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Original Research

The protective effect of *Enterococcus faecium* L-3 in experimental allergic encephalomyelitis in rats is dose-dependent

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Abstract

Recent data demonstrate that disruption of the intestinal microbiocoenosis might be the cause of several neurological diseases, including multiple sclerosis (MS). MS is a chronic disease of the central nervous system, where the nervous tissue is damaged by the individual's own immune system. The autoimmune character of MS is largely supported by the positive effects of treatment aimed at suppressing the activity of aggressive immune cells. Since probiotics have an immunomodulatory effect, they can not only correct dysbiosis, but also provide a complex immunomodulatory effect on MS. *Enterococcus faecium* L-3 is one of the probiotic strains which has been shown to have a protective effect on the course of the disease induced in the animal model of MS (known as experimental allergic encephalomyelitis; EAE).

The purpose of this study was to compare the effects of different doses of *E. faecium* L-3 on the course of EAE in rats. EAE was induced by a single subcutaneous injection of a homologous spinal cord emulsified into complete Freund's adjuvant.

It is shown that the extent of the protective action of *E. faecium* L-3 depends on the dose used, with the greatest effect on reducing the severity of EAE observed with a dose of 8.0 lg CFU/ml. Amelioration of the course of EAE in rats receiving the probiotic was accompanied by decreased inflammation and reduced damage to neurons in the spinal cord. Research shows that the use of *E. faecium* L-3 at an optimum concentration of bacteria may be beneficial for patients with MS.

Keywords: Experimental allergic encephalomyelitis, inflammation, intestinal microbiota, multiple sclerosis, neuron damage, probiotics

Introduction

Probiotics are traditionally used to correct mainly intestinal dysbiotic conditions and dysfunctions of the gastrointestinal tract. However, their range of usage in clinical practice is currently expanding. The evidence that taking probiotics can be an effective strategy for the prevention and treatment of allergic and chronic autoimmune diseases is constantly accumulating. In the last decade, the role of the intestinal microbiota in the development and function of the immune and nervous systems has been revealed, and an assumption has been made about the presence of the gut–microbiota–brain axis [1-4]. Therefore, probiotics have been tried in the treatment of a number of psychiatric and neurological diseases, including multiple sclerosis (MS) [5-7].

MS is a severe autoimmune demyelinating disease of the CNS, affecting not only young adults, but also children. Among all neurological diseases, MS is the main cause of disability [8]. Current methods of MS treatment are aimed at suppressing immune system activity. However, these approaches can only modify the disease course and not provide the complete cure. Therefore, the search for new methods of treating MS is an urgent problem.

The aetiology of MS is complex and has not yet been completely understood. Recently, the intestinal microbiota has been considered to be one of the important aetiological factors of this disease. Study of the role of the intestinal microbiota in MS became particularly important after it was shown that the development of the disease in the animal model of MS – experimental allergic encephalomyelitis (EAE) – requires the presence of an intestinal microbiota [9]. It has been shown that both animals with EAE [10] and MS patients [11, 12] develop intestinal dysbiosis in the course of the disease. The presence of intestinal dysbiosis not only contributes to gastrointestinal dysfunction, which is detected in 70–90% of patients [12, 13], but may affect the course of MS, due to a shift in immunity towards proinflammatory responses [14]. This indicates the need to adjust the composition of the intestinal microbiota in MS, including the use of probiotics for this purpose.

Probiotics are defined as "living microorganisms that when administered in adequate amounts confer a health benefit" [15].

An immunomodulatory effect provided by probiotics is able to stimulate different subtypes of T helper (Th) cells

– Th1, Th2 or Treg, followed by the type of immune response. A number of studies carried out with the EAE model using probiotic lactobacilli showed strain-specific effects on the course of the disease in animals [16]. There is evidence that some strains of probiotic lactobacilli and bifidobacteria can even worsen the condition of the laboratory animals and aggravate the course of EAE [17]. However, in most studies there was a positive effect when using both mono-and multicomponent probiotic preparations which contained lactobacilli and bifidobacteria [18-22]. The protective effect in the EAE model was demonstrated after using other bacteria, in particular, other lactic acid bacteria such as *Pediococcus acidilactici* R037 [23], *Enterococcus faecium* L-3 [24], or even the gram-negative enterobacteria *Escherichia coli Nissle* [25].

