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Effect Of The Functional Status Of Rats Of The Yogurts With Enzyme Modified Starches

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Abstract: *The use of novel food additives to improve the texture and stability of dairy products requires not only technological substantiation. Experimental studies of changes in the functional properties of fermented milk drinks with starch stabilizers should allay the fears of many consumers about the feasibility of such a technological solution. Cow skimmed milk and different types of potato starch including native and enzyme-modified one (AM-0.05 and BL-0.05) were used to prepare yoghurt. These yoghurts were added to the rats' diet for six weeks. The rats' blood was analysed for hematological and biochemical parameters, and their faeces were analysed for microbiological parameters. It was found that consumption of yoghurts with EMS significantly reduced body weight gain, improved feed efficiency, and reduced blood levels of cholesterol, triglycerides and low-density lipoprotein (LDL). In addition, these yogurts showed antioxidant properties, reducing markers of oxidative stress in the liver. Microbiological analysis showed a decrease in coliform bacteria and staphylococci, while maintaining high levels of beneficial lactobacilli. The findings suggest that yoghurts with EMS not only improve the nutritional profile of the product but also provide health benefits, including improved lipid metabolism and gut health. Summarising the results, it is concluded that enzyme-modified starches, in particular BL-0.05, can be a valuable addition to yoghurt formulations, providing functional and health benefits without adverse effects. This study contributes to the growing body of evidence supporting the feasibility of enzyme-modified starches in functional foods.*

Keywords: *yoghurt, potato starch, enzyme modification, health benefits, lipids, gut microflore*

1. INTRODUCTION

Starches from various plant sources are widely used as thickeners in the production of fermented dairy products, such as corn starch [1,2], kudzu starch [3], water yam starch [4], tapioca starch [5], and potato starch [6]. Their use significantly improves the properties of yogurt by correcting its textural deficiencies, especially in the case of fat-free or low-fat versions of yogurt and other beverages [7]. Due to its ease of application in the process cycle of fermented milk beverages and because it is more economical compared to other hydrocolloids such as gelatin, pectin, starch has become one of the most commonly used thickeners for yogurt production [8]. Obviously, different types of hydrocolloids have different physicochemical and functional properties. The main effect of starch is the positive influence on textural, structural-mechanical and technological properties, the introduction of this hydrocolloid reduces the syneresis of fermented milk drinks [9]. Moreover, the addition of starches from various botanical sources positively affects the viability of lactic acid bacteria during storage [10-11].

Yogurt is considered to be a nutritious fermented milk product that contributes to the improvement of human health. Regular consumption of yogurt supports gut health by promoting the growth of beneficial bacteria, which may enhance digestion and immune function. Many studies have been conducted to establish the health benefits of these beverages [12-14]. During the fermentation process, milk transformed into yogurt acquires a whole complex of nutrients. It contains many trace elements such as riboflavin, vitamins, calcium, zinc, potassium and magnesium, and their amounts are higher than in the original milk [15]. Additionally, yogurt's probiotics and bioactive peptides may help reduce inflammation and support metabolic health, making it a functional food with multifaceted benefits. Proteolysis of casein protein takes place during the curdling process, resulting in the release of amino acids and short peptides. Some of these casein-derived peptides have antimicrobial, antithrombotic, and immunomodulatory properties [16]. During the process of milk coagulation, exopolysaccharides accumulate, the beneficial properties of which have been reported by many researchers [17].

Studies on the effects of yogurt on the health of mammals, humans are quite extensive, and metadata on this subject are also collected, studied and analyzed. However, there are few studies on the effects of specifically starches in the composition of yogurts on the health and functional utility of consuming such a product. This means that the use of hydrocolloids in fermented dairy products still involves a huge layer of unexplored issues. In this regard, the aim of the present study was to evaluate the effect of non-fat yogurt with enzymatically modified potato starches (EMS) added as a thickener on a set of health parameters of Wistar rats, to identify changes of the usefulness of yogurt due to the presence of EMS.

