Research Paper: Oral Administration of Probiotic *Enterococcus durans* to Ameliorate Experimental Autoimmune Encephalomyelitis in Mice

Seyed Abdollah Samani[1], Mohammad Raman Moloudi[2], Rashid Ramezanzadeh[3], Mohammad Abdi[1,4], Bahram Nikkhoo[5], Esmael Izadpanah[1,6], Daem Roshani[7], Alina Abdolahi[1], Parisa Esmaili[8], Kambiz Hassanzadeh[1,6*]

1. Cellular and Molecular Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran.
2. Liver and Digestive Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran.
3. Department of Microbiology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.
4. Department of Clinical Biochemistry, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.
5. Department of Pathology and Clinical Laboratory Sciences, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.
6. Department of Medical Physiology and Pharmacology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.
7. Department of Epidemiology and Biostatistics, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.
8. Department of Immunology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.

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ABSTRACT

Introduction: Probiotics, including lactobacilli, have immunomodulatory activities with promising effects on inflammatory diseases. In this study, we evaluate the effect of *Enterococcus durans* (Edu) and three various strains of lactobacilli (Lacto-mix), including *L. rhamnosus*, *L. casei*, and *L. plantarum*, to prevent Experimental Autoimmune Encephalomyelitis (EAE) features

Methods: C57BL/6 female mice were inoculated with Myelin Oigodendrocyte Glycoprotein (MOG_{35-55}) in CFA (complete Freund's adjuvant) to induce EAE. Five groups (n=6 in each group) of animals received saline or probiotics by oral gavage with 200 µL of lactobacilli (1.5×10^8 CFU/mL) for 2 weeks before the immunization and during the test for one month.

Results: Histopathological studies showed an increase in infiltration of inflammatory cells and destruction of the myelin membrane in the EAE group but a decrease in inflammatory cells in the probiotic-treated animals. Pro-inflammatory cytokines (Interleukin [IL]-17 and Interferon [IFN]-γ) concentration in the supernatant of the brain and spinal cord tissues showed a significant increase in the EAE compared with the normal saline group ($P<0.01$). While in the spinal cord tissue, there was a decrease in IL-17 in those animals treated with the Lacto-mix and Edu + Lacto-mix ($P<0.01$) and a significant decrease in IFN-γ in those animals that received Edu ($P<0.05$). Western blot analysis of matrix metalloproteinase-9 and myelin basic protein showed a decrease and increase in treatment and EAE groups, respectively, compared to the normal control group.

Conclusion: Our data suggest that probiotic *Enterococcus durans* and Lacto-mix prevents EAE, but further studies are needed to clarify the exact mechanisms and their application in preclinical and clinical trials.

*** Corresponding Author:**
Kambiz Hassanzadeh, PhD.
Address: *Cellular and Molecular Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran.*
Tel: *+98 (914) 4156941*
E-mail: *kambizhassanzadeh@gmail.com*

Highlights

• Dysfunction of the blood-brain barrier, migration of inflammatory cells into the Central Nervous System (CNS), and an increase in the pro-inflammatory factors, are the hallmarks in the pathogenesis of Multiple Sclerosis (MS) and Experimental Autoimmune Encephalomyelitis (EAE).

• The optimal effects of probiotic strains may involve the simultaneous use of more than one strain.

• Probiotic *Enterococcus durans* and Lacto-mix have a preventive effect against EAE.

Plain Language Summary

Multiple Sclerosis (MS) is a myelin-degenerating autoimmune disease in the central nervous system. Experimental Autoimmune Encephalomyelitis (EAE), due to its similar clinical and pathologic features to MS, is widely used in many model studies of this disease. The microbiome refers to a genomic set of germs (bacteria, arches, fungi, and viruses), a commensal flora that lives in the intestine and niche of humans and other mammals. The microbiome affects the host's physiological system, especially the balance between health and disease. Additionally, the importance of the microbiome is evident in regulating the intestine-brain axis, or the coordination of the digestive and the central nervous system. In this regard, probiotics, including lactobacilli, have antioxidant and anti-inflammatory properties in vitro and in vivo. Probiotic strains have a wide range of health-improvement effects, and a combination of strains with specific properties provides a broader range of antimicrobial spectrum and stronger anti-inflammatory effects. Considering the critical role of probiotics in the immune system, this study aimed to investigate the possible role of *Enterococcus durans* alone or in combination with Lactobacillus mixture (*L. rhamnosus*, *L. casei*, and *L. plantarum*) on the EAE animal model of MS.

