THE ROLE OF PROBIOTICS IN THE REGULATION OF EXPRESSION OF GENES SUPPORTING ANTIOXIDANT STATUS AND FUNCTIONALITY OF MOUSE TESTES IN LPS-INDUCED INFLAMMATORY PROCESSES

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Systemic lipopolysaccharide (LPS)-induced inflammation has a significant impact on various organs, including the male reproductive system. In this study, we have demonstrated that LPS-induced inflammation causes oxidative stress in mouse testes, reduces expression of genes encoding the catalytic subunit of glutamate-cysteine ligase (*Gclc*) and superoxide dismutase 2 (*Sod2*). Inflammation suppressed transcription of genes involved in differentiation and metabolic regulation of testicular cells and sperm maturation: in the LPS group, the expression of the *Amh*, *Lepr*, *Eif2b4* genes was approximately 3 times lower compared to the control group. The intake of probiotic microorganisms caused a decrease in the intensity of lipid peroxidation, which was manifested in a decrease in the level of conjugated dienes (CD) compared to the LPS group, contributed to maintaining the level of expression of genes supporting the antioxidant status, as well as genes supporting the functionality of the mouse testes. The data obtained suggest that probiotics may be considered as potential tools for maintaining male reproductive function under conditions of inflammatory processes.

Keywords: probiotics; spermatogenesis; lipopolysaccharides; inflammation; oxidative stress; lipid peroxidation

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INTRODUCTION

During the period from 2000 to 2018, the total number of men with infertility more than doubled in Russia from 22,348 to 47,886. The number of patients with primarily diagnosed male infertility increased by 82% [1]. Inflammation has a significant impact on the male reproductive system, causing serious impairments in testicular function. Inflammation can be caused by bacterial and viral pathogens, as well as by autoimmune processes. Experimentally, inflammation is usually induced by injections of lipopolysaccharides (LPS), which are components of the cell wall of gram-negative bacteria that inhabit the intestinal microflora. Normally, the body's systems are able to neutralize LPS molecules entering the bloodstream, but if the intestinal barrier function is impaired or massive destruction of bacterial cells occurs, the systemic level of LPS increases, thus causing intoxication of the body [2].

During the development of systemic testicular inflammation, Sertoli cells respond by activation of proinflammatory genes, which are also involved in intercellular communication. These include genes encoding interleukin 1-alpha (*Il1a*), interleukin 6 (*Il6*) [3, 4]. Interleukins also participate in regulation of spermatogenesis processes, Sertoli cell activity and their cellular organization. When inflammatory mediators enter the testes from the blood, the functioning of spermatogenic cells is disrupted and this leads to inhibition of spermatogenesis [5]. Cells of the innate immune system produce reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, which are aimed at destroying pathogens. A long-term inflammatory process increases the ROS production, causing the development of oxidative stress (OS) [6]. ROS are one of the significant factors in pathologies of the male reproductive system; according to various estimates, they can cause up to 80% of structural damage to spermatozoa [7].

Currently, compounds with antioxidant activity, such as folic acid, L-carnitine, L-arginine, N-acetylcysteine, zinc, selenium, vitamin E, inositol derivatives, etc., are used to reduce OS in the male testes [8]. Probiotic mixtures have a significant antioxidant potential, as they have their own antioxidant systems and also produce corresponding metabolites that increase the body's defense against ROS [9]. Probiotics are live non-pathogenic microorganisms used to improve microbial balance, often in the gastrointestinal tract, due to their ability to imitate the homeostatic effects of intact microbiota [10]. A significant portion of the data obtained indicates the anti-inflammatory effect of probiotics, which, in turn, reflect the immune tolerance that exists between

the host and its microbiota [11, 12]. Many studies on the gut-brain axis have confirmed the influence of gut microbiota on brain function and activity [13–15], and the hypothalamic-pituitary-testicular (HPT) axis is considered as a classic neural regulatory pathway in the process of steroidogenesis [16, 17]. In this regard, we suggest that the gut-microbiota-testes axis may be an important pathway regulating the function of the male reproductive organs, and probiotics, in turn, can be used as a therapeutic strategy to reduce inflammation and OS in tissues responsible for spermatogenesis.

