


RESEARCH

# Comparative study of probiotic effects of *Lactobacillus* and *Bifidobacteria* strains on cholesterol levels, liver morphology and the gut microbiota in obese mice

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Received: 2 July 2017 / Accepted: 18 August 2017 / Published online: 10 October 2017  
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## Abstract

**Background** Microbiome-modulating interventions are promising for treatment and prevention of metabolic syndrome. The number of probiotic strains demonstrated ability to decrease cholesterol level in vivo, however, it was poorly confirmed in a clinical setting. The aim was to study the effects of *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB separately and in various compositions on the level of serum cholesterol, gut microbiota contents and liver morphology on a high-calorie-induced obesity model in BALB/c mice.

**Materials and methods** We used for the study female BALB/c mice 6–8 weeks old (18–24 g). Experimental animals were fed by a fat-enriched diet (FED), and 8 experimental groups were formed (12 mice in each group) to test strains of probiotic bacteria *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis*

VKB and compositions. We used ultrasound for in vivo assessment of the liver and visceral (mesenteric) fat size. In the blood serum of the obese mice, the level of cholesterol was estimated. The liver morphology and gut microbiota of obese mice were studied.

**Results** We revealed that after treatment with all of the studied probiotic bacteria and compositions of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280, the weight of obese mice decreased, and cholesterol and its fraction levels in serum were reduced. The size of the liver slightly decreased after treatment with *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB or probiotic compositions; we observed reduction of the mesenteric fat size after injection of all these probiotic bacteria (separately) and probiotic compositions. We defined the strain-dependent effects on serum lipid profiles, liver morphology and the gut microbiota. The *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition effectively recovered the liver morphological structure of obese mice. The number of *Lactobacillus* spp., *Bifidobacterium* spp. and coliform bacteria increased, the number of staphylococci and streptococci reduced, and the number of microscopic fungi significantly decreased in the gut of obese mice after treatment with *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* (separately) or their compositions.

**Conclusion** *L. casei* IMV B-7280 (separately) and a composition of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 are effective at decreasing the weight of obese mice, decreasing cholesterol level, restoring the liver morphology and beneficially modulating the gut microbiome in high-calorie-induced obesity.

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**Keywords** Predictive preventive personalized medicine · Metabolic syndrome · Obesity · Lactobacillus ·

Bifidobacterium · Cholesterol · Liver · Gut microbiota ·  
 Mouse model: mesenteric fat · Ultrasound

## Overview

### Microbiota and metabolic syndrome: Evidence of probiotic interventions efficacy for hypercholesterolemia, liver disease and the gut microbiota in obesity

Metabolic syndrome (MetS) is a violation of metabolism that leads to the development of obesity, liver disease, hypertension, dyslipidemia, hyperglycemia and insulin resistance and still is a large global challenge [1–4]. MetS is associated with altered metabolism of lipids, carbohydrates, insulin, violations of antioxidant systems, and development of inflammatory reactions [5–7]. Obesity in adults and children is a global epidemic in many countries worldwide, is often associated with hyperglycemia, hypertriglyceridemia, dyslipidemia and hypertension and is considered as the main risk factor for cardiovascular diseases [7, 8]. The World Health Organization (WHO) has predicted cardiovascular diseases (CVD) to remain the leader in causes of death, and by 2030, will affect up to 23.6 million people worldwide [9].

The development/updating of a panel of biomarkers of MetS [4, 10] is needed for diagnosis and prediction of metabolic diseases, prevention and personalized treatment. The importance of the prognostic and diagnostic significance of total cholesterol and its fractions was extensively proved by experimental and clinical studies [11] to be a major risk factor for coronary heart disease. Up to date cholesterol management requires statin therapy in increasing the target level for a low-density lipoprotein (LDL) cholesterol level of 4.9 mmol/L in patients with atherosclerotic CVD [12].

Microecological violations in various organs are triggers for development of metabolic diseases and related pathological processes, based on altered lipid metabolism. In particular, a number of experimental and clinical studies revealed the link between cholesterol metabolism disorder and other substances in such metabolic diseases as obesity and changes in gut microbiota [13–16], which is manifested primarily in increasing the number of Gram-positive *Firmicutes* and a decrease in the number of Gram-negative *Bacteroidetes* [13, 14].

The key role of gut microbiota in the development of metabolic diseases has been previously confirmed in the obesity models of mice. Thus, colonization of sterile mice by cecal microbiota of mice with obesity resulted in greater weight gain and fat accumulation compared to ordinary mice (Turnbaugh, 2008) [17]. The human gut microbiome can be modified by diet alteration, potentially facilitating adaptation to new dietary habits [18]. Probiotic strains demonstrated effectiveness in cholesterol lowering under high-cholesterol diet [19], and

the role of cholesterol intake remain debatable vs. gut microbiota as a main trigger of obesity development and atherosclerosis. The mechanisms have been defined to explain the influence of gut microbiota on cholesterol metabolism [20–31]. The broad associations of liver function and development of nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) with gut microbiota was rigorously studied [32–34].

Microbiome-modulating interventions (mostly via probiotic strains) is a promising approach for treatment and prevention [35–42]. However, definitive evidence in many aspects is lacking for their use, and the legislative process is complex and not effective enough [43]. A number of probiotic strains have demonstrated the ability to decrease cholesterol levels in vivo in studies from the last decades [44–47]. Thus, recent in vivo studies showed that use of the probiotics and/or prebiotics can effectively reduce serum/plasma cholesterol levels, improving overall lipid profiles [47]; in particular *Lactobacilli* demonstrates a clear hypocholesterolemic effect in vivo [47–52]. The meta-analysis performed by Shimizu et al. showed that probiotic supplementation could be useful in the primary prevention of hypercholesterolemia and may lead to reductions in risk factors for cardiovascular disease. In addition, this meta-analysis showed that long-term (over 4 weeks) probiotic consumption resulted in a statistically significant decrease in cholesterol levels [53]. The meta-analysis of controlled short-term probiotic intervention studies (as many as 2000), excluding open-label studies, showed a decrease of total cholesterol and LDL cholesterol after fermented yoghurt product use. Long-term studies are still needed to confirm the sustained effects of probiotics on lipid profiles [54].

Different strains of *Lactobacillus* (LAB) affect not only the metabolism of cholesterol and triglycerides, but also normalize gut microbiota [23], beneficially modulate liver function [22], and decrease accumulation of fat [55, 56]. Clinical studies showed some decrease in the level of total cholesterol and LDL cholesterol after use of probiotics, including *Lactobacillus*, *Bifidobacterium*, *Pediococcus*, *Leuconostoc* and *Enterococcus* [57, 58]; however, very few confirmed its efficacy evidentially, likely due to bias imposed on the designs. On the other hand, other past studies and meta-analyses have also shown that probiotics and prebiotics had insignificant effects on lipid profiles, debating its hypocholesterolemic potential [53, 59–63]. Despite the ability for *L. acidophilus* to reduce cholesterol in vitro, no effect was seen in volunteers [63].

An important criterion for selection of probiotic strains for development of such drugs is their ability to normalize the metabolism of lipid spectrum components, mostly cholesterol. In various experimental models of metabolic diseases and in vitro, it was shown that some strains of *Lactic acid bacteria* (LAB) and *Bifidobacteria* had effective hypocholesterolemic activity associated with enzymatic degradation of bile acids, direct binding of cholesterol by cell walls of bacteria [20–25],

and changes in expression of several genes involved in lipid metabolism [20].

There are two challenges to achieve an evidence-based and personalized approach in the use of probiotics for MetS:

- the selection of the most effective relevant strain among existing strains; also challenging is the discovery of new strains for next-generation probiotics and development of live biotherapeutic products [64];
- the appropriate stratification of the host responder according to age, gender and phenotype, using dynamic monitoring of a set of translational biomarkers for early detection and prevention of MetS via nutritional modulation [65]. Specific high-risk groups such as infants, the elderly and the immuno-compromised [59] require highly individualized prescriptions of probiotics and/or prebiotics.

We speculate that immune-centered theory [66, 67], claiming that gut microbiota can influence immune function beyond the gut, would be crucially helpful for choosing appropriate probiotic bacteria in the personalized clinical setting [42, 68]. Therefore, the selected probiotics should improve lipid metabolism by reducing the level of total cholesterol and LDL cholesterol. It is important to consider carbohydrate metabolism, insulin, the development of inflammatory reactions and maintaining a long-term effect after diet and weight loss. and considering long-term diet programming in cases of metabolic diseases [69].

We previously found that the probiotic strains *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKL and *B. animalis* VKB (separately) and in various compositions had effective hypocholesterolemic activity in vitro [52]. These probiotic strains have a high level of ability to balance the immune response via induction of various cytokines and modifying gut microbiota (Lazarenko et al., 2017) [68], and were effective for monosodium glutamate-induced obesity [70]. Our recent data demonstrated strong correlations between bacteria wall elasticity with immune-modulatory properties that facilitate selection of the most effective probiotic drugs [71].

Thus, based on the data discussed above, here we hypothesize that:

- the strains demonstrating efficacy in vitro and having high immune modulatory activity, namely *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB, separately and in compositions should demonstrate beneficial effects on serum level of cholesterol, on the gut microbiota and liver morphology in obese mice;
- selecting strains for human applications is effective under appropriate assessment of reliable markers to evaluate obesity and MetS, including imaging data [42, 70, 72];

ultrasound of visceral (mesenteric) fat is representable, fast and most relevant for screening small animals like mice;

- the efficacy of selected strains should receive rigorous confirmation in the clinical trials if properly designed.

The aim was to study the effects of *L. acidophilus* IMV B-7279, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB separately and in various compositions on the level of cholesterol in the blood serum, on the gut microbiota contents and morphology of the liver in a high-calorie-induced ultrasound-assisted obesity model on BALB/c mice; and to overview the up-to-date evidence on using probiotics for MetS manifestations.

## Materials and methods

The research was conducted in compliance with the standards of the Convention on Bioethics of the Council of Europe's 'Europe Convention for the Protection of Vertebrate Animals' used for experimental and other scientific purposes' (1997), the general ethical principles of animal experiments, approved by the First National Congress on Bioethics Ukraine (September 2001) in compliance with the Law of Ukraine of 21.02.2006 № 3447-IV «On protection of animals from abuse», and with other international agreements and national legislation in this field. Animals were kept in a vivarium that was accredited in accordance with the 'standard rules on ordering, equipment and maintenance of experimental biological clinics (vivarium)'. Instruments used in research were under metrological control. The number of animals used was as minimal as necessary; in vivo ultrasound allowed minimization of animal use with the highest informativeness. No human subjects were involved in the study.