1. Introduction

Multiple Sclerosis (MS) is an autoimmune disease in the central nervous system. The disease is characterized by the inflammatory responses of the immune cells (mainly T-cell $CD4^+$) to the myelin components of neuronal cells. The degradation of the myelin sheaths can delay or even eliminate the transmission of the neural message and eventually sensory-motor impairment (Gold, Linington, & Lassmann, 2006; Mokarizadeh, Delirezh, Morshedi, Mosayebi, Farshid, & Mardani, 2012). Experimental Autoimmune Encephalomyelitis (EAE), due to its clinical and pathological characteristics in patients with MS, has been reported as an effective and similar model in many studies of this disease (Gold et al., 2006). In MS and EAE, the transmission of self-reactive cells from the permeable Blood-Brain Barrier (BBB) leads to the activation of inflammatory responses, production of Interleukin (IL)-17 and Interferon (IFN)-γ, stimulation of inflammatory cells, higher expression of inflammatory chemokines (Monocyte Chemoattractant Protein [MCP]-1, Macrophage Inflammatory Protein-2 [MIP-2], and C-X-C motif chemokine Ligand 10 [CXCL10]) in the central nervous system, leukocyte infiltration, degradation of nerve cell myelin sheath, and finally impaired nerve signals transmission (Gold et al., 2006; Tran, Kuziel, & Owens, 2000). Recent research studies have shown that inflammatory responses and oxidative chain reactions in active microglial and macrophages play an essential role in the demyelination of the central nervous system and the pathogenesis of MS. The production of free radicals of oxygen and nitrogen, as well as inflammatory cytokines in the inflammation zone, actively contributes to the development of disease. Because inflammation and oxidative stress can have a two-way causality relationship, a continuous cycle develops (Ortiz et al., 2013). The activation of oxidative stress reactions and the production of free radicals of oxygen by macrophages causes demyelination and axonal degeneration in both MS and EAE. The findings also indicate that the weakening of the antioxidant defense system in the central nervous system of MS patients increases their vulnerability to oxidative stress anxiety. Therefore, effective antioxidant treatment can theoretically prevent the spread of tissue damage and improve neurological damage (Gilgun-Sherki, Melamed, & Offen, 2004).

Recent studies demonstrate the role of the intestinal microbial system in the development and management of the immune system (Bhargava & Mowry, 2014). The

microbiome refers to a genomic set of germs (bacteria, arches, fungi, and viruses), a commensal flora that lives in the intestine and niche of humans and other mammals. The microbiome affects the host's physiological system, especially the balance between health and disease (Bhargava & Mowry, 2014; Wang & Kasper, 2014). In addition to modifying the immune system, the intestinal microbiome seems to have significant metabolic functions such as dietary sugar regeneration, short-chain fatty acid production, bile acid deconjugation, and drug metabolism (Bhargava & Mowry, 2014; Correa-Oliveira, Fachi, Vieira, Sato, & Vinolo, 2016; Kahouli et al., 2015). Additionally, the importance of the microbiome is evident in regulating the intestine-brain axis, or the coordination of the balance system between the digestive and the central nervous system. The microbiome is an important factor for environmental peripheral hemostatic immunity and determines the host's sensitivity to various autoimmune diseases (Wang & Kasper, 2014). Therefore, modulation and resetting of gut microbiota could be considered an adjunctive therapeutic strategy for treating MS (He et al., 2019; Tankou et al., 2016). In this regard, probiotics, including lactobacilli, have antioxidant and anti-inflammatory properties in vitro and in vivo (Avram-Hananel, Stock, Parlesak, Bode, & Schwartz, 2010; Ortiz et al., 2013).

Probiotic strains have a wide range of health-improvement effects. Also, a combination of strains with specific properties has been suggested to provide a wider range of antimicrobial spectrum and stronger anti-inflammatory effects (Lavasani et al., 2010; Timmerman et al., 2007; Timmerman, Koning, Mulder, Rombouts, & Beynen, 2004). Thus, probiotics could be used as adjuvant therapy to treat immune-mediated diseases (Bron et al., 2017; Rumah, Vartanian, & Fischetti, 2017). *Enterococcus durans* (A probiotic with anti-oxidant and anti-inflammatory properties) belongs to the large family of lactobacillus, a branch of the phylum Firmicutes; this gram-positive bacterium is often diplococcus and is hardly detected by the physical characteristics of streptococci but plays an essential role in the environment, nutrition and clinical microbiology (Avram-Hananel et al., 2010; Pieniz, Andreazza, Anghinoni, Camargo, & Brandelli, 2014). Although various inflammatory and autoimmune models have shown the ability to modify immunity through various sets of probiotics, such as *L. rhamnosus*, *L. casei*, and *L. plantarum*, it seems that the optimal effects of probiotic strains may include simultaneous use of more than one strain (Lavasani et al., 2010). Considering the important role of probiotics in the immune system, this study aimed to investigate the possible role of *Enterococcus durans* alone or in combination with Lactobacillus mixture on the EAE animal model of MS.

2. Methods

Animals and experimental groups

Inbred female C57BL/6 mice (8-10 weeks old) were purchased from the Laboratory Animal Center Pastor Institute of Iran, Tehran. Mice were maintained in pathogen-free conditions at animal houses at Kurdistan University of Medical Sciences and randomly divided into five groups: EAE, saline, and probiotics (Edu, Lacto-mix, and Edu + Lacto-mix) (n=6 in each group). All experiments were conducted following the Animal Care Guidelines for the care and use of laboratory animals (National Institutes Health Publication No. 85–23, revised in 1985) and approved by the Research Ethics Committee of Kurdistan University of Medical Sciences.