The aim of our work was to study the effect of various probiotic organisms on the expression level of inflammation markers and OS in the testes, as well as their impact on the hormonal regulation of spermatogenesis under conditions of LPS-induced inflammation.

MATERIALS AND METHODS

Animals

The experiment was performed using two-month-old male C57Bl/6 mice obtained from the Stolbovaya nursery, the Branch of the Scientific Center for Biomedical Technologies (Moscow, Russia). The animals were kept under standard vivarium conditions at 25°C, relative humidity of at least 40% and a 12-h light/dark cycle, with free access to food and water.

Experimental Design

At the beginning of the experiment, 40 male mice were randomly divided into seven groups. Animals of the first group (Control) (n=6) received a standard laboratory diet for 3 weeks. On week 4, control mice were injected intraperitoneally with 0.2 ml saline (Grotex, Russia) for 7 days. Animals of the second group (LPS) (n=6) received a standard laboratory diet. On week 4 of the experiment, mice were injected intraperitoneally with lipopolysaccharides (LPS) (Pyrogenal, Gamaleya National Research Center of Epidemiology and Microbiology, Russia) at a dose of 375 μg/kg/day in a volume of 0.2 ml. Animals of the third (n=6), fourth (n=5), fifth (n=6), sixth (n=5), and seventh (n=6) groups received a laboratory diet mixed with probiotic lactic acid bacteria: *Weissella confusa*, *Weizmannia coagulans*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, and a commercial probiotic product (CPP), respectively, in a ratio of 1×10^8 CFU/g of diet. CPP is a fermented milk product that includes *Lactobacillus delbrueckii* subsp. *Bulgaricus*, *Lactiplantibacillus plantarum*, *Lactobacillus acidophilus*, and *Limosilactobacillus fermentum*. During the entire experiment, the average daily food consumption of the mice varied and was 5.1±0.08 g; this corresponded to the average daily consumption of 5.1×10^8 bacteria by each mouse. Groups 3–7 of mice also received LPS injections on week 4 of the experiment for 7 days at a dose of 375 μg/kg/day.

Measurement of Lipid Peroxidation (LPO) Products

The content of conjugated dienes (CD) [18] and malondialdehyde (MDA) [19] was measured spectrophotometrically using a Hitachi U-2900 spectrophotometer (Hitachi High-Technologies, Japan). The test material was pre-weighed and homogenized in a Bioprep-6 automatic homogenizer (Allsheng, China) in 1 ml of 0.1 M phosphate buffer, pH 7.4. To measure the CD, 0.125 ml saline (MOSFARM, Russia), 1.5 ml heptane, and 1.5 ml isopropyl alcohol (RFK, Russia) were added to 0.125 ml homogenate. The resulting mixture was centrifuged at 3000 *g* for 10 min at 4°C. Then distilled water was added to the supernatant in a ratio of 2:1 and mixed. After phase separation the upper, heptane phase, was transferred to a clean test tube and 0.5 ml ethanol was added in a ratio of 1:2. 96% ethyl alcohol served as control. The CD level was measured at 233 nm.

To measure the MDA level, the pre-prepared supernatant (0.4 ml) was collected in test tubes and 0.6 ml of 20% trichloroacetic acid was added. The samples were incubated in the cold for 30 min with stirring every 10 min. Then the samples were centrifuged at 10,000 *g* for 10 min at 4°C. 300 μl-aliquots of the supernatant were collected in new test tubes, 3 ml of trichloroacetic acid and 1 ml of 0.8% thiobarbituric acid were added. The contents were mixed and the test tubes were placed in a water bath at 100°C for 40 min. After cooling the MDA level was measured at 530 nm and 580 nm.