2. MATERIALS AND METHODS

2.1. Yogurt Making

Sterile skim cow's milk (fat content 0.05%, Valio, Russia) was used to make yogurt. The total fat content of the milk is 0.16%, protein is 3.18%, and lactose is 4.7%. Potato starch used in the experiment: native GOST 32902-2014 (LLC "Granat"), potato enzyme-modified starch AM-0.05, obtained under the action of different concentrations of Amylosubtilin® (Berdsky Plant of Biological Preparations (Sibbiopharm), Russia; G3x (A - 1500 U/g), and potato enzyme-modified starch BL-0.05, obtained under the action of different concentrations of bacterial amylase Bacillus licheniformis, and described previously [18]. Commercial freeze-dried starter "Yogurt" ("Lactosintez", Russia) (composition: Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus) was used.

Every five days a new batch of yogurt was made, for which 0.1 g/l of dry starter was added to milk heated to 40 °C (milk with starch) and the milk was fermented at 40 °C to pH 4.5, 8-10 hours on average. The yogurt was then cooled and stored at 2-4 °C and used for administration to rats. To make yogurt with starch, 1% of the appropriate starch was added to the milk, the milk was heated to 95 °C and pasteurized for 15 min, the milk was cooled to 40 °C and the starter was introduced, and the yogurt was leavened as described above.

2.2. Animal Experiment Design

Thirty-five male Wistar rats weighing 150-200 g were used in the experiments. The study was approved by the local ethics committee of the Kazan Federal University (protocol No. 40 dated March 9, 2023), on the basis of which the research was conducted. All adaptation rats were kept at 20-24°C in a room with 60-65% humidity and 12-hour light/dark cycle, with free access to standard chow and tap water for 1 week. Commercial diets for laboratory rats and mice (DeltaFids, "BioPro", Novosibirsk, Russia) were used (metabolizable energy - 2500 kcal/kg, crude protein - 19%. Composition: two-component cereal mixture, high-protein components (vegetable and animal proteins), vegetable oil, amino acids, organic acids, vitamin and mineral complex, fiber). Yogurt was administered through a probe.

The rats were also randomly divided into five groups of 7 animals each. To determine the beneficial effects, each group was fed as follows:

(Control) group received basal diet and drinking water without restriction;

(Yog) group received basal diet + low-fat yogurt (2 g/animal) and water;

(Yog+Nat) group received basal diet + low-fat yogurt with native starch (2 g/animal) and water;

(Yog+AM-0.05) group received basal diet + low-fat yogurt with EMS AM-0.05 starch (2 g/animal) and water;

(Yog+BL-0.05) group received basal diet + low-fat yogurt with EMS BL-0.05 starch (2 g/animal) and water.

The experiment was conducted for six weeks, followed by euthanasia by carbon dioxide, blood sampling and analysis, liver sampling for oxidation analysis, and sterile fecal sampling from the colon for microbiological analysis. Body weight and daily food consumption at 8:00 a.m. were recorded weekly during the study period. Feeding efficiency was calculated as the ratio of body-weight gain to total feed intake at the end of the feeding period.

2.3. Analysis of hematologic and biochemical parameters

Blood samples for analysis (hematological and biochemical) were collected from the celiac vein. Hematological blood parameters were analyzed on an Abacus Junior 5 VET hematology analyzer (Diatron Messtechnik GmbH, Austria). Determination of biochemical parameters: cholesterol, total lipids, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), Alkaline phosphatase (AP), cholinesterase (ChoE), total protein, albumin, creatinine, glucose and bilirubin were performed on a ChemWell2902 device (Awareness Technology, USA) using kits or reagents Spinreact S. A. (Spain).

2.4. Liver Tests

Peroxide number. Liver samples (3 g) were weighed into a 100 mL glass Erlenmeyer flask with a stopper. The flask was then heated in a water bath at 60°C for 3 minutes to melt the fat. The flask was then thoroughly stirred for 3 min with 30 mL of acetic acid and chloroform solution (3:2 v/v) to dissolve the fat. Whatman No. 1 filter paper was used to remove beef particles from the filtrate. After the addition of saturated potassium iodide solution (0.5 mL), starch solution was added to the filtrate as an indicator. The titration was continued against standard sodium thiosulfate solution. POV was calculated using the following equation and expressed as milliequivalents of peroxide per kilogram of sample:

$$POV\ (mEq/kg) = \{(S \times N) / W\} \times 100$$

wherein "S" is the titration volume (ml), "N" is the normality of the sodium thiosulfate solution (N=0.01), and "W" is the sample weight (g).

