatment. The present study aimed to assess the effects of probiotic supplementation on neuropathic pain and oxidative stress biomarkers in diabetic rats' serum. Forty-eight rats (200-250 g) were randomly divided into four groups (n = 12 per group) to examine the effects of the probiotics mixture as follows: the control group (CO), and the diabetic groups received 1 ml probiotics mixture (DP) containing *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, and *Bifidobacterium bifidum* (10^9 CFU of each), 100 mg/kg Gabapentin (DG), or normal saline (DM) daily. The study used animals with

plasma glucose concentrations between 70 and 100 mg/dl. Behavioral tests, including mechanical allodynia, cold allodynia, and thermal hyperalgesia, were used to evaluate the pain on days 1, 4, 7, 14, and 21 of the study. After that, the serum's biochemical analysis was completed. Taking the probiotics mixture decreased mechanical and cold allodynia as well as thermal hyperalgesia. The probiotics group also showed significant reductions in lipid peroxidation levels and increases in total antioxidant capacity (TAC) and glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities compared to the DM group. Our results showed that supplementation with the probiotics mixture could reduce pain-related behaviors in diabetic rats by enhancing the antioxidant capacity in their serum.

Keywords
Diabetic neuropathy; pain; probiotics; antioxidant activity; rats

1. Introduction

Diabetes mellitus (DM) is a chronic, metabolic, silent malady that demands comprehensive scientific management and a holistic therapeutic outlook [1]. As a result of an insufficient secretion or action of insulin, or both, DM results in increased fasting and postprandial blood glucose [2]. The disturbance presents a significant health problem that is one of the foremost reasons for mortality in the world. DM influences a patient's life quality with multiple signs, including impotence, ataxia, weakness, pain, and sensory loss [3]. DM, as a chronic condition, progresses over time and causes several consequences, such as hepatopathy, retinopathy, cardiomyopathy, nephropathy, and neuropathy [4]. The most common and difficult consequence of DM, diabetic neuropathy (DN), causes significant morbidity and mortality. It accounts for 50% to 75% of nontraumatic amputations and is the most prevalent type of neuropathy in the world. Various clinical disorders, together known as DN, can affect distinct parts of the nervous system [5]. DN is a multifunctional disease involving multiple signaling pathways simultaneously, making the pathogenic system highly complex [6, 7]. Indeed, a particular method of preventing diabetic neuropathy has yet to be discovered.

The pathogenesis of diabetic neuropathy is very complex. Hyperglycemia, dyslipidemia, and insulin resistance trigger a cascade of responses, activating pathways such as the polyol, glycolysis, hexosamine, and advanced glycation end-product pathways [8]. These activations enhanced oxidative stress and inflammatory signals, leading to endoplasmic reticulum stress, mitochondrial dysfunction, DNA damage, and elevated inflammatory factor levels [8]. One of the key links between diabetes mellitus and diabetic complications has been suggested to be hyperglycemia-induced nitrosative and oxidative stress [9]. Because of the autoxidation of glucose and the glycosylation of proteins, hyperglycemia ultimately generates free radicals. The pathophysiology of DN is influenced by increased free-radical organization and/or weakened antioxidant defenses, which results in oxidative stress [10]. Several studies on animal models discovered treatment with antioxidants, including NADPH oxidase inhibitors [11], dimethylthiourea [12], superoxide dismutase mimetics [13], and xanthine oxidase inhibitors [14], might reduce diabetic neuropathy complications through oxidative stress inhibition. Since oxidative stress is a critical component of the etiology of

neuropathic pain in people with diabetes, antioxidants that scavenge free radicals can be employed as a therapy to treat DN.

The microbiome is an ecosystem affected by various agents, including metabolism, geography, age, genetics, diet, antibiotic treatment, and stress [15]. The emerging body of research on the connection between GM and neuropathic pain (NP) highlights the intricate interplay between the gut and the nervous system. The bidirectional interplay happens through the Vagus nerve, immune mediators such as the inflammasome, and metabolites such as short-chain fatty acids, aromatic amino acids, bile acids, and even neurotransmitters [16]. These results documented that alteration of gut microbiota could lead to the up-regulation and down-regulation of cytokines and chemokines simultaneously, which may affect the occurrence of NP [17]. A potential way to harness GM to treat NP may be in probiotics, fecal microbiota transplantation, diet, and supplements such as vitamin D and palmatine [16]. Probiotics have recently gained a lot of attention for their potential to lower metabolic profiles [18], and biomarkers of oxidative stress [19]. Probiotics appear to reduce oxidative stress and inflammation by increasing glutathione (GSH) levels [20], lowering superoxide and hydroxyl radicals [21], and suppressing interleukin-6 (IL-6) production in adipocytes [22]. Researchers found that *L. casei* and *L. acidophilus* have antidiabetic properties and can reduce oxidative stress in animal models of diabetes [23]. Additionally, according to several studies, administration of probiotics intensely diminished diabetic rats' fasting blood glucose, hemoglobin A1C, and malondialdehyde (MDA) levels [23]. Besides, other evidence indicated that the gut microbiota substantially affects types of pain [24], such as functional abdominal pain [25], inflammatory pain induced by formaldehyde [26], and chemotherapy-induced pain [27]. The interaction between probiotics and neuropathic and inflammatory pain has received little research. We have recently shown that treatment of rats with neuropathic pain (chronic constriction injury model) with a probiotic mixture of *Lactobacillus plantarum, Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus rhamnosus,* and *Bifidobacterium bifidum* reduces cold and mechanical allodynia and also thermal hyperalgesia. In addition, our results have shown that this mixture reduces lipid peroxidation and increases total antioxidant capacity, superoxide dismutase, and glutathione peroxidase activity in the sciatic nerve of rats [28]. Huang and colleagues have demonstrated that receiving daily oral doses of *Lactobacillus plantarum* for 28 days before and 14 days after CCI effectively reduces neuropathic pain by enhancing the release of anti-inflammatory cytokines in the injured nerve [29].

