

PRODUCING OF BACTERIAL CONCENTRATES WITH HIGH CHOLESTEROL LOWERING ACTIVITY

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Abstract: The screening of 8 probiotic strains (*Propionibacterium*, *Lactobacillus* and *Bifidobacterium*) characterized by high cholesterol-lowering activity was carried out in the scientific research laboratory of East Siberia University of Technology and Management (Ulan-Ude, Russia). In order to research biotechnological potential of probiotic microorganisms and to conduct statistical analysis we used a mix of standard and modern physicochemical, biochemical and microbiological methods. The strains *Lactobacillus helveticus* 3₅₋₁, *Propionibacterium shermanii* AC-2503 and *Bifidobacterium longum* DK-100 were found out to have the highest cholesterol-lowering properties. It can be seen that cocultivation of *Lactobacillus helveticus* 3₅₋₁ and *Propionibacterium shermanii* AC-2503 strains shows high population density, cholesterol-metabolizing, antimutagenic, adhesive properties and exopolysaccharide biosynthesis. It indicates stable symbiotic bonds between cultures, which served as the basis for making a combined ferment. The analysis of biotechnological potential of *Lactobacillus helveticus* 3₅₋₁ and *Propionibacterium shermanii* AC-2503 strains and the study of organoleptic, physicochemical, probiotic properties and cholesterol metabolizing activity of the combined ferment have allowed to establish an optimum culture ratio in the ferment – 5 : 95. Optimum cultivation conditions of the continuous culture *Lactobacillus helveticus* 3₅₋₁ and the combined ferment were selected, process parameter of biologically active supplement and frozen ferments of direct loading were substantiated.

Keywords: bifidobacteria, propionic bacteria, lactobacteria, cholesterol-lowering activity, exopolysaccharide, bacterial concentrate

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INTRODUCTION

Nowadays cardiovascular diseases (CVD) are one of the main causes of mortality, disability, social and economic losses. According to World Health Organization (WHO), 23% of early death cases are caused by high blood cholesterol [1].

Increase in cholesterol is connected with the development of the most common disease – atherosclerosis of arterial vessel. Atherosclerosis and its concomitant diseases rank first in illness distribution and adult mortality worldwide [2, 3].

The analysis of reference data indicates that the following factors can cause hypercholesterolemia: exogenous cholesterol load exceeding compensation homeostasis abilities of this sterol in a body, increased cholesterol synthesis in host's organs and tissues, poor transit of cholesterol through digestive tract, poor transformation of cholesterol into bile acids and steroid hormones, poor inclusion of cholesterol into animal cell and microorganism membranes [1, 4, 5].

Reference data indicates that host's microflora is able to participate actively in realization of the processes

mentioned above and to control normal blood cholesterol levels [6–10]. Considering an important role of microorganisms in cholesterol maintenance, the use of probiotic bacteria pertaining to intestinal microflora microorganisms for lowering the level of this sterol in blood serum in case of hypercholesterolemia is of practical and academic interest.

The changes in blood lipids are always affected by profound microecological disorders in intestine. They present in an increased amount of aerobic bacteria, colibacilli, staphylococci, and fungi alongside with the lowering of lactobacteria in feces. Microecological disorders in a human body should be considered as a releaser of a lipid storage disease [11].

Host's microflora synthesizes, transforms and destroys exogenous and endogenous sterols and participates actively in the process of cholesterol metabolism. A significant amount of data on this topic has been stored for the last years. It let us consider host's microflora as the most important metabolic and regulatory organ taking part in the cooperation with cells in maintaining of cholesterol homeostasis [8, 9, 10, 12, 13].

The perspective of probiotic bacteria use for producing innovative pharma products is stipulated by their ability not only to synthesize different wholesome products but also to dissolve and lower a wide range of poisons.

The analysis of reference data on biologically active compounds produced by probiotic microorganisms revealed that biotechnological potential of anaerobic bifidus bacteria, *Propionibacterium*, and lactobacteria microorganisms practically hasn't been used so far. Lactic bacteria have been drawing biotechnologists' attention for a long time since they are potentially significant for health, prevention and treatment of many diseases. Meanwhile, it is needless to say that these microorganisms are a new industrial production source of essential metabolites in biobased product. The importance of this research regarding bacterial ecology and the study of cholesterol metabolism by probiotic microorganisms are determined by the need in producing biobased products for public consumption, which can provide good competition with traditional medicinal products in order to perform health care activities.

The mechanism of probiotics medical and preventive effect is really comprehensive and is stipulated not only by high living cells but also by synthesis of extracellular metabolites enhancing probiotic effect.

