

## Beneficial effects of probiotic supplementation on glucose and triglycerides in a mouse model of metabolic syndrome

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### ABSTRACT

The present study aimed to examine the effect of *Lactobacillus rhamnosus* Rosell 11 and *Lactobacillus helveticus* Rosell 52 on glucose (blood level and tolerance), lipids (cholesterol and triglycerides), transaminases (AST and ALT), ALP, urea, and creatinine, along with body weight, food intake, liquid consumption, and gross pathology in a mouse model of metabolic syndrome. Male C57BL/6J mice were fed a high-fat high-sucrose diet and treated by oral gavage with a probiotic mixture in three different concentrations ( $10^7$ ,  $10^8$ , and  $10^9$  CFU/mL) once daily for 2 months. Probiotic supplementation, particularly at a concentration of  $10^9$  CFU, significantly decreased blood glucose and serum triglyceride levels, improved glucose tolerance, and promoted body weight loss in mice fed a high-fat high-sucrose diet. According to the obtained results, probiotic supplementation is useful for controlling glucose and triglyceride levels and could be used as an adjunctive therapeutic approach in patients with metabolic syndrome.

### 1. Introduction

Metabolic syndrome (MS) is a complex condition that includes dyslipidemia (elevated triglycerides and apolipoprotein B-containing lipoproteins such as low-density lipoproteins (LDL), and decreased high-density lipoproteins (HDL)), dysregulated glucose homeostasis, affected liver and kidney function, the elevation of arterial blood pressure, as well as overweight, abdominal obesity and/or insulin resistance (Rochlani, Pothineni, Kovelamudi, & Mehta, 2017). This condition increases the risk of heart disease, stroke, and type 2 diabetes. There is evidence to support an aggressive approach to the identification and treatment of people, not only those with hyperglycemia but also those with associated risk factors for cardiovascular disease to significantly reduce morbidity and mortality (Diabetes Canada, 2018; Saklayen, 2018). MS results from an energy imbalance favoring fat accumulation in different tissues. The molecular alterations implicated in this condition include impaired or reduced mitochondrial oxidative capacity and dysregulated cellular redox state; altered insulin signaling, resulting in impaired glucose transport, and dysregulated lipolysis, all of which turn

into altered lipid and carbohydrate metabolism (James, Collins, Logan, & Murphy, 2012; McCall, 2019).

The interactions between probiotics and metabolic diseases as well as the underlying mechanisms remain unclear. According to the definition of the Food and Agriculture Organization–World Health Organization (FAO-WHO), probiotics are defined as live microorganisms that, when taken into the body in appropriate numbers, have a beneficial effect on the health of the host (Hill et al., 2014).

In contemporary medicine, to prevent as well as reduce risk factors for MS, alternative strategies with the application of probiotics with proven effects are being considered. Although the results of studies with *Lactobacillus rhamnosus* GG (LGG), as the most extensively studied probiotic strain, showed that this strain has an antihyperglycemic effect on several rodent models, the basic mechanism of action of these probiotic bacteria has not yet been elucidated (Papizadeh, Nahrevanian, Rohani, Hosseini, & Shojaosadati, 2016). As a result of the administration of LGG probiotics in the treated group of mice, glucose tolerance was significantly increased. The results show that the antidiabetic effect of LGG in db/db mice is associated with alleviated endoplasmic reticulum stress

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and suppressed macrophage activation, resulting in increased insulin sensitivity. These findings point to the therapeutic potential of probiotics for the prevention and treatment of type 2 diabetes (Park, Kim, & Hyun, 2015). By influencing biochemical markers of the liver and kidney, probiotics are also a promising tool in the prevention and treatment of their impaired function (Sharma, Garg, & Aggarwal, 2013; Fagundes, Soder, Grokoski, Benetti, & Mendes, 2018). Finally, they exert overweight reduction and anti-inflammatory activity contributing prevention of dysmetabolic complications (Ferrarese, Ceresola, Preti, & Canducci, 2018).

Clinical trials have been conducted in Indonesia to examine the effect of the mixture of two strains *L. helveticus* Rosell-52 and *L. rhamnosus* Rosell-11 on the level of lymphocytes. The results of these studies have shown a significantly increased number of lymphocytes in the group receiving probiotics at a concentration of  $10^8$  CFU/day (Wahyuningsih, Darmono, & Margawati, 2014). *L. rhamnosus* has been officially reclassified to *Lactocaseibacillus rhamnosus* as of April 2020, so the full strain name may also be referred to as *Lactocaseibacillus rhamnosus* Rosell-11 (Zheng et al., 2020). In general, it is known to be the best-documented strain conducted in approximately 800 studies and 250 clinical trials (Probiotic database, 2022). Several studies suggested that strains of *L. helveticus* can affect several different aspects of the host's physiology. *In vivo* studies in murine models showed that *L. helveticus* could prevent gastrointestinal infections, enhance protection against pathogens, modulate host immune responses, and affect the composition of the intestinal microbiota (Taverniti & Guglielmetti, 2012).