### Bacterial strain, media and growth conditions

*L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *L. casei* IMV B-7280, *B. animalis* VKL and *B. animalis* VKB allocated from associated culture during laboratory studies of the fermented biological material from the intestines of humans were used in our study. *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 and *L. casei* IMV B-7280 are deposited in the Ukrainian collection of microorganisms (D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine, Kyiv, Ukraine). The study was performed using bacteria lyophilized in a Cuddon freeze dryer FD1500 (New Zealand). Before each experiment, the viability of lyophilized strains of LAB and *Bifidobacteria* were tested by monitoring their growth on the Man–Rogosa–Sharpe (MRS) or bifidum-agar media, respectively, at 37 °C for 24–48 h.

## Animals, diets and design of experiment

Experimental studies were conducted on female BALB/c mice at the age of 6–8 weeks (18–24 g). During the experiment, animals were kept in standard vivarium conditions, in plastic cages in a separate room at a steady temperature (20–22 °C), they were provided with the full mixed feed and had free access to automatic water bowls.

The mice were fed a fat-enriched diet (FED) composed of 30% fats, 40% proteins and 30% carbohydrates during 21 days to model obesity. From the 22nd day, these mice received standard full mixed feed and *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *L. casei* IMV B-7280, *B. animalis* VKL, *B. animalis* VKB (each strain separately) or *B. animalis* VKB/*B. animalis* VKL (ratio 1:1) or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 (ratio 1:1:2) compositions. The probiotic bacteria were suspended in sterile phosphate-buffered saline (PBS). Mice received these probiotic bacteria and probiotic compositions in the dose of 500 µl and  $1 \times 10^6$  cells per animal, orally daily during 7 days.

Eight experimental groups were formed from BALB/c mice (12 mice in each group): 1) intact mice that received standard diet (control); 2) mice that received FED; 3) mice that received FED and *L. casei* IMV B-7280; 4) mice that received FED and *L. delbrueckii* subsp. *bulgaricus* IMV B-7281; 5) mice that received FED and *B. animalis* VKB; 6) mice that received FED and *B. animalis* VKL; 7) mice that received FED and composition *B. animalis* VKB/*B. animalis* VKL; 8) mice that received FED and composition *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280.

Blood samples were taken from the tail vein on the 7th, 15th and 21st days from the beginning of FED and on the 4th, 9th, 15th, 21st and 30th days from the beginning of probiotic strains injection. All liver tissue depots were removed on the 30th day, rinsed with PBS and weighed.

## Measuring body weight and ultrasonic studies of the liver and omentum

Body weight was measured once a week. Before sacrifice, mice were fasted for 12 h and anesthetized with diethyl ether.

## Ultrasound examination of mice

We performed ultrasonography (US) of mice using linear 5–12-MHz frequency probes of an ultrasound scanner (Philips/ATL HDI 5000, Netherlands). For screening and examination of the mice, we evaluated the reliable ultrasound parameters, as follows (Fig. 1):

- 1) for screening all mice involved in the study on the longitudinal (sagittal) ultrasound probe position, we measured the thickness of mesenteric fat (omentum, largest part of

visceral fat; the threshold was considered as 1.5 mm) and collected records of longitudinal and transverse organo-complex scans (in sagittal and transverse probe positions) and measured the largest longitudinal liver size (via a subcostal approach);

- 2) the additional parameters, which were accessible to evaluate in small animals (in few mice) of all groups – liver echogenicity, kidneys structure, spleen length, visceral vessels, muscle thickness at the midfemoral level.

## Cholesterol study

The concentration of free and ester cholesterol in the blood serum was determined according to the modified colorimetric method of Zlatkis-Zak with ferric chloride. The principle of this method is that free or ester cholesterol is oxidized by ferric chloride in the presence of acetic, sulfuric and phosphoric acids with the formation of unsaturated red-violet color products. Determination of the concentration of free and ester cholesterol was carried out according to the calibration chart on the spectrophotometer “MultiScan EX” with the optical wave length  $\lambda = 530$  nm. The concentration of cholesterol is expressed in mg/ml.

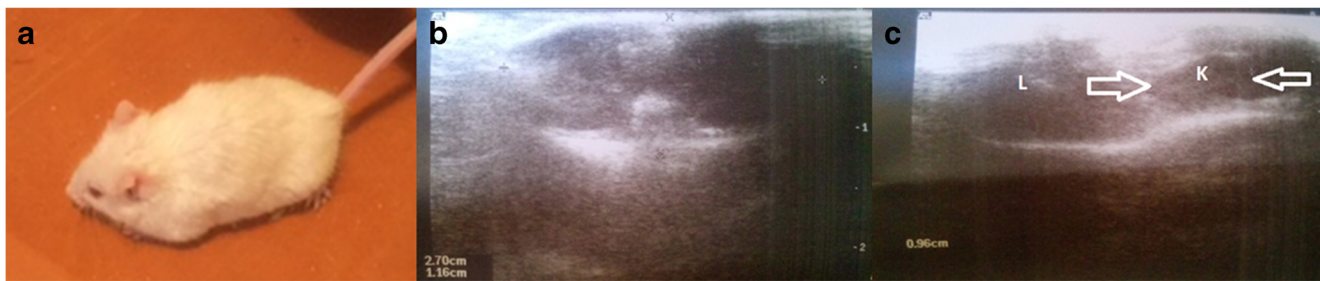
## Analysis of fecal microbiota

The number of LAB, *Bifidobacteria*, staphylococci, streptococci, coliform bacteria and microscopic fungi was determined in the intestinal contents. Different dilutions of aliquots of intestinal contents were plated on specific agar mediums to determine the quantity of different taxonomic groups of microorganisms. We counted the number of colony-forming units (CFU) of LAB, *Bifidobacteria* by seeding samples on MRS agar (MRSA) and bifidum agar (BA) medium, respectively, microscopic fungi on Sabouraud agar, coliform bacteria on ENDO, staphylococci on Baird–Parker agar (Merck, Germany), and streptococci on KF Streptococcus agar (Merck, Germany), given that one such colony corresponds to one bacterium. LAB, microscopic fungi, coliform bacteria, staphylococci and streptococci were cultured in aerobic conditions, and *Bifidobacteria* in anaerobic conditions, at 37 °C for 48 h.

## Histological analysis

Liver samples from each mouse were rinsed with sterilized PBS, fixed in 10% formalin/PBS and then embedded in paraffin for staining with hematoxylin and eosin (H&E). Microscopic images (AxioObserver Z1, Germany) were received at magnifications of  $\times 200$ ,  $\times 400$  and  $\times 1000$ .





**Fig. 1** Ultrasound examination of mice along the experiment; (A) obese mouse (general view); (B) cross-section abdominal scan (transverse organo-complex record); (C) organs evaluation on US, L liver, K kidney

### Statistical analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA), using Epi Info software (USA, version 8.0) and Microsoft Office Excel. Numeric data were presented as mean arithmetic values and their standard deviations ( $M \pm m$ ). For single comparisons, values of  $P$  were determined using Student's  $t$  test. Differences between groups were defined significant if  $P < 0.05$ .

The sample sizes were estimated based on previously obtained data and predicted differences between two means and standard deviations and considered expected death of animals.

### Results

#### The changes of mice weight and ultrasound assessment of the liver and mesenteric fat

On the 7th day from the start of FED, we observed the changes in mice behavior: animals became lethargic, lost appetite and their body temperature increased. The results of weight studying of obese mice that received probiotic bacteria or probiotic compositions are presented in Table 1.

The weight of mice that received FED increased by 6 g compared with mice that received a standard diet, confirming the development of obesity. But, the weight of obese mice that received *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB, *B. animalis* VKL (separately) or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 and *L. casei* IMV B-7280/*L. delbrueckii* subsp. *bulgaricus* IMV B-7281 probiotic compositions was decreased compared with obese mice that did not receive probiotic bacteria (control group). The weight of obese mice decreased more efficiently after injection of *B. animalis* VKB (separately) or *L. casei* IMV B-7280/*L. delbrueckii* subsp. *bulgaricus* IMV B-7281 composition. However, the *B. animalis* VKB/*B. animalis* VKL composition did not alter the weight of obese mice.

Concurrently, we observed an increasing of the size of liver and mesenteric fat in obese mice compared with intact mice receiving a standard diet (Figs. 1, 2, and 3 and Table 2).

Registration of longitudinal ultrasound scans of visceral organo-complex records of experimental mice allowed us to conduct fast screening measurements in a large number of animals (over 100) and precise post-processing analysis (Fig. 2). The size of the liver significantly increased on US after FED ( $p < 0.05$ ), and slightly decreased after *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB or probiotic composition injection into obese mice. Reduction of the mesenteric fat (omentum) size was observed in response to all these probiotic bacteria separately and the probiotic compositions. Femoral muscle thickness at the mid-femoral level was rather stable on US; therefore, fat/muscle ratio depended on visceral fat thickness. This allowed us to avoid bias, e.g. mass might be altered by gut hyperactivity induced by probiotics and/or changes of animals' appetite, etc. We observed insignificant changes in kidney structure, but it is rather hard to make significant conclusions in the current study.

#### The cholesterol level in serum of obese mice that received probiotic bacteria

The level of total, free and ester (esterified) cholesterol was increased in the serum of obese mice (within 21 days); we also revealed a reduction of the esterification index on the 21st day compared with intact mice (Fig. 4a).

Note that the concentration of free, total and ester cholesterol in serum of obese mice remained at a high level over the next 30 days, when they already received the standard diet (Table 3, Fig. 4b).

It was established that *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKL and *B. animalis* VKB (separately) or probiotic compositions significantly decreased the level of total and free cholesterol in the serum of obese mice compared with control group (Table 3).

The hypocholesterolemic effect of probiotic strains of LAB and *Bifidobacteria*, which we investigated, was definitely strain-dependent. Thus, *L. casei* IMV B-7280 was most efficient among probiotic bacteria and probiotic compositions which we investigated; the level of serum total cholesterol on the 15th, 21st and 30th days after *L. casei* IMV B-7280 administration in obese mice was even lower compared with intact mice.

