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Original Article

Effects of the Oral Ingestion of Probiotics on Brain Damage in a Transient Model of Focal Cerebral Ischemia in Mice

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Abstract

Background: Probiotics are microorganisms that may influence brain function via altering brain neurochemistry. New research evidence suggests that probiotic bacteria might protect tissue damage through diminishing the production of free radicals and/or inflammatory cytokines. Therefore, this study was designed to evaluate the effects of probiotic bacteria on the prevention or reduction of brain damage in an experimental model of stroke in mice.

Methods: In this study, 30 male BLC57 mice were randomly divided into 6 equal groups. Focal cerebral ischemia was induced via middle cerebral artery occlusion for 45 minutes, followed by 24 hours of reperfusion, in the mice. Probiotics at a concentration of 10^7 CFU/mL were administered by oral gavage daily for 14 days before ischemia. Infarct size, neurological outcome, and biochemical markers were measured 24 hours after brain ischemia. Statistical analysis were performed using the one-way ANOVA and/or Kruskal–Wallis ANOVA on rank by Sigma Stat (2.0; Jandel Scientific) software.

Results: Our results indicated that pretreatment with probiotics significantly reduced infarct size by 52% (P=0.001) but could not improve neurological function (P=0.26). Moreover, the administration of probiotics significantly decreased the malondialdehyde content (P=0.001) and the tumor necrosis factor-alpha level (P=0.004) in the ischemic brain tissue.

Conclusion: The findings of the present study showed that probiotic supplements might be useful in the prevention or attenuation of brain ischemic injury in patients at risk of stroke. Probiotics may open new therapeutic alternatives for the prevention of stroke. More preclinical and clinical studies are, however, needed to clarify their efficacy in cerebral stroke.

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Keywords ● Probiotics ● Focal cerebral ischemia ● Tumor necrosis factor-alpha ● Mice

What's Known

- Effects of the oral intake of probiotics on the prevention of cerebral ischemic injury are not clear.

What's New

- Oral consumption of probiotic bacteria considerably reduced brain damage in mice.
- This effect may be mediated via inhibiting the synthesis of TNF-α and oxidative stress pathway.
- Probiotics may open new therapeutic alternatives for the prevention of stoke.

Introduction

New evidence has revealed that gut microbacteria communicate with the central nervous system through neural, endocrine, and immune pathways and may have a direct effect on brain chemistry.[1] In this regard, there has been much evidence showing that regular feeding with the lactobacillus strain probiotics may be able to alter the expression of the receptors

for the brain-derived neurotrophic factor (BDNF) and the neurotransmitters of serotonin and gamma-aminobutyric acid (GABA) in the brain.[2-4] These data have verified that probiotics might have the potential to alter brain function and mood. Preclinical and clinical studies have suggested that probiotics may have a role in the management of anxiety, depression, cognitive impairment, chronic pain, and inflammatory diseases.[5-8]

Recent research has indicated that probiotics can attenuate tumor necrosis factor-alpha (TNF-α) production, upregulate anti-inflammatory cytokines, and enhance the activities of antioxidant enzymes in vivo and in vitro.[5,8,9] Divyashri et al.[9] suggested that probiotic bacteria might protect tissue damage via diminishing the production of free radicals and inflammatory cytokines. Additionally, probiotic bacteria in the gut may have the potential to change the patterns of DNA methylation and other epigenetic changes that have critical effects on the development of the brain.[10] Moreover, it has been recently reported that probiotics have a protective effect against ischemia damage in intestinal and cardiac tissues in rats.[11-13] Tan et al.[14] indicated that the daily oral intake of probiotics for 21 days was able to adjust immunological imbalance and result in recovering neurological function in patients with traumatic brain injury. More recently, Sun et al.[15] reported that *Clostridium butyricum* pretreatment was able to reduce cerebral ischemia injury via suppressing the apoptosis and amplification of antioxidant enzyme activity in a global model of brain ischemia in mice. However, it would be of great interest to investigate whether the oral consumption of *Bifidobacterium breve, Lactobacillus casei, Lactobacillus bulgaricus,* and *Lactobacillus acidophilus* probiotics for 2 weeks can reduce the severity of injury in transient focal cerebral ischemia in mice.

Materials and Methods

Animals

This experimental study was conducted on 30 male BLC57 mice (25±30 g), obtained from a breeding colony at Semnan University of Medical Sciences, Semnan, Iran. The animals were kept in standard cages with free access to food and water. The study protocol was approved by the institutional research ethics committee (ethical code number: IR.SEMUMS.REC.1394.98), and the experiments were performed in accordance with the national guidelines for conducting animal studies.