Free fatty acid content. The sample (5 g) was dissolved in 30 ml of chloroform using a homogenizer at 10,000 rpm for 1 min. Filter paper was used to remove bovine particles from the filtrate. After adding five drops of 1% phenolphthalein in ethanol as an indicator to the filtrate, it was titrated with 0.01 N ethanol-potassium hydroxide solution and the free fatty acids (FFAs) were determined.

$$FFA\ (\%) = (mL\ KOH \times normal\ KOH \times 28.2) / g\ sample.$$

Thiobarbituric acid number. Samples (2 g) were mixed with 10 ml of 20% trichloroacetic acid solution (200 g/L trichloroacetic acid in 135 ml/L phosphoric acid solution) in a homogenizer for 30 s. The homogenized samples were filtered through filter paper to remove liver particles. Then, 2 ml of 0.02 M aqueous TBA solution (3 g/L) was added to 2 ml of the filtrate in a test tube. The test tubes were then incubated at 100°C for 30 min and cooled with running water. The absorbance of the supernatants was measured at 532 nm on a UV-VIS spectrophotometer (SF-2000, Russia). TBA levels were calculated using a standard curve and expressed as mg malonaldehyde per kilogram (MA/kg) of liver sample.

2.5. Microbiological Analyses

For microbiological analysis, colonic contents were collected in a sterile 15 mL tube. Analysis was performed no more than 5 hours after fecal collection. Samples were stored at 4°C until analysis. From the fecal masses, 1 g was sterile weighed and serially diluted tenfold with sterile physiological solution. The drop –plate method was used. The number of different groups of microorganisms was determined by serial dilutions, the following nutrient media were used: for LAB (lactobacilli) - MRS agar (Himedia, India), the total number of heterotrophic bacteria - nutrient agar (Microgen, Russia), coli-forming bacteria and E.coli - Endo medium (Microgen, Russia), yeast - Saboura medium (Microgen, Russia), staphylococci - staphylococcal agar (Microgen, Russia). Cultivation after inoculation was carried out at 36 ° C for 1-5 days, depending on the type of microorganisms.

2.6. Statistical Analysis

All analyses were performed in three repetitions. Statistical significance of the results was determined at the $P < 0.05$ level. Data were analyzed for statistical significance using the Mann-Whitney or Kruskal-

Wallis criteria in GraphPad Prism software at a significance level of $P < 0.05$. Visualization of the principal component analysis (PCA) was performed on a bivariate P1/P2 map using Statistica10 (Statsoft), which allowed us to identify relationships between variables at a significance level of $P < 0.05$.

3. RESULTS AND DISCUSSION

Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

During 6 weeks, animals were fed yoghurt daily, during the experiments no animals fell down, all the individuals did not show any deviations in behavior. Regarding the functional health parameters, it was found that the inclusion of yogurt with starches, especially EMS, in the diet led to a decrease in weight gain and an increase in feed consumption efficiency (Table 1). At the macroorganism level, yogurt with starches contributed to a decrease in body weight accumulation, which is a positive effect considering the age of the rats (18 weeks old, sexually mature individuals). Observation of the animals showed that they remained active, had good appetite, and there were no signs of behavioral depression or general toxic effects.

Table 1: Absolute weight gain of rats, weight of feed eaten and feed consumption efficiency over 42 days of feeding in the experiment with the introduction of non-fat yogurt with different starches into a balanced diet. Asterisks show significant difference at $p < 0.05$.

Animal groups	Parameters		
	Absolute weight gain, g/animal	Weight of feed eaten, g total for 7 animals	Feed assimilation efficiency, % (per all animals)
1	2	3	4
Control	111.4±16.6	2965	962.3
Yog	109.0±7.1	3411	901.7
Yog+Nat	62.2±12.1 [a]	4700	1165.6
Yog+AM-0.05	79.2±2.7 [a]	4251	1028.8
Yog+BL-0.05	69.8±5.2 [a]	4136	1200.3

An indicator of the general state of the organism is blood analysis with leukocyte formula. The analysis results showed that in the groups receiving low-fat yogurt and yogurt with added BL-0.05, the leukocyte level decreased (Table 2). This decrease was mainly caused by a decrease in lymphocytes, which may indicate a decreased intensity of inflammatory processes and an improved state of the immune system. The positive effect of yogurt on the immune system is regularly reported [19].