Induction and evaluation of EAE

EAE was induced in C57BL/6 female mice by subcutaneous injections of 200 µg of Myelin Oligodendrocyte Glycoprotein $(MOG)_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK, 95% purity) (Ana Spec, USA) emulsified 1:1 volume in Complete Freund's Adjuvant (CFA) supplemented with 5 mg/mL heat-killed H37Ra. The strain of Mycobacterium tuberculosis (Sigma Aldrich, USA) was simultaneously injected intraperitoneally on days 0 and 2 with 400 ng of pertussis toxin (Sigma Aldrich, USA) (Bittner, Afzali, Wiendl, & Meuth, 2014). Mice were weighed and evaluated daily for disease symptoms, using a standard scoring system: 0, without apparent changes in motor functions; 1.0, limp tail; 2.0, limp tail and wobbly gait; 3.0, bilateral hind limb paralysis; 4.0, complete hind limb and partial forelimb paralysis; 5.0, death. At the end of the experiments (27 days after induction), the mice were anesthetized, separated by heart puncture, and various organs were dissected. All stages of this research were confirmed by the Research Ethics Committee of Kurdistan University of Medical Sciences.

Bacterial strains and treatment

Enterococcus durans IBRC-M 10753 (Lyophilized ampoules), Lactobacillus plantarum subsp. Planetarium IBRC-M 10817 (Lyophilized ampoules), Lactobacillus rhamnosus IBRC-M 10754 (Lyophilized ampoules), and Lactobacillus casei IBRC-M 10711 (Lyophilized ampoules) from the National Center of Genetic and Biological Reserves of Iran were purchased. All strains were kept at -70°C in a Brain Heart Infusion (BHI) medium containing 20% sterile glycerol, and new colonies of bacteria were cultured in MRS (de MAN, ROGOSA, and SHARPE) agar medium were used daily. For pro-

phylactic treatment (2 weeks before the immunization and during the test for one month), the mice were daily received (regular oral administration) 200 µL of lactobacilli, 1.5×10^8 Colony-Forming Units/mL (CFU/mL) of each suspension bacterium in normal saline solution. It is approximately 0.5 standard Mc Farland turbidity in bacteriology, including *Enterococcus durans* or Lactomix (Lactobacillus Plantarum subsp. Planetarium, Lactobacillus rhamnosus, and Lactobacillus casei). In contrast, control mice were treated with sterile physiological saline. The acceptable dose (1.5×10^8 CFU) is selected as the optimal dose for this study and is comparable to other probiotic studies in rodents and humans (10^6–10^{12} CFU/d) (Lavasani et al., 2010).

ELISA for cytokine detection

After animal anesthesia with an intraperitoneal injection of ketamine and xylazine (injection of 0.1 mL per 10 g of body weight, i.e., 100 mg/kg ketamine, 16 mg/kg xylazine). The brain and spinal cord obtained from animals were homogenized by lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, fresh protease inhibitors) and centrifuged at 12000 × g for 10 min, and the supernatant was stored at -70°C. Concentrations of IFN-γ and IL-17 cytokine were measured in serum or supernatants from homogenized tissues (brain and spine) using commercial proprietary ELISA kits (Thermo Fisher, USA, cat No#1731679A for IFN-γ and cat No#1859317A for IL-17) according to the manufacturer's instructions. The absorption rate was measured with an ELISA reader (synergy HTX, multi-mode reader, Biotek, USA). The cytokine content in supernatants was determined when the data were calculated within the standard linear curve of the cytokine values.

Pro-oxidant Antioxidant Balance (PAB) measurement

Measurement of the oxidant-antioxidant balance in the CNS was performed using a Pro-oxidant Antioxidant Balance (PAB) method and calculated by a spectrophotometric colorimetric method (ELISA). An improved PAB was used according to the previously described method (Ebrahimi, Oryan, Izadpanah, & Hassanzadeh, 2017; Tavana et al., 2016). HK is an optional unit based on the percentage of adsorption of hydrogen peroxide in a standard solution.

Immunoblotting

Proteins were extracted from the supernatant homogenized brain and spinal cord tissues in the cold chain and protease inhibitor (Sigma Aldrich, USA). Then, their concentration was determined by an absorbance assay (Total Protein kit, Pars Azmun Co, Iran, cat No# 96002), and 40 µg of specimens were placed on a 12% SDS-polyacrylamide gel and subjected to electrophoresis. The separated proteins on a PVDF (Polyvinylidene Difluoride) membrane (Roche, Germany) were electrotransferred using a mini trans-blot apparatus (ATTA, Japan). The membrane was then blocked with skimmed milk and tested with specific primary antibodies (anti-Matrix Metallo Proteinase [MMP]-9 and anti-Myelin Basic Protein [MBP]) (Abcam, USA). The PVDF membranes were subsequently incubated for 1 h at room temperature with a secondary antibody. The optimum dilution of primary and secondary antibodies was 20 µL/10 mL and 3 µL/10 mL of TBS buffer, respectively. Detection steps were performed using a commercially available kit (BM Chemiluminescence Western Blotting, Mouse/Rabbit, Roche, Germany, cat No. 16191700), according to the manufacturer's instructions. Proteins detected by antibodies were visualized on x-ray film using Enhanced Chemiluminescence (ECL). The autoradiographs were measured by densitometry (program ImageJ, 1.46 r) and displayed several times to ensure the linearity of the band intensities.