Experimental Model of Stroke

The mice were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg i.p.). Transient middle cerebral artery occlusion (MCAO) was induced by the intraluminal filament method as described previously.[16] Briefly, the right common carotid artery and its branches were exposed through a midline neck incision. Under laser Doppler flowmetry guide, a silicone-coated 8-0 monofilament was inserted into the internal carotid artery and advanced until the regional cerebral blood flow was reduced to less than 15%–20% of the baseline. The filament was removed after 45 minutes, and reperfusion was conducted for 24 hours.

Experimental Protocol and Design

Effects of probiotics on infarct size and neurological outcome

Fifteen animals were divided randomly into 3 groups. In the 1st group (n=5), sham-operated surgery was done without MCAO. For the 2nd group (n=5), saline as vehicle was given by oral gavage daily for 14 days. In the 3rd group (n=5), the mice were pretreated with probiotics at a concentration of 10^7 CFU/mL by oral gavage daily for 14 days. In the 2nd and 3rd groups, neurological deficit and infarct size were evaluated 24 hours after MCAO and in the 1st group 24 hours after surgery.

Effects of probiotics on the malondialdehyde and tumor necrosis factor-alpha level

Fifteen mice were divided into 3 groups. In the 1st group (n=5), the mice were sham-operated. In the 2nd group 2 (n=5), saline as vehicle was given by oral gavage daily for 14 days. In the 3rd group (n=5), the mice were pretreated with probiotics at a concentration of 10^7 CFU/mL by oral gavage daily for 14 days. Brain tissue was homogenized and centrifuged 24 hours after ischemia. Then, the supernatant was used for the measurement of the malondialdehyde (MDA) content (as an oxidative stress biomarker) and the TNF-α level.

Probiotics strain and preparation

In this study, we used a combination of 4 viable probiotic bacteria strains, namely *Bifidobacterium breve, Lactobacillus casei, Lactobacillus bulgaricus (Lactobacillus delbrueckii subsp. bulgaricus),* and *Lactobacillus acidophilus*, with a 10^9 CFU/g colony count. They were prepared on a laboratory scale at the Neurophysiology Research Center of Shahid Behest University of Medical Sciences, Tehran, Iran. The concentration of 10^7 CFU/mL was provided by dissolving 1 g of probiotics in 50 mL

of saline. Half milliliter of this solution was given to the mice via oral gavage daily for 14 days.

Biochemical Parameter and Tumor Necrosis Factor-Alpha Assay

The supernatants of the ischemic brain tissues were used for biochemical analyses. The MDA content (as a lipid peroxidation index) was measured as described previously.[15,17] The level of TNF-α was measured via the enzyme-linked immunosorbent assay (ELISA) method using a TNF-α ELISA kit (Biorbyt, United Kingdom).

Infarct Size

The mice were sacrificed under deep anesthesia 24 hours after brain ischemia. The brains were removed and cut coronally into five 2-mm-thick slices using a brain matrix. The slices were immersed in 2% of a 2, 3, 5-triphenyl tetrazolium chloride solution (Sigma, Germany) at 37 °C for 10 minutes. The slices were then immersed in 10% buffered formalin for 24 hours. After pictures were taken with a digital camera at high resolution (Cannon, Japan), the infarct areas were measured using image analyzer software (NIH Image Analyzer). The infarct volume of each slice was computed by multiplying the infarct area of the slice by its thickness, and the total infarct volume of each brain was calculated by summing the infarct volumes of the 5 brain slices. Infarct volume was edema-corrected as explained formerly.[18]

Neurobehavioral Testing

Neurobehavioral examination was performed 24 hours after MCAO using a modified neurological test (table 1). Neurological function was graded on a scale of 0–14 (normal score=0; maximum deficit score=14). A score of 10–14 is severe; 5–9, moderate; and 1–4, mild.[19,20]

Beam-Balance Testing

The beam-balance test is used to evaluate slight deficits in motor skills and balance.[21] Each animal was individually placed on a beam-walking apparatus, comprising a wooden bar (100 cm long, 1.2 cm wide, and 50 cm high). Response to placement and posture on the beam and at the time before dropping was assessed. The motor performance of the mice was scored on a scale ranging from 0 to 6 (table 2).