The indicators of hemoglobin and the number of erythrocytes remained unchanged when the fermented milk product was included in the diet. However, it is worth noting the decrease in platelet count compared to the control group, although the platelet count remained within the normal range. This may indicate a reduced risk of thrombosis with regular consumption of the fermented milk product. The addition of starch to the yogurt did not affect the manifestation of this effect.

Table 2: Effect of yogurt on hematologic indices of blood of rats after 6 weeks of experiment. Asterisks show significant difference at $p < 0.05$.

Indicator	Reference values for rats	Control	Yog	Yog +Nativ	Yog +AM-0.05	Yog +BL-0.05
leukocytes, 10^9/L	2.1-19.5	10.26±4.1	4.26±2 [a]	10.83±2.2	7.41±1.7 [a]	5.81±1.5 [a]
lymphocytes, 10^9/L	1.3-14.1	7.02±3.3	3.18±1.7 [a]	7.09±3.3	5.58±3.1	5.28±1.4 [a]
sum: neutrophils, monocytes, ejosinophils, basophils, 10^9/L	0-0.98	0.27±0.04	0.19±0.03 [a]	0.16±0.04 [a]	0.25±0.06	0.10±0.01 [a]
granulocytes, 10^9/L	0-7.5	1.33±0.09	0.66±0.02	1.32±0.15	1.09±0.4	0.44±0.10 [a]

			[a]			
lymphocytes %	25-97	77.8±8.5	84±6.3 [a]	71.7±11.8	75.3±8.7	91.3±1.5 [a]
sum: neutrophils, monocytes, ejosinophils, basophils, %	0-7	2.8±1.3	3.6±2.7	2±0.4	4.4±2.9	1.7±1.2 [a]
granulocytes, %	0-75	19.5±6	12.4±5.1	25±11.4	21.3±7.4	7.3±1 [a]
erythrocytes, 10^{12}/L	4.5-10	12.33±2.3	10.06±2.5	12.8±2.9	10.17±4.3	7.58±1.8 [a]
hemoglobin, g/L	120-180	130±24	122±17.7	124±29.3	122±15.5	129±9
hematocrit, %	35-52	52.0±11.4	47.0±12.4	62.1±14.3	55.4±24.6	45.2±10.9
average erythrocyte count, ft	50-96	55±1.6	55±2.7	56±3.1	59±2.8 [a]	60±0.9 [a]
erythrocyte population distribution, %	–	17.6±0.9	17.3±1.2	17.9±1.1	16.2±1.8 [a]	14.5±0.8 [a]
platelets, 10^{9}/L	150-1370	786±169	221±82 [a]	130±14 [a]	266±78 [a]	414±97 [a]
thrombocrit, %	0-15	0.51±0.20	0.17±0.10 [a]	0.19±0.04 [a]	0.24±0.05 [a]	0.28±0.10 [a]
average platelet volume, ft	–	6.6±0.5	6.3±0.6	6.6±1	6.6±0.5	6.8±0.2
platelet population distribution latitude, %	–	32.6±2.2	33.4±2.2	33.4±3.1	32.6±1.9	32.2±0.4

Levels of cholesterol, triglycerides, and high and low-density lipoproteins were reduced in rats after daily consumption of natural low-fat yogurt compared to the control (Fig.1). In the case of EMS in yogurt, there was a significant reduction in the levels of triglycerides compared to the native starch variant. Importantly, there was a decrease in cholesterol levels when yogurt or yogurt with starch was consumed. Total lipid levels decreased in the yogurt and yogurt with EMS varieties, while lipid levels in the native starch varieties were similar to the control. Many studies have investigated the effects of the reduction of cholesterol levels in the blood of mammals by the consumption of a variety of fermented dairy products [20,21]. In our case, we see a similar picture, but with an increased effect in the case of the EMS that is used in the yogurt. Probably there is a protective effect of EMS on the microbiota of the lactic acid product, a kind of prebiotic effect.