As probiotics are now well recognized as powerful dietary ingredients with multiple health-promoting functions, along with their ability to fight specific diseases, they are currently the main focus of attention worldwide to be explored as potential biotherapeutics in treating several metabolic disorders (Dong, Xu, Chen, & Bhochohibhoya, 2019). Therefore, this research aimed to examine the potential impact of this *Lactobacillus* species on mice fed a high-fat high-sucrose (HFHS) diet, primarily on glucose (blood level and tolerance), lipids (cholesterol and triglycerides), transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)), alkaline phosphatase (ALP), urea, and creatinine, as well as body weight, food intake, liquid consumption, and gross pathology. The findings of this study should reveal the ability of a mixture of two probiotic strains (*L. rhamnosus* Rosell-11 and *L. helveticus* Rosell-52) to modulate the main biochemical markers of MS and indicate the possible benefits of using this probiotic supplement in patients with MS.

## 2. Materials and methods

### 2.1. *In vitro* testing

#### 2.1.1. Probiotic samples

Nine packages of Probiodrops® (commercial dietary product, Hemofarm, Serbia) from the same batch production (lot number) were provided (one for quality control and eight for treatment), and each of them consisted of a bag with lyophilized powder of *L. rhamnosus* Rosell-11 and *L. helveticus* Rosell-52 weighing 1.4 g, a dark glass bottle with 10 mL of vegetable oil, and a glass dropper (pipette). The product has been reconstituted according to the manufacturer's instructions.

#### 2.1.2. Sample preparation

For sample preparation of lactobacilli suspensions, the content of the bag of Probiodrops® was poured into the bottle with vegetable oil, and then the bottle was closed with the supplied dropper. After that, the bottle was shaken vigorously to obtain a uniform suspension. For experimental work, a stock suspension was prepared by reconstitution of the lyophilisate in vegetable oil and stored at 2–8 °C for 7 days. Every day, serial dilutions (ten-fold) were made from the stock suspension by adding the vegetable oil and the number of live lactobacilli was determined by the indirect method (Taylor, 1962).

### 2.1.3. Medium preparation

Semi-selective de Man, Rogosa, and Sharpe agar (MRS agar) is a medium for the cultivation and enumeration of *Lactobacillus* spp. Commercial MRS agar (Torlak, Belgrade, Serbia) was rehydrated in distilled water according to the manufacturer's instructions, and 1 M HCl was used to adjust the pH of the medium to 6.2. The agar media were sterilized by autoclaving at 121 °C for 15 min., cooled at 47 °C, and poured into sterile Petri dishes of 90 mm diameter (12 mL/plate), left to solidify, and stored at 4 °C until use.

### 2.1.4. Enumeration of viable lactobacilli cells

The test was done according to SRPS ISO 7889:2011 standard (MRS agar, pH 6.2 was used for growth and enumeration of the number of living lactobacilli cells). Suitable decimal dilutions (volume of 0.1 mL) of the bacterial suspension were plated by pour plate technique using MRS agar; then, dilutions were incubated at 37 °C for 48 h under anaerobic conditions using an anaerobic jar (Gas Pak Anaerobic Systems, Biomerieux) and colonies were counted. The results are reported as Colony Forming Units (CFU)/mL of bacteria. The CFU/mL was calculated using the formula: CFU/mL = number of colonies (N)/dilution factor (R) × volume of sample plated (V).

The quality control was performed 10 days before starting the treatment by determination of the total number of viable lactobacilli related to the declared number and simulation of lactobacilli stability during 7-day storing of the reconstituted product at 2–8 °C. The test of lactobacilli stability was performed by determination of the total number of viable lactobacilli immediately ( $12.2 \times 10^9$  CFU) and 7 days ( $11.6 \times 10^9$  CFU) after opening and reconstitution of the product. Since no significant differences were observed in this test, one vial/reconstituted suspension was used for 7 days.

## 2.2. *In vivo* testing

### 2.2.1. Ethics statement

The study was performed according to the regulations and standards of the national (Serbian) Law on the Experimental Animals Treatment and European Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes).

### 2.2.2. Animals

Eight-week-old male C57BL/6J mice, average weight 18–22 g, were obtained from Vivarium, Galenika a.d. (Belgrade, Serbia). The mice were housed in standard cages under environmentally controlled conditions: temperature of  $22 \pm 2$  °C, relative humidity of  $60 \pm 5\%$  and a 12/12 h light/dark cycle, and given free access to food and water throughout the experiment, unless indicated otherwise. Before commencement of the study, all mice were quarantined for 7 days and evaluated for body weight gain and any gross signs of disease or injury.