Among the probiotic microorganisms, enterococci are easier to cultivate and they can survive for longer in the body not only in the distal, but also in the proximal intestine [26]. In addition, unlike *E. coli*, *Enterococcus* spp. do not contain lipopolysaccharide.

The *E. faecium* L-3 strain was successfully used to correct intestinal dysbiosis in both experimental models and in humans [26]. We have previously found that the protective effect of *E. faecium* L-3 in the EAE model is comparable to the effects of an immunomodulator (glatiramer acetate), but is mediated by other immune

mechanisms [24]. We also observed that endogenous enterococci have a protective effect in this model, if their intestinal abundances are large enough (8.0 lg CFU/g); that is, in a quantity comparable to the probiotic enterococci used. Since the decrease in the level of endogenous *Enterococcus* spp. in the intestine of rats even by one order caused the loss of the protective effects in the EAE model it was necessary to check whether *E. faecium* L-3 can provide a positive curative effect on the course of EAE at lower doses.

The purpose of this study was to compare the effects of different doses of *E. faecium* L-3 on the course of EAE in rats.

Materials and methods

Animals

88 female Wistar rats weighing 230–250 g were used. Rats were obtained from the nursery 'Rappolovo' (Rappolovo, Russian Federation). Rats were housed with free access to food and water under a 12-hour light/dark cycle in ventilated rooms at 23°C, and acclimatized for 21 days before EAE induction.

EAE induction and probiotic treatment

EAE was induced by a single subcutaneous inoculation of an encephalitogenic mixture, containing a homologous spinal cord emulsified in Freund's complete adjuvant (FCA; Sigma) at two sites on the tail in a total volume of 400 .l, as previously described [10].

After EAE induction, rats were allocated into four groups (22 rats in each group) as follows: group 1 was considered the EAE negative control, which was subjected to EAE induction and treated with saline. Groups 2–4 were considered the *E. faecium* L-3-treated groups, which were subjected to EAE induction and treated with a dose of 6.0 lg CFU/ml, 7.0 lg CFU/ml or 8.0 lg CFU/ml, respectively. Probiotic or saline was administered to rats by gavage at a volume of 0.5 ml once a day from day 2 to 16, as described earlier [24].

Probiotic strain

The probiotic strain *E. faecium* L-3 from the collection of the All-Russia Research Institute for Agricultural Microbiology and the International Collection (LMG P-27496; Laboratorium voor Microbiologie, Universiteit Gent, Belgium) was used in this study. The genome of the *E. faecium* L-3 (GenBank No. SUB167269) has a size of 2,629,318 base pairs and contains 2717 genes. The genome of the strain is free from genes of known enterococcal virulence factors and vancomycin resistance and carries the genes: entA, entB, enxA and enxB, encoding four different bacteriocins (enterocin) [27].

Scoring of EAE

To assess the severity of clinical symptoms of EAE in rats, a previously developed scale was used, taking into account the degree of limb lesions (weakness, paresis or paralysis) and the number of affected limbs (1–4 and tail) [10]. Weakness of one limb corresponded to 0.5; paresis to 1.0; and paralysis to 1.5. The daily clinical score (CS) was calculated as the sum of the points for all affected limbs. In the absence of clinical manifestations, CS was taken as 0, and in a lethal outcome, CS was taken as 6.0.

In addition, to assess the severity of EAE, the following criteria were used: the duration of the inductive phase (the number of days before the first symptoms appeared), the duration of the disease, the CS maximal (at the peak of the disease), the CS cumulative (the sum of CSs over the period of the disease), the CS mean (CS cumulative/disease duration) and CS convalescence (CS in the stage of recovery). Animals with CS = 0.5–2.0 were considered slightly sick; with CS = 2.5–3.5, moderately sick; and with CS > 4.0, severely sick.