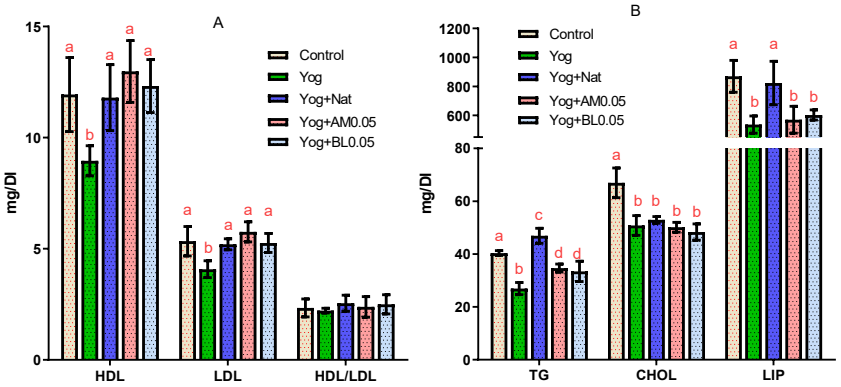

Figure 1. Biochemical indices of lipid metabolism in rat serum after 42 days of experiment: A - high density lipoproteins (HDL), low density lipoproteins (LDL), HDL/LDL ratio; B – triglycerides (TG), cholesterol (CHOL), total lipids (LIP). Asterisks show significant difference at $p < 0.05$.

In the EMS (AM0.05 and BL0.05) varieties, the evaluation of the effect of yogurt on the protein metabolism (albumin, total protein) of rats showed an increase in the albumin level (Fig. 2). Such an

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increase within the normal range may indicate an improvement in the protein diet of the rats, better protein digestion. The creatinine index evaluates the state of kidney function, and an increase in creatinine was detected in the native starch variant, indicating a slight increase in organ stress. In the YOG and YOG_AM0.05 and _BL0.05 EMS diets, the serum creatinine level decreased compared to the control. The blood glucose level of the animals was not significantly different in the experimental groups (receiving yogurt), but was lower than in the control. According to Momoh et al. (2022) [22], consumption of unsweetened yogurt can improve glucose metabolism to some extent, but it is more effective in treating gastrointestinal complications, possibly improving protein digestibility and increasing albumin in the blood.

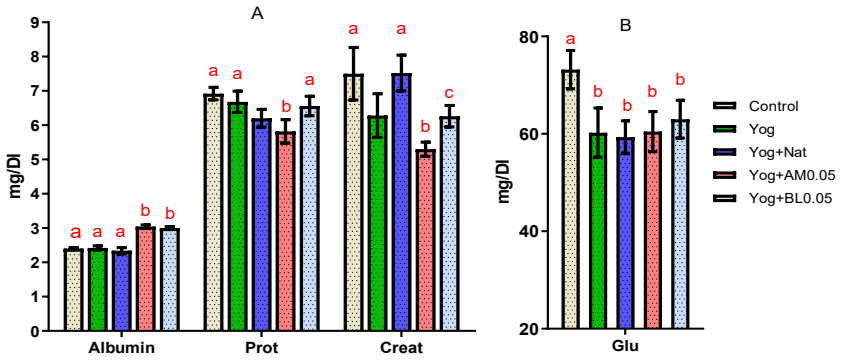

Figure 2. Biochemical parameters of protein metabolism (A – albumin, total protein (prot), creatinine (Creat) and glucose (B – Glu) in serum of rats after 42 days of experiment. Asterisks show significant difference at $p < 0.05$.

The level of activity of serum enzymes: cholinesterase, alkaline phosphatase, AST and ALT, was within the normal range in all variants of the experiment, however, trends of increase in the level of enzymatic activity were found in a number of variants (Fig. 3). For example, in the pure yogurt variant, the animals had higher alkaline phosphatase activity, which may indicate a more active process of calcium assimilation from the diet. The level of AST activity was slightly higher than in the control in the variant with yogurt and in the yogurt with BL-0.05 starch, probably, it is associated with an increase in cardiac activity, improved physical activity. Serum cholinesterase activity is higher in case of using yogurt with native starch in animals' diet than in control group. An increase in the activity of this enzyme within the normal range may indicate an increase in the synthetic activity of the liver.