Exometabolites of probiotic microorganisms inhibit pathogenic flora development and exhibit radioprotective, antitumour, dismutagenic, immune correcting, and cholesterol metabolising properties [8–10, 12–15].

The number of articles on the ability of some lactobacteria strains to show a cholesterol lowering effect has been increasing through the past few years [9, 16, 17, 18]. Nevertheless, the question on producing food with cholesterol lowering activity is still open.

That is why the study of cholesterol metabolism by probiotic microorganisms and producing of biobased products for public consumption in order to provide health care activities is still a promising direction.

The aim of the research is to produce bacterial probiotic concentrates with cholesterol metabolizing properties.

OBJECTS AND METHODS OF STUDY

The targets of the research are the pure cultures of propionibacteria *Propionibacterium freudenreichii* subsp. *shermanii* AC-2503, *Propionibacterium freudenreichii* subsp. *freudenreichii* AC-2500, *Propionibacterium cyclohexanicum* Kusano AC-2559, *Propionibacterium cyclohexanicum* Kusano AC-2560, received from the stocks of Institution of Biochemistry and Microbial Physiology (Moscow), the strain *Lactobacillus helveticus* 3₅₋₁ and Bifidobacteria cultures *Bifidobacterium longum* DK-100, *Bifidobacterium longum* B379M, *Bifidobacterium bifidum* 8₃, received from All-Russian industrial microorganisms collection of State Research Institute of Genetics and Selection of Industrial Microorganisms and activated by a biotechnological method developed in East Siberia

State University of Technology and Management [19].

Bacteria were cultured in growth medium with the following composition: curd whey, microbiological agar, magnesium chloride, trisodium citrate dehydrate, potassium monophosphate, ascorbic acid.

Purified blood serum was used as a source of cholesterol.

Titrate acidity was determined by GOST 3624-92 (all-Union State Standard) by titration 0.1 N NaOH with phenolphthalein and expressed in Turner degrees.

The measure of active acidity was determined by potentiometric method with the help of pH-222.2 device according to GOST 3624-87.

The growth of active biomass was determined according to the extinction of cell-rich fluid with the help of photocolometric method on KF-77 upon $\lambda = 550$ nm.

The number of propionibacteria cells was determines by limiting dilution culture method in MH (maleic hydrazide) or HMS according to TS 10-10-02-789-192-95.

The number of bifidobacteria cells was determines by limiting dilution culture method in MH-1 according to MG 4.2.999-000.

The number of lactobacteria cells was determined by limiting dilution culture method in solid agar medium MRS according to TS 10-10-02-789-192-95.

Bacteria morphology was studied through Gram staining and the following microscopic examination in immersion system with 90x zoom. The micropictures of cells were taken with the help of digital microscope USB "BIOR".

The concentration of cholesterol in nutritional medium was measured by a fermentation method [20, 21, 22]. The main principle is that cholesterol ethers degrade into cholesterol and fatty acids under the influence of cholesterol esterase ferment. Then cholesterol affected by cholesterol superoxide dismutase gives coloured compound and hydrogen peroxide. Staining intensity in reactor feed is in direct proportion to cholesterol concentration in a sample. Then we measured the absorbency of a test sample (E) and a calibration sample (E_k) against the reagent consisting of ferment mixture at the wavelength 450 nm. The concentration of cholesterol was determined by a computational method. The measurements of test samples of intense green color concede twofold dilution of samples with physiological saline. The data received were divided by 2.

$$C = \frac{E}{E_k} \cdot 4.65, \quad (1)$$

where C is the concentration of cholesterol in a sample, mmole/L; E is the extinction of test samples; E_k is the extinction of calibration samples; 4.65 is the concentration of cholesterol in calibrator, mmole/L.

The selection of cultures in combined ferment was exercised considering cholesterol metabolizing and probiotic properties.

Exopolysaccharide concentration was measured by an anthrone method [23]. The definition is based on the fact that furfural, 5-methylfurfural or 5-hydroxy-methylfurfural when interacting with anthrone give the

product of intensive green or teal color and they are formed in the process of strong sulphuric acid attacking carbohydrates.

Experimental procedure was the following: 1 volume of microbial consortium was added to 4 volumes of deionized water and 10 volumes of freshly mixed anthrone reagent. The mixture was incubated for 10 minutes at 100°C in water bath. The concentration of endoplasmic reticulum was measured by a spectrometric method at the wavelength 620 nm. Glucose solutions of different concentrations were used as a standard.