Evaluation of mRNA expression fom inflammatory Chemokines MCP-1(CCL2) and CXCL10 in brain and spinal cord cells

Quantitative real-time PCR analysis

Total RNA was extracted from brain and spinal cord tissue using RNX-PLUS (CinnaGen, Iran). According to the manufacturer's instructions, the first-strand cDNA was synthesized using a Prime-Script TM RT reagent kit (Takara, Japan). The mRNA expression was performed using quantitative real-time PCR. Real-time PCR was performed using SYBR Green Master Mix (Takara, Japan) under standard thermocycler (Corbett RG-6000, Australia) (94°C for 30 s, 60°C for 30 s, 72°C for 45 s, 40 cycles) conditions. The data collected were analyzed using LinRegPCR software (version 11.0). The Results are presented as the fold increase relative to the expression of the housekeeping gene (β-actin). Table 1 lists the compliance with PCR primers.

Histopathological changes of the brain and spinal cord

Samples of the brain (corpus callosum and periventricular white matter area) and the spinal cord (lumbar area) taken from the mice were kept in histopathological examination of 10% formalin. The paraffin-embedded tissue sections (8-10 µ) were stained with Hematoxylin

Table 1. Specific primers used in real-time PCR analysis

Molecule	Primer	Sequence
β-actin	Forward	5-CTTGGGTATGGAATCCTGTG-3
	Reverse	5-ACTGTTGGATAGAGGTC-3
MCP-1 (CCL2)	Forward	5-TCAGGCAGATGCAGTTAACG-3
	Reverse	5-TCTTTGGGACACCTGCTGC-3
CXCL10	Forward	5-CCACGTGTTGAGATCATTGCC-3
	Reverse	5- TCCATCCATCGCAGCACC-3

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and Eosin (H&E) to determine the infiltration of the inflammatory cell, while Luxol Fast Blue (LFB) staining was used to detect the loss of myelin sheath (Gibson-Corley et al., 2016). The tissue sections were observed with an inverted microscope (Olympus IX71, Japan; 40× and 100×magnification).

Statistical analysis

The obtained data were analyzed using SPSS version 20 (SPSS, IBM Statistics 20). To analyze the statistical significance in two or more comparisons, the dependent t-test was used. Western blotting image data were compared using 1-way analyses of variance (ANOVA). In all analyses, the statistical significance ($P<0.05$) was determined by ANOVA followed by Tukey's post hoc test.

3. Results

We present the effect of oral administration of probiotics on experiments in the EAE model.

Histopathological analysis

Our histopathological findings revealed that the EAE group showed the presence of mononuclear cells in the spinal cord compared with the healthy group controls (Figure 1, G and H). At the same time, the Edu-treated group showed a relative decline in mononuclear cells infiltration compared to the EAE animals (Figure 1, I and H). These results indicate that probiotics treatment can reduce the development and growth of EAE. In parallel with the changes in the infiltration of inflammatory cells, myelin changes in the groups were also studied. The spinal cord sections obtained from normal, EAE, and probiotic (Edu) treated mice were analyzed by LFB staining to assess demyelination on day 27 post-immunization. The EAE group showed relative demyelination compared to the normal control group, while in the Edu-treated group, improvement was observed to decrease demyelination compared with the EAE and normal groups (Figure 1, A, B, C, D, E, and F).

The effect of treatment with probiotics on cytokines IFN-γ and IL-17 in the EAE model

Investigating the pro-inflammatory cytokine concentration of IL-17 in the supernatant in the brain and spinal cord homogenized tissues showed its significant increase in EAE (control) compared to the normal saline group. There was no significant difference between the probiotics group and the control group in the brain, while in the spinal cord tissue, the IL-17 value was decreased in animals treated with the Lacto-mix and Edu + Lacto-mix (Figure 2). This decrease was statistically significant when compared to the control. The measurement of pro-inflammatory cytokine concentration of IFN-γ in the supernatant of the brain and spinal cord homogenized tissues showed a relative increase in EAE compared to the saline group. However, no significant difference was found between the probiotics and control groups in the brain. However, in the treated groups with probiotics, there was a significant difference in the spinal cord tissue only in the Edu group compared to the control (EAE) group (Figure 2).

Analysis of data obtained from the PAB in different groups

According to Table 2, in the supernatant derived from homogenized brain tissue, PAB values were significantly higher in the EAE group compared to the saline group. While in the treated groups with the Edu, Lacto-mix, and Edu + Lacto- mix, data analysis showed a significant difference with the control group (EAE), but there was no significant difference between the probiotics group and the control group in the spinal cord.