**Table 1** Weight change of mice that received fat-enriched diet and probiotic bacteria or probiotic compositions

| Group of animals   | Mice weight, g/term of observation |              |              |              |               |
|--|------------------------------------|--------------|--------------|--------------|---------------|
|  | Day 0                              | Day 7        | Day 14       | Day 21       | Day 30        |
| Mice that received FED   | 20.3 ± 1.3                         | 23.4 ± 0.5   | 25.7 ± 0.1   | 26.1 ± 0.2   | 26.3 ± 0.7 ** |
| Mice that received FED and <i>L. casei</i> IMV B-7280  | 23.6 ± 0.9                         | 24.7 ± 1.1   | 27.4 ± 1.9** | 27.2 ± 1.1** | 23.5 ± 1.4#   |
| Mice that received FED and <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281                             | 23.6 ± 1.0                         | 29.5 ± 0.8** | 26.9 ± 0.7** | 26.8 ± 0.2** | 23.6 ± 1.5#   |
| Mice that received FED and <i>B. animalis</i> VKB  | 20.5 ± 4.6                         | 22.4 ± 2.0   | 23.4 ± 1.0   | 23.9 ± 0.9** | 18.2 ± 1.9*#  |
| Mice that received FED and <i>B. animalis</i> VKL  | 21.2 ± 0.8                         | 22.2 ± 0.4   | 23.3 ± 0.8   | 24.2 ± 0.9** | 20.4 ± 0.6*#  |
| Mice that received FED and <i>B. animalis</i> VKB/ <i>B. animalis</i> VKL  | 21.0 ± 2.0                         | 22.2 ± 1.2   | 23.4 ± 1.9   | 23.3 ± 1.7   | 23.2 ± 3.2    |
| Mice that received FED and <i>B. animalis</i> VKL/ <i>B. animalis</i> VKB/ <i>L. casei</i> IMV B-7280            | 22.8 ± 1.4                         | 24.6 ± 1.8   | 24.8 ± 1.6   | 26.9 ± 0.6** | 22.8 ± 1.6#   |
| Mice that received FED and <i>L. casei</i> IMV B-7280/ <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281 | 18.5 ± 2.8                         | 18.9 ± 2.7   | 19.45 ± 0.9  | 22.5 ± 1.0** | 17.0 ± 3.9*#  |

Significant differences with the mice weight (all together) on the 21st day of FED are represented by \* ( $P < 0.05$ ); significant differences with the weight of mice that received FED on the 14th day after probiotic bacteria administration are presented by # ( $P < 0.05$ ); significant differences with mice weight before FED (day 0) are presented by \*\* ( $P < 0.05$ )

As shown in Table 3, the level of free serum cholesterol in obese mice reduced more efficiently after administration of two LAB strains: *L. casei* IMV B-7280 and *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 (separately) compared with *B. animalis* VKL and *B. animalis* VKB (separately) throughout the observation period ( $P < 0.05$ ) and *B. animalis* VKB/*B. animalis* VKL and *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 compositions on the 4, 9 and 15th days. The level of free serum cholesterol in obese mice that received *L. casei* IMV B-7280 or *B. animalis* VKB/*B. animalis* VKL composition on the 21st and 30th days was even lower than in intact mice. The impact of these strains of LAB and *Bifidobacteria* and their compositions on the level of ester serum cholesterol varied. *L. casei* IMV B-7280, *B. animalis* VKL, *B. animalis* VKB/*B. animalis* VKL composition (throughout the observation period), *B. animalis* VKB or *B. animalis* VKB/*B. animalis* VKL/*L. casei* IMV B-7280 composition (on the 15–30th days) decreased the level of ester cholesterol in the serum compared with control group and even intact mice (Table 3). *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 differed from other strains by the fact that under

its influence, the level of ester cholesterol in obese mice, on the contrary, significantly increased. The esterification ratio (the ratio of ester cholesterol to total cholesterol) rose throughout the observation period in mice receiving *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, and on the 4th day after administration of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition to obese mice compared with mice of all other groups (Table 1). In the serum of obese mice that received probiotic bacteria or probiotic compositions, the ratio of esterification was decreased compared with control group and even with intact mice.

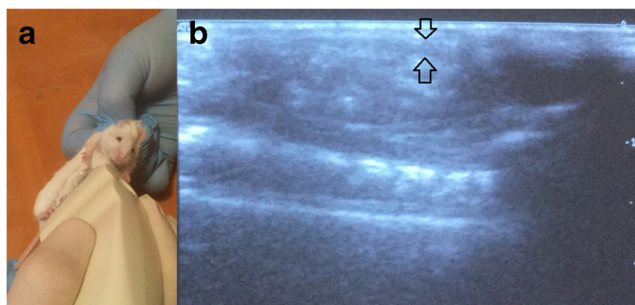
Note that the level of serum cholesterol remained reduced on the 9–30th days, i.e. after obese mice received these probiotic bacteria or probiotic compositions.

### Morphology of the liver

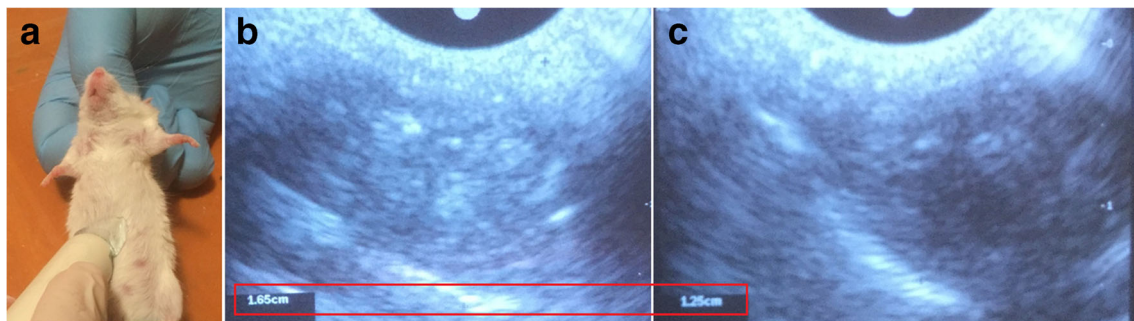
The liver tissue structure was unchanged in intact mice; pathomorphological changes were not revealed (Fig. 5). In the liver of obese mice, we found hemorrhages, hepatocytes fatty degeneration and necrosis (Fig. 5).

The morphological changes of liver tissue of obese mice that received *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKL or *B. animalis* VKB, as well as *B. animalis* VKL/*B. animalis* VKB or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 compositions are presented in the Fig. 6.

We revealed that *L. casei* IMV B-7280, *B. animalis* VKL or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition recovered the liver structure of obese mice. After injection of this probiotic composition to obese mice, the dystrophic changes in the liver were not detected, while partial necrosis and fatty degeneration of hepatocytes remained in the liver of obese mice treated with *L. casei* IMV B-7280 or *B. animalis* VKL. Fatty



**Fig. 2** Ultrasound examination (A) to obtain longitudinal scans of the abdominal cavity of mice along the experiment (sagittal organo-complex records); B measurement of visceral (mesenteric) fat



**Fig. 3** Ultrasound examination of the liver (A) during the experiment; (B) the enlarged liver of obese mouse; (C) liver size decreasing after treatment

degeneration and necrosis of hepatocytes decreased after treatment with these probiotic bacteria or probiotic compositions. Yet, hemorrhages in the liver were not found in obese mice treated with *L. casei* IMV B-7280 or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition. However, after injection of *B. animalis* VKB, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 or *B. animalis* VKL/*B. animalis* VKB composition to obese mice, we found necrosis and fatty degeneration of hepatocytes.

The treatment with *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition effectively recovered the liver morphological structure in obese mice. *L. casei* IMV B-7280 and *B. animalis* VKL (separately) restored the liver morphological structure of obese mice to a lesser degree. *B. animalis* VKB or *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 (separately) and *B. animalis* VKL/*B. animalis* VKB composition were ineffective.

### The changes of the gut microbiota of obese mice that received probiotic strains or probiotic compositions

As demonstrated on the Table 4, obesity in mice was associated with increasing the total number of microorganisms (on the 4th–15th days) and changes in spectrum of gut microbiota. Thus, we observed decreasing the number of *Lactobacillus* spp. (throughout the observation period), *Bifidobacterium*

spp. (on the 4th and 9th days) and coliform bacteria (on the 9th–30th days). However, the number of Gram-positive cocci (staphylococci, streptococci; throughout the observation period) and fungal species (on the 4th and 15th days) increased compared with intact mice.

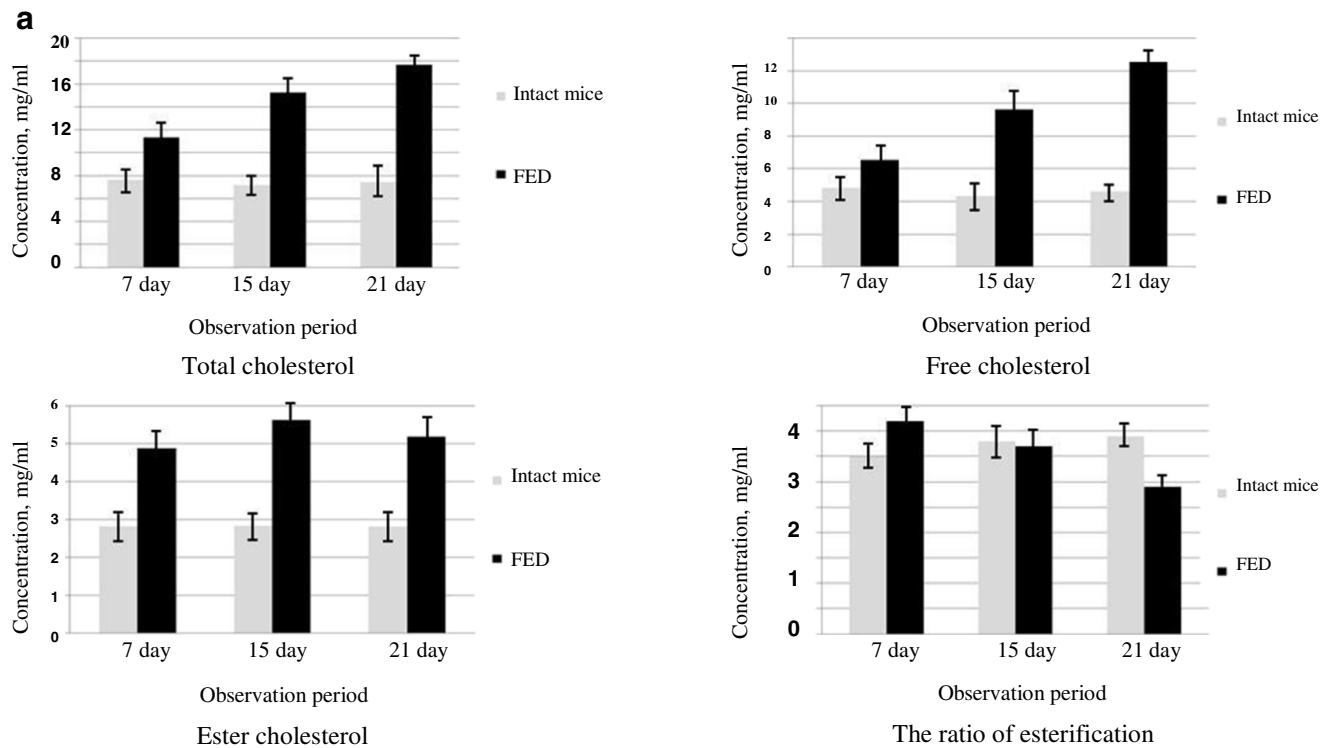
The number of *Lactobacillus* spp., *Bifidobacterium* spp. and coliform bacteria increased in the gut of obese mice that received *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB or *B. animalis* VKL (separately) or probiotic compositions, and the number of staphylococci and streptococci in most cases were reduced in different periods of observation compared with control or intact mice, although the total number of bacteria remained high.