Our study aimed to investigate whether treatment with a probiotics mixture would improve DN and antioxidant status in streptozotocin (STZ)-induced diabetic rats.

2. Methods

2.1 Animals

The present study was conducted by the Ethical Committee, Deputy of Research and Technology, Kashan University of Medical Sciences (IR.KAUMS.MEDNT.REC.1396.105) guidelines. Animal House of Kashan University of Medical Sciences provided us with young male Wistar rats (200-250 g). The rats were kept in a controlled temperature (22 ± 2°C) and 12 h-12 h light-dark cycle, with free access to food and water.

2.2 Experimental Groups

In this study, four experimental groups (12 animals per each group) were formed: the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or Gabapentin (DG).

2.3 Chemicals and Reagents

The drugs used were streptozotocin (ZellBio, Germany), probiotics (Probiotics International Ltd, United Kingdom), and Gabapentin (Actoverco, Iran). Streptozotocin was dissolved in citrate buffer 0.1%. The probiotic solution was produced using the probiotics mixture (500 mg) and saline (1 ml). Gabapentin dissolved in normal saline and 100 mg/kg of gabapentin was administered via intragastric gavage.

2.4 Induction of Diabetes

Before diabetes was induced, a standard glucometer measured plasma glucose levels in all animals (Bionime, Rightest GM110, GmbH). The study used animals with plasma glucose concentrations between 70 and 100 mg/dl. A single intraperitoneal dose of Streptozotocin (65 mg/kg) was injected for diabetes induction [30]. The levels of plasma glucose were evaluated after one week of STZ administration. Studies on diabetic neuropathy were conducted on animals with plasma glucose levels exceeding 250 mg/dL [31].

2.5 Probiotic Supplementation

The flavorless and colorless multispecies probiotic powder contained a combination of *Bifidobacterium bifidum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Lactobacillus delbrueckii* (Probiotics International Ltd, United Kingdom). The total CFU per gram of the supplement was 2×10^9. The supplementation was given using intragastric gavage, and the probiotic solution was produced by combining the probiotics mixture (500 mg) with saline (1 ml) [28]. Gavage was performed daily on the animals 7 days post-STZ injection for three weeks. Rats in the DG group were given a 100 mg/kg dose of gabapentin via intragastric gavage 30 minutes before the pain evaluation tests [32].

2.6 Behavioral Studies

All behavioral experiments were administrated in a blinded manner. Behaviorally, the neuropathic pain score was determined using the von-Frey, radiant heat plantar, and acetone tests [33]. The behavioral tests of neuropathic pain were done before the probiotic gavage (day 1) and 30 minutes after the gavage on the experiment's 4, 7, 14, and 21 days.

2.6.1 Mechanical Allodynia (Von-Frey Test)

Rats were kept for 15 minutes in a plastic container with a wire mesh bottom to help them adapt. Mechanical allodynia was evaluated by assessing the left hind paw withdrawal response to von-Frey filament. To measure mechanical thresholds, von-Frey filament with bending forces of 2 to 60 grams (Stoelting Inc., Wood Dale, IL) were applied to the central region of the plantar surface of the left

hind paw. Pushing down on the rat's rear paw three times in a row until the rat retracted it or the fiber bowed served as the stimulation. During three consecutive stimulations, the withdrawal threshold was defined as the smallest filament size that obtained at least two withdrawal responses [34].

2.6.2 Thermal Hyperalgesia (Plantar Test)

The Hargreaves method was used to evaluate thermal hyperalgesia. In this procedure, the rats were placed on an elevated clear, square, and bottomless acrylic box on an elevated glass platform and given an extra 15-25 minutes to get used to their surroundings before testing. A plantar test device was used to measure the latency of paw withdrawal in response to radiant heat (Ugo Basile, Varese, Italy). A high-intensity projector lamp was turned on and directed under the hind paw mid-plantar surface. The stimulus was administered three times with a 5-minute break between each exposure to prevent the hind paw sensitization. To prevent tissue injury, a 22-second cutoff was applied. Each foot's average withdrawal response latency was calculated [34].

2.6.3 Cold Allodynia

The acetone test was used to detect cold allodynia (evaporation-evoked cooling). On the hind paw skin’s plantar surface, without touching the skin, 250 µl of acetone was applied five times (at intervals of five minutes) to rats lying on a wire mesh floor. (The number of paw withdrawals/Total Number of Trials) × 100 was used to express the frequency of the paw withdrawal reflex [34].