Ames test was used to determine antimutagenic activity [24]. Test strain *Salmonella enteritidis* was used for determining antimutagenic activity. The principle of this method is that histidine revertants, the number of which reveals mutagenic effect, develop under the influence of mutagen. Subsequently, antimutagenes lower the number of induced revertants.

Experimental procedure was the following: 2 ml of top agar consisting 0.5 mm of histidine/biotin were added to 0.1 ml of fresh culture *S. enteritidis*, 0.1 ml of mutagen under test and 0.1 ml of sample used as a mutagen. The mixture was stirred fast and then powered to the surface of minimal agarized medium (lower agar) into Petrie dishes. Through rapid mixing top agar was equally spread on the surface of lower agar. The dishes were incubated for 48 hours at 37°C. At the same time we put positive control when mixture contained mutagen and no antimutagen, and negative control when there was no mutagen but potential antimutagen was present. The total volume of the mixture was brought to 0.4 ml with the help of 0.2 M of phosphate buffer, pH 7.4. The choice of mutagen concentrates was determined after trial dose-response experiment. Mutagen concentrates from lineal parts of the dose-response curves were used. For sodium azidemutagene it was 3.0 mkg/dish, for nitro-soguanidine – 10 mkg/dish. In some cases we pre-incubated mutagen and antimutagen for 20 minutes at 37°C.

After incubation we counted the number of revertants in the dishes. The experiments were carried out thrice and then statistical data were processed.

Antimutagen activity is determined by the formula:

$$\text{Inhibition (\%)} = \frac{(a - b) \cdot 100}{a - c}, \quad (2)$$

where a is a number of histidine revertants, induced under the influence of mutagen; b is a number of histidine revertants, induced under the influence of mutagen in the presence of antimutagen; c is the number of revertants, developing in the presence of only one antimutagen.

In all cases the number of spontaneous revertants was taken into account and subtracted.

Adhesion properties were studied on formalinized red blood cells by a detailed method of V.I. Brilis [25]. A mixture of formalinized human red blood cells O(I) Rh+ blood type and microorganism suspension ($1 \cdot 10^9$ cells/ml) was incubated at 37°C during 30 minutes and was regularly stirred. Then we prepared a sample, dried, fixed and stained by Romanowsky-Giemsa method. The study of adhesion was conducted under light microscope, the count was kept considering in all no less than 50 red blood cells. In characterizing adhesive properties we used the following criteria: average adhesion score (the average number of microorganisms, having attached to one red blood cell); adhesion index (the percent of red blood cells that have adhesive microorganisms on their surface); microorganisms adhesion index (an average number of microbial cells on red corpuscles). Only those red blood cells that took part in the adhesive process were taken into account. Microorganisms were considered non-adhesive in case of microorganisms adhesion index was from 1.76 to 2.50, middle-adhesive – from 2.51 to 4.00, and highly adhesive in case of microorganisms adhesion index being ≥ 4.1 . Red blood cells from only one donor O(I) were used for abundance by the standard terms.

Statistical data were proceeded with the help of program package “Statistica 6”. We used Mann-Whitney rank test for comparing independent subsets. The differences were considered significant in case of error probability $p \leq 0.05$.

RESULTS AND DISCUSSION

At the first step, we studied cholesterol metabolizing activity of different probiotic microorganisms strains in the process of cultivation. The results of the research are presented in Table 1.

Table 1 data analysis shows that all the probiotic microorganisms strains studied in the process of cultivation in growth medium laced with blood serum as a source of cholesterol lower the level of this steroid compound.

Table 1. Cholesterol metabolizing activity of probiotic microorganisms

Strain name	Cholesterol concentration in growth medium, mmole/L							The rate of cholesterol destruction, %
	culture time, h							
	0	4	8	12	16	20	24	
<i>P. Freudenreichii</i> AC-2500	4.92	4.92	4.81	4.48	3.98	3.32	2.92	40.64
<i>P. Kusano</i> AC-2559	4.92	4.92	4.89	4.68	4.10	3.36	2.89	41.36
<i>P. Kusano</i> AC-2560	4.92	4.92	4.86	4.53	3.91	3.21	2.74	44.22
<i>P. shermanii</i> AC-2503	4.92	4.91	4.68	4.25	3.67	2.93	2.64	46.32
<i>B. longum</i> B379M	4.92	4.92	4.90	4.76	4.19	3.68	3.02	38.68
<i>B. longum</i> DK-100	4.92	4.92	4.88	4.61	4.03	3.28	2.85	42.13
<i>B. bifidum</i> 8 ₃	4.92	4.92	4.91	4.81	4.31	3.87	3.10	37.08
<i>L. helveticus</i> 3 ₅₋₁	4.92	4.90	4.54	4.06	3.36	2.54	2.38	51.70