Morphology

On the 17th day, three animals from each group were examined morphologically. Animals under anaesthesia (exposure to ether vapour) were decapitated and the lumbar spinal cord thickening was removed. Spinal cord tissue was fixed in a solution of zinc–ethanol–formaldehyde within 1 day, dehydrated and embedded in paraffin. Transverse paraffin sections of the spinal cord with a thickness of 7μm were stained with haematoxylin– eosin and thionin using the Nissl method as previously described [28].

Statistical analysis

As this was an explorative study, no power analysis was performed. Statistical analysis and plotting were carried out using the Statistica 10.0 software package (StatSoft, USA) and Excel (Microsoft). Data for each treatment group were reported with descriptive statistics. All data were expressed as the mean±SEM. The differences between variables were analyzed using one-way ANOVA followed by the Tukey HSD post hoc test. Repeated measures ANOVA followed by Bonferroni comparison was also utilized to analyze clinical scores during the study period. For comparison of percentages, the .2 test was used. Differences were considered significant when the p values were less than 0.05.

Results

The effects of different doses of *E. faecium* L-3 on the course of EAE in rats

After administration of the encephalitogenic mixture, EAE of varying severity developed in 95.5% of rats within the control group. The course of the disease in the rats of the control group was similar to that previously described [10, 24]. Characteristics of EAE in rats are shown in Table 1.

Probiotic enterococci modulated the course of EAE in rats in a dose-dependent manner, while significant differences from the control group were observed only in the group of rats receiving a high dose (8.0 lg CFU/ml) of microorganisms.

The administration of *E. faecium* L-3 to rats at a high dose had a significant protective effect. In this group, the symptoms of EAE appeared 5 days later and lasted 5 days less than in the control group.

The severity of the disease in rats treated with a high dose of *E. faecium* L-3 also decreased, as indicated by CS maximal, CS cumulative and CS mean **(Table 1)**, as well as the increase in the percentage of animals without clinical manifestations (CS = 0) and slightly sick rats (CS = 0.5–2.0) **(Fig. 1)**. The severity of the disease in this group was reduced compared to the control rats almost throughout the experiment, as can be seen from the dynamics of the severity of the disease **(Fig. 2)**.

The only parameter for which there was no difference was the convalescence CS (Table 1). Thus, as seen previously [10, 24], the course administration of *E. faecium* L-3 at a dose of 8.0 lg CFU/ml had a positive effect on the course of EAE.

In rats treated with lower doses of *E. faecium* L-3 (6.0 and 7.0 lg CFU/ml), all of the studied parameters were comparable, and the differences compared with the control group did not reach the level of statistical significance. It should be mentioned that no significant differences were observed between the group of rats receiving a high dose of probiotic and other groups receiving probiotics **(Table 1)**.

However, when analyzing the dynamics of the severity of the disease **(Fig. 2)**, it turned out that at the peak of the disease in rats treated with probiotic enterococci at lower doses (6.0 and 7.0 lg CFU/ml), the severity of the disease was reduced compared to the rats within the control group – by 17–20 days after EAE

Group	Inductive phase (days)	Duration of disease (days)	Clinical score			
			maximal	cumulative	mean	convalescence
Saline	12.6 ± 0.8	17.7 ± 1.2	4.0 ± 0.3	61.4 ± 8.0	3.1 ± 0.3	2.4 ± 0.4
E. faecium L-3 6.0 lg CFU/ml	14.0 ± 1.5	14.8 ± 1.5	3.0 ± 0.5	42.2 ± 8.8	2.3 ± 0.4	1.9 ± 0.5
E. faecium L-3 7.0 lg CFU/ml	14.2 ± 0.8	14.0 ± 1.1	2.8 ± 0.4	40.9 ± 6.6	2.0 ± 0.3	1.7 ± 0.4
E. faecium L-3 8.0 lg CFU/ml	17.9 ± 1.8 *	12.2 ± 1.9 *	2.4 ± 0.5 *	31.5 ± 7.6 *	1.8 ± 0.4 *	1.5 ± 0.5

**p<0.05 – E. faecium* L-3 8.0 lg CFU/ml compared with saline; one-way ANOVA followed by Tukey HSD post hoc test

Table 1 Effects of different doses of *Enterococcus faecium* L-3 on experimental allergic encephalomyelitis duration and clinical score

Figure 1
The percentage of animals with differing severity of EAE

******p<0.05 E. faecium* L-3 8.0 lg CFU/ml compared with saline, χ2 test;

*p<0.05 E. faecium* L-3 7.0 lg CFU/ml compared with saline, χ2 test.