We noted the higher number of *Lactobacillus* spp. in the gut of obese mice treated with *L. casei* IMV B-7280 (on the 9th–30th days), *B. animalis* VKL (on the 21st and 30th days), as well as treated with *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 (on the 15th–30th days) and *B. animalis* VKB/*B. animalis* VKL (on the 30th day) compositions compared with all other groups of mice (including intact mice;  $P < 0.05$ ). The number of *Bifidobacteria* was higher ( $P < 0.05$ ) even compared with all other groups of mice (including intact ones) after treatment with *L. casei* IMV B-7280 (on the 4th–30th days), *B. animalis* VKB or *B. animalis* VKL

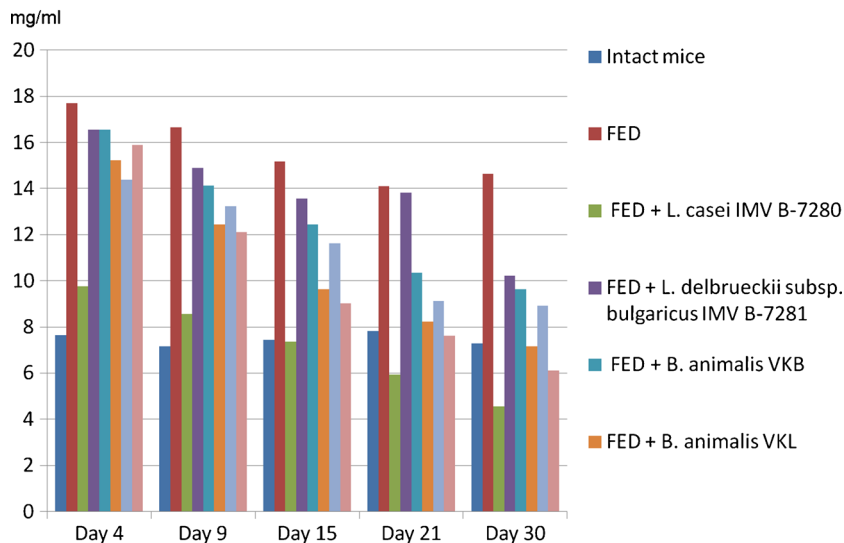
**Table 2** Results of ultrasound studies of the liver and mesenteric fat of mice that received fat-enriched diet and probiotic strains of bacteria

| Group of mice  | Liver size, mm | Mesenteric fat size, mm |
|--|----------------|-------------------------|
| Intact mice  | 1.17 ± 0.02    | 0.13 ± 0.02             |
| Mice that received FED   | 1.56 ± 0.02*   | 0.18 ± 0.01*            |
| Mice that received FED and <i>L. casei</i> IMV B-7280  | 1.50 ± 0.02    | 0.13 ± 0.02#            |
| Mice that received FED and <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281                             | 1.40 ± 0.02#   | 0.10 ± 0.02#            |
| Mice that received FED and <i>B. animalis</i> VKB  | 1.30 ± 0.02#   | 0.08 ± 0.02#            |
| Mice that received FED and <i>B. animalis</i> VKL  | 1.70 ± 0.02    | 0.09 ± 0.02#            |
| Mice that received FED and <i>B. animalis</i> VKB/ <i>B. animalis</i> VKL  | 1.45 ± 0.02#   | 0.10 ± 0.02#            |
| Mice that received FED and <i>B. animalis</i> VKL/ <i>B. animalis</i> VKB/ <i>L. casei</i> IMV B-7280            | 1.45 ± 0.02#   | 0.11 ± 0.02#            |
| Mice that received FED and <i>L. casei</i> IMV B-7280/ <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281 | 1.45 ± 0.02#   | 0.11 ± 0.02#            |

Significant differences of intact mice are presented by \* ( $P < 0.05$ ); significant differences of obese mice are presented by # ( $P < 0.05$ )



**b**  
Concentration of total cholesterol during experiment



**Fig. 4** Cholesterol levels; (A) the concentration of total, free and ester cholesterol in the blood serum of BALB/c line mice that received FED; (B) changes of total cholesterol levels in the blood serum during the experiment in all groups

(on the 9th–30th days), and *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 or *B. animalis* VKB/*B. animalis* VKL (on the 4th–30th days) compositions. A significant increase in the number of both *Lactobacillus* spp. and *Bifidobacterium* spp. in the gut was observed after administration of *L. casei* IMV B-7280 and *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition to obese mice; the lesser increasing was observed after treatment with

*B. animalis* VKL (only on the 21st–30th days) or *B. animalis* VKB/*B. animalis* VKL composition (on the 30th day).

The number of coliform bacteria in the gut of obese mice that received *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 (on the 9th–21st days) or *B. animalis* VKB (on the 15th day) or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition (on the 9th–30th days) was even higher than in intact mice.



**Table 3** Concentration of total, free and ester cholesterol in the blood serum of BALB/c mice treated with probiotic bacteria or probiotic compositions

| Group of mice   | Concentration of cholesterol (mg/ml) term of observation after probiotic bacterial strains injection |               |               |               |               |
|---|--|---------------|---------------|---------------|---------------|
|   | Day 4  | Day 9         | Day 15        | Day 21        | Day 30        |
| Concentration of total cholesterol (sum of free and ester cholesterol), mg/ml                       |  |               |               |               |               |
| Intact mice   | 7.64 ± 0.35  | 7.16 ± 0.31   | 7.43 ± 0.17   | 7.83 ± 0.11   | 7.28 ± 0.14   |
| Mice that received FED  | 17.71 ± 0.76*  | 16.66 ± 0.28* | 15.18 ± 0.37* | 14.09 ± 0.70* | 14.63 ± 0.53* |
| Mice that received FED + <i>L. casei</i> IMV B-7280   | 9.77 ± 0.06*   | 8.56 ± 0.15*  | 7.37 ± 0.25*  | 5.93 ± 0.48*  | 4.56 ± 0.23*  |
| Mice that received FED + <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281                  | 16.55 ± 0.15*  | 14.89 ± 0.48* | 13.56 ± 0.38* | 13.81 ± 0.81* | 10.23 ± 0.63* |
| Mice that received FED + <i>B. animalis</i> VKB   | 16.55 ± 0.65*  | 14.12 ± 0.36* | 12.45 ± 0.61* | 10.35 ± 0.45* | 9.63 ± 0.63*  |
| Mice that received FED + <i>B. animalis</i> VKL   | 15.22 ± 0.38*  | 12.45 ± 0.74* | 9.64 ± 0.64*  | 8.22 ± 0.39*  | 7.15 ± 0.47*  |
| Mice that received FED + <i>B. animalis</i> VKB/ <i>B. animalis</i> VKL                             | 14.38 ± 0.21*  | 13.22 ± 0.08* | 11.63 ± 0.19* | 9.13 ± 0.59*  | 8.91 ± 0.10*  |
| Mice that received FED + <i>B. animalis</i> VKL/ <i>B. animalis</i> VKB/ <i>L. casei</i> IMV B-7280 | 15.88 ± 0.26*  | 12.11 ± 0.35* | 9.03 ± 0.35*  | 7.62 ± 0.95*  | 6.11 ± 0.69*  |
| Concentration of free cholesterol   |  |               |               |               |               |
| Intact mice   | 4.82 ± 0.36  | 4.34 ± 0.31   | 4.61 ± 0.18   | 5.01 ± 0.11   | 4.46 ± 0.14   |
| Mice that received FED  | 12.33 ± 0.77*  | 11.27 ± 0.29* | 9.77 ± 0.37*  | 8.68 ± 0.71*  | 9.21 ± 0.53*  |
| Mice that received FED + <i>L. casei</i> IMV B-7280   | 8.41 ± 0.06*   | 7.19 ± 0.15*  | 5.99 ± 0.25*  | 4.53 ± 0.49*  | 3.15 ± 0.23*  |
| Mice that received FED + <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281                  | 7.12 ± 0.55*   | 6.89 ± 0.62*  | 5.15 ± 0.37*  | 5.59 ± 0.84*  | 4.85 ± 0.17*  |
| Mice that received FED + <i>B. animalis</i> VKB   | 11.13 ± 0.82*  | 10.67 ± 0.45* | 8.11 ± 0.81*  | 8.22 ± 0.37*  | 7.78 ± 0.55*  |
| Mice that received FED + <i>B. animalis</i> VKL   | 10.46 ± 0.71*  | 9.79 ± 0.43*  | 8.56 ± 0.33*  | 7.16 ± 0.62*  | 6.02 ± 0.27*  |
| Mice that received FED + <i>B. animalis</i> VKB/ <i>B. animalis</i> VKL                             | 9.72 ± 0.22*   | 8.54 ± 0.08*  | 6.93 ± 0.19*  | 4.41 ± 0.60*  | 4.19 ± 0.10*  |
| Mice that received FED + <i>B. animalis</i> VKL/ <i>B. animalis</i> VKB/ <i>L. casei</i> IMV B-7280 | 8.65 ± 0.35*   | 8.12 ± 0.41*  | 6.52 ± 0.89*  | 6.03 ± 0.63*  | 4.45 ± 0.40*  |
| Concentration of ester cholesterol  |  |               |               |               |               |
| Intact mice   | 2.82 ± 0.04  | 2.83 ± 0.03   | 2.82 ± 0.05   | 2.82 ± 0.03   | 2.84 ± 0.01   |
| Mice that received FED  | 5.31 ± 0.09*   | 5.45 ± 0.14*  | 5.44 ± 0.04*  | 5.43 ± 0.06*  | 5.38 ± 0.08*  |
| Mice that received FED + <i>L. casei</i> IMV B-7280   | 1.31 ± 0.02*   | 1.37 ± 0.04*  | 1.42 ± 0.02*  | 1.45 ± 0.02*  | 1.43 ± 0.03*  |
| Mice that received FED + <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281                  | 9.41 ± 0.05*   | 8.12 ± 0.21*  | 8.37 ± 0.18*  | 8.25 ± 0.16*  | 5.33 ± 0.09*  |
| Mice that received FED + <i>B. animalis</i> VKB   | 5.47 ± 0.11*   | 3.38 ± 0.04*  | 4.24 ± 0.03*  | 2.17 ± 0.03*  | 1.83 ± 0.01*  |
| Mice that received FED + <i>B. animalis</i> VKL   | 4.73 ± 0.13*   | 2.65 ± 0.08*  | 1.11 ± 0.01*  | 1.05 ± 0.01*  | 1.19 ± 0.02*  |
| Mice that received FED + <i>B. animalis</i> VKB/ <i>B. animalis</i> VKL                             | 4.67 ± 0.11*   | 4.62 ± 0.07*  | 4.75 ± 0.07*  | 4.71 ± 0.07*  | 4.79 ± 0.07*  |
| Mice that received FED + <i>B. animalis</i> VKL/ <i>B. animalis</i> VKB/ <i>L. casei</i> IMV B-7280 | 7.33 ± 0.04*   | 4.12 ± 0.03*  | 2.55 ± 0.03*  | 1.59 ± 0.02*  | 1.69 ± 0.01*  |
| The ratio of esterification (the ratio of ester cholesterol to total cholesterol)                   |  |               |               |               |               |
| Intact mice (control)   | 0.35 ± 0.02  | 0.38 ± 0.01   | 0.39 ± 0.02   | 0.37 ± 0.02   | 0.40 ± 0.03   |
| Mice that received FED  | 0.28 ± 0.03  | 0.33 ± 0.01   | 0.37 ± 0.01   | 0.38 ± 0.03   | 0.36 ± 0.01   |
| Mice that received FED + <i>L. casei</i> IMV B-7280   | 0.13 ± 0.05  | 0.16 ± 0.02   | 0.19 ± 0.09   | 0.24 ± 0.04   | 0.31 ± 0.03   |
| Mice that received FED + <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281                  | 0.59 ± 0.04*   | 0.57 ± 0.03*  | 0.64 ± 0.03*  | 0.63 ± 0.04*  | 0.54 ± 0.04*  |
| Mice that received FED + <i>B. animalis</i> VKB   | 0.34 ± 0.01  | 0.22 ± 0.02*  | 0.34 ± 0.02   | 0.22 ± 0.01*  | 0.17 ± 0.01*  |
| Mice that received FED + <i>B. animalis</i> VKL   | 0.32 ± 0.03  | 0.23 ± 0.02*  | 0.12 ± 0.01*  | 0.14 ± 0.01*  | 0.16 ± 0.01*  |
| Mice that received FED + <i>B. animalis</i> VKB/ <i>B. animalis</i> VKL                             | 0.32 ± 0.09  | 0.34 ± 0.01   | 0.40 ± 0.08   | 0.51 ± 0.02   | 0.54 ± 0.09   |
| Mice that received FED + <i>B. animalis</i> VKL/ <i>B. animalis</i> VKB/ <i>L. casei</i> IMV B-7280 | 0.47 ± 0.02*   | 0.36 ± 0.01   | 0.27 ± 0.02*  | 0.20 ± 0.02*  | 0.29 ± 0.02*  |