The *L. helveticus* 3₅₋₁ strain has the highest cholesterol lowering activity. This culture binds up to 51.7% of cholesterol. The level of propionibacteria cholesterol degradation is ranged within 40–46% of cholesterol source quantity put into cultivation medium. *P. shermanii* AC-2503 strain has the highest cholesterol lowering activity among propionibacteria. It binds 46.32%. *P. freudenreichii* AC-2500 has the lowest cholesterol metabolizing activity (40.64%). Also it's worth noting that bifidobacteria have high cholesterol metabolizing activity as well (up to 37–42%). *B. longum* DK-100 strain is the best cholesterol destructive (42.13%). *B. bifidum* 8₃ strain is the less active one (37.08%).

As we can see in table 1, the amount of cholesterol for the first 8 hours of cultivation decreases insignificantly, then it is being intensively destroyed and in 24 hours the destruction reaches its peak.

The analysis of the research conducted let us come to a conclusion that all the probiotic microorganism strains analyzed are characterized by reasonably high cholesterol metabolizing properties which depend on species and strains.

At the next step of the research we studied culture association of *Lactobacillus helveticus* 3₅₋₁ and *Propionibacterium shermanii* AC-2503 that possesses the highest cholesterol lowering properties.

Numerous studies prove that multiple strain starters are resistant to unfavourable medium factors and have higher biotechnological potential. That's why the top priority in culture selection is to study symbiotic relationship between *L. helveticus* 3₅₋₁ and *Pr. shermanii* AC-2503.

Considering high acid forming ability of *L. helveticus* 3₅₋₁, co-culturing was carried out at comfort for propionibacteria growth temperature (30°C).

The choice of microorganism's optimum ratio in the combined ferment was conducted according to biochemical, probiotic and organoleptic properties of

cultures and their associations.

The results of the research are presented in Table 2.

The data received indicate rising cholesterol metabolizing activity in case of co-culturing. The highest degree of cholesterol lowering is observed by the ratio of 20 : 80 (67.0%), but in this case hyperacidity is noticed and it has an adverse impact on organoleptic indicator of a combined ferment. The best organoleptic indicators were observed by cultures ratio of 5 : 95. In this case the degree of cholesterol lowering falls insignificantly (63.0%).

The results presented in Table 2 let us regard the observed microorganisms as the potential producers of micronutrients possessing antimutagen and adhesive properties, the concentration of which increases under the conditions of co-culturing.

High population density of *L. helveticus* 3₅₋₁ and *Pr. shermanii* AC-2503 was noted. It indicated good compatibility and microorganisms symbiosis. It can be probably explained by high proteolytic activity of *L. helveticus* 3₅₋₁ culture which splits protein components with the formation of peptides, amino acids and creates favorable conditions for propionibacteria growth.

It is worth noting that nowadays, microbiological paradigm is gradually changing. We are moving beyond the idea of microorganisms monocellularity to the idea of microbial colonies being integral epiorganisms. It reflects in the growing interest for microbial colonies form, picture, and macro- and microstructure. That is why the following research is dedicated to bacterial morphology in a combined ferment. The morphology of ferments is presented in Fig. 1.

As we can see on Fig. 1, *L. helveticus* 3₅₋₁ cells have a form of single bacilli and *P. shermanii* cells have a form of long and short chains. Cell aggregation can be observed in a combined ferment of propionibacteria and lactibacteria, showing cell cooperation – aggregation (cohesion).

Table 2. The choice of cultures optimum ratio in a combined ferment

Indexes	Characteristics if ferment				
	<i>L. helveticus</i> 3 ₅₋₁	<i>P. shermanii</i> AC-2503	Variant of combined ferment <i>L. helveticus</i> 3 ₅₋₁ : <i>P. shermanii</i> AC-2503		
			20 : 80	10 : 90	5 : 95
Cholesterol metabolizing activity, %	51.70	46.32	67.00	65.10	63.00
Acidity, °T	93–95	68–70	90–91	88–89	82–83
Active acidity, pH	4.68	4.98	4.69	4.71	4.72
VFA content, mg/100g	2.8	2.4	3.8	3.9	3.9
Fermentation activity, h	6–8	12–14	6–7	8–9	8–10
Cell count, CFU/cm ³					
<i>L. helveticus</i> 3 ₅₋₁	2·10 ⁹	–	1·10 ⁹	1·10 ⁹	1·10 ⁹
<i>P. shermanii</i> AC-2503	–	1·10 ⁹	2·10 ⁷	1·10 ⁸	2·10 ⁹
Adhesive activity:					
AAS*	4.5	4.6	4.7	4.9	5.1
ECF**, %	85	85	86	87	88
MAI***	5.29	5.4	5.51	5.6	5.69
Exopolysaccharide biosynthesis, ug/ml	0.578	1.88	1.90	1.92	2.01
Antimutagenic activity (inhibition), %	50.2	50.3	52.5	53.6	53.9

