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Effect of pro- and metabiotic *Lactobacillus delbrueckii D5* strain on myocardial resistance to ischemia – reperfusion injury in the rat model of systemic inflammatory response

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ABSTRACT

Aim. To study the effect of lyophilized *L. delbrueckii D5*, as well as its inactivated culture, during intragastric administration on myocardial resistance to ischemia – reperfusion injury (IRI), markers of inflammation, and intestinal epithelial permeability.

Materials and methods. The experiments were performed on male Wistar rats with a model of systemic inflammatory response syndrome (SIRS). Myocardial IRI was reproduced on an isolated Langendorff heart.

Results. A significant increase in the levels of tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6, and lactoferrin in SIRS was revealed. The introduction of both inactivated and lyophilized culture of *L. delbrueckii D5* resulted in normalization of these changes. Normalization of the increased blood level of lipopolysaccharide in SIRS was also noted with the introduction of both inactivated and lyophilized *L. delbrueckii D5*. However, the inactivated culture had no effect on the myocardial infarct size, which was increased in the SIRS group compared to the controls, whereas the introduction of the lyophilized strain led to a significant decrease in this parameter.

Conclusion. The inactivated culture of *Lactobacillus delbrueckii D5* has a pronounced anti-inflammatory effect, but does not impact myocardial resistance to IRI, unlike the lyophilized strain, which requires further research.

Keywords: myocardium, ischemia – reperfusion injury, infarct size, *Lactobacillus delbrueckii D5*, systemic inflammatory response syndrome, leukocytes, cytokines, probiotics

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

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Влияние про- и метабиотической формы штамма *Lactobacillus delbrueckii D5* на устойчивость миокарда к ишемии-реперфузии в условиях системного воспалительного ответа у крыс

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РЕЗЮМЕ

Цель. Изучение эффекта лиофилизированного штамма *L. delbrueckii D5*, а также его инактивированной формы при внутрижелудочном введении на устойчивость миокарда к ишемическому-реперфузионному повреждению (ИРП), маркеры воспаления и проницаемость эпителиального барьера кишки.

Материалы и методы. Эксперименты выполнены на самцах крыс стока Вистар с улучшенным конвенциональным статусом на модели синдрома системного воспалительного ответа (ССВО). Ишемически-реперфузионное повреждение миокарда воспроизводили на изолированном сердце, перфузируемом по Лангендорфу.

Результаты. Отмечено значимое повышение уровня фактора некроза опухоли альфа, интерлейкина (ИЛ) 1β, ИЛ-6 и лактоферрина при ССВО. Введение как инактивированного, так и лиофилизированного штамма *L. delbrueckii D5* приводило к нормализации указанных изменений. Также отмечена нормализация повышенного при ССВО уровня липополисахарида в крови при введении как инактивированного, так и лиофилизированного штамма *L. delbrueckii D5.* Однако инактивированный штамм не оказывал влияния на размер инфаркта миокарда, который был увеличен при ССВО по сравнению с контролем, тогда как при введении лиофилизированной формы имелось значимое снижение размера инфаркта.

Заключение. Инактивированная культура *Lactobacillus delbrueckii D5* обладает выраженным противовоспалительным свойством, но не влияет на устойчивость миокарда к ИРП, в отличие от лиофилизированного штамма, что требует дальнейших исследований.

Ключевые слова: миокард, ишемическое-реперфузионное повреждение, размер инфаркта, *Lactobacillus delbrueckii D5*, синдром системного воспалительного ответа, лейкоциты, цитокины, пробиотики

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

The search for signaling molecules that increase myocardial resistance to ischemia – reperfusion injury (IRI) is of undoubted interest for experimental and clinical medicine due to systemic inflammatory response syndrome (SIRS). Recently, data on myocardial IRI reduction induced by various changes in the composition of gut microbiota (GMB) have appeared in the literature. Attempts to identify a single molecular mediator linking the GMB with immune and cardiovascular systems of the superorganism are most likely doomed to failure [1]. Given the infinite diversity of GMB composition and its involvement in all physiological and pathological processes of the macroorganism, the hypothesis of exclusive properties of the bacterium determining the health of the host organism and its microbiota also sounds unlikely. The most probable condition for the formation and maintenance of health seems to be a harmonious combination of key signaling factors of immunity and metabolic parameters to provide an adequate and balanced relationship between the organism, consumed food, and GMB composition.