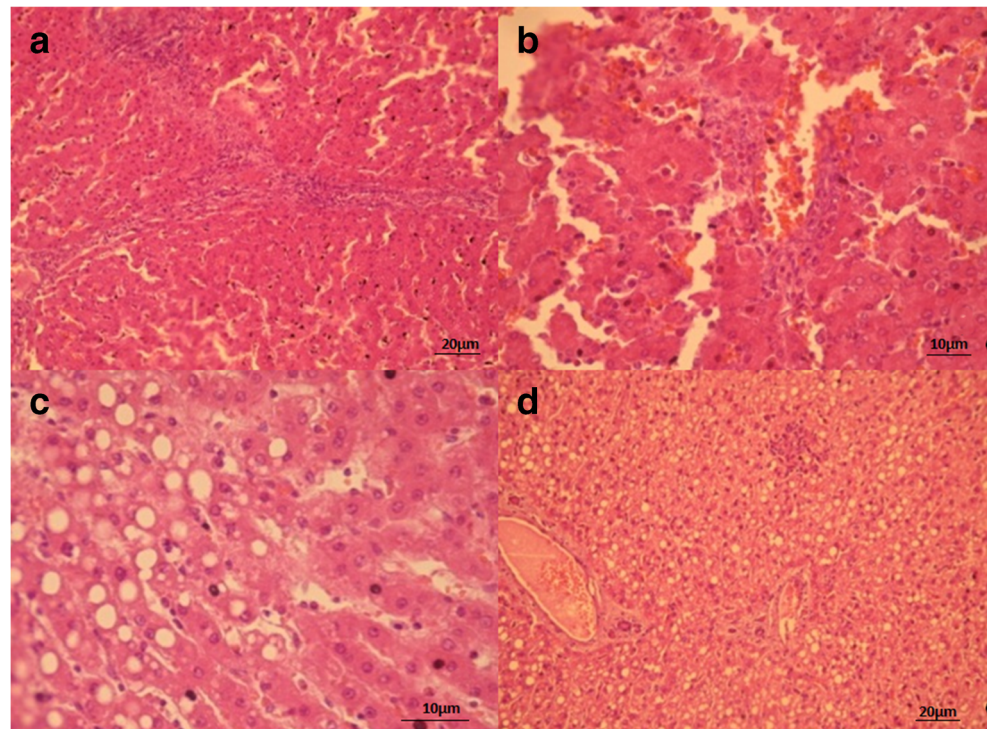
Significant differences with intact mice are represented by \* ( $P < 0.05$ ); significant difference with obese mice that did not receive probiotic bacteria are presented by • ( $P < 0.05$ )

The treatment with *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB and *B. animalis* VKL or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition significantly decreased the number of microscopic fungi in the gut of obese mice compared with the control and/or intact mice. The number of microscopic fungi in the gut was significantly lower after obese mice received *L. casei* IMV B-7280 or

*B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition ( $P < 0.05$ ).

On the other hand, the treatment with *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB or *B. animalis* VKL (separately) and *B. animalis* VKB/*B. animalis* VKL or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 compositions did not normalize the total number of bacteria and the number of

**Fig. 5** Liver tissue of intact (A, B) and obese (C, D) mice. (A, B) structure of liver tissue is preserved as normal, pathologic changes not detected. (C, D) fatty degeneration/necrosis of hepatocytes (A – x200 magnification, B – x400 magnification)



Gram-positive bacteria in the gut of obese mice; however, the number of *Lactobacillus* spp., *Bifidobacterium* spp. and Gram-negative bacteria (coliform bacteria) significantly increased.

## Discussion

The normalization of lipid metabolism and treatment of gut microbiota violations using probiotics is a pivotal treatment and preventive strategy for patients with obesity and metabolic diseases. Our results are supposed to add evidence to the issue and are promising to facilitate clinical application.

Probiotics affect physiological functions and metabolic processes directly or through the normalization of microbiocenosis of mucous membranes of various organs and body systems; however, the range of their biological activity is a strain-dependent characteristic (Arora, 2012, 2013 Guo, 2011, Cho, 2015, Lumeng, 2013) [57, 58, 73–75].

Thus, bacterial strains have different probiotic effects on metabolic disease and obesity.

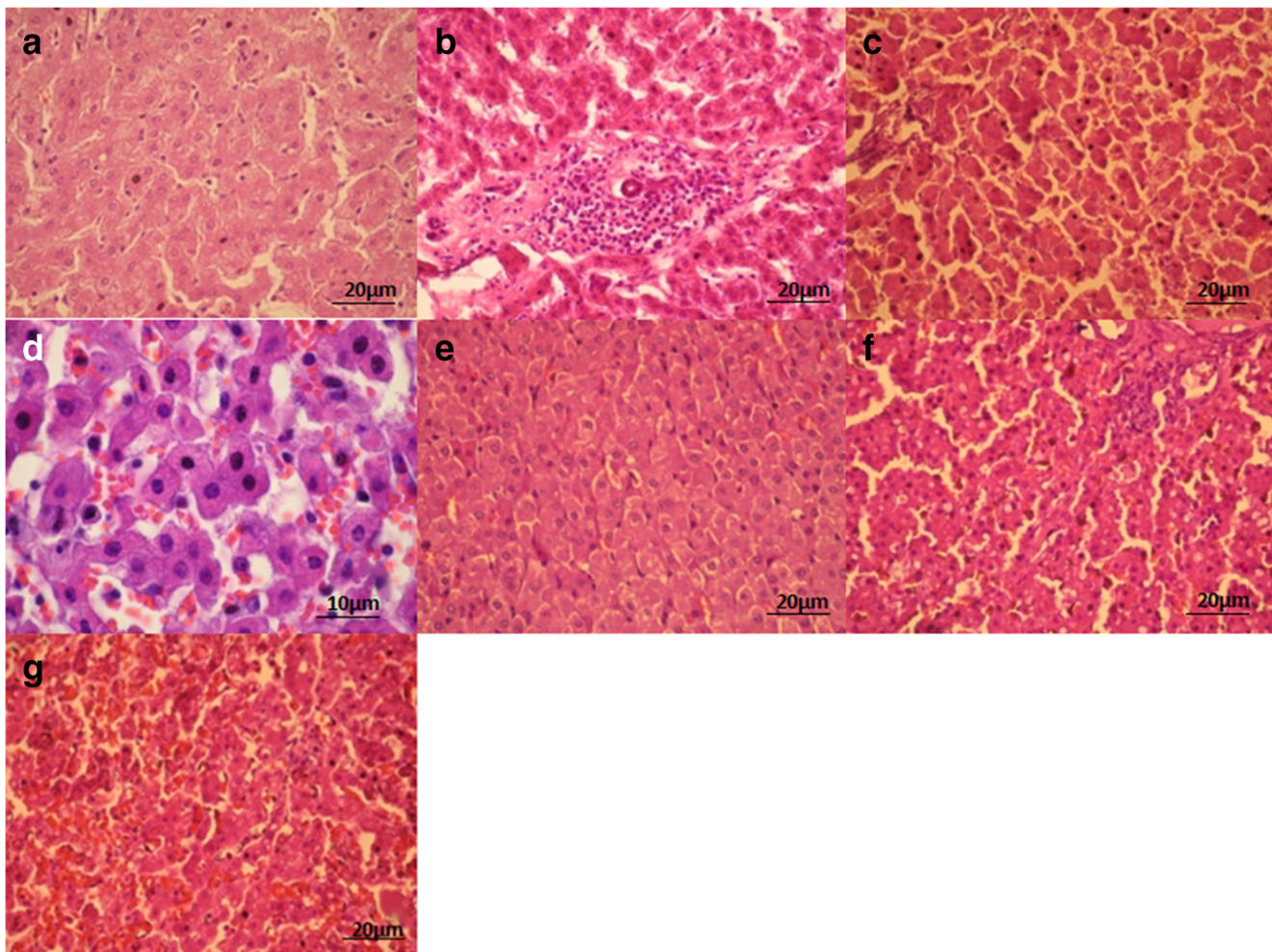
For example, in clinical and experimental studies, probiotic bacteria *L. plantarum* and *L. gasseri* reduced the body weight (Million, 2012; Wu, 2015) [31, 76] and cholesterol level [77] but, on the contrary, *L. acidophilus*, *L. fermentum* or *L. ingluviei* increase the body weight [76], and *L. acidophilus* NCDC 13 had no impact on obesity [73].

In the current study, we have defined that the probiotic bacteria *L. casei* IMV B-7280, *L. delbrueckii* subsp.