### 2.2.3. Diets

After 7 days of acclimatization, mice were randomly divided into five groups ( $n = 8$  per group): three experimental, one positive (HFHS), and one negative (C) control group. An HFHS group had continuous access to a dish with saturated fat (a high-fat diet, the fat was mixed and added into a pellet) and a bottle of 20% sucrose solution (sucrose mixed from commercial-grade sugar and tap water), while a negative control group was on a standard diet and tap water which were available to animals *ad libitum* throughout the experiment. Three experimental groups were offered continuous access to the dishes with saturated fat and bottles of 20% sucrose solution. The probiotic mixtures in different concentrations ( $10^7$ ,  $10^8$ , and  $10^9$  CFU/mL) were given to them in equal volumes (0.5 mL) by oral gavage once daily for 2 months and were accordingly designated as HFHS +  $10^7$  CFU, HFHS +  $10^8$  CFU, and HFHS +  $10^9$  CFU group, respectively. The vial with the prepared probiotic mixture was shaken immediately before each use.

During the 2 months of the experiment, the high-fat diet was changed every day to prevent the oxidation of fat to produce odor which affected the mice to eat. All sucrose bottles were cleaned and refilled daily. The activity, behavior, and general health of the mice were monitored daily, while the food intake, liquid (20% sucrose solution or water) consumption, and body weight of the mice were recorded weekly. At the end of the experiment, all mice were removed from the diet for 12 h and then were anesthetized and sacrificed. All efforts have been made to minimize the number of animals used and their suffering.

#### 2.2.4. Monitoring blood glucose level

The blood glucose level was monitored monthly throughout the experiment period. Multiple serial blood glucose level determinations in individual mice were performed on small blood samples (~20 µL) collected from the tail vein on the 30th and 60th day after the start of the experiment. Glucose concentration was measured on a GlucoSure Plus apparatus according to the instruction of the manufacturer (Prizma, Kragujevac, Serbia). Results were expressed in mmol/L.

#### 2.2.5. Oral glucose tolerance test

Oral Glucose Tolerance Test (OGTT) was performed in overnight-fasted mice by oral administration of glucose solution (2 g glucose/kg body weight) at the end of the last week of the diet. A blood sample was collected from the tail vein (~20 µL). Glucose concentration was determined prior and 30, 60, 90, and 120 min after glucose administration. Glucose concentration was measured as described previously above (see section 2.2.3.).

#### 2.2.6. Serum biochemical analyses

Blood samples were collected by cardiac puncture in sterile without anticoagulant tubes for biochemical tests. They were centrifuged at 5000 rpm for 10 min and the serum was separated.

Biochemical parameters including cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, and creatinine were determined in certified biochemical laboratory Biomedica (Belgrade, Serbia), by automatic Architect CI 8200 integrated serum-plasma analyzer (Abbot Laboratories, Illinois, USA) according to the manufacturer instruction.

#### 2.2.7. Gross pathology observation

At the end of the experiment, after a cardiac puncture, all mice were sacrificed by cervical dislocation and a complete necropsy (external and internal) was performed on each mouse to identify any gross abnormalities. The external examination included the appearance of fur, skin, eyes, ears, muzzle, mouth, tongue, teeth, anal and genital openings, limbs, and joints, as well as checking the existence of growths. The internal examination included the esophagus, stomach, small and large intestines, liver, spleen, kidneys, lungs, heart, large lymph glands (parotid, axillary, inguinal), muscles, and subcutaneous tissue. Organs and tissues were examined from the outside and then cut in more places and several layers. The criteria of the gross pathological examination were based on the weight, position, shape, size, color, and consistency of the organs.

### 2.3. Statistical analysis

All experimental data were displayed as mean ± standard error of the mean (SEM). Analysis of the data was carried out using SPSS 22.0 software (IBM, New York, USA). The values of p for serum cholesterol, triglycerides, AST, ALT, ALP, urea, and creatinine were determined using one-way analysis of variance (ANOVA) and for blood glucose, glucose tolerance, and body weight using two-way ANOVA with repeated measures, followed by a *post hoc* LSD test. P values < 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Colony morphology and enumeration of lactobacilli on selective MRS agar media