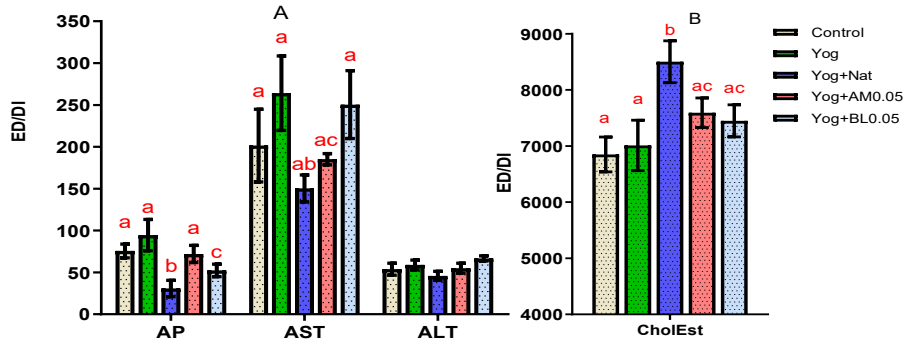

Figure 3. Activity of some enzymes (A - alkaline phosphatase (AP), aspartateaminotransferase (AST), alanineaminotransferase ALT), B – cholinesterase (CholEst)) in serum of rats after 42 days of experiment. Asterisks show significant difference at $p < 0.05$.

Oxidation of liver tissues (Fig. 4) was studied in addition to biochemical markers. The amount of free

fatty acids formed under the action of lipases in yogurt variants is lower than in control, and the lowest level was observed in starch variants. The lowest level of peroxide number in the variant with pure yogurt and yogurt with starch BL-0.05. Malonic dialdehyde decrease was observed in all yogurt varieties. Some authors have shown the positive effect of lactobacilli on lipid metabolism, cholesterol level and liver function [23-25].