In accordance with our previous results obtained on the rodent model of SIRS developed within the concept of probiotic-induced cardioprotection, the following intermediate conclusions can be made. In SIRS, which occurs with a combination of primary visceral obesity (PVO), antibiotic-induced dysbiosis (AID), and chemically induced colitis, there is a decrease in myocardial resistance to IRI [2], and administration of some probiotic strains is accompanied by a decrease in myocardial IRI [3].

In addition to the generally recognized and established cardiotropic markers of systemic inflammation (tumor necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), etc.), metabolic products (short-chain fatty acids (SCFAs), bile acids (BA)), and leukocyte population related to changes in the composition of GMB [4], changes in endothelial and epithelial permeability and acute phase reactants, in particular haptoglobin (Hp) and lactoferrin (Lf), are of interest. The elucidation of physiological and molecular mechanisms regulating the effect of GMB and its metabolites on inflammation and myocardial resistance to IRI is of particular interest. In order to identify promising targets and mechanisms, it is necessary to experimentally substantiate the general and specific features of the effect of live and inactivated strains, called metabiotic, on the macroorganism [5].

In the present work, we studied the effect of *Lactobacillus delbrueckii D5* and its inactivated culture on changes in animal body weight, feed and water consumption, hematological and immunological parameters, hemodynamic characteristics, and myocardial resistance to IRI using an isolated Langendorff heart on the previously developed rat model of SIRS in the vivarium (Wistar rats). The working hypothesis was the assumption on differences in the efficacy of inactivated culture compared to live bacteria.

MATERIALS AND METHODS

The experiments were performed on male Wistar rats weighing 320–370 g in accordance with the European Council Directive (86/609/EEC) on the protection of animals used for scientific purposes and the protocol approved by the Commission for the control over care and use of laboratory animals at

Almazov National Medical Center. Modeling of SIRS was described in detail earlier [2].

The animals were randomly distributed into one of five groups (n = 10 in each group): 1) controls: rats received standard feed and drinking water *ad libitum*; 2) SIRS: in addition to the standard diet, rats received 2 g of polyunsaturated fat and 1 g of sucrose daily per os for 28 days, followed by modeling of chemically induced colitis. For this purpose, the animals were rectally injected once with 1 ml of a 3% acetic acid solution + ethanol. Starting from this day, the animals were intragastrically administered a mixture of antimicrobial drugs (AMD) – amoxicillin, metronidazole, and clarithromycin: 1 ml of antimicrobial solution at a daily dose of 15 mg of each AMD per rat for three days and 1 ml of normal saline (NS) for 8 days; 3) SIRS + *L. delbrueckii D5* – all manipulations corresponded to those described for the SIRS group, but instead of 1 ml of NS, the animals were administrated 1 ml of *L.delbrueckii D5* suspension at a concentration of 10*8 CFU per animal; 4) SIRS + inactivated culture – rats of this group were administered 1 ml of probiotic *L.delbrueckii D5* suspension after its pasteurization at 85–90 °C for 1 minute.

Under combined anesthesia, the heart of the rats was removed from the chest by thoracotomy after the end of drug administration (zoletil 20 mg / kg, IM, isoflurane 1.5–2%) and mounted on the Langendorf apparatus. Retrograde perfusion was performed through the aorta with the oxygenated Krebs – Henseleit buffer at a constant pressure of 80 mm Hg. The temperature of the heart and solutions was maintained at 37 °C throughout the experiment. A polyethylene balloon was inserted into the left ventricle for recording isovolumetric pressure. Diastolic pressure and pulse pressure in the left ventricle were recorded on the personal computer using the PhysExp 3.0 program. Heart rate (bpm) was estimated from the left ventricular pressure curve using the software-based method. Coronary flow (CF) in ml / min was also determined by measuring perfusate flow velocity from the pulmonary artery.

The study protocol included the following steps: 1) baseline monitoring of functional indices after a 10 min stabilization period; 2) induction of global ischemia for 30 min; 3) reperfusion through the aorta with the oxygenated Krebs – Henseleit buffer for 60 min with readings every 15 min. After completing 60-minute reperfusion, we performed planimetry of infarct size by staining heart slices with triphenyltetrazolium chloride (TTC) [6].