2.7 Blood Collection

After the behavioral examination, the animals were placed in a standard CO_2 chamber for 3-5 minutes for anesthesia, and blood was drawn from the jugular vein and left at room temperature to clot. It was then centrifuged at 2500 RPM. After being separated, the serum was kept at -80°C until quantified.

2.8 Biochemical Studies

The malondialdehyde (MDA) level, a byproduct of lipid peroxidation, was assessed using the thiobarbituric acid (TBA) reaction. 0.5 ml of serum and 1 ml of trichloroacetic acid (20%) were combined, and the mixture was centrifuged at 2500 g for 10 minutes. Then, 0.5 ml of the serum was combined with 1 ml of TBA 0.67%. The resultant mixture was centrifuged at 3000 g after being chilled in ice and heated at 100°C for 25 minutes. Finally, a spectrophotometer measured the transparent supernatant's absorbance at 532 nm. The concentration of MDA was given as nmol/mg protein. The Lowry et al. technique determined the samples protein content. The activity of superoxide dismutase (SOD) and the total antioxidant capacity (TAC) (ZX-44108 and ZX-44109, ZellBio, Germany) and glutathione peroxidase (GPx) (Nagpix-96, Navand Salamat, Iran) in the sample was measured using standard commercial laboratory ELISA kits.

2.9 Statistical Analysis

All analysis were conducted using GraphPad Prism (San Diego, CA, USA). All results were presented as mean S.E.M. $P < 0.05$ was considered a significant difference. Repeated-measures analysis of variance and Tukey's test were used to analyze the behavioral data statistically. Data from biochemical testing were evaluated using a two-way ANOVA with a Tukey's correction for multiple comparisons.

3. Results

3.1 Effect of Probiotics Mixture on STZ-Induced Diabetes Symptoms in Rats

The intraperitoneal injection of STZ successfully induced the rat model to study the function of probiotics in diabetic neuropathy. The body weight of the rats was determined and recorded. On days 4, 7, 14, and 21 following therapy, the STZ-treated group rats were lighter than the control group ($P < 0.001$). On days 14 and 21 of probiotic administration, the body weight significantly increased in comparison to the DM rats ($P < 0.01$). However, still, the body weight on days 4 to 21 was lower than the CO group ($P < 0.001$) (Figure 1A).

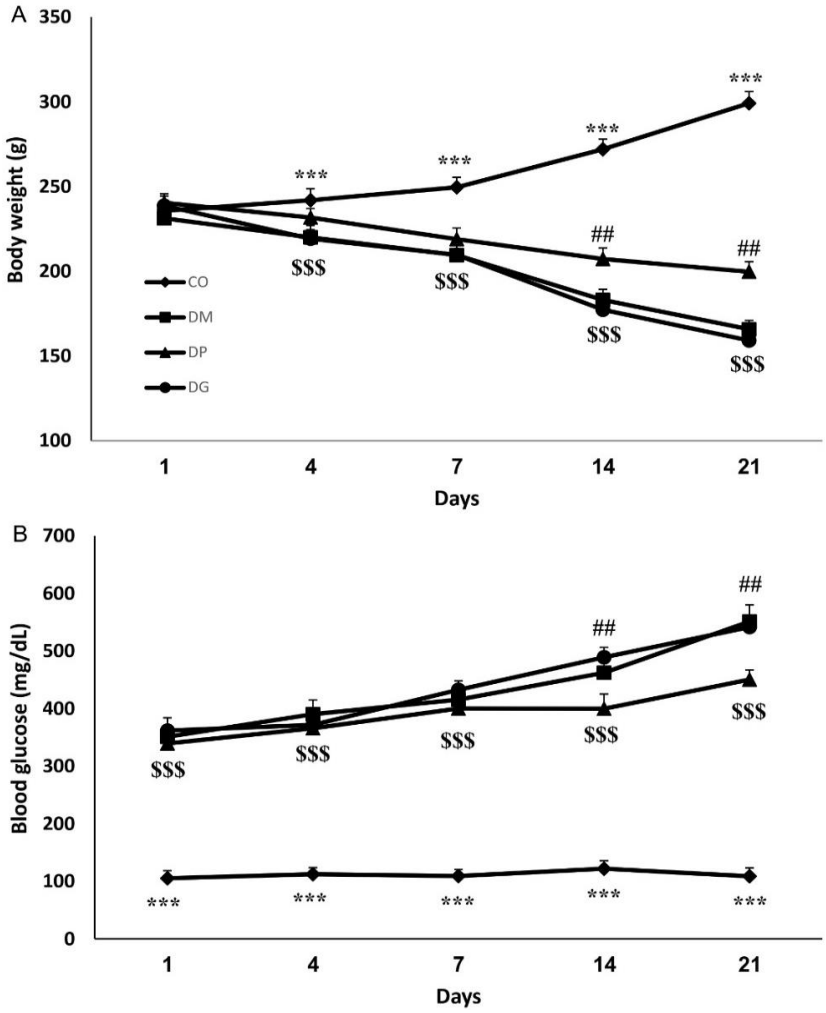

Figure 1 Effect of different treatments on body weight (A) and blood glucose (B) of the animals at days 1, 4, 7, 14, and 21 of the experiment. Results are expressed as Mean ±

SEM. The experimental groups (12 animals per each group) include the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or Gabapentin (DG). *** P < 0.001 the CO group vs. the DM group. ## P < 0.01 the DP group vs. the DM group. $$$ P < 0.001 the DP group vs. the CO group.