Notes. AAS* – average adhesion score; ECF** – erythrocyte contribution factor; MAI*** – microorganisms adhesion index.

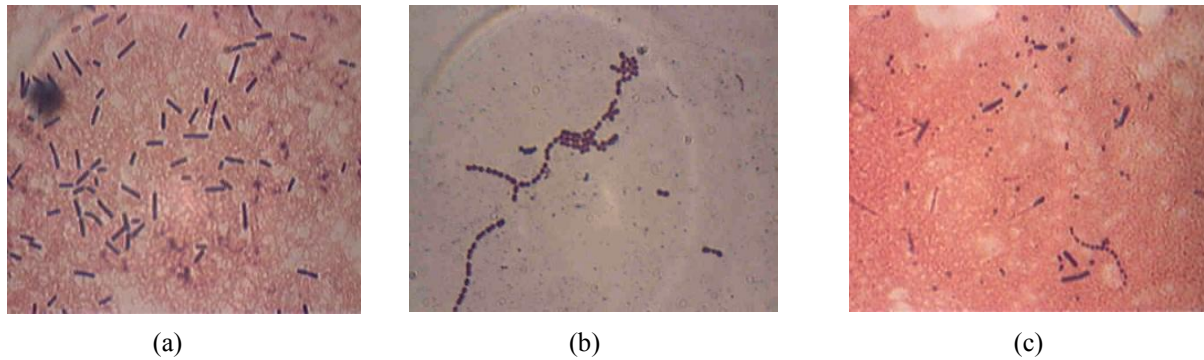


Fig. 1. Morphology of *L. helveticus* 3₅₋₁ ferment (a), of *P. shermanii* AC-2503 ferment (b) and of combined ferment (c) on milk.

From reference data [26, 27] we know that cohesion is not just a number of cells but a special supraorganismal system similar but not identical to multicellular organism. The special feature of such system is the cooperation of certain cells when their common activity targets achieving the same results. The property of such system is cooperation of certain cells when their concerted activity is aimed to achieving one and the same result. One of the cooperation mechanisms is communication (signals and information exchange by means of extracellular metabolites functioning, which regulates bacteria activity). The formation of such cooperation provides adaptive, physiological cell resistance to the influence of ambient negative conditions.

As can be seen from the above, optimum culture balance 5 : 95 that provides high cholesterol metabolizing activity, extracellular metabolites synthesis and also fine organoleptical properties, was chosen.

Starter activity plays an important role in the process of microorganism cultivation. The influence of inoculum dose on cell growth was studied on the next step. *L. helveticus* 3₅₋₁ culture, *L. helveticus* 3₅₋₁ and *P. shermanii* AC-2503 combined ferments in proportion 5 : 95 were used as inoculum. Inoculum dose varied from 1 to 3%. The received data are presented on Fig. 2 and 3.

As we can see from Fig. 2 and 3, more active microorganisms growth is observed upon stepping up the inoculum dose from 1 to 2%. Further ferment dose increase up to 3% doesn't lead to significant growth.

Then the growth of probiotic microorganisms biomass and the changes of cholesterol level in the process of cultivation were studied next. The results of the research is presented on the Fig. 4.

On the picture 4a we can see that the most active growth of bacterial mass was observed in the process of combined inoculum cultivation, it indicates mutual culture stimulation. It was proved that active cholesterol lowering takes place in the process of cultivation (Fig. 4b). It was also noted that for the first 6 hours the amount of cholesterol decreases insignificantly. This period of microorganisms growth is called lag growth phase, during which the culture adapts to the new environment. Then follows exponential growth phase characterized by the highest speed of culture growth. Cell number dose depends linearly on time. The growth medium peters out as the

result of culture growth, metabolism products accumulate and start to lower cholesterol intensively. This period starts after 6 hours of cultivation and lasts up to 18 hours. Phase of growth declining cells regression (stationary phase) is observed after the intensive microorganism growth when spatial delimitation appears upon culture biomass increase and leads to weakening of microbial cell contact with the growth medium [14, 28]. As the result, microorganism cholesterol lowering activity decreases.