*bulgaricus* IMV B-7281, *B. animalis* VKB and *B. animalis* VKL (separately) or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 and *B. animalis* VKB/*B. animalis* VKL compositions decreased the weight of obese BALB/c mice, which was associated with decreased cholesterol level in serum and partial normalization of intestinal biocenosis. This was manifested in the increased number of *Lactobacillus* spp., *Bifidobacterium* spp. and coliform bacteria. Furthermore, a slight decreasing of the liver size and mesenteric fat thickness in obese mice was also observed under the effect of these probiotic bacteria and probiotic compositions. Other strains of LAB have also demonstrated hypocholesterolemic activity in vivo on mice models of metabolic diseases. The level of cholesterol decreased in obese mice after administration of *L. plantarum* strain K21 [31, 77], *L. acidophilus* NS1 [30], *L. curvatus* HY7601 and *L. plantarum* KY1032 [22]. *L. rhamnosus* CCFM1107 decreased the level of cholesterol in the liver and serum of mice with alcoholic impact of the liver [28] After administration of *L. acidophilus* to obese mice with damaged livers after a cholesterol-enriched diet, a reduction of cholesterol level both in serum and liver was observed [29]. *L. acidophilus* ATCC 4356 protected mice from atherosclerosis by reducing the level of cholesterol in blood plasma [23], and *L. plantarum* CAI6 and *L. plantarum* SC4 had a protective effect in models of cardiovascular disease in hyperlipidemic mice by reducing the level of total and low-density lipoprotein cholesterol [24].

Recently, the “bile salt hydrolase hypothesis” (BSH) has been proposed [48, 78–80]. Deconjugated bile acids are





**Fig. 6** Liver tissue of mice that received FED and *L. casei* IMV B-7280 (A), *B. animalis* VKL (B), *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 (C), *B. animalis* VKB (D, E), *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 (F), *B. animalis* VKB/*B. animalis* VKL composition (G). (A) restoring liver structure, lipid inclusions are detected in hepatocytes. Partial necrosis and fatty degeneration of hepatocytes (x400 magnification). (B) restoring structure, lipid inclusions in hepatocytes and existing isolated sites with lymphocytes

and macrophages infiltrates are detected; partial necrosis and fatty degeneration of hepatocytes (x400 magnification). (C) restoring structure, degenerative changes not detected. Partial necrosis of hepatocytes (x400 magnifications). (D, E) partial necrosis and fatty degeneration of hepatocytes (D – x1000 magnification, E – x400 magnification). (F) necrosis and fatty degeneration of hepatocytes (x400 magnification). (G) necrosis and fatty degeneration of hepatocytes (x400 magnification)

transformed into secondary bile acids by colonic microbes, which is most acceptable to explain cholesterol-lowering mechanics of probiotics together with direct binding to cholesterol, producing propionic and butyric acids and reducing the synthesis of cholesterol in the liver. Gut microbiota can regulate cholesterol metabolism via the following mechanisms: 1) direct effects on enzyme systems in liver cells and other organs that produce endogenous cholesterol; 2) increasing the regeneration rate of the intestinal villi cells, which produce endogenous cholesterol; 3) impact on absorption of cholesterol from the intestine, which depends on the transit of neutral sterols through the intestine, the concentration of ions (mainly calcium ions), the affinity of cell receptors to lipoproteins or microorganisms, that are involved in the transformation of cholesterol; 4) reduction of cholesterol level via the hydrolysis

system of deconjugation of bile acids and direct binding of cholesterol by cell walls; 5) degradation and transformation of bile acids and steroid hormones, the concentration change of which can lead to strengthening or inhibition of cholesterol synthesis; 6) decreasing pH value in the gut, which ultimately leads to a decrease of bile acid synthesis in the liver and inhibition of cholesterol synthesis [20–31, 78–80]. Bosch et al. [78] concluded that the above characteristics suggest that the strain is an excellent candidate for reducing high blood cholesterol levels.

We have defined the clear strain-dependent hypocholesterolemic effect of the studied strains. Thus, *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB and *B. animalis* VKL (separately) or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 and

**Table 4** Spectrum of gut microbiota of obese mice that received probiotic bacteria or probiotic compositions, M ± m

| Group of mice   | Day | Number of microorganisms, that were sowed on the nutrient mediums, Lg CFU/mg |                       |               |               |               |                |               |
|---|-----|--|-----------------------|---------------|---------------|---------------|----------------|---------------|
|   |     | Baird–Parker agar  | KF-Streptococcus agar | MRSA          | BA            | ENDO          | Sabouraud agar | MPA           |
| 1   | 2   | 3  | 4                     | 5             | 6             | 7             | 8              | 9             |
| Intact mice   | –   | 2.65 ± 0.09  | 3.25 ± 0.05           | 2.68 ± 0.02   | 2.15 ± 0.02   | 4.15 ± 0.09   | 2.77 ± 0.05    | 3.11 ± 0.09   |
| Mice that received FED  | 4   | 4.11 ± 0.11*   | 6.75 ± 0.12*          | 0.52 ± 0.06*  | 1.44 ± 0.06*  | 3.75 ± 0.09   | 3.19 ± 0.07*   | 4.47 ± 0.07*  |
|   | 9   | 4.22 ± 0.09*   | 6.58 ± 0.05*          | 1.35 ± 0.02*  | 1.25 ± 0.04*  | 3.42 ± 0.08*  | 3.22 ± 0.09*   | 4.74 ± 0.06*  |
|   | 15  | 4.13 ± 0.03*   | 5.12 ± 0.06*          | 1.10 ± 0.01*  | 2.06 ± 0.07   | 3.11 ± 0.04*  | 3.07 ± 0.11    | 4.32 ± 0.03*  |
|   | 21  | 4.44 ± 0.05*   | 5.67 ± 0.08*          | 1.22 ± 0.05*  | 2.12 ± 0.09   | 2.67 ± 0.02*  | 3.11 ± 0.06    | 3.76 ± 0.04*  |
|   | 30  | 3.92 ± 0.12*   | 6.47 ± 0.03*          | 1.41 ± 0.03*  | 2.11 ± 0.03   | 2.52 ± 0.03*  | 3.16 ± 0.08    | 3.98 ± 0.03*  |
| Mice that received FED + <i>L. casei</i> IMV B-7280   | 4   | 3.89 ± 0.06*   | 5.91 ± 0.05*          | 2.77 ± 0.06•  | 2.92 ± 0.07*• | 3.90 ± 0.08   | 2.60 ± 0.11•   | 4.26 ± 0.09*  |
|   | 9   | 3.87 ± 0.12*   | 5.11 ± 0.07*•         | 3.49 ± 0.08*• | 3.32 ± 0.03*• | 4.22 ± 0.11•  | 0*•            | 4.17 ± 0.06*• |
|   | 15  | 3.52 ± 0.09*•  | 5.22 ± 0.02*          | 4.78 ± 0.11*• | 3.11 ± 0.05*  | 4.40 ± 0.15•  | 0*•            | 4.56 ± 0.07*  |
|   | 21  | 2.99 ± 0.03*•  | 4.46 ± 0.10*•         | 3.52 ± 0.05*• | 3.65 ± 0.04*  | 4.13 ± 0.09•  | 1.65 ± 0.06*•  | 4.89 ± 0.10*• |
|   | 30  | 2.45 ± 0.08•   | 4.21 ± 0.07*•         | 3.57 ± 0.09*• | 2.99 ± 0.08*  | 4.86 ± 0.09*• | 1.98 ± 0.02*•  | 4.23 ± 0.08*  |
| Mice that received FED + <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> IMV B-7281                  | 4   | 4.45 ± 0.09*   | 6.62 ± 0.09*          | 1.16 ± 0.06*• | 1.65 ± 0.03*  | 4.40 ± 0.09•  | 3.65 ± 0.09*   | 5.22 ± 0.09*• |
|   | 9   | 4.61 ± 0.11*   | 5.89 ± 0.11*•         | 2.11 ± 0.04*• | 2.16 ± 0.03•  | 5.06 ± 0.08*• | 2.79 ± 0.07    | 5.37 ± 0.12*• |
|   | 15  | 3.87 ± 0.07*   | 6.03 ± 0.13*•         | 2.25 ± 0.11*• | 2.27 ± 0.09   | 5.11 ± 0.11*• | 2.86 ± 0.11    | 5.62 ± 0.09*• |
|   | 21  | 4.14 ± 0.09*   | 5.63 ± 0.09*          | 2.67 ± 0.09•  | 2.08 ± 0.01   | 4.98 ± 0.09*• | 3.01 ± 0.09    | 5.64 ± 0.08*• |
|   | 30  | 3.12 ± 0.03*•  | 5.49 ± 0.07*•         | 2.53 ± 0.12•  | 2.19 ± 0.05   | 4.55 ± 0.12•  | 2.92 ± 0.12    | 5.76 ± 0.09*• |
| Mice that received FED + <i>B. animalis</i> VKB   | 4   | 4.12 ± 0.11*   | 5.92 ± 0.12*•         | 1.52 ± 0.03*• | 2.22 ± 0.09•  | 4.02 ± 0.12   | 2.17 ± 0.06*•  | 4.78 ± 0.06*  |
|   | 9   | 3.51 ± 0.04*•  | 5.16 ± 0.09*•         | 1.99 ± 0.05*• | 3.14 ± 0.06*• | 4.14 ± 0.04•  | 1.63 ± 0.03*•  | 5.21 ± 0.08*• |
|   | 15  | 2.74 ± 0.03•   | 4.22 ± 0.03*•         | 2.11 ± 0.04*• | 3.92 ± 0.07*  | 4.96 ± 0.07*• | 1.26 ± 0.02*•  | 5.23 ± 0.09*• |
|   | 21  | 2.98 ± 0.06•   | 4.11 ± 0.05*•         | 2.60 ± 0.02•  | 3.62 ± 0.07*  | 4.32 ± 0.09•  | 2.54 ± 0.07•   | 5.11 ± 0.12*• |
|   | 30  | 3.12 ± 0.07*•  | 4.07 ± 0.09*•         | 2.65 ± 0.09•  | 3.13 ± 0.03*  | 4.07 ± 0.04•  | 2.82 ± 0.04    | 4.98 ± 0.06*• |
| Mice that received FED + <i>B. animalis</i> VKL   | 4   | 3.61 ± 0.03*•  | 6.17 ± 0.15*          | 2.15 ± 0.05*• | 2.10 ± 0.05•  | 3.80 ± 0.06*  | 2.99 ± 0.10    | 4.87 ± 0.05*  |
|   | 9   | 3.41 ± 0.07*•  | 5.03 ± 0.06*•         | 2.66 ± 0.09•  | 3.42 ± 0.06*• | 3.96 ± 0.08•  | 2.76 ± 0.04•   | 4.61 ± 0.06*  |
|   | 15  | 4.06 ± 0.12*   | 4.48 ± 0.02*•         | 2.98 ± 0.12•  | 3.65 ± 0.11*  | 4.67 ± 0.11*• | 2.54 ± 0.07•   | 4.17 ± 0.03*  |
|   | 21  | 3.21 ± 0.08*•  | 3.62 ± 0.03•          | 3.16 ± 0.06*• | 2.95 ± 0.06*  | 3.72 ± 0.05*• | 2.80 ± 0.11    | 3.89 ± 0.04*  |
|   | 30  | 3.10 ± 0.05*•  | 3.44 ± 0.05•          | 3.25 ± 0.04*• | 2.63 ± 0.04*  | 3.31 ± 0.09*• | 3.06 ± 0.09    | 3.51 ± 0.02•  |
| Mice that received FED + <i>B. animalis</i> VKB / <i>B. animalis</i> VKL                            | 4   | 4.10 ± 0.11*   | 6.12 ± 0.18*          | 1.77 ± 0.07*• | 3.79 ± 0.06*• | 3.89 ± 0.08   | 2.20 ± 0.07*•  | 4.77 ± 0.07*  |
|   | 9   | 3.78 ± 0.13*   | 5.93 ± 0.11*•         | 2.16 ± 0.11*• | 4.26 ± 0.07*• | 4.20 ± 0.09•  | 2.11 ± 0.05*•  | 5.12 ± 0.12*  |
|   | 15  | 3.77 ± 0.06*•  | 5.11 ± 0.14*          | 2.38 ± 0.10•  | 4.61 ± 0.03*  | 4.30 ± 0.04•  | 1.96 ± 0.03*•  | 5.34 ± 0.11*• |
|   | 21  | 3.11 ± 0.06*•  | 4.16 ± 0.09*•         | 2.55 ± 0.09•  | 5.14 ± 0.06*  | 3.58 ± 0.03*• | 2.63 ± 0.08•   | 4.98 ± 0.09*• |
|   | 30  | 2.62 ± 0.03•   | 3.41 ± 0.04•          | 3.06 ± 0.03*• | 5.03 ± 0.07*  | 3.97 ± 0.09*• | 2.79 ± 0.06    | 4.33 ± 0.05*• |
| Mice that received FED + <i>B. animalis</i> VKL/ <i>B. animalis</i> VKB/ <i>L. casei</i> IMV B-7280 | 4   | 3.61 ± 0.02*•  | 5.11 ± 0.14*•         | 2.17 ± 0.03*• | 2.86 ± 0.07*• | 4.12 ± 0.08•  | 1.60 ± 0.03*•  | 4.78 ± 0.08*  |
|   | 9   | 2.87 ± 0.02•   | 5.23 ± 0.12*•         | 2.77 ± 0.07•  | 4.14 ± 0.11*• | 4.55 ± 0.03*• | 0*•            | 4.35 ± 0.07*  |
|   | 15  | 2.45 ± 0.04•   | 4.46 ± 0.03*•         | 3.58 ± 0.09*• | 5.10 ± 0.09*  | 4.92 ± 0.09*• | 1.20 ± 0.04*•  | 3.87 ± 0.05*• |
|   | 21  | 2.97 ± 0.09•   | 4.05 ± 0.08*•         | 4.13 ± 0.05*• | 4.83 ± 0.13*  | 5.16 ± 0.12*• | 1.11 ± 0.01*•  | 3.65 ± 0.06   |
|   | 30  | 3.14 ± 0.06*•  | 3.86 ± 0.05*•         | 4.19 ± 0.08*• | 4.22 ± 0.17*  | 5.22 ± 0.16*• | 1.62 ± 0.03*•  | 3.27 ± 0.07•  |