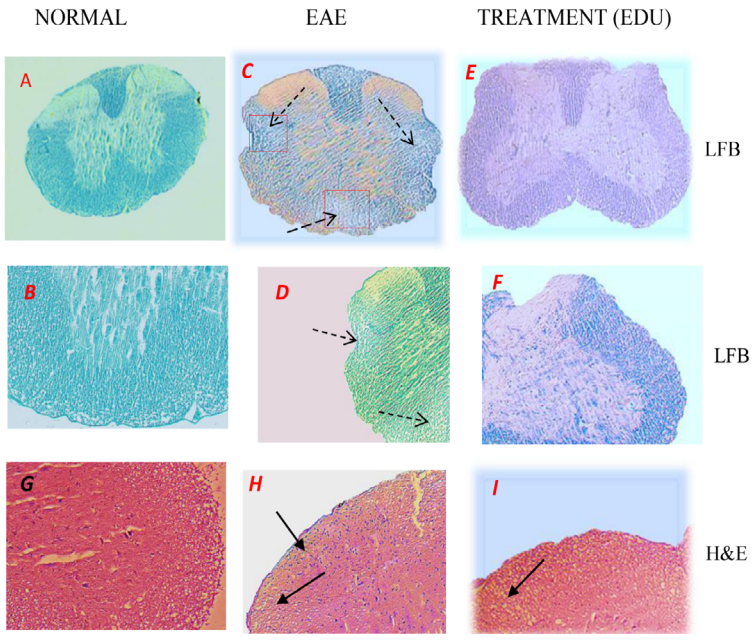

Figure 1. Histopathological staining of spinal cord tissue

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Spinal cord sections were obtained from normal control, EAE, and probiotic *Enterococcus durans*-treated mice on day 27 post-immunization and were analyzed by H&E staining (G, H, and I) for inflammation and LFB staining (A, B, C, D, E, and F) for demyelination.

Solid arrows indicate areas of inflammation, and dotted arrows indicate areas of demyelination. The tissue sections were observed and analyzed by an inverted microscope (40× and 100× magnification).

Western blot

Western blotting data showed that the EAE induction had a relatively high increase in the MMP-9 (an indicator protein for increasing the permeability and failure of the BBB in inflammatory CNS diseases) in the brain and spine of the EAE group compared to the control that was not statistically significant. In addition, animals treated with probiotics (Edu, Lacto-mix, and Edu + Lacto-mix) after inoculation with MOG showed lower expression of MMP-9 in the brain and spinal cord than in the EAE group, which was not statistically significant (Figure 3. A and C). In parallel with the evaluation of MMP-9, an assessment of Myelin Basic Protein (MBP) levels in the brain and spinal cord was performed. The highest reduction in MBP was observed in the brain of the EAE group

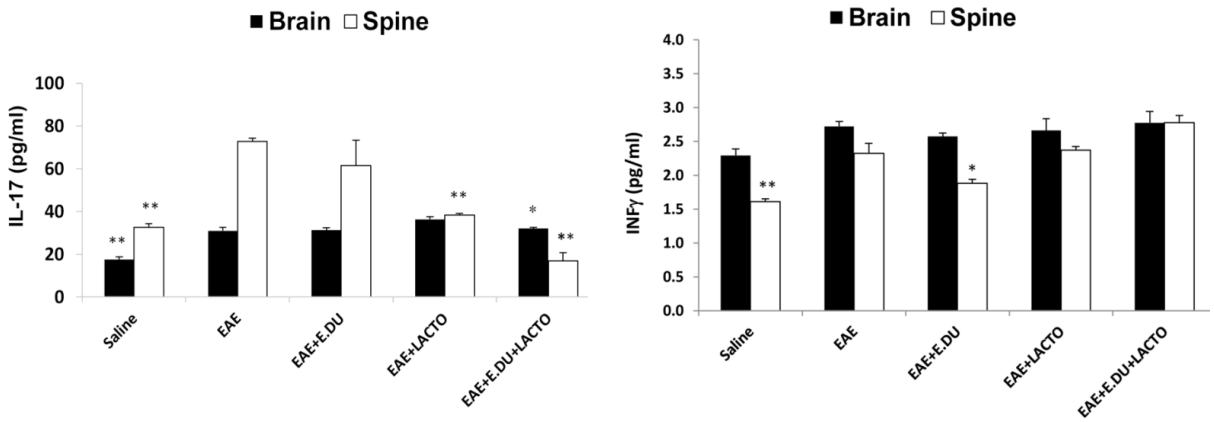

Figure 2. Brain and spine pro-inflammatory cytokines IL-17 and IFN-γ content

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* P<0.05 and **P<0.01 represent a significant difference between the experimental groups compared with the control group (EAE) using 1-way ANOVA (n=6 per group).

EAE: Experimental Autoimmune Encephalomyelitis; EDU: *Enterococcus durans*; LACTO: Lacto-mix.