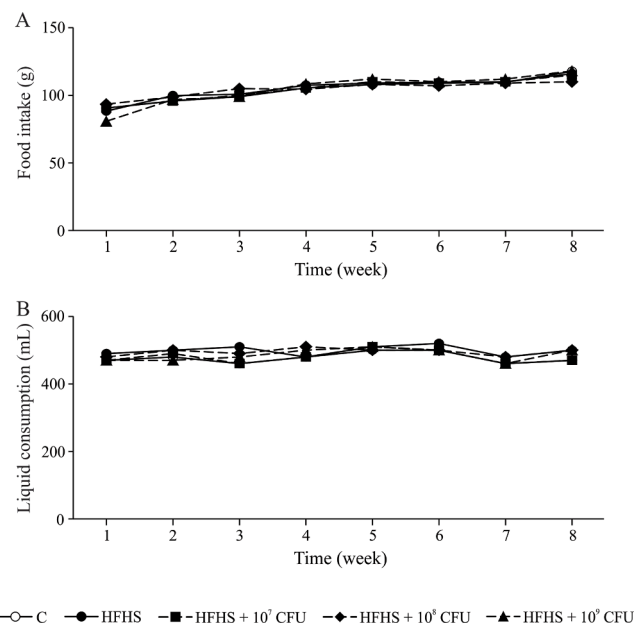
The tested lactobacilli were subjected to growth on selective MRS agar media at pH 6.2 under anaerobic conditions and produced a round shape with an external ring, off-white to cream color, and shiny colonies that were similar to the reference *Lactobacillus* spp. grown on MRS agar media. Colonies were counted at the end of incubation. The number of viable lactobacilli was  $12.2 \times 10^9$  CFU.

### 3.2. Effect of probiotic supplementation on food intake and liquid consumption in mice fed an HFHS diet

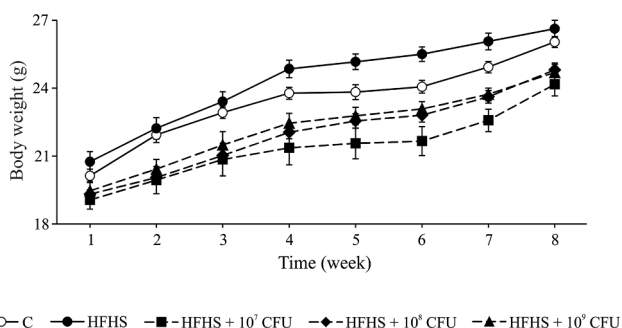
Mice of all experimental groups had similar food intake (Fig. 1A) and 20% sucrose solution or water consumption (Fig. 1B), with no observed difference between any probiotic-treated group (HFHS +  $10^7$  CFU, HFHS +  $10^8$  CFU, and HFHS +  $10^9$  CFU), as well as between them and HFHS and C group during the whole experimental period.

### 3.3. Effect of probiotic supplementation on body weight of mice fed an HFHS diet

Two-way ANOVA with repeated measures revealed a significant main effect of treatment ( $F = 9.160$ ,  $df = 4$ ,  $p < 0.001$ ), time ( $F = 644.969$ ,  $df = 7$ ,  $p < 0.001$ ), and treatment × time ( $F = 3.968$ ,  $df = 28$ ,  $p < 0.001$ ) for body weight. Monitoring of body weights within each group revealed that, as anticipated, mice fed an HFHS diet gained weight at a faster rate than those receiving the standard diet (Fig. 2). The mice fed an HFHS diet and treated with probiotics (HFHS +  $10^7$  CFU, HFHS +  $10^8$  CFU, and HFHS +  $10^9$  CFU groups) displayed a similar growth pattern, and body weights significantly less compared to the HFHS group and even more so to the C group (Table 1).



**Fig. 1.** Effect of probiotic supplementation on food intake and liquid (20% sucrose solution or water) consumption in mice fed an HFHS diet. C – negative control (standard diet and tap water); HFHS – positive control (high-fat diet and 20% sucrose solution); HFHS +  $10^7$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^7$  CFU/mL; HFHS +  $10^8$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^8$  CFU/mL; HFHS +  $10^9$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^9$  CFU/mL.



**Fig. 2.** Effect of probiotic supplementation on body weight of mice fed an HFHS diet. Results are presented as mean ± SEM (*n* = 8 mice per group). C – negative control (standard diet and tap water); HFHS – positive control (high-fat diet and 20% sucrose solution); HFHS + 10<sup>7</sup> CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of 10<sup>7</sup> CFU/mL; HFHS + 10<sup>8</sup> CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of 10<sup>8</sup> CFU/mL; HFHS + 10<sup>9</sup> CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of 10<sup>9</sup> CFU/mL. Between-group significant differences in body weight are presented in Table 1.