Complete blood count was performed on the automated veterinary hematology analyzer (URIT-3000 Vet Plus, URIT Medical Electronic, China). The analysis of the leukocyte count in the blood was performed on three leukocyte populations: LYM (lymphocytes), MID (total number of monocytes, eosinophils, basophils, and blasts), and GRAN (granulocytes). The levels of lipopolysaccharide (LPS), TGFβ1, TNFα, IL-1β, IL-6, Hp, and Lf were assessed by the enzyme-linked immunosorbent assay (MR-96A, Mindray, China). Throughout the experiment, the clinical status of the animals, feed and water consumption, and body weight were assessed daily from 9:00 to 10:00 AM.

Statistical processing of the experimental data was carried out using the STATISTICA 12.0 software package. The statistical analysis of discrete values was performed using the nonparametric Kruskal – Wallis H test to detect statistically significant differences, followed by post hoc comparisons using the Mann – Whitney U test. The tables presented the median (Me) and the lower and upper quartiles ($Q_{25\%}$;$Q_{75\%}$). The differences were considered statistically significant at $p < 0.05$.

RESULTS

Body weight, feed and water consumption

The body weight of the rats in the control group throughout the follow-up increased on average by 1.25 ± 0.33 g / day, while in the SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, we noted a decrease in the animal body weight by 3.63 ± 1.21, 3.18 ± 0.95, and 3.25 ± 0.97 g / day, respectively ($p < 0.05$ compared to the controls). Water consumption over the same period per 100 g of body weight was 8.8 ± 1.1 ml / day in the control group and 7.3 ± 0.1; 10.1 ± 1.5, and 9.3 ± 0.3 ml / day for the SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, respectively. Feed consumption in these groups was 1.2 ± 0.2; 1.4 ± 0.3, and 1.5 ± 0.1 g / day, respectively, which was 2.8, 2.4, and 2.2 times lower ($p < 0.05$) than in the control group (3.3 ± 0.4 g / day).

Complete blood count

The leukocyte count ($\times 10^9$ / l for all populations) in the control group was 5.3 (5.2; 5.4); in the SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, it was 7.9 (6.6; 10.1) ($p < 0.05$), 7.5 (5.3; 10.4) ($p < 0.05$), and 6.2 (5.8; 7.3), respectively. The granulocyte population (GRAN) made the main contribution to the increase in the leukocyte count

compared to the control group (2.6 (2.3; 2.8)) and was 5.9 (4.2; 6.8) ($p < 0$ .05); 5.5 (4.8; 6.2) ($p < 0.05$), and 4.6 (4.3; 5.5) ($p < 0.05$) in the experimental groups, respectively. The MID values were 0.2 (0.2; 0.28), 0.4 (0.2; 0.5), 0.30 (0.2; 0.49), and 0.20 (0.2; 0.4) in the control, SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, respectively. The LYM population accounted for 1.2 (1.1; 1.3), 1.6 (1.4; 2.0), 1.3 (0.9; 1.9), and 1.2 (1.1; 1.6) in the control, SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, respectively.

Immunological parameters

The study revealed a significant increase in the blood LPS concentration by more than 3 times ($p < 0.05$) in the SIRS group compared to the controls, with no increase in other groups. The levels of inflammatory markers and hormones are presented in Table 1.

Table 1

Results of assessing plasma levels of cytokines, haptoglobin, lactoferrin, and lipopolysaccharide, pg / ml, *Me* ($Q_{25\%}$; $Q_{75\%}$)

Parameter	Controls	SIRS	SIRS + *L. delbrueckii D5*	SIRS + inactivated culture
TNFα	10.4 (8.7; 11.4)	19.0 (15.0; 21.5)*	11.2 (10.0; 13.3)#	14.8 (12.8; 14.6)
IL-1β	4.6 (2.2; 4.9)	9.6 (7.4; 10.2)*	4.9 (4.3; 5.2) #	6.6 (6.0; 7.6)
IL-6	1.5 (1.4; 1.8)	2.2 (2.0; 2.5)*	1.3 (1.2; 2.5)	1.4 (1.0; 2.1)
TTGFβ	3.2 (2.5; 4.4)	6.2 (5.1; 11.1)*	4.5 (3.1; 7.3)	3.8 (3.2; 4.9)#
Lactoferrin	86 (58; 121)	107 (90; 109)	133 (119; 145)*	55 (37; 63)#
Haptoglobin	31 (24; 37)	76 (64; 102)*	24 (22; 30)#	35 (26; 40)#
LPS	52 (42; 65)	163 (91; 168)*	60 (54; 71)#	63 (50; 69)#

* $p < 0.05$ compared to the control group, # $p < 0.05$ compared to the SIRS group.