Figure made in Excel.

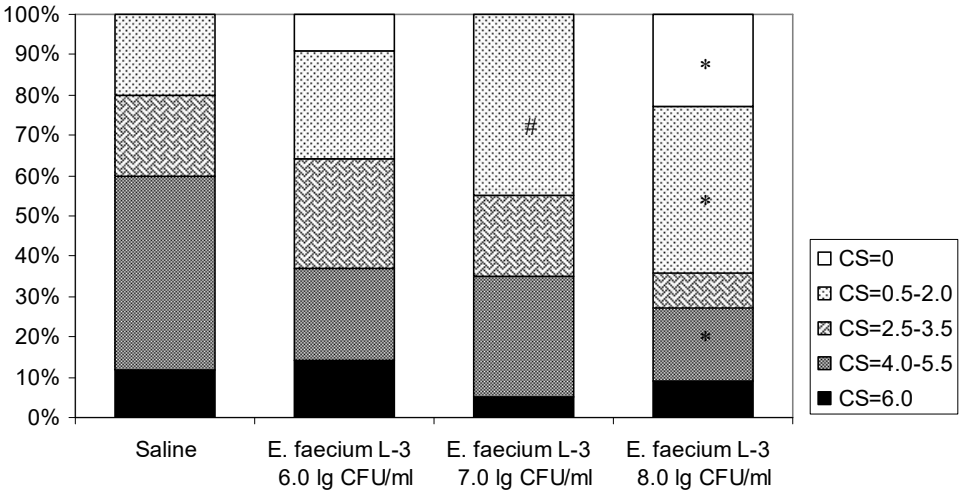


Figure 2
Modification of EAE severity by administration of various doses of probiotic enterococci

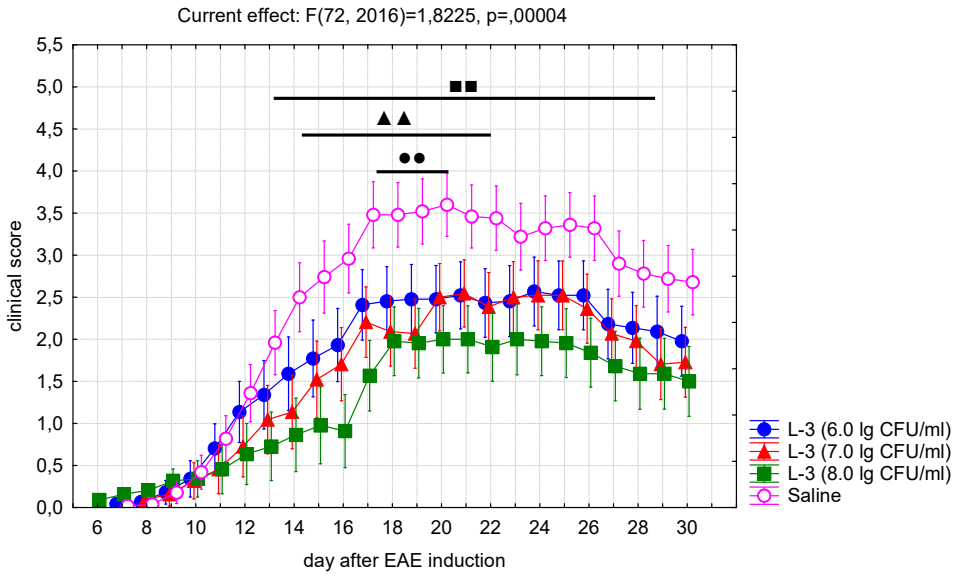
■■ *p<0.05 E. faecium* L-3 8.0 lg CFU/ml compared with saline;

▲▲ *p<0.05 E. faecium* L-3 7.0 lg CFU/ml compared with saline;

●● *p<0.05 E. faecium* L-3 6.0 lg CFU/ml compared with saline.

Repeated measures ANOVA with Tukey HSD post hoc test.

