Biotechnology of starting culture capable of cholesterol metabolism

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Abstract. The article discusses the results of screening strains of probiotic bacteria by their ability to metabolize cholesterol during development on different nutrient media. Cholesterol content was determined by the method of Zlatkis-Zack. The results on the possibility of creating starter cultures from proven strains capable of lowering cholesterol in vitro and taking into account technological properties were presented. Four starter cultures were created, which included microorganisms belonging to two genera: Lactobacillus, Bifidobacterium (strains were deposited). The created starter cultures showed a higher activity for cholesterol reduction than each strain separately. To confirm the revealed effect, studies of starter cultures were carried out in vivo on the SHK line both sexes white mice. The research results showed that when using the created starter cultures in the nutrition of white mice, against a background of elevated cholesterol, there was a decrease in the concentration of total cholesterol in the animal blood by 38.5 % maximum, compared with mice fed with high cholesterol but without starter cultures. The obtained research results allow us to draw to a conclusion about the possibility of participation of the created starter cultures in cholesterol metabolism and recommend them for use in biotechnology of functional foods.

1. Introduction

The importance of starter cultures in the biotechnology of fermented animal products has been proven by numerous studies of scientists around the world, and has been tested in practice for decades [1,2,3]. Starter cultures perform various functions that positively affect the nutritional value, formation of organoleptic, physical, chemical and microbiological indicators of finished products. Starter cultures ferment carbohydrates to form lactic, acetic, propionic and other organic acids, aromatic and other compounds. The functions shown by starter cultures are determined by the properties and nature of the interaction between the strains of microorganisms, which they include. Scientific information indicates that strains are able to exhibit individual properties, such as synthesis of growth inhibitors of pathogenic and opportunistic microorganisms, production of vitamins, exopolysaccharides, superoxide dismutase enzyme, biologically active peptides and other substances [4,5]. It is the manifestation of various functions by starter cultures that is necessary for the formation of not only regulated quality indicators, but also specific properties determining dietary preventive and functional orientation of the resulting...
products. The development of this product segment is associated with an increase in the number of alimentary diseases, such as atherosclerosis, type 2 diabetes, obesity, digestive tract chronic diseases, chronic infections, autoimmune diseases and others, among the population. Timely correction and maintenance of the composition of the intestinal microbiota, which plays a key role in the pathogenesis of many diseases, are an essential factor in supporting vital functions of the human body [6,7,8]. Scientists show more and more interest in studying and increasing the spectrum of new strains of probiotic bacteria included in the list of microorganisms recommended by the IDF/EFFCA. Some probiotic strains may participate not only in the recovery of beneficial intestinal microbiota, but also in the metabolism (including metabolism of cholesterol) in the human body [9,10,11,12,13]. In this regard, there is an objective need for the development and production of food products using starter cultures that include probiotic bacteria with specific properties.

The objective of the studies is to screen the strains of probiotic bacteria for their ability to metabolize cholesterol when developing on different nutrient media and to develop a biotechnology of a starter culture based on them.

The objects of the study were probiotic bacteria strains: Lactobacillus acidophilus AGN-3 (VKPM B-8552), Lactobacillus acidophilus ANT-7 (VKPM B-9471), Lactobacillus acidophilus LA-TR4 (VKPM B-9466), Lactobacillus helveticus LH-4 (VKPM B-9472), Bifidobacterium bifidum GSB-15 (VKPM S-1539), Bifidobacterium adolescentis B-1 (VKPM Ac-1243), Bifidobacterium longum VGB-21 (VKPM S-1540). All strains are deposited in the All-Russian Collection of Industrial Microorganisms (VKPM), NRC "Kurchatov Institute" - GosNIInetekta. Starter cultures created on the basis of the studied strains with the ability to reduce cholesterol.