Statistical Analysis

The groups were compared regarding their infarct size, TNF-α, and MDA using the one-way analysis of variance (ANOVA) and the Dunnett post-hoc test. The Kruskal–Wallis ANOVA on rank and the Dunn method as post-hoc tests were used to analyze the neurological scores. The results are presented as mean±standard error of the mean (SEM). Differences were considered significant at the level of P<0.05 (Sigma Stat 2.0; Jandel Scientific, Erkrath, Germany).

Table 1: Neurological examination score after focal cerebral ischemia in the mice

Behavioral test	Score
Motor tests	
Raising the mouse by the tail:	
Flexion of the forelimbs	1
Flexion of the hind limbs	1
Head moved>10° to vertical axis within 30 seconds	1
Placing the mouse on the floor:	
Inability to walk straight	1
Circling toward the paretic side	1
Falling down to the paretic side	1
Abnormal movements	
Immobility and staring	1
Tremor (wet dog shakes)	1
Myodystony, irritability, seizures, and myoclonus	1
Sensory tests	
Visual and tactile placing (limb placing test to detect visual and superficial sensory)	
Moving the mouse laterally toward the table:	
Reaching the table slowly with the help of limbs or cannot move at all	1
Proprioceptive test (deep sensory) Pushing the paw against the table edge to stimulate limb muscles:	
Losing the resistance	1
Reflexes	
Absence of pinna reflex (a head shake when touching the auditory meatus)	1
Absence of corneal reflex (an eye blink when lightly touching the cornea with cotton)	1
Absence of startle reflex (a motor response to a brief loud noise from snapping a clipboard paper)	1
Maximum points	14

Table 2: Motor skills and balance assessment score after focal cerebral ischemia in the mice

Beam balance test	Points
Balances with steady posture	0
Grasps the side of the beam	1
Hugs the beam and 1 limb falls down from the beam	2
Hugs the beam and 2 limbs fall down from the beam (>60 s)	3
Attempts to balance on the beam but falls off (>40 s)	4
Attempts to balance on the beam but falls off (>20 s)	5
Falls off: no attempt to balance or hang on to the beam (<20 s)	6
Maximum points	6

Results

Regional Cerebral Blood Flow

Laser Doppler flowmetry monitoring of the local cerebral blood flow demonstrated that inducing ischemia diminished the blood flow to less than 20% of the baseline throughout the 45 minutes' MCAO in all the groups, whereas the blood flow did not change in the sham-operated group (figure 1). There were no significant differences between the groups *vis-à-vis* the local cerebral blood flow before MCAO, during MCAO, and after reperfusion (P=0.12) (figure 1).

Effects of Pretreatment of Probiotics on Infarct Size and Neurobehavioral Function Outcome

Pretreatment with probiotic bacteria significantly decreased infarct size compared with that in the saline group (63±9 vs. 28±5) (P=0.001) (figures 2A-C). The administration of probiotics reduced infarct area noticeably in sections 2 to 5 (except section 3) in the posterior part of the MCA region, where the cortical (i.e., penumbral) tissue predominates (P=0.01) (figures 2B-C). There was no statistically significant difference between the neurological score (5.66±0.88 vs. 3.38±1.04) (P=0.26) and the beam-balance score (3.28±0.72 vs. 3±0.64) (P=0.31) in the saline and probiotics groups (figures 3A-B). There was no damage or neurological change in the sham-operated group (figure 2A-C, figures 3A-B).

Effects of Pretreatment with Probiotics on the Malondialdehyde Content and the Tumor Necrosis Factor-Alpha Level

Ischemic stroke significantly enhanced the MDA content in the ischemic hemisphere cortex of the saline group (2.06±0.09) as compared with the sham-operated group (1.32.06±0.05) (P=0.001). Pretreatment with probiotics for 14 days caused a significant reduction in the MDA content of brain tissue (1.52.06±0.08) (P=0.001) (figure 4).

MCAO significantly enhanced the TNF-α level in the ischemic hemisphere cortex of the saline group (10.7±0.5) as compared with the sham-operated group (5.3.06±0.7) (P=0.004). The consumption of probiotics for 2 weeks led to a significant reduction in the TNF-α level of brain ischemic tissue (7.3±0.9) as compared with the saline group (P=0.004) (figure 5).