Gene Expression Assay

Total RNA was isolated from testicular tissue using the commercial ExtractRNA RNA kit (Eurogen, Russia) according to the manufacturer's protocol. Reverse transcription was performed using the REVERTA-L cDNA reagent kit (AmpliSens, Russia) according to the attached protocol.

Gene expression levels were assessed using quantitative PCR analysis. The reaction mixture (20 μl) contained: 4 μl qPCRmix-HS SYBR, 1 μl forward and reverse primer mixture, 1 μl cDNA, 14 μl mQ. The reaction conditions were: total denaturation 95°C for 3 min; denaturation at the beginning of the cycle at 95°C for 30 s; primer annealing 59°C for 30 s; elongation 72°C for 30 s. The number of cycles was 45. The primers were selected for genes that were responsible for the regulation of the body antioxidant defense, participated in the regulation of spermatogenesis, or were markers of inflammatory processes in the testes. The primer sequences are given in Table 1. The primers were developed in the Primer-BLAST program.

The results were normalized to *Gapdh* gene expression.

Table 1. The sequences of primers used for gene expression analysis

#	Gene name	Sequences of primers
1	*Gapdh*	F: 5′-CATCACTGCCACCCAGAAGACTG-3′; R: 5′-ATGCCAGTGAGCTTCCCGTTCAG-3′
2	*Eif2b4*	F: 5′-GCTTGCAACAGGTAGCTTGT-3′; R: 5′-CCCCTCACTCACCTTGACAT-3′
3	*Crisp4*	F: 5′-ATGGATGTGGGTATGGCAGT-3′; R: 5′-GCAGCTGAACTCCAACTCAC-3′
4	*Lepr*	F: 5′-CTTTCCTGTGGACAGAACCAGC-3′; R: 5′-AGCACTGAGTGACTCCACAGCA-3′
5	*Amh*	F: 5′-CCGCTATTTGGTGCTAACCGTG-3′; R: 5′-AAGGCTTGCAGCTGATCGATGC-3′
6	*Gclc*	F: 5′-GGGGTGACGAGGTGGAGTA-3′; R: 5′-GTTGGGGTTTGTCCTCTCCC-3′
7	*Sod2*	F: 5′-CAGACCTGCCTTACGACTATGG-3′; R: 5′-CTCGGTGGCGTTGAGATTGTT-3′
8	*Prdx3*	F: 5′-GTGGTTTGGGCCACATGAAC-3′; R: 5′-TGGCTTGATCGTAGGGGACT-3′
9	*Nfe2l2*	F: 5′-CTCTCTGAACTCCTGGACGG-3′; R: 5′-GGGTCTCCGTAAATGGAAG-3′
10	*Il1b*	F: 5′-TTGACGGACCCCAAAAGATG-3′; R: 5′-AGAAGGTGCTCATGTCCTCA-3′
11	*Il6*	F: 5′-CGGAGAGGAGACTTCACAGAG-3′; R: 5′-CATTTCCACGATTTCCCAGA-3′
12	*Tnf*	F: 5′-TATGGCTCAGGGTCCAACTC-3′; R: 5′-GGAAAGCCCATTTGAGTCCT-3′
13	*Gfap*	F: 5′-CCACGTTAAGCTAGCCCTGGACAT-3′; R: 5′-CTCACCATCCCGCATCTCCACAGT-3′
14	*Ptgs2*	F: 5′-AGTCCGGGTACAGTCACACTT-3′; R: 5′-TTCCAATCCATGTCAAAACCGT-3′

Statistical Analysis

Statistical analysis was performed using the Statistica 12 program. The results were expressed as the mean ± standard error of the mean. The data were analyzed using one-way analysis of variance (ANOVA). The Bonferroni test was used to determine the level of significance. In this paper, only statistically significant differences ($p<0.05$) are discussed.