2. Materials and methods
The ability of probiotic strains to reduce cholesterol levels in vitro was studied in the following media: lactobacilli - MRS broth (Merck KGaA, Germany), medium for lactobacilli (State Research Center for Applied Microbiology & Biotechnology (SRC AMB), Obolensk, Russia); bifidobacteria - MRS broth with 0.05 % L-cysteine (Serva Electrophoresis GmbH, Germany), Blaurocca medium (SRC AMB, Obolensk, Russia). The studies were carried out using the modified method of M. Ziarno [14]. Anaerobic conditions were created using the AnaeroPack anaerobic jar (Mitsubishi Gas Chemical Company, Japan), GENbox Anaer gas generators (bioMérieux, France). The presence of anaerobic conditions was controlled by anaerobic indicators (Oxoid Ltd., England). The strains of probiotic bacteria under study were grown for 24 hours in the liquid nutrient media specified above with the addition of cholesterol (cholesterol LS for biochemistry, 95 % of the main substance, Panreac) at a final concentration of 70 mg per 100 cm³. The seed dose of microorganisms, determined using the McFarland optical turbidity standard, was 107 CFU/cm³ for all strains, the final concentration after 24 h of incubation was 6.0x108 CFU/cm³ for lactobacilli, and (4.0-7.0) x109 CFU/cm³ for bifidobacteria. Since cholesterol in its pure form is insoluble in water, it was dissolved by heating in a mixture of 99 % ethanol and Tween-80 in a ratio of 3:1 to the concentration in solution of 3 mg/cm³. The cholesterol concentration in culture fluid was determined spectrophotometrically by the Zlatkis-Zak method. 3 indicators of optical density were used during the study: a liquid nutrient medium (blank sample); a liquid nutrient medium with cholesterol and Tween-80 (control); a liquid nutrient medium with cholesterol after cultivation of bacterial strains or starter cultures in it and released from cells by centrifugation (experimental samples). The decrease in cholesterol level (in %) was calculated using the Zlatkis-Zak formula [15]. The method of determination is based on the reaction of cholesterol with FeCl₃ in the presence of concentrated sulfuric and glacial acetic acids to form a yellow-colored complex. A 0.1 cm³ sample was dissolved in 3.0 cm³ of glacial acetic acid, then 2.0 cm³ of a color reagent was added (a 10% solution of iron (III) chloride in 100 % glacial acetic acid, diluted 100 times with concentrated sulfuric acid) and carefully mixed, avoiding the formation of bubbles. The obtained solutions were cooled at room temperature and the extinction of experimental samples was measured against the control one (without cholesterol) at a wavelength of 560 nm in cuvettes with an optical path length of 1 cm. A
sample containing only cholesterol (background), without any added cultures under study, was used as zero point. The cholesterol content (D, %) in the samples under study was calculated using the formula:

\[ D = 100 - \frac{E_{\text{exp}}}{E_{\text{zero}}} \times 100\% \]

where, D was a decrease in cholesterol level, %; \( E_{\text{exp}} \) was the extinction of the experimental sample, the unit of optical density; \( E_{\text{zero}} \) was the extinction of the zero sample, the unit of optical density.

The effect of the created starter cultures with probiotic bacteria on cholesterol metabolism in vivo was studied in white SHK mice of both sexes. The animals were divided into 4 groups: those fed a standard diet - group 1, with an increased cholesterol level - group 2; with an increased cholesterol level and developed starter culture SK-X3 - group 3; with an increased cholesterol level and developed starter culture SK-X4 - group 4. Clinically healthy, well-fed, active, agile, well-haired, white mice with a normal color of the mucous membranes and formed stool were selected for the study. To establish the initial lipid profile of the laboratory animals under study and initial indicators of intestinal microbiocenosis, the "Background" group of mice was selected using the free sample method prior to the start of the study. Blood lipids of the animals were determined using the SAPHIRE-400 biochemical analyzer (TOKYOBOEKI, Japan). Fatty acids were determined using the Chromatec Crystal-5000 gas chromatograph (Chromatec SDB JSC, Yoshkar-Ola). Cholesterol BioChemica, AppliChem (Germany), was used in all studies.

Experiments on the effect of starter cultures on cholesterol reduction in vitro were performed in 3 replications. Statistical processing of the results was performed based on the normal distribution.

3. Results and discussion

The ability to reduce cholesterol under in vitro conditions during the development of probiotic bacterial strains under study was determined during their development on nutrient media of different composition. This is due to the fact that the composition of the nutrient medium and cultivation conditions affects the metabolism of microorganisms, including cholesterol-metabolizing activity. When studying the target function of lactobacilli strains, nutrient media that differ in several types of components were used. First, these media differ in the source of amine nitrogen: proteose peptone in MRS and pancreatic casein hydrolysate in the medium for lactobacilli. Second, they differ in redox-lowering components: in MRS, it is sodium citrate, and in the medium for lactobacilli, it is ammonium citrate. Third, the MRS medium and the medium for lactobacilli differ in the composition of salts and index of active acidity. The results of studying the ability of lactobacilli strains to reduce cholesterol level under in vitro conditions on nutrient media that differ in the composition are shown in table 1.