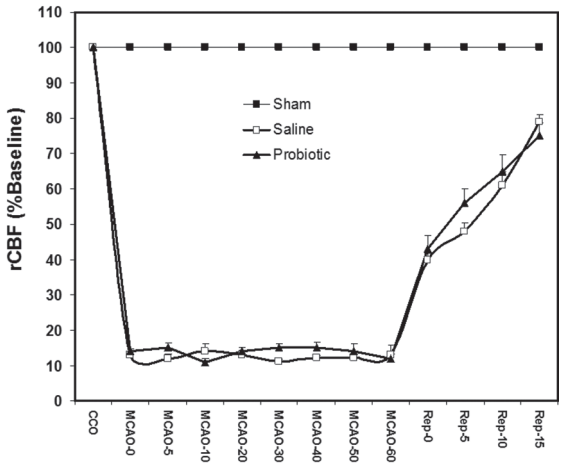

Figure 1: Regional cerebral blood flow (rCBF; % from baseline) in the sham-operated group, 15 **min**utes before middle cerebral artery occlusion (MCAO), during 45 **min**utes of MCAO, and 15 **min**utes after reperfusion in the control (saline) and probiotics-treated groups. Values are mean±SEM.

Discussion

The aim of the present study was to determine the effects of pretreatment with probiotics on brain injury, neurological function, and pro-inflammatory cytokine of TNF-α, and MDA content, as an oxidative stress biomarker, in a transient model of focal cerebral ischemia in mice. Our findings exhibited that the consumption of 4 viable probiotic bacteria for 2 weeks attenuated the severity of brain injury probably via inhibiting the synthesis of TNF-α and oxidative stress damage in the mice. These data suggest that probiotics may open a new therapeutic opportunity for the treatment or prevention of stroke.

The results of the present study verified that the simultaneous ingestion of probiotics *Bifidobacterium breve*, *Lactobacillus casei*, *Lactobacillus bulgaricus*, and *Lactobacillus acidophilus* at a concentration of 10^7 CFU/mL was able to effectively reduce brain damage by 52%, compared with the control group. This finding is consistent with a recent investigation indicating that pretreatment with only *Clostridium butyricum* had a neuroprotective effect in a global model of cerebral ischemia that was induced with bilateral common carotid artery occlusion in mice.[15] Likewise, a new research demonstrated that the intragastric administration of *Clostridium butyricum* at a concentration of 0.5×10^9 CFU/mL for 6 weeks after brain ischemia reduced neuronal hippocampus damage and cognitive impairment in diabetic mice, which further supports the findings of the present study.[22] Additionally, there is considerable information showing that probiotics have cardioprotective effects in the rat model of myocardial infarction, which somewhat confirms the results of our study.[12,13]

In addition, our results showed that despite reducing infarct size, pretreatment with probiotics could not improve neurological