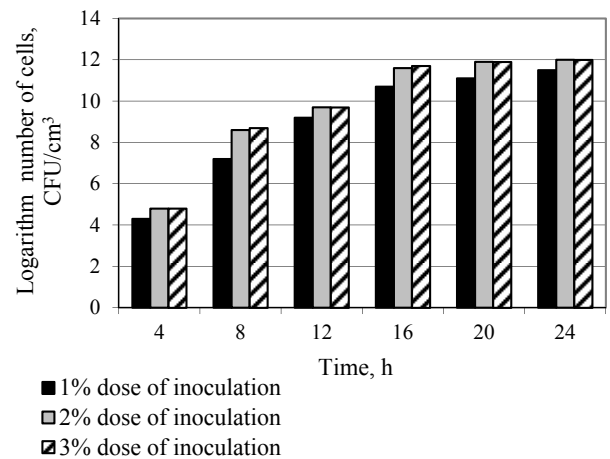


Fig. 2. The influence of *L. helveticus* 3₅₋₁ inoculum dose on cell growth.

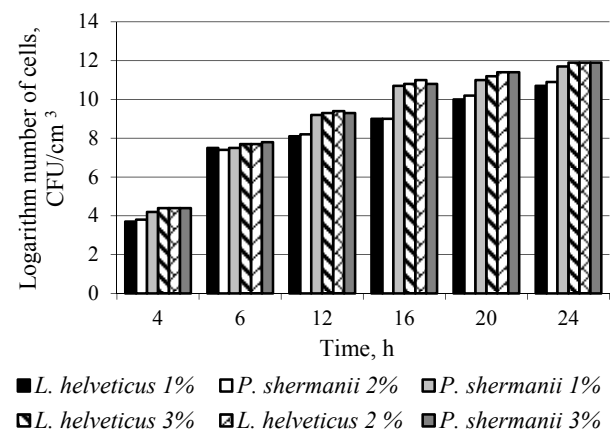
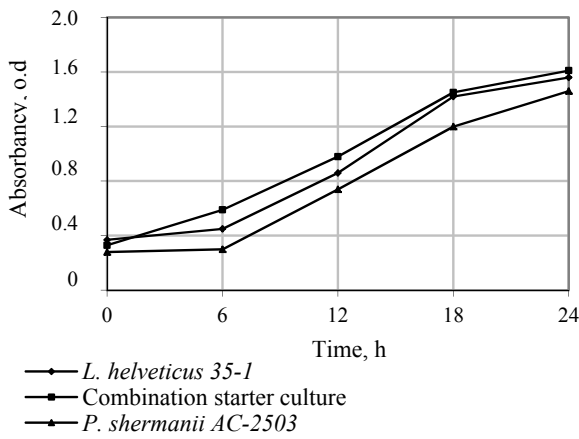
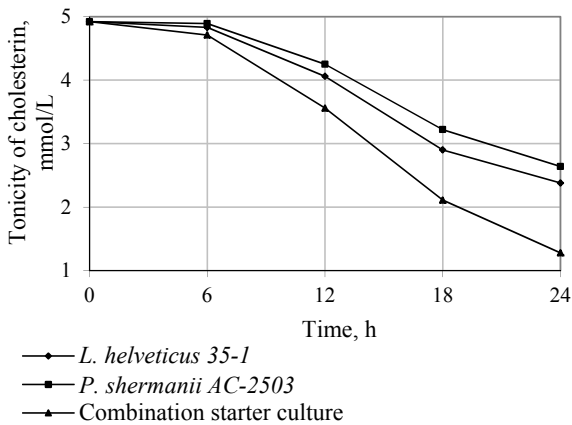


Fig. 3. The influence of *L. helveticus* 3₅₋₁ and *P. shermanii* AC-2503 inoculum dose on cell growth.



(a)



(b)

Fig. 4. The growth of probiotic microorganisms biomass (a) and the change of cholesterol level (b) in the process of cultivation. Combination starter culture.

It is worth saying that the biggest amount of cholesterol is destroyed at the end of exponential growth phase after 18 hours of cultivating. This is due to the fact that enough amount of biologically active compounds is accumulated in the growth medium, and the biomass increase reaches its maximum level. The highest cholesterol metabolizing activity is observed upon combined inoculum cultivation (63.0%). At that, the number of living cells in continuous culture *L. helveticus* 35-1 and in combined ferment reaches 10^{12} cm³.

The active growth of propionibacteria in a combined ferment was elicited (Fig. 5).