**Table 1**  
Between-group significant differences in body weight.

	Week	1	2	3	4	5	6	7	8
C vs.	HFHS					*	*		
	HFHS + 10 <sup>7</sup> CFU		**	**	***	***	***	***	**
	HFHS + 10 <sup>8</sup> CFU		**	**	**	*	*	*	*
	HFHS + 10 <sup>9</sup> CFU		*	*	*			*	*
HFHS vs.	HFHS + 10 <sup>7</sup> CFU	**	***	***	***	***	***	***	***
	HFHS + 10 <sup>8</sup> CFU	*	***	***	***	***	***	***	**
	HFHS + 10 <sup>9</sup> CFU	*	**	**	***	***	***	***	**
	CFU								

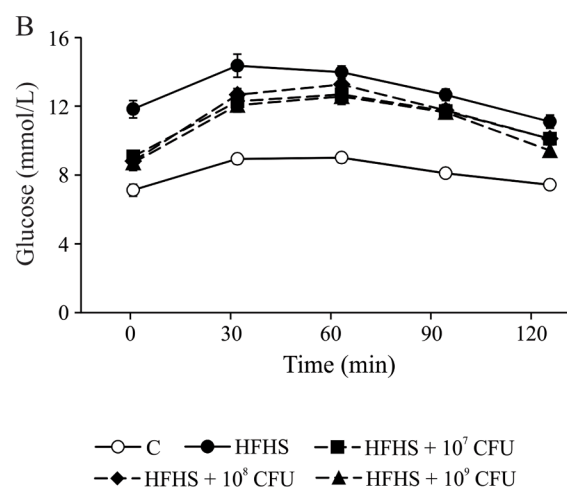
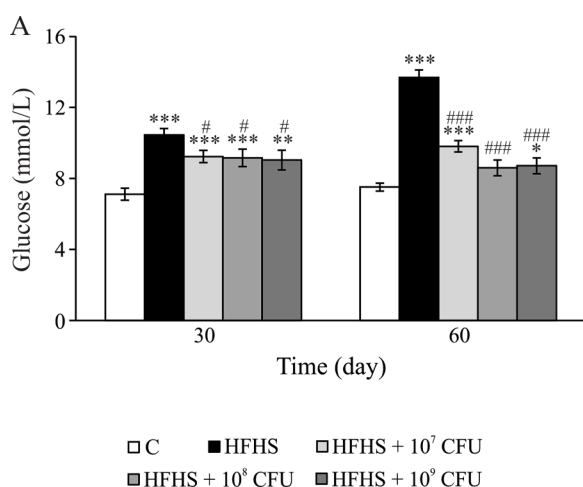
**3.4. Effect of probiotic supplementation on blood glucose level and glucose tolerance in mice fed an HFHS diet**

Analyzing blood glucose levels, two-way ANOVA with repeated measures revealed a significant main effect of treatment (*F* = 19.785, *df* = 4, *p* < 0.001), time (*F* = 43.454, *df* = 1, *p* < 0.001), and treatment × time (*F* = 45.117, *df* = 4, *p* < 0.001). The blood glucose level in the HFHS group was significantly higher compared to the C group on the 30th (by 48%) and even more (by 83%) on the 60th day after the start of the experiment (Fig. 3A). It has been noticed that probiotic supplementation, particularly at a concentration of 10<sup>9</sup> CFU, significantly reduced HFHS diet-induced increased blood glucose levels both on the 30th and 60th day. Observed decline in the blood glucose level was similar in all probiotic-treated groups on the 30th day and more pronounced in the HFHS + 10<sup>8</sup> CFU and HFHS + 10<sup>9</sup> CFU groups than in the HFHS + 10<sup>7</sup> CFU group on the 60th day, but still significantly increased compared to the C group.

Two-way ANOVA with repeated measures revealed a significant main effect of treatment (*F* = 35.897, *df* = 4, *p* < 0.001), time (*F* = 131.050, *df* = 4, *p* < 0.001), and treatment × time (*F* = 3.346, *df* = 16, *p* < 0.001) for data obtained in the OGTT test. As shown in Figure 3B, a significant increase in blood glucose levels was observed in the HFHS and all probiotic-treated groups (HFHS + 10<sup>7</sup> CFU, HFHS + 10<sup>8</sup> CFU, and HFHS + 10<sup>9</sup> CFU) compared to the C group during the whole period of monitoring, with an apparent peak at 30 min after oral administration of glucose. In mice fed an HFHS diet, probiotic supplementation, especially at a concentration of 10<sup>9</sup> CFU, improved glucose tolerance (Table 2). In all probiotic-treated groups, this effect was the most pronounced in the first 60 min after oral administration of glucose.