RESULTS AND DISCUSSION

The effect of Probiotics on The Expression of Genes Responsible for the Hormonal Regulation of Spermatogenesis

In LPS-treated mice, the expression of the *Amh*, *Lepr*, *Eif2b4* genes was approximately three times lower compared to the control group (all $p<0.05$) (Table 2). The *Amh* gene encodes the AMH protein, which binds to the anti-Mullerian hormone receptor type 2 and thus causes regression of the Mullerian ducts in the male embryo; this protein also plays a role in the differentiation and functioning of Leydig cells [20]. The *Lepr* gene encodes the receptor for leptin, an adipose tissue hormone that regulates energy and neuroendocrine metabolic processes. LepR is found in all tissues of the male reproductive system thus indicating its key role in reproductive function maintenance [21]. The *Eif2b4* gene encodes the delta subunit of the eukaryotic transcription initiation factor 2B protein, and normal expression levels of this gene are necessary for spermatogenesis [22]. Impaired spermatogenesis and decreased testosterone levels have been previously reported in the context of systemic inflammation [4, 23].

LPS causes various structural changes in testicular tissue, such as a sharp increase in the number of convoluted seminiferous tubules with destructive changes, and a decrease in the number of spermatogonia and spermatocytes [23]. All of these structural changes in the testes were accompanied by metabolic disorders in the cells of the spermatogenic epithelium [23]. The presence of pathogenic molecules penetrating from the bloodstream during systemic inflammation can disrupt the normal function of Sertoli cells and spermatogenic cells; this leads to overall disruption of the spermatogenesis process [4]. However, we did not find any significant differences in sperm motility between control group animals and LPS-treated animals [24]. However, our data indicate that induced inflammation in the testes causes changes in spermatogenesis at the level of transcriptional regulation, which could potentially be the cause of decreased reproductive function.

A study of biologically active substances of the endocrine system of the body has shown that certain hormones involved in steroidogenesis are actively synthesized in the colon by the intestinal microbiota [25, 26]. It also directly produces androgens, so changes in the composition of microorganisms can modulate the metabolism of male hormones. Intestinal glucuronidated testosterone and dihydrotestosterone excreted by the liver can be effectively reduced to the active form by the intestinal microbiota, since this form is not used for excretion from the body; most androgens are present in the distal intestine [27]. In addition to deglucuronidase activity, some taxa are able to express enzymes involved in the metabolism of steroid hormones. Among the representatives of the intestinal microbiota, such bacteria as *Butyricicoccus desmolans*, *Clostridium cadaveris*, *Propionimicrobium lymphophilum*, *Clostridium scindens*, and *Clostridium innocuum* express steroid-17,20-desmolase, 20β-HSDH, 20α-HSDH, 3α-HSDH, or 5β-reductase [28]. Thus, the intestinal microbiota is involved in androgen metabolism, and its specific role requires a more detailed study.

We found signs of a positive effect of probiotics on the hormonal regulation of spermatogenesis. The *Crisp4* gene expression was significantly increased in the group of mice receiving *L. salivarius* ($p<0.05$),

Table 2. Relative gene expression level (mean ± SEM)

Control	LPS	LPS + *L. plantarum*	LPS + *L. salivarius*	LPS + *W. confusa*	LPS + *W. coagulans*	LPS + CPP
Eif2b4						
1.99±0.57	0.66±0.24*	0.56±0.43	0.48±0.13	0.57±0.54	0.22±0.25	3.39±2.65
Crisp4						
0.69±0.35	0.78±0.43	0.53±0.42	2.87±0.82*	0.41±0.31	5.45±2.53	1.69±1.60
Lepr						
2.75±0.89	1.00±0.29*	1.94±0.95	2.10±0.67	1.42±0.43	1.11±0.92	1.36±0.47
Amh						
1.99±0.47	0.69±0.30*	0.90±0.54	1.79±0.52	0.55±0.44	0.52±0.60	3.39±2.66
Gclc						
4.29±1.16	0.95±0.41**	1.67±1.01*	2.93±0.72	1.24±0.74	1.36±1.07	1.63±0.68*
Sod2						
2.74±0.61	1.00±0.33**	1.59±0.93	2.37±0.60	1.05±0.62*	1.52±0.86	2.25±0.46
Prdx3						
2.35±0.68	1.00±0.47	1.89±1.17	1.21±0.29	0.78±0.39	0.75±0.47	1.37±0.52
Nfe2l2						
3.46±1.46	1.00±0.53	1.49±0.99	1.95±0.61	1.13±0.57	0.89±0.59	1.89±0.63
Il1b						
0.37±0.13	0.48±0.28	2.13±1.19$^{\#}$**	0.23±0.07	1.38±0.81*	0.23±0.11	0.57±0.35
Il6						
0.63±0.31	0.73±0.4	0.43±0.38	1.00±0.59	0.78±0.36	0.99±0.28	0.41±0.24
Tnf						
0.67±0.22	1.18±0.76	1.29±0.86	0.91±0.36	1.02±0.38	1.13±0.24	0.79±0.47
Gfap						
1.27±0.53	1.18±0.55	1.38±0.77	0.71±0.23	0.99±0.63	0.38±0.22	1.06±0.57
Ptgs2						
0.24±0.09	0.63±0.37	0.39±0.22	1.00±0.35	0.65±0.32	0.91±0.29	0.63±0.41