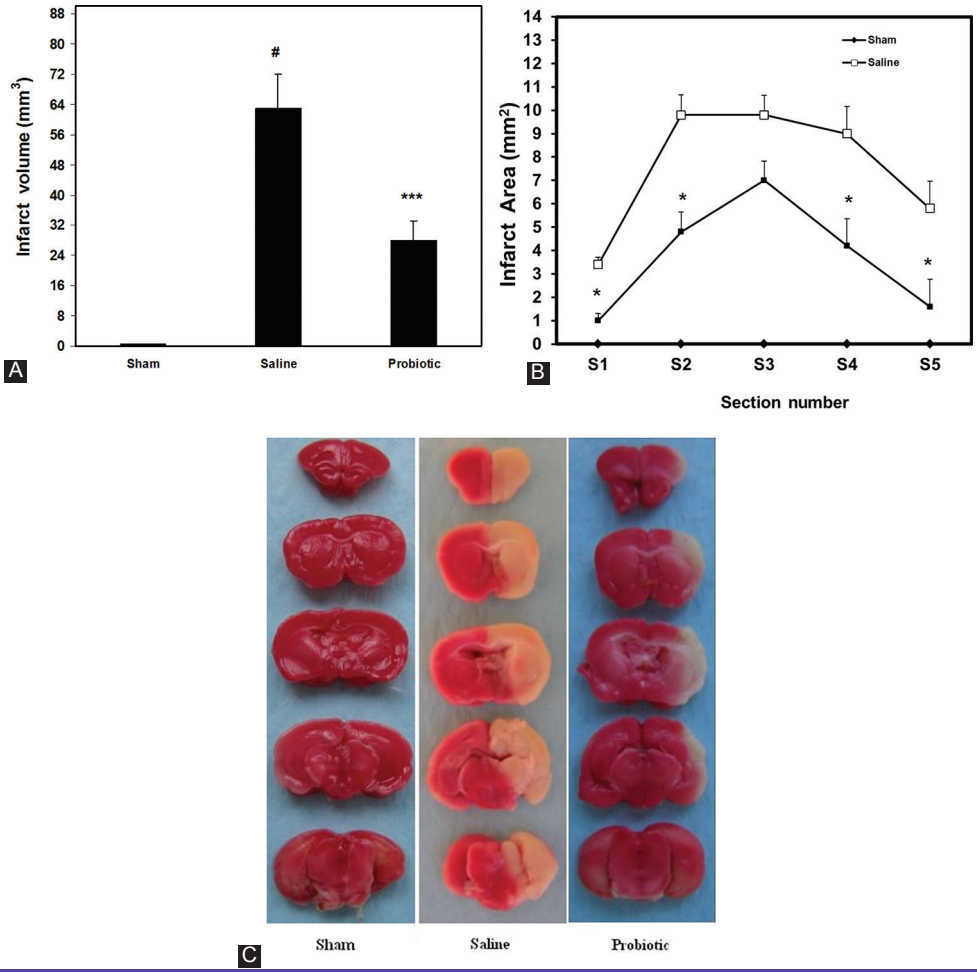

Figure 2: Infarct size (A), infarct area (B), and 2, 3, 5-triphenyl tetrazolium chloride staining photograph (C) in the sham-operated, control (saline), and probiotics-treated groups at 24 hours after cerebral ischemia or surgery in the mice. Red color is the normal area and white color is the infarct area. Colorless region relates to the occluded middle cerebral artery region. Values are mean±SEM. ***P<0.001, compared to the saline group. #P<0.05 from the respective sham-operated group.

outcome. This finding should be interpreted cautiously because our sample size was small. We suggest that further studies with larger numbers of animals be conducted to arrive at a more accurate conclusion. In contrast with our results, Sun et al.[15] reported that the oral intake of *Clostridium butyricum* for 14 days significantly improved neurobehavioral function in a mouse model of global cerebral ischemia. This discrepancy may be related to differences in the model of ischemia or injury, strains of probiotics, and/or different methods of neurological function outcome measuring. In the current study, neurological function was evaluated via an accurate method with a 14-point score, while Sun et al.[15] used a simple method with a 4-point score for the assessment of neurological impairment. Moreover, previous research has demonstrated that a direct correlation between infarct size and the neurological score is not always observed in animal models of stroke.[23] Barone et al.[24] demonstrated that a large reduction in brain injury was essential for recovery in neurological function in an animal model of focal stroke.

The mechanisms by which probiotics can reduce the risk of brain damage are not completely understood. The overproduction of pro-inflammatory cytokines and free radicals has a critical role in the development of ischemic damage following cerebral stroke.[16,25] Therefore, suppressing TNF-α and free radicals may lead to decreased neuronal damage after cerebral ischemia.[16,25] In the gut epithelium, probiotics may interact with toll-like receptors and cause the activation of the innate immune system, which can result in alterations in the circulating levels of pro-inflammatory and anti-inflammatory cytokines.[26] This may directly affect brain function. Our study demonstrated that treatment with probiotics for 2 weeks conferred a significant reduction in the level of TNF-α and in the oxidative stress biomarker MDA in the brain tissue. These data are consistent with other studies reporting that probiotics have antioxidant activity and can

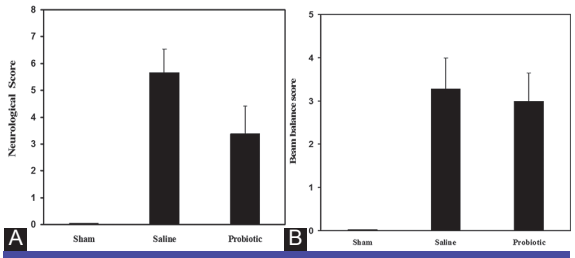

Figure 3: Neurobehavioral (A) and beam-balance (B) scores in the sham-operated, control (saline), and probiotics-treated groups 24 hours after cerebral ischemia or surgery in the mice. Values are mean±SEM.