Besides, animals injected with STZ exhibited a significant elevation in blood glucose levels when compared to the control animals from one week after STZ injection to day 21 of the experiment (P < 0.001). Still, this increase was suppressed by probiotics mixture when compared to the STZ animals on days 14 and 21 of treatment (P < 0.01). Nonetheless, the blood glucose levels of the DP group remained considerably higher than those of the CO group throughout the experiment (P < 0.001) (Figure 1B).

3.2 Effects of Probiotics Mixture on STZ-Induced Diabetic Neuropathic Painful Responses

3.2.1 Cold Allodynia

The results of cold allodynia are shown in Figure 2. Paw withdrawal latencies to cold stimuli significantly decreased in diabetic rats (P < 0.001). Probiotics treatment significantly declined withdrawal frequency compared to the DM group on days 14 and 21 of the experiment (P < 0.001). When compared to the DM group on days 7, 14, and 21 of the research, gabapentin, an effective neuropathic pain medication, dramatically decreased withdrawal frequency (P < 0.001). However, a statistical difference was seen between the paw withdrawal frequency in the DP group and the CO group on days 7, 14, and 21 of the experiment (P < 0.001). Moreover, no significant difference was observed between the DP and the DG group in withdrawal frequency on any study day (P = 0.695).

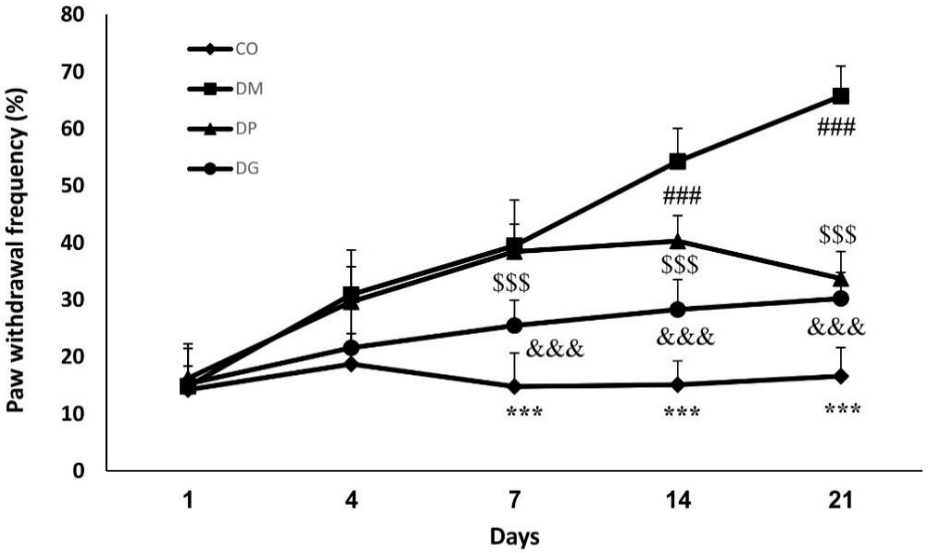

Figure 2 Graphs show the response frequencies of foot withdrawals. Cold stimuli were applied to the hind paw with acetone. The behavioral responses were determined at days 1, 4, 7, 14, and 21 of the experiment. Results were expressed as Mean ± SEM. The experimental groups (12 animals per group) include the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or

Gabapentin (DG). *** P < 0.001 the CO group vs. the DM group. ### P < 0.001 the DP group vs. the DM group. $$$ P < 0.001 the DP vs. the CO group. &&& P < 0.001 the DG group vs. the DM group.

3.2.2 Mechanical Allodynia

The withdrawal threshold significantly decreased in the DM group compared to the CO group (P < 0.001). Accordingly, on days 4 to 21 after STZ induction of diabetes in rats, the hind paw became sensitive to mechanical stimulation, even using a weaker von-Frey filament test. The withdrawal threshold was raised in the rats treated with a probiotic mixture on days 14 and 21 (P < 0.001), albeit it did not entirely return to the level of the control group (P < 0.01). Data analysis also showed that gabapentin administration significantly increased the paw withdrawal threshold compared to the DM group from the seventh day until the end of the study (P < 0.001). Additionally, statistical analysis revealed no difference between the DP and DG groups on any of the research days (P = 0.804) (Figure 3).