In the SIRS group, the level of TNFα, IL-1β, IL-6, TGFβ, Hp, and LPS increased by 83, 108, 47, 94, 146, and 214%, respectively ($p < 0.05$), compared to the control group. In the SIRS + inactivated culture group, there was a decrease by 63, 94, and 117% in TGFβ, Lf, and Hp, respectively ($p < 0.05$), compared to the SIRS group. In the SIRS + *L. delbrueckii D5* group, the values of all parameters were close to the control ones, except for a significant increase in Lf by 133% ($p < 0.05$).

Histologic examination of the colon

The histologic examination of the corresponding section of the colon in the rats of the control group revealed normal histologic design of the tissue. In the SIRS group, large areas with ulcers and erosions with purulent exudate were identified on the colonic mucosa at the site of inflammation (5–7 cm from the anus). The mucosa and submucosa were replaced with granulation tissue. We detected edema and severe inflammatory infiltrate with prevalence of lymphocytes; however, macrophages, polymorphonuclear leukocytes, and eosinophils were also present in fairly large numbers. Plethora of microcirculatory and lymphatic vessels was noted. The muscular layer was relaxed, inflammatory infiltrate of varying severity was determined. The bottom of the ulcerative lesion had characteristic changes in the form of alternating layers of fibrinoid necrosis, granulation tissue, and fibrous tissue. The mucosa around extensive ulcerative lesions was ulcerated, with impaired histologic design, pronounced inflammatory infiltrate, and necrosis of crypts by more than 2/3.

In the SIRS + *L. delbrueckii D5* and SIRS + inactivated culture groups, extensive ulcerative lesions were also identified, but there were more pronounced signs of tissue regeneration, re-epithelialization at the borders of ulcerative lesions of up to 40%, predominance of the fibrinoid component, restoration of histologic design in the muscular layer and submucosa, and a lack of mucopurulent discharge. In the SIRS + inactivated culture group, the superficial layers of ulcerative lesions were characterized by formation of immature connective tissue in place of granulation tissue as well as by decreased inflammatory infiltrate. When the mucosa and submucosa were replaced with granulation tissue, signs of regeneration were noted. In the SIRS + *L. delbrueckii D5* group with similar changes in some samples, re-epithelialization by single-layered intestinal epithelium was detected at the borders of ulcerative lesions, indicating accelerated regeneration.

Morphofunctional characteristics of the isolated heart

The infarct size in the control group was 41% (38; 45), and in the SIRS group – 54% (52; 57), which was significantly greater ($p < 0.05$) compared to the controls. In the SIRS + *L. delbrueckii D5* group, the infarct size was 42% (36; 48), which was significantly smaller than in the SIRS group. In the SIRS + inactivated culture group, only a trend toward a decrease in infarct size was revealed compared to the SIRS group (47% (44; 58), $p = 0.6061$).

Table 2 presents a correlation between the values of the studied parameters and myocardial infarct size.

The figure shows significant differences between infarct size, TNF**α**, IL-1β, TGFβ, and Hp values in the groups.

Table 2

Spearman's correlation between plasma parameters (infarct size, TNFα, IL-1β, TGFβ, haptoglobin, and lactoferrin) in all the rats in the experiment, $p < 0.05$

Parameter	TNFα	IL-1β	TGFβ	Lactoferrin	Haptoglobin	Infarct size
TNF**α**, pg / ml	1.0000	0.4570*	0.2209	–0.0269	0.3077	0.4654*
IL-1β, pg / ml	0.4570*	1.0000	0.3944*	–0.3455*	0.4159*	0.5063*
T TGFβ, pg / ml	0.2209	0.3944*	1.0000	–0.0477	0.2638	0.3657*
Lactoferrin, pg / ml	–0.0269	–0.3455*	–0.0477	1.0000	–0.2164	–0.2102
Haptoglobin, pg / ml	0.3077	0.4159*	0.2638	–0.2164	1.0000	0.4244
Infarct size, %	0.4654*	0.5063*	0.3657*	–0.2102	0.4244*	1.0000

* $p < 0.05$ compared to the control group.