**Table 2**  
Between-group significant differences in glucose tolerance.

	Min	0	30	60	90	120
C vs.	HFHS		***	***	***	***
	HFHS + 10 <sup>7</sup> CFU		***	***	***	***
	HFHS + 10 <sup>8</sup> CFU		**	***	***	***
	HFHS + 10 <sup>9</sup> CFU		**	***	***	***
HFHS vs.	HFHS + 10 <sup>7</sup> CFU	***	***	*		
	HFHS + 10 <sup>8</sup> CFU	***	**			
	HFHS + 10 <sup>9</sup> CFU	***	***	**		**
	CFU					

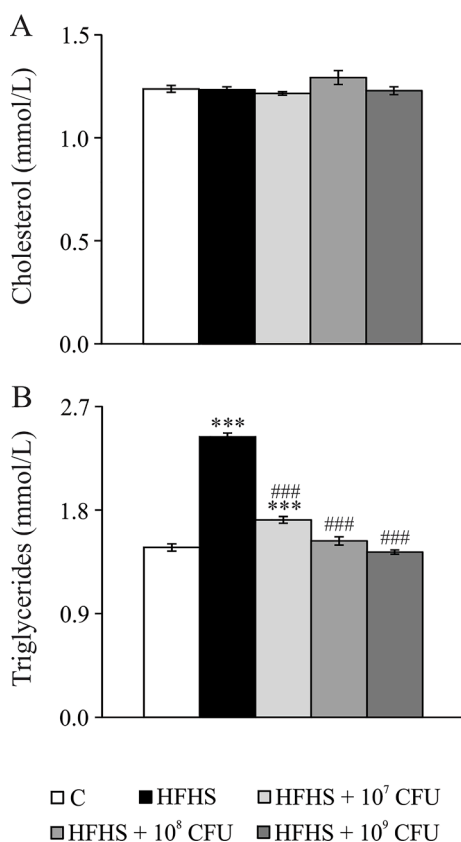


**Fig. 3.** Effect of probiotic supplementation on blood glucose level and glucose tolerance in mice fed an HFHS diet. Results are presented as mean ± SEM (*n* = 8 mice per group). C – negative control (standard diet and tap water); HFHS – positive control (high-fat diet and 20% sucrose solution); HFHS + 10<sup>7</sup> CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of 10<sup>7</sup> CFU/mL; HFHS + 10<sup>8</sup> CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of 10<sup>8</sup> CFU/mL; HFHS + 10<sup>9</sup> CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of 10<sup>9</sup> CFU/mL. \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 compared to the C group; #*p* < 0.05 and ###*p* < 0.001 compared to the HFHS group (LSD test). Between-group significant differences in glucose tolerance are presented in Table 2.

### 3.5. Effect of probiotic supplementation on serum lipids in mice fed an HFHS diet

The results of one-way ANOVA revealed no significant main effect of treatment on cholesterol level ( $F = 2.179$ ,  $df = 4$ ,  $p = 0.09$ ). Surprisingly, serum cholesterol level in the HFHS group after a two-month feeding period was not increased compared to the C group, and it was similar as in probiotic-treated groups (HFHS +  $10^7$  CFU, HFHS +  $10^8$  CFU, and HFHS +  $10^9$  CFU) (Fig. 4A).

In contrast to cholesterol, one-way ANOVA revealed a significant main effect of treatment on serum triglyceride level ( $F = 190.650$ ,  $df = 4$ ,  $p < 0.001$ ). As expected, the HFHS diet significantly increased (by 65%) serum triglyceride level after a two-month feeding period compared to standard diet feeding (Fig. 4B). Probiotic supplementation applied for the whole feeding period had a significant suppressive effect on HFHS diet-induced increase in serum triglyceride level, particularly at a concentration of  $10^9$  CFU, reducing it to the level as in the C group. Comparing serum triglyceride levels between probiotic-treated groups, they were significantly lower in HFHS +  $10^8$  CFU and HFHS +  $10^9$  CFU groups compared to the HFHS +  $10^7$  CFU group ( $p < 0.001$ ), as well as in HFHS +  $10^9$  CFU group compared to the HFHS +  $10^8$  CFU group ( $p < 0.05$ ).



**Fig. 4.** Effect of probiotic supplementation on serum lipids in mice fed an HFHS diet. Results are presented as mean  $\pm$  SEM ( $n = 8$  mice per group). C – negative control (standard diet and tap water); HFHS – positive control (high-fat diet and 20% sucrose solution); HFHS +  $10^7$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^7$  CFU/mL; HFHS +  $10^8$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^8$  CFU/mL; HFHS +  $10^9$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^9$  CFU/mL. \*\*\* $p < 0.001$  compared to the C group; ### $p < 0.001$  compared to the HFHS group (LSD test).