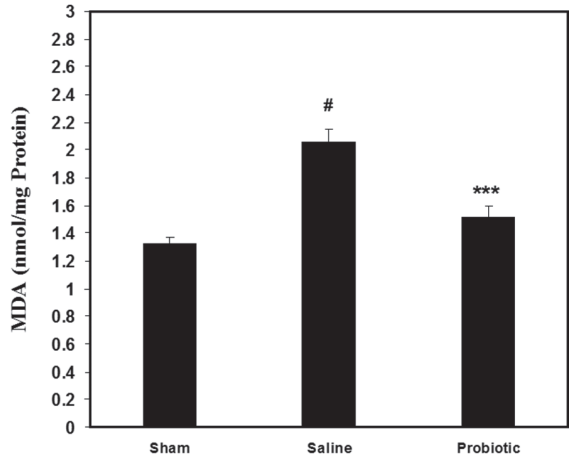

Figure 4: The malondialdehyde content in the sham-operated, control (saline) and probiotics-treated group 24 hours after cerebral ischemia or surgery in the mice. ***P<0.001, compared to the saline group. Values are mean±SEM. #P<0.05 from the respective sham-operated group.

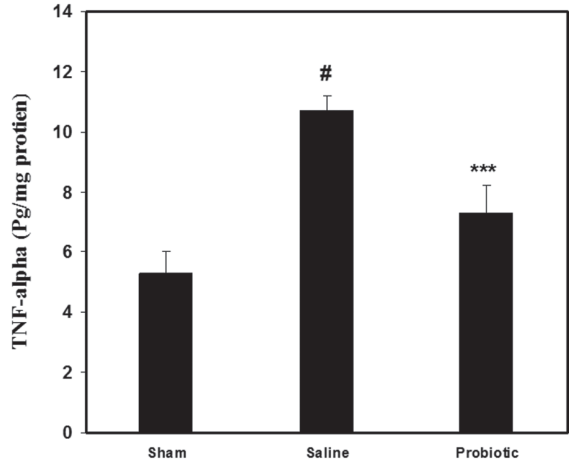

Figure 5: Tumor necrosis factor-alpha level in the sham-operated, control (saline), and probiotics-treated group 24 hours after cerebral ischemia or surgery in the mice. Values are mean±SEM. ***P<0.001 from the respective saline value. #P<0.05 from the respective sham-operated group.

suppress pro-inflammatory cytokines.[5,8,9,13,27] Additionally, our results indicated that the MDA content in the brain tissue increased after acute stroke and pretreatment with probiotics restored it to near the baseline level. The increase in the MDA content may be due to an increase in free radical formation and a decrease in the level of antioxidant enzymes following acute stroke. In the current study, the activity level of antioxidant enzymes was not measured. Nevertheless, a previous study showed that pretreatment with *Clostridium butyricum* (as a probiotic bacterium) significantly magnified antioxidant enzyme activity after cerebral stroke.[15] Therefore, we suggest that at least part of the decrease in stroke injury by probiotics may be related to augmentation of the antioxidant defense system and attenuation of pro-inflammatory cytokines in neuronal cells. In addition, it has been shown that probiotics could increase BDNF[3] and reduce apoptosis[15] in the brain of mice. Preclinical studies have shown that the increase of brain BDNF[28,29] and/or the inhibition of apoptosis[30] can have a protective effect against stroke damage. Therefore, the neuroprotective activity of probiotics, which was observed in the current study, may in part relate to the increase of BDNF and/or the reduction of apoptosis, and this requires further research. However, more animal and clinical studies are necessary to clarify other possible mechanisms and their therapeutic efficacy in cerebral stroke.

The probiotics used in the present study, namely *Bifid bacterium breve, Lactobacillus casei, Lactobacillus bulgaricus*, and *Lactobacillus acidophilus*, are well known and safe and belong to the anaerobic bacteria of the human gastrointestinal tract.[14,27] It has been proven that probiotics have potent anti-inflammatory and antioxidant properties and protective effects against oxidative stress injury in various animal tissues.[13,14,27] Also, a new clinical study has reported that the administration of supplement containing the probiotics *Lactobacillus acidophilus, Lactobacillus casei*, and *Bifidobacterium* for 12 weeks in diabetic hemodialysis patients significantly decreased plasma inflammatory and oxidative stress biomarkers.[31] Therefore, according to the above evidence, probiotics were chosen in the current study.

Altogether, the current study showed that 2 weeks' pretreatment with probiotics was able to alleviate the severity of brain ischemic damage in an animal model of stroke. However, the small number of our animal experimental groups and the incomplete assessment of the underlying mechanisms of the neuroprotective activity of the probiotics are important limitations of the present study and they should, as such, be taken into account in future research.

Conclusion

The findings of the present study suggest that probiotic supplements might be useful in the prevention or reduction of the risk of cerebral stroke. Further preclinical and clinical studies are needed to elucidate this assumption and to consider its possible therapeutic effects in stroke patients.

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Conflict of Interest: None declared.

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