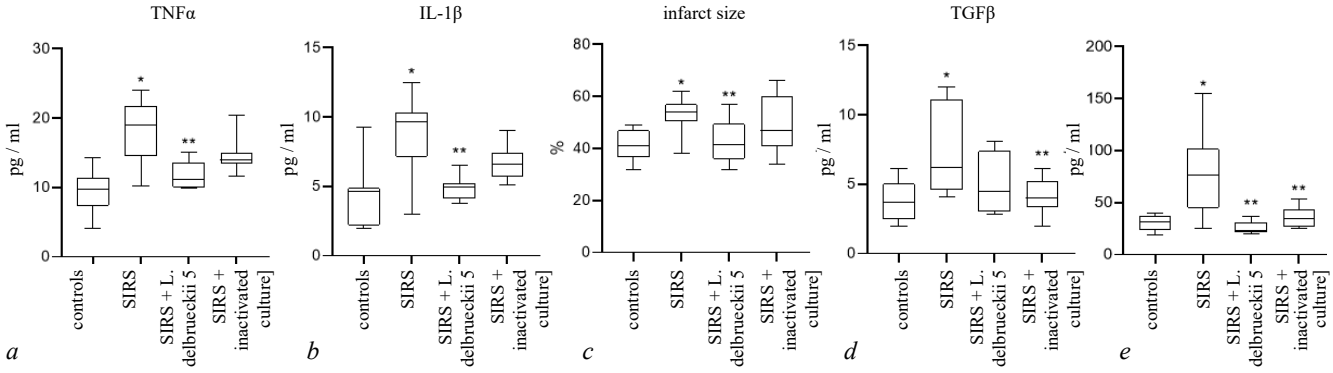

Figure. TNFα (*a*), IL-1β (*b*), infarct size (*c*), TGFβ (*d*), Hp (*e*) in the blood plasma: * $p < 0.05$ compared to the control group, ** $p < 0.05$ compared to the SIRS group

Due to limited possibilities of leukocyte differentiation into subpopulations and by maturity, it can be indirectly stated that neutrophils played the main role in the increase in the GRAN population in modeling SIRS. In the SIRS group, there was a decrease in the lymphocyte count and the MID population, which included monocytes, eosinophils, basophils, and blasts. In the SIRS + inactivated culture group, minimal MID and LYM values were noted. Administration of live and inactivated culture to the rats in the SIRS group resulted in normalization of the total leukocyte count and a decrease in the LYM, GRAN, and MID populations compared to SIRS.

In the SIRS group with a rise in TNFα, IL-1β, IL-6, and Hp in the blood and an increase in the GRAN population, a decrease in the LYM and MID populations was observed, indicating a causal role of SIRS in the production and migration of leukocytes. Regulation of lymphocyte proliferation and differentiation was affected by cytokines, intensively formed during exposure to various infectious and non-infectious antigens by lymphocytes and monocytes, in particular IL-2, IL-4, IL-6, and IL-7. In the SIRS group, degenerative and destructive changes in the intestinal wall with impairment of barrier function were noted, with a significant increase in the level of LPS in the blood, which triggered systemic inflammation. The main, but probably not the only reason for the decrease in LPS levels in the groups receiving probiotic and metabiotic therapy for SIRS may be competitive reduction of signs of opportunistic microbiota and / or a decrease in the permeability of

the intestinal wall to LPS. Each mechanism, either individually or in combination, may make a decisive contribution to endotoxinemia, since suppression of phagocytic and immunocompetent host cells is found only in individual strains.