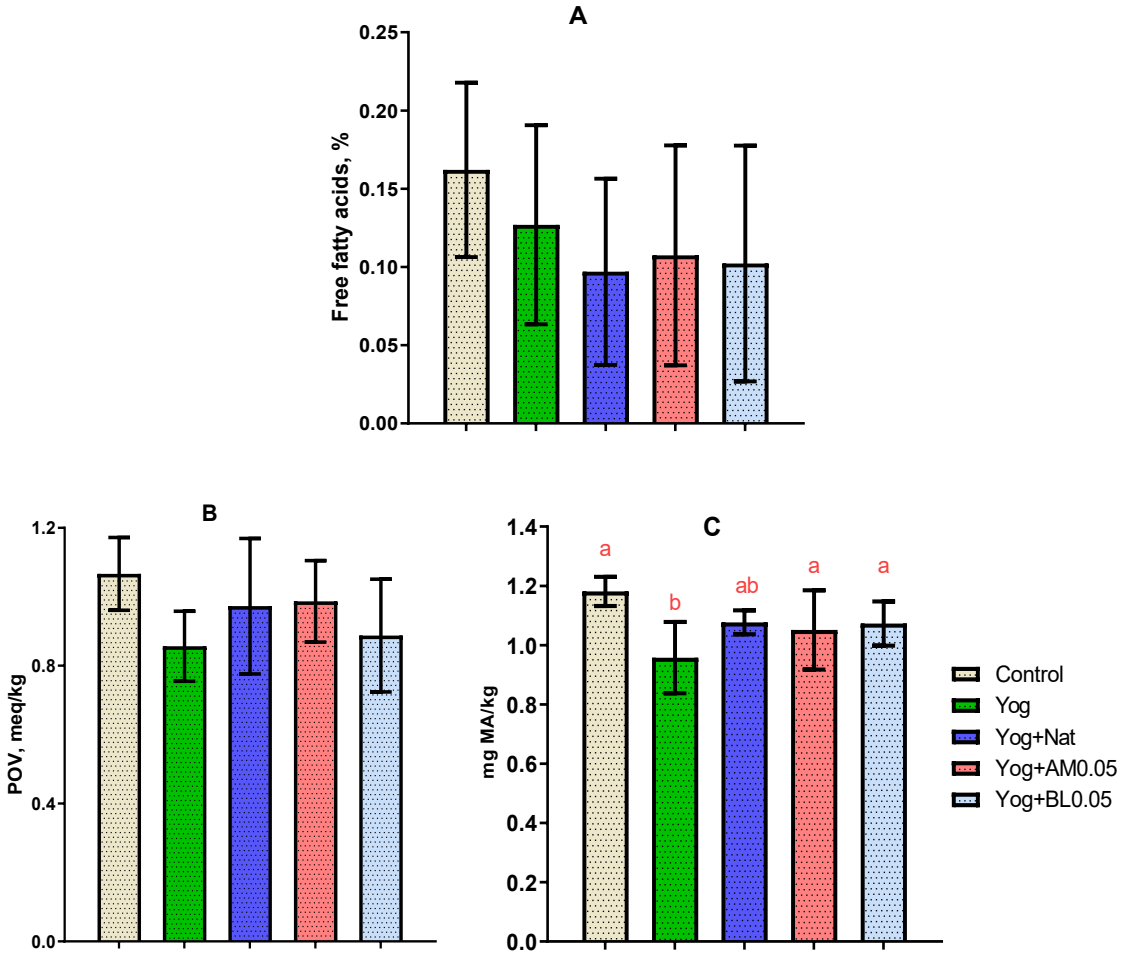

Figure 4: Oxidation indices (A – free fatty acids, B – peroxide number, C – thiobarbituric acid reactive substance (TBARS)) of rat liver after 42 days of experiment. Asterisks show significant difference at $p < 0.05$.

The high antioxidant potential of Lactobacillus has been reported in many studies [26]. LAB can reduce the level of reactive oxygen radicals due to its antioxidant enzyme system [27]. The ability to scavenge peroxides and free radicals has been demonstrated not only in culture broth, but also in fermented milk products [28] and in selected protein components isolated from fermented milk products [29]. The contribution to the overall antioxidant potential of fermented milk products is also made by EMSs, for which we have previously identified antioxidant properties, in particular reducing and radical scavenging activity [30].

Shifts in some important bacterial groups were observed in microbiological studies of rat feces. In particular, a decrease in coliform bacteria, including Escherichia coli, was observed (Figure 5). In addition, yeast and Staphylococcus aureus counts were almost an order of magnitude lower in the yogurt groups with EMS, especially in the Bl-0.05 samples, compared to the control and low-fat yogurt groups. At the same time, the number of lactobacilli remained at a stable high level, above 10^8 CFU/g, in all the groups

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studied, with no significant differences between the variants. The stable positive effect of yogurt with Bl-0.05 starch may be due to its increased resistance to amylase, as previously shown [31]. In fact, a number of studies have shown a positive effect of starches on the abundance of lactic acid bacteria in yogurt. For example, resistant starch from lotus seeds has a strong prebiotic effect [32]. Corn starch in a thermostat yogurt system promotes the growth of beneficial microorganisms such as bifidobacteria and lactobacillus [33]. A second reason for the increased abundance of lactic acid bacteria has also been suggested. Lactic acid bacteria can use starch as a carbon source to support their growth by converting it to lactic acid during fermentation [34].