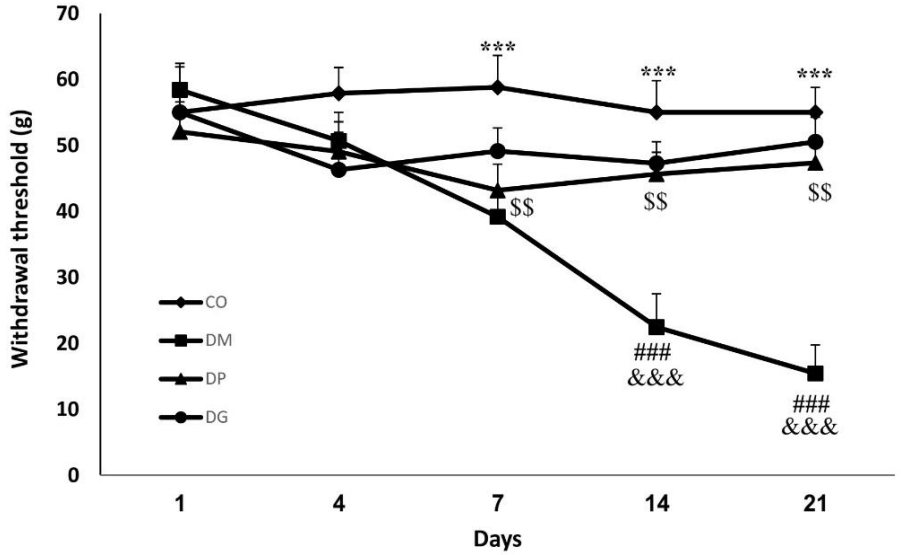

Figure 3 Graphs show the response paw withdrawal threshold (gram) with stimuli applied by a series of von-Frey filament. The behavioral responses were determined 1, 4, 7, 14, and 21 days of the experiment. The results are expressed as Mean ± SEM. The experimental groups (12 animals per group) include the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or Gabapentin (DG). *** P < 0.001 the DM group vs. the CO group. ### P < 0.001 the DP group vs. the DM group. $$ P < 0.01 the DP group vs. the CO group. &&& P < 0.001 the DG group vs. the DM group.

3.2.3 Thermal Hyperalgesia

A significant increase in the paw withdrawal latency to thermal stimuli was observed in the diabetic rats from day 7 to day 21 of the study, as shown in Figure 4 (P < 0.001). Consecutive treatment of the animals with a probiotic mixture significantly reversed the elevated withdrawal latency (P < 0.01). Gabapentin treatment significantly diminished the withdrawal latency of the

diabetic rats compared to the DM group (P < 0.001). Additionally, data analysis revealed that the anti-nociceptive effect of the probiotics mixture is comparable to that of gabapentin. Therefore, the difference between the DP and the DG groups was not statistically significant (P = 0.087).

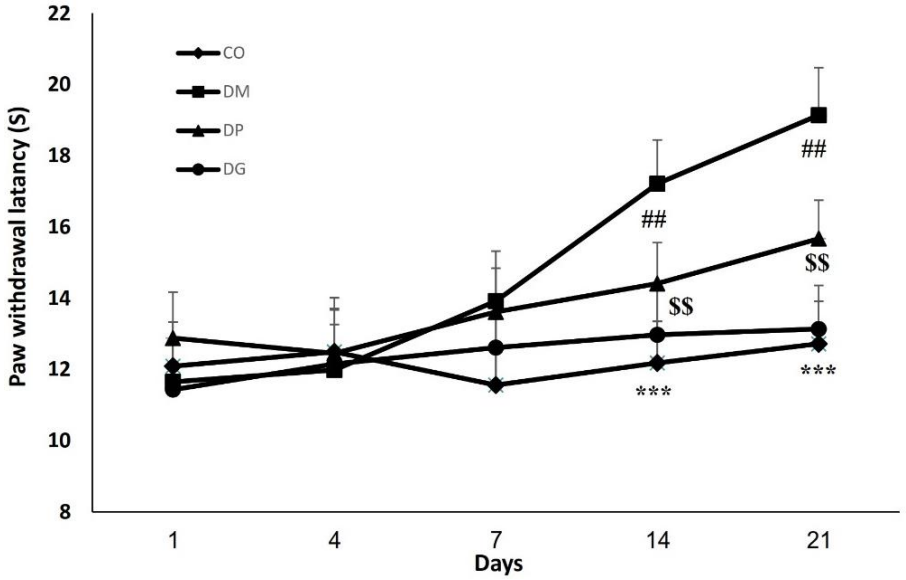

Figure 4 Heat hyperalgesia measured by noxious radiant heat paw withdrawal latency (S) on different experiment days. The behavioral responses were determined 1, 4, 7, 14, and 21 days of the experiment. The results are expressed as Mean ± SEM. The experimental groups (12 animals per group) include the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or Gabapentin (DG). *** P < 0.001 the DM group vs. the CO group. ## P < 0.01 the DP and DG groups vs. the DM group. $$ P < 0.01 the CO vs. the DP group.

3.3 Effects of Probiotics Mixture on Serum Oxidative Stress Biomarkers in STZ-Induced Diabetic Rats

3.3.1 Lipid Peroxidation

Using calorimetry technique, changes in the MDA levels of different groups were studied (Figure 5). Data analysis revealed that different treatments affect the study groups' malondialdehyde levels ($F_{3,44}$ = 194.542, P < 0.0001). The acquired data demonstrated that, in comparison to the CO group, the MDA level in the DM group was considerably higher (P < 0.001). Consecutive treatment with probiotics decreased the elevated MDA expression induced by STZ administration (P < 0.001). It should be noted that the MDA level did not fully increase to the level of the control group (P < 0.001). The levels of MDA in the gabapentin group did not statistically differ from those in the DM group (P = 0.725). Furthermore, it was found that the level of MDA in the DM group was significantly lower than that in the DG group (P < 0.001).