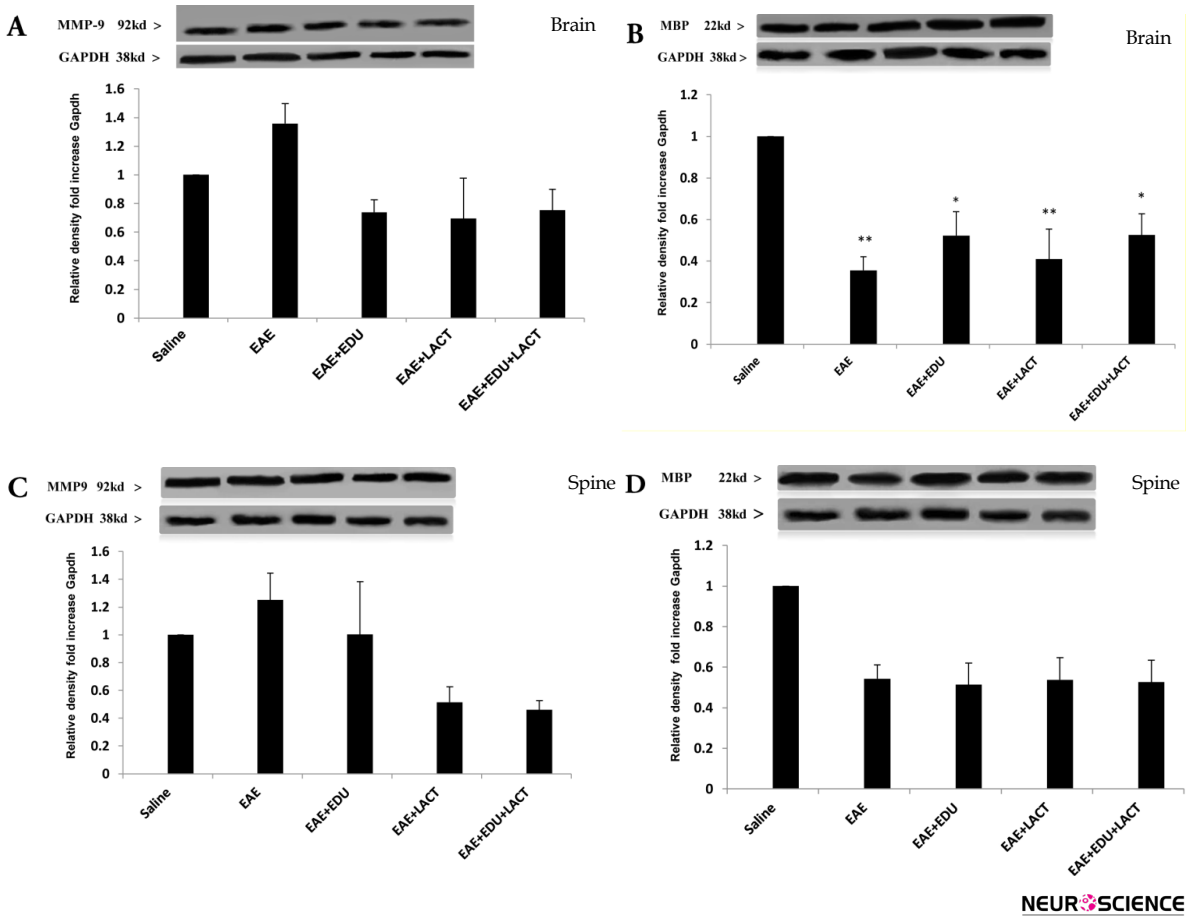

Figure 3. MMP-9 and MBP levels measured by the Western blot method in the brain tissue homogenates (A and B), spinal cord (C and D), from saline (lane 1), EAE mice (lane 2) and treated mice (lanes 3, 4, and 5)

Band densities of MMP-9, MBP, and GAPDH protein were quantified by densitometry and expressed as ratios of MMP-9 and MBP/GAPDH. The bands normalized to the GAPDH, and data expressed as a relative density fold increase/GAPDH.

*P<0.05, **P<0.01; n=6 per group.

MMP-9: Matrix Metalloproteinase-9; MBP: Myelin Basic Protein; EAE: Experimental Autoimmune Encephalomyelitis; EDU: *Enterococcus durans*; LACTO: Lacto-mix.

compared to the control group (P<0.01), while animals treated with probiotics after inoculation with MOG had higher MBP expression in the brain and spinal cord than the EAE group. These differences were significant in brain homogenates but not significant in the spinal cord. The highest treatment effect was observed in the Edu + Lacto-mix group (P<0.05) (Figure 3, B and D).

Polymerase Chain Reaction (PCR)

Now we discuss the expression of the *CCL2* and *CXCL10* genes of the brain and spinal cord in different groups. Parts A and B of Figure 4 show that CCL2 and CXCL10 (chemokines expressed in MS lesions) appear to be pivotal in the chemoattraction of T cells and monocytes into the CNS increased in the EAE group compared to the saline group, while in the treated animals with Edu, Lacto-mix, and Edu + Lacto-mix, the expression levels of CCL2 and CXCL10 decreased, compared to the control group. The highest reduction of *CCL2* expression in the brain was related to the Lacto-mix group and the Edu + Lacto-mix group in the spinal cord tissue, respectively. Also, the highest reduction of *CXCL10* expression in the brain was related to the Edu + Lacto-mix group and was correlated with the Lacto-mix group in the spinal cord. All these differences in the brain and spinal cord were not statistically significant.

4. Discussion

The failure of tolerance mechanisms to prevent the expansion of inflammatory autoreactive T cells may lead to autoimmune diseases such as Multiple Sclerosis (MS). Therefore, the main challenge in MS is finding a new

Table 2. Comparing Pro-oxidant Antioxidant Balance (PAB) values (HK units) among groups

Group	Brain		Spine	
	Mean±SEM	P-Value	Mean±SEM	P-Value
Saline	28.26±1.28	0.007	48.11±1.45	0.025
EAE	36.98±1.85	-	55.51±0.33	-
EAE+EDU	20.24±0.51	0.0001	57.58±0.46	0.925
EAE+LACTO	23.70±1.93	0.0001	58.84±0.97	0.637
EAE+EDU+LACTO	28.93±1.69	0.014	53.507±1.48	0.934

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Significant differences (means) were determined by Tukey's post hoc test. P-values of <0.05 compared to the EAE group were considered statistically significant. HK is an optional unit based on the percentage of adsorption of hydrogen peroxide in a standard solution (n=6 per group).