The analysis of the received data indicates that upon chosen cultivation process parameters *L. helveticus* 35-1 and *P. shermanii* AC-2503 strains develop well in the growth medium on the basis of milk whey. On the basis of the conducted research we chose optimum conditions for cultivation of *L. helveticus* 35-1 and combined ferment: inoculum dose – 2%, cultivation temperature – 30°C and the duration – 18–20 h.

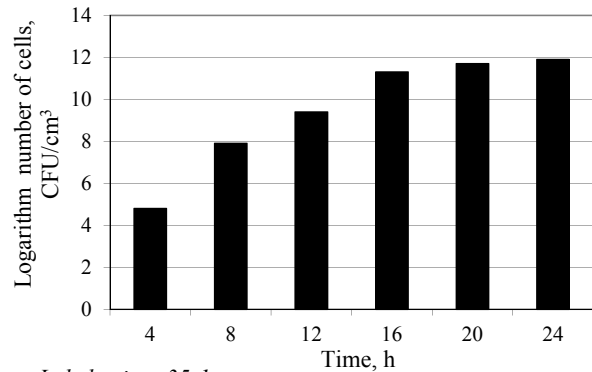
Received experimental data revealed that *L. helveticus* 35-1 is characterized not only by marked cholesterol lowering activity, but also by fine probiotic properties that served as a methodological foundation for producing ferments. The research of structure-functional foundation of *L. helveticus* 35-1 and

P. shermanii AC-2503 cooperation proves the perspectives of combined ferment use in bacterial concentrates production.

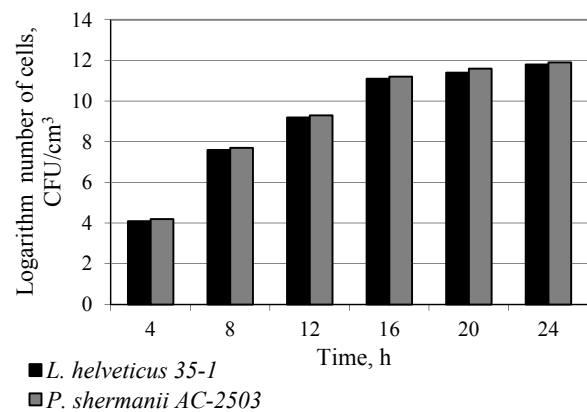
As the result of conducted research we chose optimal technological parameters of getting concentrates. The technology of getting concentrates includes such operations as growth medium preparation, introduction of inoculum and upstream. After the process of cultivation culture liquid is segregated from biomass. This liquid concentrate can be used as biologically active additives. For production of frozen concentrated ferments of direct loading biomass is decanted to the complete segregation of culture liquid and is mixed with the protective medium. The protective medium aims to decrease cell damages in the process of freezing. It consists of water, sucrose and Tri-Sodium citrate dehydrate. The protective effect can be explained by the ability of sucrose to hydrate, to decrease the point of freezing water and to slow down the speed of crystallization.

The researches indicate that after the freezing process the number of probiotic microorganism living cells decreases insignificantly and biochemical activity is preserved. The mixture is flaked, frozen after blending with the protective medium and is kept until realization.

The storage life of BAA (biologically active additives) is 120 days and the storage life of frozen ferments of direct loading is 240 days during which the high amount of living cells and biochemical activity are preserved. Qualitative description of concentrates is presented in Table 3.



(a)



(b)

Fig. 5. Growth rates of *L. helveticus* 35-1 (a) and the combined ferment (b) in growth medium.

Table 3. Qualitative characteristic of bacterial concentrates

Index	Characteristic of biologically active supplement		Characteristic of frozen ferments of direct loading	
	BAA on the basis of <i>L. helveticus</i> 3 ₅₋₁	BAA on the basis of acombined ferment	Ferment of direct loading <i>L. helveticus</i> 3 ₅₋₁	Combined ferment
Flavor and aroma	Clean, slightly sour, tasteless and odorless			
Consistency	Homogenous with subtle whey residuum		A small pillar of frozen suspension	
Color	From white to slightly yellow			
pH limit	4.2–4.4	4.6–4.8	5.4–5.6	5.8–6.0
Fermentation activity, h	–	–	6–8	8–10
Cholesterolmetabolizing activity, %	51.73	63.00	49.45	62.30
Antimutagenic activity (inhibition), %	50.2	53.9	48.3	51.5
Adhesive activity:				
AAS	4.5	4.8	4.1	5.1
ECF, %	85	86	83	88
MAI	5.29	5.58	5.21	5.69
Exopolysaccharide, ug/ml	0.578	2.010	0.569	2.310
Outlet temperature, °C	4–6		Minus 25	
Number of cells, CFU/cm ³				
<i>L. helveticus</i> 3 ₅₋₁	4·10 ¹²	2·10 ¹²	2·10 ¹²	1·10 ¹²
<i>P. shermanii</i> AC-2503	–	4·10 ¹²	–	3·10 ¹²
The volume of product (cm ³) consisting				
Coliforms		10		10
<i>S. aureus</i>		10		10
Pathogenic microorganisms (including salmonella)		50		100
Yeasts and mold, CFU/cm ³ , no more than		10		5