| The name of the strain          | Reducing the concentration of cholesterol in the nutrient medium, % | MRS broth | Environment for lactobacilli |
|--------------------------------|---------------------------------------------------|-----------|------------------------------|
| Lactobacillus acidophilus AGN-3 | 24.4±1.5                                          | 19.1±1.8  |
| Lactobacillus acidophilus ANT-7 | 22.5±1.7                                          | 17.8±1.4  |
| Lactobacillus acidophilus LA-TR4| 30.9±1.4                                          | 24.9±1.6  |
| Lactobacillus helveticus LH-4   | 25.9±1.6                                          | 20.1±1.5  |

It was found that the lactobacilli strains under study showed the greatest ability to reduce the cholesterol level on MRS broth and differed from each other. Lactobacillus acidophilus LA-TR4 strain and Lactobacillus helveticus LH-4 strain showed a higher ability to reduce cholesterol, and
Lactobacillus acidophilus ANT-7 strain showed the lowest ability. The cholesterol-reducing ability of Lactobacillus acidophilus LA-TR4 strain was higher by 37.3 %, and that of Lactobacillus helveticus LH-4 - by 15.1 % compared to the ability of Lactobacillus acidophilus ANT-7 strain in this series of experiments. The results obtained confirm the information of other scientists that the cholesterol-reducing ability of microorganisms is a specific property peculiar for specific strains.

When studying the target function of bifidobacteria strains, we used MRS broth with the addition of 0.05 % L-cysteine and Blaurocca nutrient medium, which differ in several types of components: the source of nitrogen, carbohydrates, antioxidants, fatty acids, salts and vitamins, as well as the indicator of the active acidity of the medium. The results of studying the ability of bifidobacteria strains to reduce cholesterol in vitro on nutrient media that differ in composition are shown in table 2.

Table 2. The ability of the bifidobacteria strains under study to reduce the cholesterol level on nutrient media of various composition.

| The name of the strain                  | Reducing the concentration of cholesterol in the nutrient medium, % |
|----------------------------------------|---------------------------------------------------------------|
|                                        | MRS-broth with 0.05 % L-cysteine | Blaurock          |
| Bifidobacterium bifidum GSB-15         | 37.4±1.5                           | 43.1±1.5          |
| Bifidobacterium adolescentis B-1       | 33.5±1.4                           | 39.0±1.6          |
| Bifidobacterium longum VGB-21          | 31.7±1.7                           | 37.3±1.7          |

All studied strains of bifidobacteria in vitro showed a higher ability to reduce the cholesterol level compared to the studied strains of lactobacilli. Bifidobacteria strains showed the greatest ability to reduce cholesterol on the Blaurocca nutrient medium, which belongs to the classical media for this type of microorganisms. The studied strains of the genus Bifidobacterium differed from each other in their cholesterol-reducing ability, but they had rather high values of the target function. The analysis of the results obtained made it possible to make a conclusion about the feasibility of using Bifidobacterium bifidum GSB-15, Bifidobacterium adolescentis B-1, Bifidobacterium longum VGB-21 strains in the biotechnology of starter cultures.

Based on the studied strains able to reduce cholesterol level in vitro, 4 starter cultures, containing 4 strains of probiotic bacteria each, were developed (table 3).

Table 3. Composition of starter cultures based on probiotic bacteria strains capable of reducing cholesterol.

| Microorganisms introduced into the starting cultures |
|------------------------------------------------------|
| SK-X1       | SK-X2       | SK-X3       | SK-X4       |
| Lactobacillus | Lactobacillus | Lactobacillus | Lactobacillus |
| acidophilus AGN-3 | acidophilus ANT-7 | acidophilus LA-TR4 | helveticus LH-4 |
| Bifidobacterium | Bifidobacterium | Bifidobacterium | Bifidobacterium |
| bifidum GSB-15 | bifidum GSB-15 | bifidum GSB-15 | bifidum GSB-15 |
| Bifidobacterium | Bifidobacterium | Bifidobacterium | Bifidobacterium |
| adolescentis B-1 | adolescentis B-1 | adolescentis B-1 | adolescentis B-1 |
| Bifidobacterium | Bifidobacterium | Bifidobacterium | Bifidobacterium |
| longum VGB-21 | longum VGB-21 | longum VGB-21 | longum VGB-21 |

The results of studying the compatibility of strains using the perpendicular streak technique indicated the absence of antagonism between bacteria. Colonies of probiotic strains in the form of streaks in the zone of their intersection were uniform with no apparent zones of growth inhibition. The possibility of combining strains and joint presence in starter cultures has been established. Studies on the ability of
starter cultures to metabolize cholesterol have shown that they exhibit greater ability than strains in a monoculture. The results are given in figure 1.

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** The degree of cholesterol reduction by individual strains and compiled starter cultures in a nutrient medium (from the level of 70 mg per 100 cm$^3$).