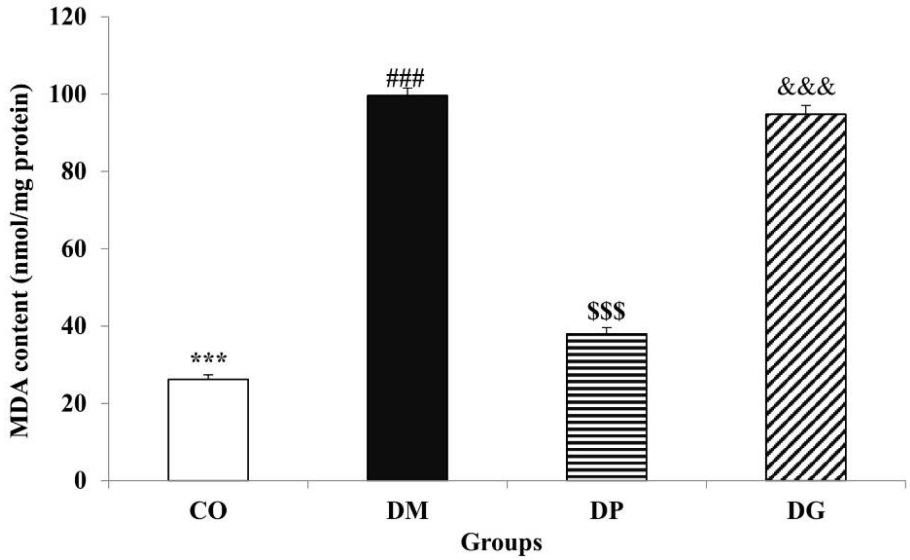

Figure 5 The malondialdehyde (MDA) level in a blood sample of animals in all groups. Values are expressed as Mean ± SEM. The experimental groups (12 animals per group) include the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or Gabapentin (DG). *** $P < 0.001$ the CO group vs. the DM, DP, and DG groups. ### $P < 0.001$ the DM group vs. the DP group. &&& $P < 0.001$ the DG group vs. the DP group. $$$ $P < 0.001$ the DP group vs. the CO group.

3.3.2 SOD and GPx Enzyme Activities

The study groups' SOD and GPx activity varied significantly, as shown by the two-way ANOVA results ($F_{3,44} = 21.058$; $P < 0.0001$, $F_{3,44} = 198.236$; $P < 0.0001$, respectively). When compared to the control group, the diabetic rats' SOD and GPx activity significantly decreased ($P < 0.001$ for both comparisons). There was also a significant increase in the activity of SOD and GPx in the DP group compared to the DM group in the study ($P < 0.001$ for both comparisons). Indeed, the probiotic treatment restored the SOD of the DP rats to a normal level so that we detected no significant difference between the DP and the CO groups ($P = 0.06$). Administration of gabapentin did not change the activity of SOD and GPx in comparison to the DM group ($P = 0.075$) (Figure 6A and Figure 6B).