Significant differences with intact mice are represented by \* ( $P < 0.05$ ); significant differences with obese mice that did not receive probiotic bacteria are presented by • ( $P < 0.05$ )

*B. animalis* VKB/*B. animalis* VKL compositions also impact the level of various fractions of serum cholesterol (total, free and ester cholesterol). The decrease of the total cholesterol level in serum occurred as a result of the effects of *L. casei* IMV B-7280, and the level of free serum cholesterol was more effectively reduced

under the effect of *L. casei* IMV B-7280 and *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, compared with *B. animalis* VKB and *B. animalis* VKL. These two strains of LAB showed more effective in vitro hypocholesterolemic activity than *B. animalis* VKB and *B. animalis* VKL. Under the treatment with *L. casei* IMV B-



7280 and *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, the assimilation of cholesterol from the gastrointestinal tract or its binding was probably more intensive. A possible explanation might be the ability of different LAB strains to decrease the pH value in the gut, evoking deconjugation of the bile acids that bind cholesterol at low pH values. However, more research is needed to elucidate this mechanism.

The best probiotic composition with hypocholesteremic activity was *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280; their cholesterol-lowering activity on the serum-free cholesterol was better from these two *Bifidobacteria* strains in monoculture.

The level of ester cholesterol and the ratio of esterification, which is an important functional indicator of the liver function, significantly increased under the effect of *L. delbrueckii* subsp. *bulgaricus* IMV B-7281 throughout the observation period and only on the 4th day after the administration of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition to obese mice. This probably occurred by reducing the level of free cholesterol under the effect of these probiotic strains and probiotic composition, and an increasing of the synthesis of ester cholesterol in the liver happens as a physiologically normal process. In the blood serum of obese mice that received *L. casei* IMV B-7280, *B. animalis* VKB and *B. animalis* VKL (separately) or *B. animalis* VKB/*B. animalis* VKL composition, the level of serum ester cholesterol and the ratio of esterification decreased. However, to obtain explanations for this, further research is required.

The rate of esterification is an indirect marker of the liver function, a site where cholesterol esters are synthesized. Furthermore, we observed that the liver morphological structure of obese mice was effectively recovered by *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition. The liver morphological structure of obese mice was restored to a lesser extent by *L. casei* IMV B-7280 and *B. animalis* VKL (separately). Other probiotic bacteria and probiotic composition were ineffective. This proved that the restoration of the morphological structure of the liver of obese mice is associated with an improvement in lipid metabolism.

Probiotics are known to improve the liver function via the so-called ‘gut-liver axis’ [32–34, 56, 81], and may delay the progression of NAFLD, likely via both decreasing endotoxemia by downregulating serum lipopolysaccharides (LPS) and liver Toll-like receptor (TLR)-4, and improving gut flora alteration [81].

The gut microbiota is associated with obesity development and is changed by the hypocholesterolemic effect of probiotics. *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB or *B. animalis* VKL (separately) and *B. animalis* VKB/*B. animalis* VKL or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 compositions modified the gut microbiota in obese mice.

The gut microbiota in humans and mice is represented mainly by two phylotypes: *Bacteroidetes* and *Firmicutes*

[82, 83]. The reduction in the number of Gram-negative *Bacteroidetes* bacteria and increase in the number of Gram-positive *Firmicutes* was revealed in the gut on different obesity models in mice and human studies with excess weight [13], although other studies have shown that the decrease in the ratio of *Bacteroidetes*:*Firmicutes* had no relationship to obesity in humans [84]. In another study on the obesity model, the reduction of *Lactobacillus* spp. and increase in *C. perfringens* was observed in the intestinal contents of mice [77]. We have found that in the intestinal contents of obese mice, the number of *Lactobacillus* spp., *Bifidobacterium* spp. and coliform bacteria decreased, and the number of Gram-positive cocci—staphylococci and streptococci—on the contrary, increased. This has proven that the intestinal microbiota can be an additional factor contributing to the development of obesity in mice.

*L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB and *B. animalis* VKL (separately) or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 and *B. animalis* VKB/*B. animalis* VKL compositions changed the microflora of the intestinal contents of obese mice (increased the number of *Lactobacillus* spp., *Bifidobacterium* spp. and coliform bacteria) against the background of reducing the level of serum cholesterol that can provide a natural, safe alternative protection from obesity.

Treatment of obese mice with *L. casei* IMV B-7280 or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition significantly increased the number of *Lactobacillus* spp. and *Bifidobacterium* spp., in comparison with mice of other groups. The levels of serum total, free and ester cholesterol were more effectively decreased under the treatment of *L. casei* IMV B-7280, and to a lesser extent under the influence of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 composition.

Importantly, *L. casei* IMV B-7280 had the best hypocholesterolemic activity in vitro, as well as the best adhesiveness to epithelial cells compared with other strains that we investigated. *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB and *B. animalis* VKL (separately) and *B. animalis* VKB/*B. animalis* VKL composition are probably less effective in cases of obesity.

### Defining causality vs. correlation: is an inflammation in focus?

Identifying the causative associations of obesity and the human microbiota is still a challenge [75, 85–90]. Communication between the microbiota and immunity alters the metabolic responses during obesity and MetS. The beneficial bacteria can induce pro-inflammatory or regulatory immune responses, depending on the individual phenotype of the gut microbiome, and dietary habits [91]. Obesity coincides with low-level chronic inflammation in metabolic tissues. This obesity-related ‘metabolic

inflammation' involving adipose tissue, liver and muscle, which are key regulators of whole-body glucose homeostasis, drives immunological underpinnings of insulin resistance and cardiovascular disease [65, 91]. Thus, the study by Fåk et al. demonstrated associations between immune modulatory and hypocholesterolemic properties of *L. reuteri* ATCC PTA 4659 probiotic strain which partly prevented diet-induced obesity in Apoe<sup>-/-</sup> mice, yet, induced no effects on blood cholesterol or atherosclerosis and likewise no effect on inflammatory markers (on macrophages or T-cell numbers in plaques) [92]. Lipopolysaccharide, the cell wall component of Gram-negative bacteria in the gut, are supposed as an important trigger of chronic inflammation associated with obesity (Cani et al.) [93].

However, the associations between immune modulatory vs. hypocholesterolemic activity has not yet been finally elucidated.

Obesity-induced endotoxemia [94] and liver dysfunction might be modulated by beneficial microbes via immune response, e.g., by TLR [81] to inhibit the cholesterol synthesis signaling pathway in the liver.

Thus, based on our preliminary data and series of in vitro and in vivo studies and data of trials that show efficacy in both settings [19], we speculate that the ability of the strain to decrease cholesterol may be associated with its immune-modulatory properties.

To tighten up the research power in order to predict outcomes for probiotic studies in a clinical setting and smart utilization of in vivo data is an important task.

### **Calls for new studies and translation: a personalized approach for microbiome-modulating interventions is needed**

Many novel treatment techniques (including probiotics), despite showing their effectiveness, still lack rigorous scientific support [42]. Many of usual, every-day practice treatments found to be effective are still not supported by level I evidence. Probiotic research and the translation thereof is a cornerstone to solve this limitation, possibly successful only via changing health care and extensive public–private partnerships and regulatory bodies [95]. Successful translation of microbiome research will require: a research community; recognition of the effects of food microbiomes and its ingredients on health; appropriate regulations; and trusted products with a clear health benefit to consumers [95].

Selection of LAB as the most effective for lowering cholesterol [61, 78] requires an effective research agenda for translation and requires high validity for prediction results in a clinical setting based on studies in vivo.