*p<0.05, **p<0.01 – statistically significant differences compared to the control group, $^{\#}p$<0.05 – statistically significant differences compared to the group of LPS-treated animals. Results are expressed as mean ± SEM.

in animals receiving *W. coagulans*, this increase was at the level of statistical tendency (p=0.09). This cysteine-rich secretory protein 4 (Crisp4) is associated with the process of sperm maturation and plays an important role in fertilization [29]. All the studied probiotics prevented the LPS-induced decrease in the expression of *Amh* and *Lepr*. *W. confusa*, *L. plantarum*, and CPP prevented the LPS-dependent decrease in the *Eif2b4* expression (Table 2).

The data described above and those obtained by us indicate the influence of probiotic microorganisms on the hormonal regulation of spermatogenesis. However, further studies are needed to investigate the pathways of regulation of processes that ensure male fertility, in particular, by adding probiotics to the diet.

Antioxidant Effects of Probiotics in LPS-Induced Systemic Inflammation

The development of OS is a serious consequence of systemic inflammation: it is manifested in the activation of LPO processes. In the testes of LPS-treated mice, there was a tendency for the increase in the level of such LPO products as CD (by 11%) and MDA (by 34%) (p>0.05). CD are toxic metabolites that can cause damage to various biologically active molecules, including lipoproteins, proteins, enzymes, and nucleic acids; they are formed as the primary LPO products [30]. Administration of CPP, *W. coagulans*, and *L. plantarum* caused a significant decrease in the CD level compared to the LPS-treated group (p<0.05). In general, a decrease in the CD level was observed in the testes of all groups

of mice treated with probiotics (Fig. 1A). A similar tendency was observed with the MDA level (Fig. 1B), another common marker of the intensity of free-radical lipid oxidation. However, according to our data, the analysis of the CD level was more informative than the MDA assay. The latter is an unstable and quite reactive compound and certain difficulties exist in evaluation of its level [30].

The antioxidant effects of probiotic could account for the decrease in the intensity of LPO in the testes of mice. Probiotic microorganisms have their own antioxidant enzymatic systems, which can contribute to the antioxidant protection of the host cells [31]. Moreover, it was previously shown that probiotics also effectively increased the activity of antioxidant enzymes [32] and regulated their expression. It is known that a number of probiotics, including lactobacilli, can stimulate the Nrf2/ARE signaling pathway, one of the main regulators of gene expression involved in antioxidant protection [33].

LPS injections significantly reduced the expression of genes encoding the catalytic subunit of glutamate-cysteine ligase (*Gclc*) and superoxide dismutase 2 (*Sod2*) in the testes of mice (both $p<0.01$). LPS tended to decrease in the expression of the peroxiredoxin 3 (*Prdx3*) gene, as well as the *Nfe2l2* gene encoding the transcription factor Nrf2 (Table 2). In mice receiving probiotics, in most groups, no statistically significant decrease in the expression of the studied genes was observed, thus indicating that probiotics could prevent LPS-induced suppression of antioxidant defense at the level of transcriptional regulation (Table 2).