The analysis of data in Table 3 indicates that developed BADS and frozen ferments have high cholesterol lowering ability. BADS are recommended to be used in practical healthcare and frozen cells can be used in the production of fermented milk products of functional nutrition.

The results received in this study show relatively high cholesterol lowering ability of probiotic microorganism which depend on specie and strain. The change of cholesterol concentration in cultivation process shows the interrelation of cholesterol level and incubation time. The highest cholesterol lowering activity was registered at the end of exponential growth phase when the highest amount of metabolites accumulates. The received results are coherent with reference data on cholesterol lowering activity of probiotic microorganisms [13, 16, 29].

Though the mechanisms of cholesterol destruction are understudied, some data point to the fact that probiotic microorganisms regulate endogenous cholesterol synthesis through the formation of short-chain fatty acids that accumulate in the process of fermentation.

It is known that propionate formed in the process of propionibacteria cultivation is able to lower cholesterol level. Rectal introduction of acetate and propionate to adults in proportion of 3 : 1 is followed by the lowering of cholesterol concentration and the increasing of triglycerides amount in blood serum. Consequently, upon accession of probiotic microorganisms into digestive tract they will synthesize volatile fatty acids and change acetate and propionate pool in a body and regulate cholesterol synthesis [27].

Probiotic microorganisms (bifidobacteria, propionibacteria and lactobacteria) showing proteolytic, hydrolytic, lipolytic or other biochemical activity are able to modify regulatory combinations or lower them controlling cholesterol formation [30, 31].

One more significant result of this work is the creation of combined ferment from *L. helveticus* 3₅₋₁ and *P. shermanii* AC-2503 cultures with high biotechnological potential. Optimal culture choice of propionibacteria and lactobacteria increases not only the degree of cholesterol destruction but also biosynthesis of exopolysaccharides, antimutagenic substances and volatile fatty acids. The formation of short-chain fatty acids, as mentioned above, regulates cholesterol synthesis. The increase of adhesive activity, exopolysaccharides and antimutagenic properties in combinations of different microorganisms will contribute to better colonization rates and adaptation of microorganisms in extreme conditions when passing through digestive tract.

In natural cenosis different microorganisms are represented by organized population clusters possessing collective functions and cooperation [32]. Nowadays we have a concept of “biological bacterial consortium” consisting of morphologically and functionally differentiated microorganisms performing a complex of metabolic process on cooperative basis [6, 33].

Adaptation to environmental factors is provided by mechanisms ensuring the stability of microbial consortium. These mechanisms include, for example, cell cooperation – bounds (cohesion) and solid attaching of cells to substrate (adhesion) [26, 30, 34].

According to our sources, morphological cell differentiation of propionibacteria and lactobacteria in combined ferment is stipulated by high exopolysaccharide potential of propionibacteria, which helps to form microcolonies.

As long as mechanisms of cell junction realization and cell attaching to substrate are provided by the same structures [35], our research proves the applicability of highly adhesive propionibacteria and lactobacteria strains as perspective for producing bacterial preparations.

Antimutagenic substances formed in combined ferments, first of all, protect microorganisms from mutations under the influence of different chemical and biological mutagens. Also, practical realization of such concentrates will enable producing of biobased products with antimutagenic properties.

Bacterial concentrate from the mixture of propionibacteria and lactobacteria cultures (*L. helveticus* 3₅₋₁ and *P. shermanii* AC-2503) with high biochemical and cholesterol metabolizing activity was developed during this research for the first time. Technological parameters of its preparing for further practical use were also substantiated.

Received in this paper data indicate that cholesterol can modify and degrade by both pure and mixed cultures. They can be used practically for product-line expansion of fermented milk products of functional nutrition.

In conclusion, the received data open wide perspectives for searching new strains of probiotic microorganisms that synthesize essential biologically active substances, and aim at sequential realization of their unique metabolism in biotechnology.

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