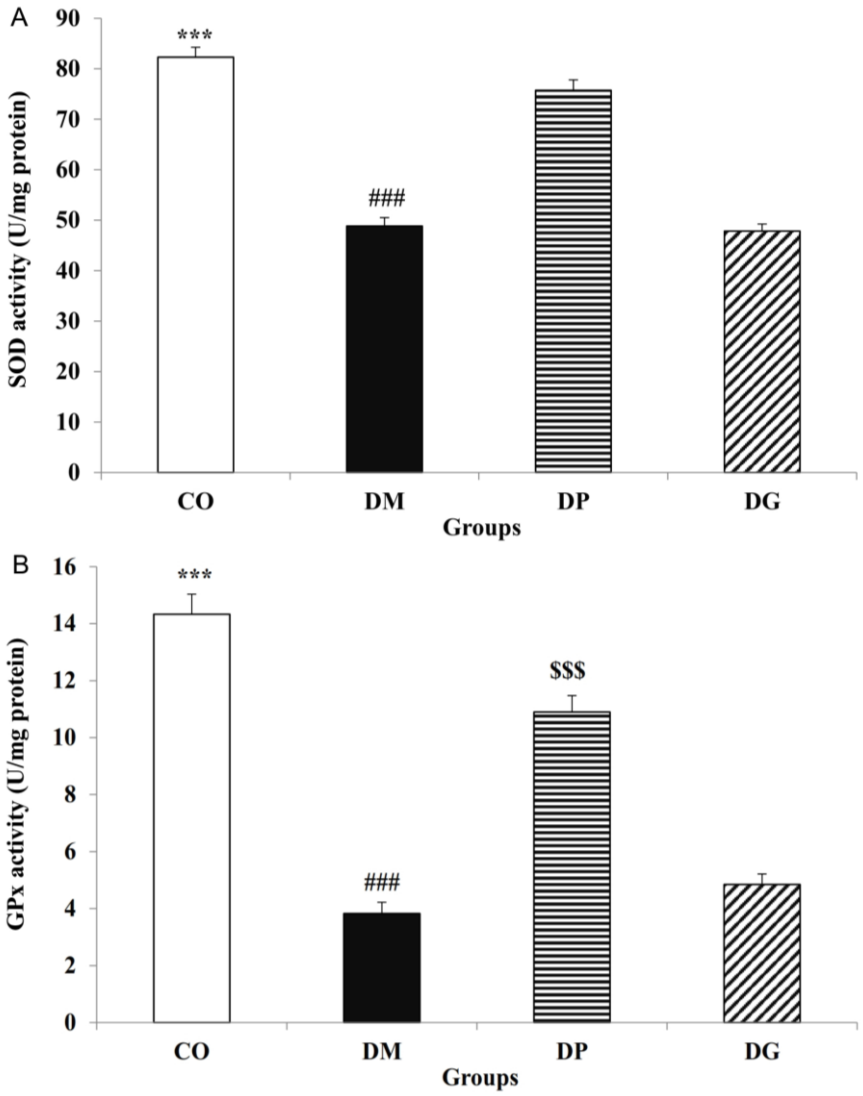

Figure 6 Superoxide dismutase (SOD) (A) and glutathione peroxidase (GPx) (B) activity in blood samples of the animals in all groups. Values are expressed as Mean ± SEM. The experimental groups (12 animals per group) include the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or Gabapentin (DG). *** $P < 0.001$ the CO group vs. the DM, DP, and DG groups. ### $P < 0.001$ the DP group vs. the DM group. $$$ $P < 0.001$ the DP vs. the CO group.

3.3.3 Total Antioxidant Capacity

Results of two-way ANOVA revealed a difference between the serum level of TAC in all the animal groups ($F_{3,44} = 118.265$; $P < 0.0001$). Moreover, the TAC level of the DM group (0.21 ± 0.02 µmol/ml) was significantly lesser than the CO group (1.20 ± 0.06 µmol/ml; $P < 0.001$). Treatment with a probiotic mixture significantly enhanced the total antioxidant capacity in comparison to the DM group ($P < 0.001$). But, the probiotics mixture could not restore the TAC level of the DP animals to a normal level ($P < 0.001$). The administration of gabapentin did not change the TAC level in comparison to the DM group ($P = 0.87$). Also, the levels of TAC in the probiotics group were significantly higher than in the gabapentin group ($P < 0.001$) (Figure 7).

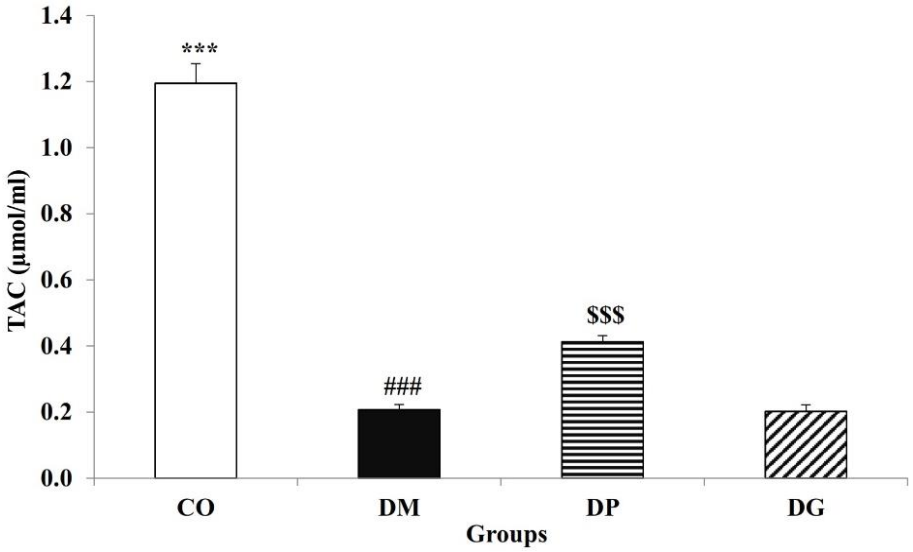

Figure 7 The total antioxidant capacity (TAC) level in the blood sample of the animals in all groups. Values are expressed as Mean ± SEM. The experimental groups (12 animals per group) include the control group (CO), and the STZ-treated (diabetic) groups received normal saline (DM), probiotics mixture (DP), or Gabapentin (DG). *** P < 0.001 the CO group vs. the DM, DP, and DG groups. ###P < 0.001 DP group vs. DM group. $$$ P < 0.001 DP group vs. DG group.

4. Discussion

In this study, we evaluated the effects of supplementation with a probiotics mixture including *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, and *Bifidobacterium bifidum* on pain-related behavior and antioxidant status in Streptozotocin-induced diabetic rats. During our research, we discovered that probiotic treatment alleviated mechanical and cold allodynia and thermal hyperalgesia in diabetic rats. Additionally, we found that probiotic treatment increases GPx and SOD activities, decreases MDA levels, and increases TAC levels in the serum of diabetic rats. Besides, as we expected, gabapentin reduced allodynia and hyperalgesia in diabetic rats and did not change GPx and SOD activity and levels of MDA and TAC in the rats' serum. According to studies, gabapentin reduces neuronal excitability and alters the release of neurotransmitters by acting on calcium channels in the central and peripheral nervous systems [35].

We evaluated the effects of probiotic supplementation on FBS and weights of rats, even though these two factors were not among the study's objectives. Our results have shown that the probiotics mixture significantly suppressed increased blood glucose in diabetic rats. Besides, we saw that the STZ-treated rats were lighter than the control group, and on days 14 and 21 of probiotic administration, the body weight significantly increased compared to that of the DM rats. Diabetes mellitus is known to disturb the uptake and utilization of glucose and the metabolism of glucose. As a result, the body's cells cannot extract glucose from the blood and use it as energy. When this happens, the body starts burning fat and muscle for energy, resulting in weight loss. In line with our results, Babashahi and her colleagues have demonstrated that probiotic soy milk fermented by Lactobacillus plantarum significantly reversed the elevation of blood glucose and reduced weight in diabetic rats [36]. Also, Mohammadi Sartang et al. have revealed that probiotic supplementation

decreases blood glucose and increases weight in Streptozotocin nicotinamide-induced diabetic rats [37].