A synergistic effect was found in the reduction of cholesterol by the studied strains included in the starter cultures SK-X1, SK-X2, SK-X3 and SK-X4. The decrease in cholesterol was observed at the level of (in %): 45.4±0.4; 43.9±0.5; 48.9±0.4; 47.7±0.6 of its initial content in the medium for starter cultures SK-X1, SK-X2, SK-X3, SK-X4, respectively. Apparently, symbiotic interactions between the strains of microorganisms contributed to the strengthening of their physiological functions and led to a faster effect on cholesterol in the nutrient medium. It was found that the starter cultures, which included strains with a high cholesterol-reducing ability, had a higher overall cholesterol-reducing ability.

The research results allowed us to determine the rational parameters of biotechnology of starter cultures capable of metabolizing cholesterol. The following sequence of operations in the biotechnology of starter cultures was proposed: obtaining an inoculate of each strain separately on a specific nutrient medium; fermentation to obtain culture fluid; obtaining a biomass by concentrating it; mixing the biomass with a cryoprotective medium; lyophilic drying of the biomass of each strain separately; mixing dry biomass of the strains being a part of the starter cultures; packaging and labeling. The starter cultures were developed in the production environment and passed the control in terms of all the regulated indicators, which met the requirements of regulatory documents.

To confirm the detected effect, studies of the starter cultures SK-X3 and SK-X4, having a higher ability to metabolize cholesterol, were conducted *in vivo* on white SHK mice of both sexes during 21 days. The animals were divided into 4 groups. The number of bifidobacterium and lactobacterium cells in the suspension was at least 1x10$^8$ CFU/cc. The suspension with starter cultures was given to the animals in a diluted form (with sterile water) replacing water in the drinkers (the suspension was replaced twice a day, in the morning and in the evening). The results of the study of the created starter cultures on the ability to participate in the metabolism of cholesterol in the blood of laboratory animals are given in figure 2.
Figure 2. Effect of starter cultures SK-X3 and SK-X4 on the concentration of total cholesterol in the blood of laboratory animals: 1 - white SHK mice of a gender of both sexes that received standard briquetted food (manufactured by Laboratorykorm LLC, balanced in amino acid composition, minerals, vitamins and water, 2 - white SHK mice of a gender of both sexes receiving briquetted food with a high cholesterol content of 2% and water, 3 - white SHK mice of a gender of both sexes receiving a briquetted feed with a high cholesterol level of 2% and a suspension with a starter culture SK-X3; 4 - white SHK mice of a gender of both sexes receiving a briquetted feed with a high cholesterol level of 2% and a suspension with a starter culture SK-X4.

The minimum level of cholesterol in the blood of animals was in group 1 (with a standard diet): 2.8 ± 0.3 mmol/dm³, the maximum one - in group 2: 3.9 ± 0.2 mmol/dm³. The total cholesterol level in group 3 (using the SK-X3 starter culture) was 2.4 ± 0.3 mmol/dm³, and in group 4 (using the SK-X4 starter culture): 2.6 ± 0.2 mmol/dm³.

The results of in vivo studies showed that when using the created starter cultures in the diet of white mice (group 3 and group 4), against the background of increased cholesterol levels in the diet, the total cholesterol level in the blood of animals decreased compared to group 2, which was fed a high-cholesterol diet, but without the use of starter cultures. The data obtained suggest that this effect will also be shown by other created starter cultures, SK-X1 and SK-X2, which showed a slightly lower cholesterol-reducing ability in vitro compared to SK-X3 and SK-X4.

4. Conclusion
In the course of the research, the ability of the studied strains of probiotic bacteria of the genus Lactobacillus and Bifidobacterium to metabolize cholesterol both in vitro and in vivo has been revealed. Nutrient media, on which the studied strains of microorganisms showed the cholesterol-reducing ability during cultivation to a greater extent, have been determined: lactobacilli, on the MRS medium, and bifidobacteria, on the Blaurocca medium. It has been found that the studied strains of microorganisms do not show antagonistic effects against each other and therefore can be used as part of starter cultures. A higher ability to metabolize cholesterol has been found in 4 created starter cultures in comparison with the action of individual strains of probiotic bacteria. The discovered effect has been tested in vivo on white SHK mice that were fed high-cholesterol food. It has been shown that the use of SK-X3 and SK-X4 starter cultures with the ability to reduce cholesterol in animal nutrition led to a decrease in the total cholesterol blood level in the animals by 38.5 % and 33.3 %, respectively. This indicates that the created starter cultures can participate in cholesterol metabolism and can be used in the biotechnology of dietary preventive and functional foods.
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