Abbreviations: EAE, Experimental Autoimmune Encephalomyelitis; EDU, *Enterococcus durans*; LACTO, Lacto-mix

treatment with maximum effect and minimal side effects to change the inflammatory responses to treatment or disease prevention. Research on the effect of intestinal microflora in chronic inflammatory and autoimmune disease have directed toward improving the microflora composition using probiotics (Fasano & Shea-Donohue, 2005; Lavasani et al., 2010; So et al., 2008; Tlaskalová-Hogenová et al., 2004). In this study, the anti-inflammatory and anti-oxidant effects of probiotic *Enterococcus durans* and a triple combination of lactobacilli (Lacto-mix), including *Lactobacillus plantarum subsp. planetarium* IBRC-M 10817, *Lactobacillus rhamnosus* IBRC-M 10754, and *Lactobacillus case*i IBRC-M 10711 with probiotic activity tested for the prevention of EAE features.

To evaluate the synergistic effects of a possible combination of these strains in the suppression of EAE, the animals were fed with a mixture of four selected probiotic strains. Based on the histopathological analysis, we found that induction of EAE resulted in the infiltration of mononuclear cells into the spinal cord (Figure 1, H) and relative demyelination compared to the normal control group (Figure 1, C and D). While pretreatment with probiotics is relatively suppressing the development of EAE and infiltration of inflammatory immune cells in the spinal cord (Figure 1, E, F, and I). This finding was in line with the Kwon et al. study, which evaluated the prophylactic and therapeutic effects of IRT5 (a mixture of 5 probiotics) in EAE. Pretreatment of IRT5 probiotics before disease induction significantly reduced EAE growth. Also, the results of H&E staining and immune-staining with an anti-CD3 antibody confirmed the decreased infiltration of CD3+ T lymphocytes into the spinal cord after IRT5 probiotics treatment (Kwon et al., 2013). Venice administration of Bifidobacterium animalis PTCC1631 were in combination with L. plantarum A7 ameliorated neuroinflammation in the EAE mouse model (Salehipour et al., 2017).

In this regard, previous studies have shown that in inflammation and brain damage such as multiple sclerosis, cerebral ischemia, Alzheimer and Parkinson, there is a significant increase in the expression of chemokines in microglial cells and active astrocytes. Also, the expression of chemokines such as IP-10 (CXCL10), MCP-1 (CCL2), lymphotoxin, MIP-2 (CXCL2), RANTES, and T cell 3 gene activity during induction of EAE in rats increases (Tanuma, Sakuma, Sasaki, & Matsumoto, 2006). In line with the findings of this study, the research on the EAE laboratory model shows that BBB failure is associated with an increase in histological inflammation and the acute phase of the disease (Tanuma et al., 2006). The apparent differences in cytokine and the chemokine profile of immunized mice may be influenced by their role in central nervous system changes and the severity of degenerative damage. In this study, the effects of probiotic (Lacto-mix, Edu + Lacto-mix, and Edu) groups on pro-inflammatory factors (IL-17, INF-γ, CCL2, and CXCL10) were evaluated. Thus, the greatest effect on the reduction of inflammatory factors in the brain and or spinal cord was observed in the Edu + Lacto-mix group. This finding was consistent with previous studies in synergistic effects of probiotic strains (Consonni et al., 2018; Kwon et al., 2013; Lavasani et al., 2010; Salehipour et al., 2017). Kwon et al. reported that oral administration of probiotics ameliorated the progression of EAE by reducing MOG-reactive T-cell proliferation and pro-inflammatory cytokine levels. In addition, the effect of mixed probiotic strains on EAE in mice was assessed

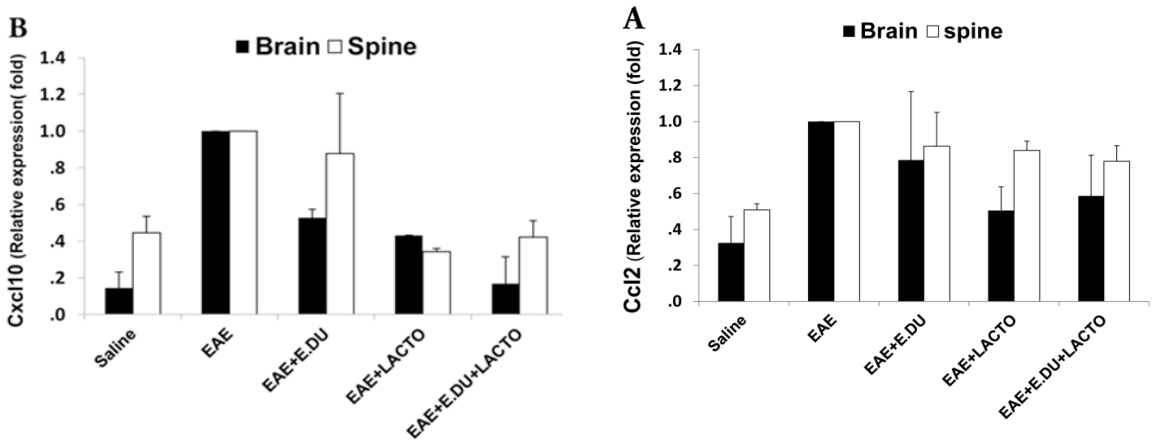

Figure 4. Valuation differential expression selected genes (*CCL2* and *CXCL10*) by real-time RT-PCR

mRNA expression levels as determined by quantitative real-time PCR analysis relative to the housekeeping gene (*β-actin*).