Figure made in Statistica.



induction (d.p.i.) and 14–22 d.p.i., respectively. That is, the protective effect of *E. faecium* L-3 was observed with these doses, but was less pronounced than when using the high dose.

It should be noted that, within each group that received probiotics, animals were found for which the treatment was poorly effective or not effective (CS > 4). This ranged from 25% (with a high dose) of animals to 35– 37% (with the middle and low doses), although in the control group, the percentage of animals with severe EAE, up to death, reached 60% **(Fig. 1)**.

Thus, the extent of the protective action of *E. faecium* L-3 in the EAE model in rats depends on the dose used.

Morphological changes in the spinal cord of rats with EAE receiving different doses of *E. faecium* L-3

To assess the neuroprotective effects of probiotic enterococci, the rat spinal cord was examined for the presence of inflammation and damage to neurons. Since we did not observe differences in the course of EAE in rats receiving *E. faecium* L-3 at doses of 6.0 and 7.0 lg CFU/ml, we only used rats receiving low and high doses of enterococci for morphological studies.

In the study of spinal cord sections from rats treated with probiotic enterococci, a dose-dependent attenuation of the morphological changes characteristic of EAE was also detected.

At the peak of the disease, in the presence of clinical symptoms of EAE, significant morphological changes were found in the spinal cord of rats within the control group, which indicated pronounced inflammation in the CNS and damage to the neurons.

Hyper-and hypochromic neurons were detected in sections of the spinal cord, around which pericellular oedema was observed **(Fig. 3a)**. At the same time, neurons came into direct physical contact; these were without visible structural changes but were hypertrophied.

Near cells with pronounced chromatolysis **(Fig. 3b)**, which had lost Nissl substance and nucleolus colouration, an accumulation of gliocytes was detected along the border of the pericellular oedematous region. Occasionally, structures resembling apoptotic bodies were observed in sections.

Moderate infiltration of monocyte-macrophage cells was recorded in the white matter and membranes of the spinal cord. The blood vessels were surrounded by clutches of mononuclear epithelial-type cells (Fig. 3c). Inside the blood vessels, lymphocyte groups were sometimes present. An accumulation of glial cells was observed in the posterior horns of the spinal cord **(Fig. 3d)**.

In rats treated with *E. faecium* L-3 at a dose of 6.0 lg CFU/ml, there were no significant differences compared to the rats of the control group. In sections of the spinal cord from this group of rats, hyperchromic neurons and cell couplings were recorded around blood vessels, and a pronounced microglial reaction and infiltration of white matter by monocyte-macrophage cells was observed **(Fig. 3e)**.

In rats receiving a high dose of *E. faecium* L-3 (8.0 lg CFU/ml), the severity of morphological changes in the spinal cord decreased as the severity of the clinical symptoms decreased. Occasionally, neurons undergoing chromatolysis were found in the posterior horns. Although small amounts of pericellular oedema were present around unchanged neurons, there was no inflammatory reaction in the membranes and around the blood vessels, and there were no clear signs of a glial reaction **(Fig. 3f)**.

Thus, the correspondence between the severity of EAE in rats and morphological changes in the spinal cord, as well as the dose-dependent attenuation of the inflammatory response and damage to neurons against the background of a milder EAE in rats treated

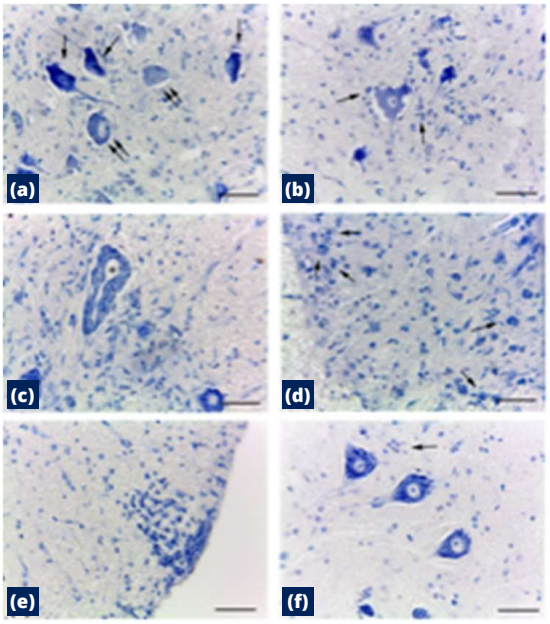


Figure 3
Inflammation and damage in neurons and glia in the spinal cord of rats after EAE induction and the modulation by Enterococcus faecium L-3 in a dose-dependent manner