Recently, we demonstrated the sequence of strains based on bacterial wall elasticity [71], which correlated with immune modulatory properties. The rigidity of the cell walls among LAB strains was distributed as follows: *Lactobacillus acidophilus*

IMV B-7279 > *Lactobacillus casei* IMV B-7280 > *Lactobacillus delbrueckii* subsp. *bulgaricus* IMV B-7281; and among the *Bifidobacteria* strains: *B. animalis* VKB > *B. animalis* VKL. We suggested the bacterial wall elasticity evaluation as a fast and accurate method to assess parameters of probiotic strains to predict their immune-modulatory properties. According to our observations, strains with the most pronounced immune-modulatory properties also demonstrate a high efficacy in decreasing cholesterol levels; the correlation between in vitro/in vivo studies in decreasing cholesterol levels has been shown, e.g., for *L. casei* IMV B-7280. There are examples of successful clinical implementation [22].

The major strains demonstrating beneficial properties for health in vivo have to be clinically effective and chosen for further studies to be tested more precisely. This approach to choose an appropriate strain would be helpful considering strong biases in the clinical trials.

Evidence might be lacking when a personalized approach (or at least individualized or person-centered) should be initially supposed, but not applied.

The recent advances in predictive, preventive and personalized medicine (PPPM) and/or so-called precision medicine (see debates on term clarification in [96]) open a new era in utilization of the microbiome in human health for patient-tailored preventative or early treatment measures. The successful implementation of PPPM has been made in many domains [42, 96–98]. The recent advances are facilitating microbiome-wide association studies, which are analogous to genome-wide association studies [99]. Personalized modulation of the microbiome via nutritional and pre-, pro- and post-biotic intervention suppose dramatic increasing of their efficacy and level of evidence [42, 100, 101].

In order to achieve this ambitious goal, a diagnostic and predictive panel with a reliable model for stratification of MetS is needed to be created via host profiling using dynamic monitoring of a set of translational biomarkers [4, 10, 65, 101, 102]. A basic panel should include data of the host's sex, age, phenotype and metabolic profile with estimation of levels of cholesterol, lipids, glucose, insulin resistance [10], uric acid, leptin, adiponectin, plasminogen activator inhibitor-1, interleukin-6, interleukin-10, tumor necrosis factor- $\alpha$ , oxidized LDL and paraoxonase-1; imaging data on liver, kidney structure/function, organs vascularity patterns, etc.

Microbiome biomarkers are those related to the etiological role of gut microbiota, like lipopolysaccharide binding protein (LBP), C-reactive protein (CRP), fasting insulin, and homeostasis model assessment of insulin resistance (HOMA-IR) [65], and other host-associated factors influencing the gut microbiota, like variation in vitamin D receptors [101].

Flammer syndrome markers (including NO, endothelin-1, questionnaire data) [102], physical activity patterns and a broad data set on dietary experience [91] should be considered.

Gender aspects for the use of probiotics are unclear; immune response was reported to have differences in both sexes

[103], as gut microbiota differs in men and women and its impact on insulin sensitivity. Therefore, women are considered to be less sensitive to gut microbiota-associated metabolic diseases than men. A low-fat diet is efficacious in reducing the concentrations of TC, HDL-C, and LDL-C but not in reducing TG and TC/HDL-C ratio in women, yet is efficacious in premenopausal women [104].

Study of microbiome under stress, physical and psychical exercises should provide a source of potential biomarkers. The emerging evidence implicating microbiota in stress-related disorders and crosslinks between lifestyle and microbiome provide an intriguing hypothesis to stratify patients according to the response on stress, sterile environment, Flammer phenotype- and hypoxia-related patterns [105–107], and also consider the biomarkers, based on physical activity, movement patterns like gait analysis and feedback-based tests [108], etc. Our recent research in Antarctica provides novel insights in these domains [107]. This early detection and stratification of patients with MetS will support treatment and prevention via nutritional and lifestyle modulation [65].

### **Dose & periodicity of probiotic treatments during studies and antiobesity programs**

The recent review of dose–responses of probiotics suggests that studying higher doses for this endpoint would be most worthwhile. The lack of a clear dose–response for the endpoints does not mean the effect does not exist. In particular, lower doses (less than  $10^8$  CFU/day) are lacking and may explain why a non-effective dose is not commonly identified [109]. Evidence-based recommendations for treatment indications for probiotics suggested a dose of  $10^9$  or higher [37]. Thus, in some cases, dose can be increased. In a volunteer study by Larsen et al. [110], the recovery of a group receiving  $10^{11}$  CFU/day of probiotic strain was demonstrated. High doses of probiotics in humans are well-tolerated [110].

Recommendations on a probiotic treatment duration, breaks between sessions and dietary regime during and after treatment [90] have not been finalized. Heinsen et al. [111] showed that beneficial changes of both gut microbiome diversity and metabolism in obese humans under weight loss intervention were not sustained during weight maintenance.

The correct selection of an optimal time frame for intervention during an antiobesity program is a critical point effecting clinical success. In our study, the cholesterol levels remained increased long after receiving FED, even on the standard diet (see Table 3). On the other hand, the level of serum cholesterol in the current study remained reduced in mice after receiving probiotic bacteria or probiotic compositions.

The recent findings suggested that the microbiome should be targeted during antiobesity programs [111]; close interplay between nutritional modulation of gut microbiota and healthy aging was demonstrated [112]. Thus, calorie restriction can

effectively lengthen the lifespan in various animal models, and has the health-promoting potential of balancing gut microbiota. This is possible due to the competition between the host and gut bacteria for nutrients, which may determine the composition of the feeding medium for homeostatic control of microbiota in the lower gut. This ‘oligotrophic condition’ is recommended to preserve one’s lifespan [112].

### **Probiotic study design**

Important to consider are appropriate designs for conducting, publishing, and communicating results of clinical studies involving probiotic applications in human participants [113, 114]. The experts of the International Scientific Association for Probiotics and Prebiotics (ISAPP) suggest [111] following four recommendations to conduct clinical studies of probiotic and/or prebiotic use: to define the end goal to reach a highest clinical effect and impact; design the study to maximize the chance of a positive response; choose which strain(s) and/or product(s) should be used and why; and carefully select the study cohort.

Nevertheless, we have to admit, that proper design of probiotic clinical trials is rather unfeasible in a large cohorts, especially when done unpersonalized.

Use of preclinical imaging can strongly extend results of an experiment. We suggested a simple and effective preclinical US imaging technique using equipment of general use, applicable on models of mice, rats and larger animals. The evaluated parameters as a cross-section area of the body, visceral fat and liver size are more reliable and much more informative than the mass of an animal, since it allows avoiding bias, induced, e.g., by congestion, gut hyperactivity induced by probiotics and an animals’ appetite, etc. Registration records of longitudinal ultrasound scan of visceral organocomplexes of experimental mice is a method of screenings for study designs with a large number of animals. Mesenteric fat is the largest deposit in the abdominal cavity [115]. We can consider the threshold of mesenteric fat (greater omentum) thickness in mice as high as 1.5 mm for obesity. Since the muscle thickness was observed to be rather stable, the muscle/fat ratio increased during the obesity model. Visceral fat is largely represented by brown adipose tissue (BAT) and can be activated by the sympathetic nervous system or hormones to produce heat [72]. Contrast-enhanced ultrasound (CEUS) can be effective for visceral fat imaging identification, since BAT is a highly vascularized tissue [72]. Additional measuring of subcutaneous fat deposits (dorso-lumbar, gluteal and subcutaneous) and assessment of fat and organic blood flow should be also relevant preclinical imaging markers.

The information regarding colonic microbiota and the colonic mucosa; muscles and nerves in colon can be obtained using non-invasive hybrid techniques, including computed tomography (CT), magnetic resonance imaging (MRI), and US [116].

## Conclusion

*L. casei* IMV B-7280 (separately) and a composition of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 are effective at decreasing the weight of obese mice, decreasing cholesterol level, restoring the liver morphology and beneficially modulating the gut microbiome in high-calorie-induced obesity in a mouse model.

These strains are most promising for creation of probiotic preparations for application in humans.

Ultrasound was feasible and informative in a large amount of small animals and extended the possibilities of the experiment.

## Outlooks and recommendations

We believe that a comprehensive approach for evaluating the efficacy of probiotic strains on an obesity model allows one to select the strains for creating effective probiotic preparations for prevention and treatment of metabolic diseases, which could be recommended for further preclinical and clinical studies.

However, further research of the impact of *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB and *B. animalis* VKL (separately) or *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 and *B. animalis* VKB/*B. animalis* VKL compositions is needed.

Microbiome-wide association studies would be the best option to follow up current research with multiparameter stratification patients with MetS, including data regarding lipids, carbohydrate metabolism, antioxidant system, inflammatory response, etc. on the largest cohorts possible.

The research focus should on potentially increasing the efficacy and the level of evidence via utilization of potentiated effects of probiotic compositions (mixtures) [117] and additional use of prebiotics. Thus, a new vision on prebiotics has been formulated by ISAPP experts in a new consensus [118], supposing that a prebiotic has no need to be involved in the broad metabolism of a beneficial microorganism but rather bias it towards the benefit of the host's health. It has been proposed that the definition of a prebiotic is 'a substrate that is selectively utilized by host microorganisms conferring a health benefit'. This opens an opportunity to test substances that were not previously considered as prebiotics and can be suggested for use with probiotic strains with synergized activity. Thus, potential candidates can be initially suggested as follows: fenugreek [94], nanomaterials, based on gold, nanoceria [119], selenium [29, 120], etc.

The use of probiotics is regulated by the guidelines of a number of organizations including WHO and Food and Agriculture Organization (FAO) [35], World Gastroenterology Organization (WGO) [36], ISAPP [37, 113, 118], European Food Safety Authority (EFSA) [121], United European Gastroenterology Organization (UEG), EPMA [96, 97, 122] and others.

The legislative process is complex and has been recently criticized; in particular, for the EU 'adjudicate claims for probiotics is severely flawed, as has been stated by many outstanding scientists, companies and organizations' [43].

Considering the anticipated rapid microbiome and probiotics research progress in the scope of PPPM, the unification of multidisciplinary approaches is needed, and should involve the EPMA to join leading regulatory bodies in this field in Europe and globally, firstly considering preparation of a 'PPPM position paper on microbiome and probiotics'.

**Acknowledgements** The study was conducted with the support of the State Agency on Science, Innovations and Informatisation of Ukraine.

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**Authors' contributions** RVB suggested the idea and design of the study, participated in experiments, did the ultrasound survey, study analysis, prepared the discussion, formulated future outlooks, and performed the second and final article drafting.

LML, LPB and VVM performed the experiments on animals and analysis of the study.

LML and LPB prepared the first draft of the manuscript, did the literature analysis, interpreted the results, and performed the statistical analysis.

OVN did the liver morphology study.

OMD participated in analysis of the study.

MYS did the organization, manuscript revision, data interpretation, and contributed to the overall development of the studied topic.

All authors read and approved the final manuscript. Funding Not applicable.

## Compliance with ethical standards

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Availability of data and material** Not applicable.

**Competing interests** Authors declare that they have no competing interests.



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