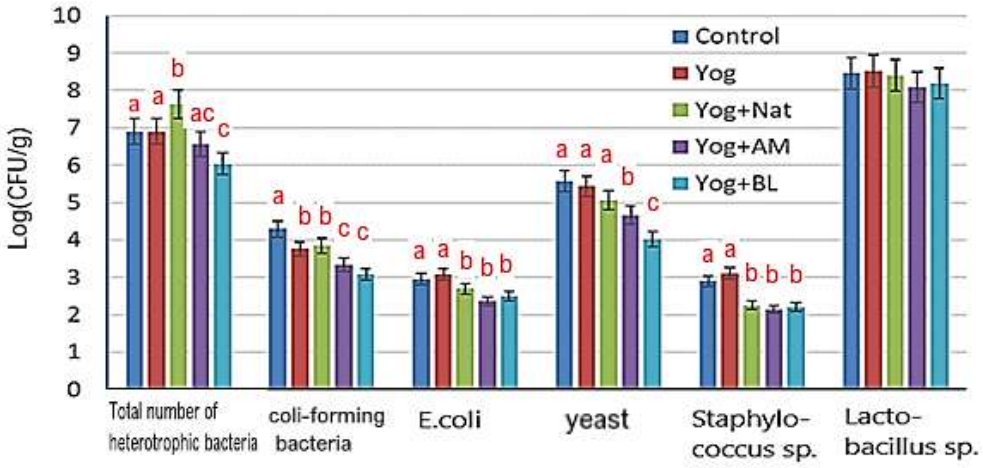

Figure 5. Effect of defatted yogurt with starches on the microflora of intestinal contents of rats (cecal intestine). Asterisks show significant difference at $p < 0.05$.

Statistical analysis by the method of principal components of the data on the state of health of animals revealed significant changes in the location of the experimental groups relative to the control group when a fermented milk drink was introduced into the diet (Fig. 6). The addition of native starch caused more pronounced changes in the totality of indicators than in the group receiving non-fat yogurt. The areas of their distribution practically did not overlap.

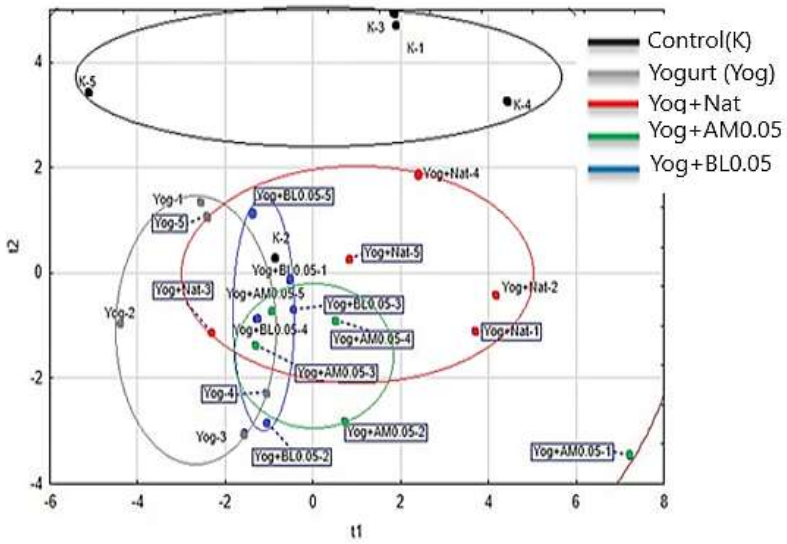

Figure 6. PCA of indicators of different groups of animals depending on the type of yogurt in the diet

The groups that received yogurt with EMS showed a partial overlap, but with insignificant differences between them. Their values are closer to the group that received plain yogurt. Thus, the totality of the results suggests that yogurt with added EMS and yogurt without stabilizer have similar effects on the mammalian organism, which is not the case with the product containing native starch. Thus, the beneficial potential of non-fat natural yogurt and yogurt made with EMS differ little in their effects on the mammalian organism.

5. CONCLUSIONS

The analysis of the obtained data indicates that adding enzyme-modified starch to yogurt can have a positive impact on the physiological and biochemical parameters of mammals. In particular, it enhances the reduction of cholesterol and triglycerides in the blood, reduces oxidative processes in the liver and improves the functional state of organs and the composition of intestinal microflora. Antioxidant properties have also been found to be better in yogurt with EMS. Interestingly, yoghurt with EMS can significantly reduce the number of harmful microorganisms. This is likely a result of its protective effect on lactobacilli as they travel through the gut, and understand their antibacterial capabilities in the large intestine. The addition of a starch-based stabilizer to yogurt has a positive effect on the health of mammals, and this is especially the case when the stabilizer has partial resistance.

AUTHOR CONTRIBUTIONS

Conceptualization, investigation, data curation, writing—original draft preparation, funding acquisition E.N.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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