Accumulating evidence in recent years suggests an important role of GMB in maintaining selective permeability of intestinal epithelium and preventing cellular barrier dysfunction leading to increased intestinal permeability. It provides nonspecific transport of proinflammatory antigens, microbes, and metabolites from the intestinal lumen to the mucosa and bloodstream, which in turn may be responsible for a variety of diseases, including inflammatory bowel disease and tumors, obesity, non-alcoholic fatty liver disease, depression, neurodegeneration, cardiovascular diseases, and type 1 diabetes mellitus [7]. Members of normal GMB apparently play a specialized role in maintaining the integrity of physical intestinal barrier, with their generation of microbial metabolites and bacterial components being the main way to achieve this goal [8].

For example, butyrate produced through bacterial fermentation of dietary fiber enhances the expression of tight junction (TJ) proteins – crucial multiprotein complexes that regulate the permeability of the intestinal barrier [9]. Microbial components, such as lipopeptides, enhance TJ function by stabilizing the levels of occludin-1 (ZO-1) and claudin by activating protein kinase C through interaction with Toll-like receptors (TLRs) on intestinal epithelial cells [10].

In this regard, a number of *in vitro* [11–15] and *in vivo* studies, including clinical trials [16, 17] on the effect of probiotic bacterial strains of the *Lactobacillus, Lactiplantibacillus, Limosilactobacillus, Bifidobacterium*, and *Streptococcus* genera on intestinal barrier function have been conducted, with promising results. Probiotic supplementation was found to markedly improve intestinal barrier function, as measured by transepithelial electrical resistance (TEER) and serum zonulin and LPS levels. Besides, it normalizes increased intestinal permeability and reduces the content of proinflammatory cytokines, including C-reactive protein (CRP), TNFα, and IL-6 in unfavorable conditions (bacterial infections, oxidative stress, high-fat diet, alcohol, chronic allergens, dysbiosis), in particular by enhancing the function of TJ in intestinal epithelial cells associated with increased synthesis of occludins [18, 19].

It should be noted that complex probiotics demonstrate higher efficacy in maintaining intestinal barrier function and preventing increased intestinal permeability compared to single strains. Thus, probiotics may play a crucial role in the treatment of autoimmune and metabolic diseases by improving intestinal barrier function. However, an exhaustive evaluation of probiotics regulating intestinal barrier function in various diseases is still lacking.

A significant inverse relationship of Lf values and a direct relationship of Hp values with IL-1β were found in the SIRS + *L. delbrueckii D5* group at high Lf concentration. Normal values of IL-1β and Hp with normal infarct size were observed, which may indicate that the live strain had specific properties compared the inactivated culture.

In this study, the administration of live and inactivated culture equally affected most of the studied parameters in the blood, indicating molecular sufficiency of pro- and metabiotic in this case. It is obvious that when the animals received live bacteria, the production of Lf increased significantly, while the production of Hp and proinflammatory cytokines decreased to control values. These trends were confirmed by significant differences between infarct size in the group of animals receiving live bacteria. It is likely that live lyophilized lactobacilli that became metabolically active had time to modulate immune responses, bringing them to control values. The obtained data indicate the expediency of probiotic therapy with live microorganisms in acute cardiac pathologies.

CONCLUSION

Lf and Hp, as acute phase proteins, showed different responses during pro- and metabiotic modulation of SIRS. Blood Hp levels were directly associated with LPS values, while Lf values were inversely correlated with the levels of the studied cytokines and myocardial infarct size.

In this study, synergistic changes in TNFα, IL-1β, IL-6, and Lf values across the groups are worth noting, with the maximum increase in the SIRS group, the intermediate increase in the SIRS + inactivated culture group, and the minimum increase in the SIRS + *L. delbrueckii D5* group. Taking into account the association of myocardial infarct size with an increase in the values of the studied parameters in the SIRS and SIRS + inactivated culture groups and a decrease in these parameters in the control and SIRS + *L. delbrueckii D5* groups, we can assume the presence of specific properties of the live probiotic culture and the corresponding mechanism.

The question remains open whether a combination of increased TNFα, IL-1β, IL-6, and Lf confirms decreased myocardial resistance to IRI. If the correlation between these markers and myocardial morphofunctional indices is established, targeted exclusion of one of the factors in the safest way is possible. Inactivated culture of *Lactobacillus delbrueckii D5* has a pronounced anti-inflammatory effect without increasing resistance to IRI, in contrast to the lyophilized strain, which requires further studies.

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