### 3.6. Effect of probiotic supplementation on biochemical markers of liver and kidney function in mice fed an HFHS diet

One-way ANOVA revealed no significant effect of treatment on serum AST ( $F = 0.365$ ,  $df = 4$ ,  $p = 0.83$ ), ALT ( $F = 0.932$ ,  $df = 4$ ,  $p = 0.46$ ), ALP ( $F = 2.229$ ,  $df = 4$ ,  $p = 0.09$ ), urea ( $F = 0.695$ ,  $df = 4$ ,  $p = 0.60$ ), and creatinine ( $F = 0.647$ ,  $df = 4$ ,  $p = 0.63$ ). The HFHS diet *per se* did not affect analyzed biochemical parameters, and their levels were similar to those observed in the C group and probiotic-treated groups (HFHS +  $10^7$  CFU, HFHS +  $10^8$  CFU, and HFHS +  $10^9$  CFU) (Table 3).

### 3.7. Gross pathology observation in mice fed an HFHS diet

No changes in appearance and relative weights of the vital organs and tissues were observed in probiotic-treated groups (HFHS +  $10^7$  CFU, HFHS +  $10^8$  CFU, and HFHS +  $10^9$  CFU) and HFHS group compared with the C group at necropsy. Since no changes were found, further pathohistological examinations of the organs and tissues were not performed.

## 4. Discussion

In the present study, carefully balanced two strains of probiotic bacteria (*L. rhamnosus* Rosell-11 and *L. helveticus* Rosell-52) were chosen to investigate their effects on the HFHS diet-induced MS. The main findings are (1) dysregulation of glucose homeostasis, dyslipidemia, and increased body weight in an established mouse model of MS, and (2) beneficial effects of probiotic supplementation applied for the whole feeding period, particularly at a concentration of  $10^9$  CFU, observed as a significantly reduced blood glucose and serum triglyceride levels, improved glucose tolerance, as well as promoted body weight loss in mice fed an HFHS diet.

Feeding mice with an HFHS diet was used as a model for MS (Schreyer, Wilson, & LeBoeuf, 1998). Glucose and lipid (cholesterol and triglycerides) levels are classical biochemical markers of metabolic function elevated in these animals (da Costa et al., 2019). OGTT is used in clinical practice and research to identify individuals with normal or impaired glucose tolerance and patients with type 2 diabetes. Impairment of glucose tolerance indicates problems with the maintenance of glucose homeostasis. Type 2 diabetes is characterized by fasting hyperglycemia resulting from the inadequate secretion of the glucose-lowering hormone insulin and/or insulin resistance. Primarily driven by overnutrition and sedentary lifestyles, type 2 diabetes is a major global health problem in both developing and developed countries

**Table 3**

Effect of probiotic supplementation on biochemical markers of liver and kidney function in mice fed an HFHS diet.

	AST (U/L)	ALT (U/L)	ALP (U/L)	Urea (mmol/L)	Creatinine ( $\mu$ mol/L)
C	215.51 $\pm$ 1.36	23.38 $\pm$ 0.78	122.12 $\pm$ 2.29	7.66 $\pm$ 0.09	36.02 $\pm$ 0.63
HFHS	218.97 $\pm$ 2.72	24.02 $\pm$ 0.90	123.47 $\pm$ 2.70	8.31 $\pm$ 0.31	35.40 $\pm$ 1.07
HFHS + $10^7$ CFU	216.80 $\pm$ 2.58	26.59 $\pm$ 1.83	122.49 $\pm$ 2.13	8.24 $\pm$ 0.40	35.75 $\pm$ 1.28
HFHS + $10^8$ CFU	218.75 $\pm$ 3.53	24.37 $\pm$ 1.38	129.13 $\pm$ 2.57	8.04 $\pm$ 0.43	35.79 $\pm$ 1.19
HFHS + $10^9$ CFU	215.97 $\pm$ 2.55	24.21 $\pm$ 1.16	118.73 $\pm$ 2.86	7.75 $\pm$ 0.39	33.91 $\pm$ 1.12

Values are expressed as mean  $\pm$  SEM ( $n = 8$  mice per group).

C – negative control (standard diet and tap water); HFHS – positive control (high-fat diet and 20% sucrose solution); HFHS +  $10^7$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^7$  CFU/mL; HFHS +  $10^8$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^8$  CFU/mL; HFHS +  $10^9$  CFU – high-fat diet, 20% sucrose solution, and probiotics at a concentration of  $10^9$  CFU/mL.

(Chatterjee, Khunti, & Davies, 2017). Hypercholesterolaemia and hypertriglyceridemia are major risk factors for cardiovascular disease and it has been found that some probiotic bacteria possess cholesterol- and triglyceride-lowering capabilities (Kumar et al., 2012; Choi et al., 2016). MS is not necessarily associated with an increase in both cholesterol and triglyceride levels and can be classified according to lipid alterations into the following groups: mixed dyslipidemia (altered triglycerides and HDL cholesterol), hypoalphalipoproteinemia: (normal triglycerides but low HDL cholesterol), hypertriglyceridemia (elevated triglycerides and normal HDL cholesterol), and without dyslipidemia (normal triglycerides and HDL cholesterol) (Pedroza-Tobias, Trejo-Valdivia, Sanchez-Romero, & Barquera, 2014). MS, as a complex condition, also includes impaired liver and kidney function (Rochlani et al., 2017), thus their biomarkers could be used as clinical predictors of risk for this disease. AST, ALT, and ALP are important enzymes present in hepatocytes and usually help to detect chronic liver diseases by monitoring their concentrations, while urea and creatinine are sensible biomarkers of kidney alterations, especially when they are increased concomitantly.