Many experimental and clinical studies have suggested that oxidative stress is crucial in diabetes pathogenesis and its complications progression. Increased oxidative stress in diabetes is a pathological condition that changes electrophysiological parameters and afflicts sensory perception due to damage to non-myelinated and myelinated nerve fibers [38]. NP is caused by hyperexcitable afferent nociceptors and central neurons that generate spontaneous impulses sent through axons and the dorsal ganglia due to oxidative stress-induced injury to peripheral nerves [39]. Furthermore, oxidative stress-related death of neuronal and Schwann cells is linked to hyperglycemia in diabetes mellitus [40]. The diabetic rats have shown unusual pain-related behaviors, such as increased sensitivity to painful stimuli (hyperalgesia) or nociceptive responses to a normally innocuous stimulus (allodynia). Besides, antioxidant defense weakness is another critical factor in the experimental DN pathogenesis that results from increased levels of radical formation [41]. Also, it has been shown that hyperglycemia can stimulate both sciatic nerve lipid peroxidation and reactive oxygen species (ROS) formation, leading to sciatic nerve dysfunction and decreasing endoneurial blood flow [42]. In addition to oxidative stress as the main factor in diabetic neuropathy genesis, hydrogen peroxide, superoxide radicals, reactive nitrogen species, and hydroxyl radicals are also involved [43]. Also, prolonged hyperglycemia and increased levels of ROS production result in increased oxidative stress with over-activation of NADPH oxidase, an essential component of metabolic syndrome [44]. The decreased serum GSH, GSH/GSSG concentrations, and increased serum GSSG level, percentage of SOD inhibition, and urine MDA level in DN rats might verify the theory that continuous hyperglycemia and accumulation of ROS productions may cause axonal atrophy, demyelination, blunted regenerative potential, and loss of peripheral nerve fibers, which mainly contribute to the pathogenesis of DN [45]. Oxidative stress plays a crucial role in the development of neuropathic pain in diabetes, so using antioxidants that scavenge free radicals could be a therapeutic strategy for treating diabetic neuropathy. Scientists have used many antioxidants to resolve nerve-related problems in animal models of diabetic neuropathy [6]. It has been shown that glutathione treatment could recover diabetic neuropathy [46]. Free radical generation increases in diabetes mellitus, resulting in lipid peroxidation and MDA generation [47]. In addition, GPx, SOD, and CAT activities that scavenge reactive oxygen species are diminished in diabetes mellitus [48]. So, enhancing these enzymes' activity is essential for removing reactive oxygen species. Improving oxidative stress status may contribute to managing diabetes [47].

It has been documented that diabetes mellitus may arise from an imbalance in the gut microbiota [49]. Larsen et al. demonstrated that the GM of diabetic patients is somewhat rich in gram-negative bacteria [50]. A meta-analysis showed that probiotics could be critical in type 2 diabetes mellitus prevention and treatment [51]. In a study by Ejtahed and colleagues, ingestion of probiotic yogurt considerably lowered HbA1c and fasting blood sugar and enhanced blood GPx and SOD activity and also TAC in comparison to regular yogurt consumption. In addition, both groups' MDA levels were significantly decreased [52]. Yadav et al. showed that probiotic Dahi could suppress oxidative destruction in the diabetic rats' pancreatic tissues by lipid peroxidation inhibition and preserving GPx, SOD, and CAT activity [53]. Studies showed that supplementation with *Lactobacillus casei* and *Lactobacillus acidophilus* weakens oxidative stress and has anti-diabetic effects in animal models [23, 54].

Besides MDA, SOD, Gpx, and TAC, evaluating changes in other oxidative stress markers, including mitochondria function markers, GSH/GSSH ratio, and NAD/NADH ratio in diabetic rats and the effects of probiotic supplementation on them could be helpful. Because of some limitations, we could not assess these factors, and we strongly suggest evaluating them in future studies.

5. Conclusions

In conclusion**,** our findings displayed that supplementing with the probiotics mixture could alleviate the cold and mechanical allodynia and thermal hyperalgesia in Streptozotocin-induced diabetic rats. This was because the probiotics mixture increased GPx and SOD activity, decreased MDA levels, and increased the TAC in the serum of rats. One limitation of the present study was that it did not examine the histopathological changes of the sciatic nerve. Evaluating these possible changes and the electrophysiological aspect of the sciatic nerve function following probiotic supplementation could help find how probiotics improve diabetic neuropathy. Besides, because taking normal doses of probiotics is safe, the effects of the present mixture of probiotics in humans with diabetic neuropathy could be evaluated.

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Author Contributions

SAT and FB contributed to supervising the project. MS, MM, EH, and FA contributed to performing the experimental sets. MS, FA, and SAT contributed to providing the initial manuscript draft and SAT revised this draft. All authors read and approved the final edition of the manuscript.

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Competing Interests

Authors state no conflicts of interest.

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