(a) Hyperchromic (indicated by arrow) and hypochromic (indicated by double arrow) neurons in the anterior horns of the spinal cord.
(b) A cell with pronounced signs of chromatolysis: the disappearance of the chromophilic substance, the clearing of the cytoplasm, the eroded outlines of the cell, which indicate its imminent death. Arrows indicate clusters of glial cells.
(c) A blood vessel surrounded by a clutch of mononuclear epithelial-type cells (the lumen of the vessel is marked with an asterisk).
(d) Clusters of glial cells in the posterior horns of the spinal cord (indicated by arrows).
(e) Moderate infiltration by cells of the monocyte-macrophage type of white matter and the lining of the spinal cord.
(f) Structurally unchanged neurons with a well-pronounced Nissl substance, weak pericellular oedema, small clusters of glial cells (indicated by arrow). **a–d** - control group; **e** - group *E. faecium* L-3 (6.0 lg CFU/ml); **f** - group *E. faecium* L-3 (8.0 lg CFU/ml). Sections of paraffin blocks with Nissl coloured by toluidine blue.

The scale distance is 50 microns.

with probiotic enterococci, especially in high doses, has been identified.

Discussion

The present study demonstrated the ability of the probiotic strain *E. faecium* L-3 to prevent the development of EAE in rats and reduce the severity/duration of the disease in a dose-dependent manner. Interestingly, both endogenous and probiotic enterococci had a pronounced protective effect on EAE at a high dose. It was also shown that reduction in the severity of EAE in rats was accompanied by suppression of the inflammatory response in the CNS and a decrease in neuronal damage. It was previously shown that the severity of morphological manifestations correlates with the severity of the disease [28].

It should be noted that the protective effects of *E. faecium* L-3 in EAE is not a unique property of this particular strain. In other studies, several strains of lactic acid bacteria, bifidobacteria and escherichia had similar effects on the immune system [16, 17, 19-23, 25]. For example, a positive effect on the course of EAE in animals was observed while using the probiotic strain *Bifidobacterium animalis* PTCC 1631 in combination with *Lactobacillus plantarum* A7 [29], or by using the strictly anaerobic intestinal bacteria *Bacteroides fragilis* [30] and *Prevotella histicola* [31]. For many probiotic strains and commensal species, the association of positive effects on the course of EAE with the ability to stimulate the differentiation of the regulatory subpopulation of CD4+CD25+FoxP3+ T cells, producing the anti-inflammatory cytokine IL-10, has been shown.

In contrast to other probiotic bacteria, the protective effect of *E. faecium* L-3 was associated with an increase in the circulation of the other subpopulation of T cells, with the phenotype CD4+CD25+FoxP3-[24]. Among FoxP3-subsets of CD4+ T cells, two regulatory subsets are known: Tr1 cells, which have a suppressor function, producing both IL-10 and TGF. [32], and Th3, which secrete a high level of TGF., but not IL-10 [33]. In addition, *E. faecium* L-3 stimulated TNK cells and suppressed B cells circulating in the blood [24].

The key points associated with trigger-

ing inflammation in the CNS are increased BBB permeability and migration in the CNS of T cells sensitized to nervous tissue antigens. However, in recent years, in the light of awareness of Th17 migration in the CNS, the role of the integrity of the intestinal barrier has also been discussed.

An increase in intestinal permeability and imbalance of Th1/Th17 and Treg cells in the intestine was detected in mice with EAE [34], and the degree of intestinal barrier disturbance correlated with the severity of the disease [25]. Intestinal barrier integrity disorders were also found in patients with MS [35].