The HFHS diet used in this study caused an increase in blood glucose and serum triglyceride levels, as well as impaired glucose tolerance. Probiotic supplementation applied for the whole feeding period alleviated these HFHS diet-induced changes, with the most pronounced effects observed at a concentration of  $10^9$  CFU. An interesting observation is that in mice fed an HFHS diet and treated with probiotics, blood glucose level and glucose tolerance were significantly improved, but still higher, while serum triglyceride level was completely restored to the level observed in mice fed a standard diet. These results are consistent with the literature data (Taranto, Medici, Perdigon, Ruiz Holgado, & Valdez, 1998; Li et al., 2016; Sohag, Paul, Al-Bari, Wahed, & Khan, 2019; Yan et al., 2020) and suggest that probiotic supplementation in conditions of the metabolic disorder may be used to control blood glucose and improve lipid metabolism by decreasing triglyceride concentration. The potential mechanism(s) underlying the observed changes in blood glucose level and glucose tolerance include the promotion of the release of glucagon-like peptide-1 and peptide YY resulting in increased insulin and decreased glucagon secretion, and suppressed appetite (Kim, Keogh, & Clifton, 2018), while changes in triglycerides could be connected with upregulation of apolipoprotein A-V (ApoA-V) playing an important role in determining plasma triglyceride levels, peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) regulating the oxidation and transport of fatty acids, and farnesoid X receptor (FXR) functioning as the bile acid receptor (Choi et al., 2016). No less important effect was significant probiotics-induced body weight loss in mice exposed to an HFHS diet, despite the lack of differences in food intake and liquid consumption between them and mice given only an HFHS or standard diet. Body weight, food intake, and liquid consumption were followed as an indicator of general toxicity. Stable food intake and liquid consumption are evidence that the applied probiotics did not exhibit significant systemic toxicity, except for body weight loss. In support of this are results of the assessment of organ hypertrophy, as a first-hand indication of the toxicity of the tested substance, showing no differences amongst standard diet, HFHS diet, and HFHS diet and probiotic supplementation groups. Increased body weight is one of the signs of MS and thus its reduction is an essential preventive and management strategy (Rochlani et al., 2017).

Recently reported data suggest that overall susceptibility to infectious agents may be reduced by probiotic supplementation. It is believed that probiotics can modulate the human immune system and its inflammatory responses by affecting the intestinal microbiota. In recent years, a range of studies in animal models has reported beneficial effects of probiotics on the host's health, such as improvement of the immune system (Palomar, Bru, Maldonado Galdeano, & Perdigon, 2017). The probiotic strain *L. fermentum* 296 attenuates cardiometabolic disorders (Cavalcante et al., 2019), as well as type 2 diabetes (Wang et al., 2020) in high-fat diet-treated mice. It is believed that the improvement of

glycemic and lipid parameters by probiotic strains is primarily associated with the restoration of intestinal barrier function by colonization.

## 5. Conclusion

Supplementation of mice, fed an HFHS diet, with two strains of probiotic bacteria (*L. rhamnosus* Rosell 11 and *L. helveticus* Rosell 52) significantly reduced blood glucose and serum triglyceride levels, improved glucose tolerance, as well as promoted body weight loss. These findings show that these two strains, especially at a concentration of  $10^9$  CFU, reduce the risk and complication of glucose and lipid metabolism-associated disorders. They could be good candidates for further research in the prevention of MS and used as an adjunctive therapeutic approach in patients with MS, but there is a need for further study of the mechanisms underlying observed metabolic benefits.

## Ethics statement

The study was performed according to the regulations and standards of the national (Serbian) Law on the Experimental Animals Treatment and European Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes).

## Data availability statement

All data generated or analyzed during this study are included in this published article.

## CRediT authorship contribution statement

**Gordana Zavišić:** Conceptualization, Writing – original draft, Formal analysis. **Slavica Ristić:** Conceptualization, Formal analysis, Resources. **Milena Rikalović:** Writing – review & editing. **Branka Petković:** Writing – review & editing, Formal analysis. **Drina Janković:** Writing – original draft. **Aleksandar Vukadinović:** Investigation, Methodology. **Saša Petričević:** Investigation, Methodology.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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