The results are presented as fold change (n=6 per group).

CCL2: Chemokine Ligand 2; CXCL10: C-X-C motif chemokine Ligand 10; MMP-9: Matrix Metalloproteinase-9; MBP: Myelin Basic Protein; EAE: Experimental Autoimmune Encephalomyelitis; EDU: *Enterococcus durans*; LACTO: Lacto- mix.

by Kwon et al. and Lavasani et al. and showed that the probiotic mixture prevents pro-inflammatory cytokines and chemokines (IL-17, INF-γ, CCL2, and CXCL10) augmentation. Also, changes in the expression of MMP9 and MBP proteins were shown in the study groups (Kandagaddala, Kang, Chung, Patterson, & Kwon, 2012; Pérez-Nievas, García-Bueno, Madrigal, & Leza, 2010).

Our findings showed that the measurement of MMP-9 and MBP proteins in the tissue homogenates of the brain and spinal cord increased and decreased, respectively, in the EAE group, compared to the control group. Confirmation of these results has already shown that MMP-9 up-regulation is associated with BBB dysfunction in multiple neuropathological scenarios. Western blot analysis showed that MBP levels in spinal cord homogenates from the EAE group decreased by 21 days after inoculation of MOG (Pérez-Nievas et al., 2010). On the other hand, treatment with probiotics (Lacto-mix, Edu + Lacto-mix, and Edu) showed a decrease and increase in the expression of MMP-9 and MBP, respectively, in the Edu+ Lacto-mix and or Lacto-mix groups compared to the control group (Figure 3. Parts A, B, C, and D). Compared with other studies, these results suggest that oral administration of a probiotics mixture effectively prevents or treats EAE (Kwon et al., 2013; Lavasani et al., 2010). Analysis of PAB concentration in homogenous brain tissue revealed that probiotics prevented the increase of PAB values in EAE animals (Table 2), suggesting the oxidative stress scavenging effects of probiotics. These results from PAB may reflect the direct antioxidant properties of probiotics (Martarelli et al., 2011; Pieniz et al., 2014) and or the induction of endogenous antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione (Dimitrijević et al., 2017).

Applying sufficient amounts of probiotics can also alter the composition of the intestinal microflora, which also indirectly modulates host immune responses (Kwon et al., 2013). It has been previously demonstrated that the effects of probiotics on EAE in mice are due to the strained-dependent, either stimulating or suppressing clinical symptoms. Cytokine-induced intestinal profiles are associated with effects on the EAE. Strains that produce proinflammatory cytokines in the intestine aggravate the cumulative disease burden, while strains that produce regulated cytokines reduce this effect (Ezendam, De Klerk, Gremmer, & Van Loveren, 2008; Maassen & Claassen, 2008). Although the effects of strain-dependent probiotics in controlling inflammatory diseases have been suggested, the essential molecular mechanism of these bacteria has not been determined yet (Maassen & Claassen, 2008; Reid, Jass, Sebulsky, & McCormick, 2003).

5. Conclusion

Our findings provide a deeper understanding of the significant immunomodulatory activities of probiotics and a synergistic effect of immunosuppressive properties of different probiotic strains for the treatment of chronic CNS inflammation with no side effects. This approach may present a suitable treatment for autoimmune diseases. Although animal models are suitable for showing an indication of the immunomodulatory properties and mechanisms, well-

designed human experiments are needed to gain more insight into the efficacy and safety of the probiotic strains.

Ethical Considerations

Compliance with ethical guidelines

All experiments were conducted following the Animal Care Guidelines for the care and use of laboratory animals (National Institutes Health Publication No. 85–23, revised in 1985) and approved by the Research Ethics Committee of Kurdistan University of Medical Sciences

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All authors equally contributed to preparing this article.

Conflict of interest

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Abbreviations: MS: Multiple Sclerosis; EAE: Experimental Autoimmune Encephalomyelitis; Edu: *Enterococcus durans*; LACTO: Lacto-mix; CNS: Central Nervous System; H&E: Hematoxylin and Eosin; LFB: Luxol Fast Blue; IP: Intraperitoneally; MOG: Myelin Oligodendrocyte Glycoprotein; CFA: Complete Freund´s Adjuvant; PVDF: Polyvinylidene Difluoride; ELISA: Enzyme-Linked Immunosorbent Assay; CCL2: Chemokine Ligand 2; MCP1: Monocyte Chemoattractant Protein-1; CXCL10: C-X-C motif Chemokine Ligand 10; CFU/mL: Colony-Forming Units per milliliter; MMP-9: Matrix Metallo Proteinase-9; MBP: Myelin Basic Protein

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