The Effect of Probiotics on the Expression of Inflammatory Markers

It is known that chronic inflammation induced by LPS injections can lead to an increase in the expression of inflammatory markers [34]. However, we showed that a weekly course of LPS injections did not cause a significant increase in the expression of these genes in the mouse testes, although a trend towards an increase in the expression of *Tnf* and *Ptgs2* was found (Table 2). Probiotic intake often did not affect the expression level of proinflammatory marker genes under conditions of LPS-induced inflammation. A trend towards a decrease in the expression of the glial fibrillary protein (*Gfap*) gene was found in the group of animals receiving *W. coagulans* ($p=0.072$). GFAP and its modified forms are a biomarker of neurotrauma and nervous tissue disorders [35]. Testicular Leydig cells, in turn, have neuroendocrine properties, producing various neuroendocrine markers, including GFAP [36]. It is known that the *W. coagulans* strain is able to reduce inflammation by inhibiting the secretion of proinflammatory cytokines and increasing the secretion of anti-inflammatory cytokines [37], including in the testes as shown in our study (Table 2). At the same time, in the testes of LPS-treated mice, which also received *W. confusa*, as well as *L. plantarum*, increased expression of *Il1b* was found (Table 2). The latter rather suggests stimulation of inflammatory processes and does not allow to make the unambiguous conclusion about anti-inflammatory effects of these strains.