Previously in EAE models, the ability of some probiotic strains to prevent the permeability of the intestinal barrier, such as *Lactobacillus rhamnosus* GG and a mixture of *Lactobacilli* and *Bifidobacteria*, *Escherichia coli* Nissle 1917, was shown [25, 36]. It is known that probiotic enterococci, and in particular, the strain *E. faecium* NCIMB 10415 were also able to prevent leaks in the intestinal barrier [37]. The regulation of intestinal permeability may be one of the mechanisms underlying the protective action of *E. faecium* L-3, which we found in this model.

One reason for the increasing gut permeability may be changes in the composition of intestinal microbiota (dysbiosis) due to the higher abundance of opportunistic and/or pathogenic microorganisms. We have previously shown that intestinal dysbiosis develops in rats during EAE [10] and *E. faecium* L-3 is able to inhibit the growth of opportunistic microorganisms, such as *Klebsiella* spp., *Proteus* spp., *Staphylococcus aureus* and *Candida* spp. [26]. It is logical to assume that *E. faecium* L-3, by suppressing the growth of opportunistic bacteria, will help preserve the integrity of the intestinal barrier. Moreover, the administration of *E. faecium* L-3 to rats in the course of EAE induced restoration of the abundance of several important intestinal inhabitants, including the major butyrate producer *Faecalibacterium prausnitzii* [10]. Indigenous *F. prausnitzii* have the same protective effect on intestinal permeability as the probiotic strain *Lactobacillus rhamnosus* CNCM I-3690 [38], and a positive correlation between the levels of *F. prausnitzii* in the mammalian gut and production of the anti-inflammatory cytokine IL-10 was also noted [39]. Probiotic enterococci themselves were also able to stimulate an increase in the levels of IL-10 in the mesenteric lymph nodes [40, 41]. We can suggest that the amelioration of EAE in rats receiving *E. faecium* L-3 may be induced by the direct and/or mediated effect of enterococci on intestinal permeability and the production of short.chain fatty acids, including butyrate.

In addition, enterococci may affect the migration of Th cells outside the intestine. Chemokines and their receptors play an important role in the migration of T cells. Previously, it was demonstrated that *E. faecium* L-3 suppresses the production of the chemokine MCP-1 in a model of antibiotic-associated dysbiosis [42]. Probably, in the EAE model, *E. faecium* L-3 also demonstrates this activity, which limits the migration ability of T cells especially effectively when using a high dose of probiotic.

The absence of differences in the severity of the disease in the later phase of EAE in rats treated with *E. faecium* L-3 (CS convalescence) from the control group may be due to the fact that we used an acute EAE model with spontaneous recovery (full or partial) of animals within the control group. Recovery in such models is associated with anti-inflammatory IL-10. These data are consistent with the previously detected increase in the expression of IL-10 mRNA in the rat spinal cord [43], an increase in the number of CD4+CD25+FoxP3+ T cells in the blood [24] and increased levels of circulating IL-10 [43], previously determined in rats at the recovery phase of EAE.

In future studies, it is necessary to find out why the treatment with probiotic enterococci was ineffective in some of the animals. One possible explanation may be related to the

microbial antagonism between the probiotic strain and individual microbiota of different animals.

We have observed similar antagonism previously between *E. faecium* L-3 and the immunomodulatory substance glatiramer acetate [10], which stimulated the same populations of immune cells in blood as lactobacilli.

Conclusion

The study showed that a sufficiently high dosage of the probiotic *E. faecium* L-3 strain is needed to implement protection in rats in the EAE model. However, additional studies are necessary to evaluate the interaction of this probiotic bacteria with members of the intestinal microbiota. This will allow us to develop criteria for the application or exclusion of the use of this probiotic strain in EAE in order to avoid possible negative consequences.

In the future, the use of probiotics on the basis of enterococci normalizing the composition of the intestinal microbiota, contributing to maintaining intestinal integrity and capable of modulating host immune functions, may be an addition or alternative to pharmacological immunomodulation not only for MS, but also for other autoimmune diseases.

Ethical standards

All experimental procedures were carried out in compliance with the principles of humanity (European Community Directives No. 86/609 of the EU), approved by the local ethics committee of the FGBNU "Institute of Experimental Medicine", SPb, Russia.

Conflict of Interest

The authors declare that they have no conflict of interest.

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