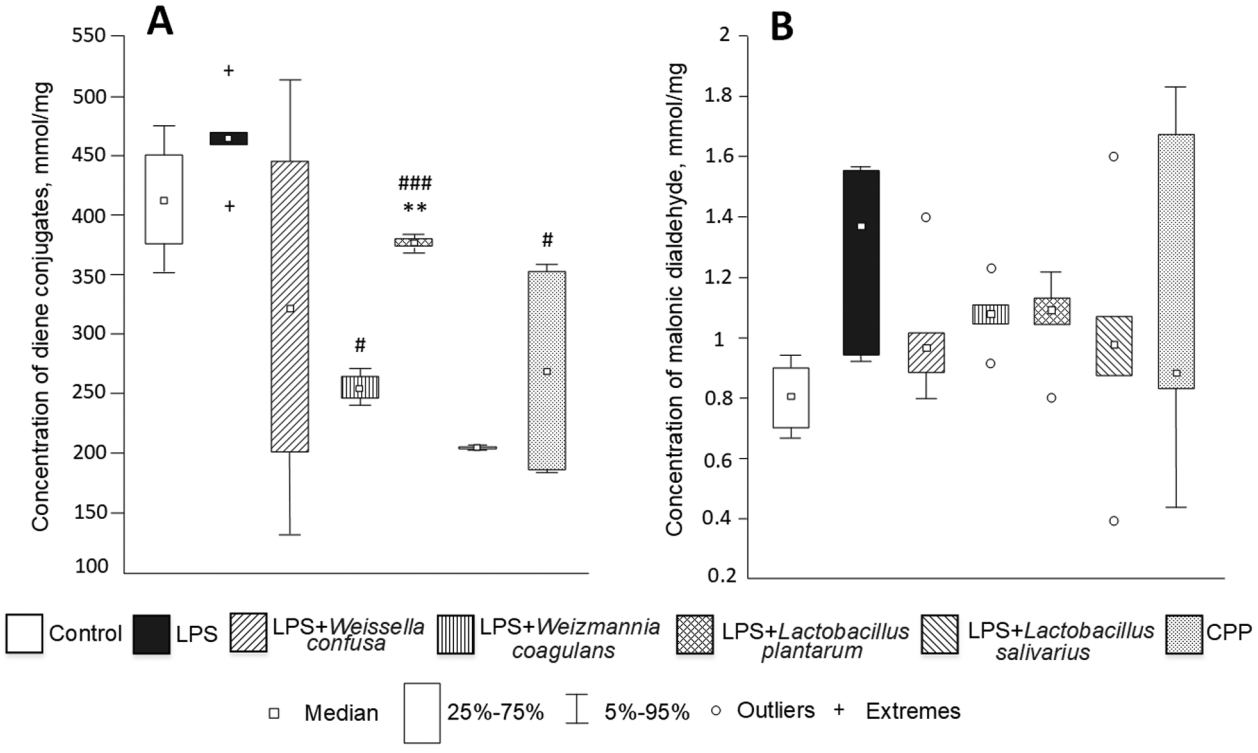

Figure 1. Concentrations of CD (**A**) and MDA (**B**) in testicular tissues. $^{**}p<0.01$ – statistically significant differences versus control group; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ – statistically significant differences versus group of LPS-treated animals.

CONCLUSIONS

Thus, LPS-induced inflammatory processes cause significant OS in the testes and reduce the expression of genes associated with hormonal regulation of spermatogenesis, *Amh*, *Lepr*, *Eif2b4* by 2.9, 2.8, 3.0 times, respectively. Probiotic microorganisms, in turn, are able to reduce OS in the reproductive organs of male mice by normalizing the expression of antioxidant genes, as well as reducing the amount of LPO products. Administration of CPP, *Weizmannia coagulans*, *Lactobacillus plantarum* reduced the CD level by 1.8, 1.7, and 2.3 times, respectively. In addition, they contributed to an increase in the expression of genes responsible for the normal functioning of Leydig cells, encoding leptin receptors and factors responsible for sperm maturation. Therefore the studied probiotics can potentially be used to prevent testicular dysfunction and male infertility caused by systemic inflammatory processes by modulating the "gut-microbiota-testes" axis.

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COMPLIANCE WITH ETHICAL STANDARDS

The animals were raised, kept and killed in accordance with the rules approved by the Ethics Committee for the Expertise of Biomedical Research at the Voronezh State University (Protocol No. 42-03 dated October 8, 2020). The rules comply with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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РОЛЬ ПРОБИОТИКОВ В РЕГУЛЯЦИИ ЭКСПРЕССИИ ГЕНОВ, ПОДДЕРЖИВАЮЩИХ АНТИОКСИДАНТНЫЙ СТАТУС И ФУНКЦИОНАЛЬНОСТЬ СЕМЕННИКОВ МЫШЕЙ, ПРИ ВОСПАЛИТЕЛЬНЫХ ПРОЦЕССАХ, ИНДУЦИРОВАННЫХ ЛИПОПОЛИСАХАРИДАМИ

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Известно, что системное индуцированное липополисахаридами (ЛПС) воспаление затрагивает целый ряд органов, в том числе и мужскую репродуктивную систему. В данном исследовании мы продемонстрировали, что индуцированное инъекциями ЛПС воспаление вызывает окислительный стресс в семенниках мышей, снижает экспрессию генов, кодирующих каталитическую субъединицу глутамат-цистеинлигазы (*Gclc*) и супероксиддисмутазы 2 (*Sod2*). Воспаление подавляло транскрипцию генов, участвующих в дифференциации и метаболической регуляции клеток семенников и созревании сперматозоидов — в группе ЛПС экспрессия генов *Amh*, *Lepr*, *Eif2b4* была примерно в 3 раза ниже по сравнению с контрольной группой. Приём пробиотических микроорганизмов вызывал снижение интенсивности перекисного окисления липидов, что проявлялось в снижении уровня диеновых конъюгатов (ДК) по сравнению с группой ЛПС, способствовал поддержанию уровня экспрессии генов, поддерживающих антиоксидантный статус, а также генов, поддерживающих функциональность семенников мышей. Полученные данные позволяют рассматривать пробиотики в качестве потенциального средства для поддержания репродуктивной функции мужчин на фоне воспалительных процессов.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: пробиотики; сперматогенез; липополисахариды; воспаление; окислительный стресс; перекисное окисление липидов

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