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Effects of Polyunsaturated Fatty Acids of Cedar Oil on Quality of the Dietary Supplements Based on Bifidobacteria

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Abstract

The article deals with the study of effects of polyunsaturated fatty acids of cedar oil on cholesterindegrading activity of bifidobacteria. It is revealed that adding cedar oil in the concentration of 1.5% into a nutrient solution in the course of cultivation of all bifidobacteria strains leads to active degradation of cholesterol compared to control. But the greatest hypocholesterolemic effect is peculiar to a strain of *Bifidobacterium longum DK-100*, which in the biomass scale-up process in the nutrient solution with cedar oil destroys 68% of the total cholesterol. Analysis of fatty acid composition of the bifidobacteria biomass has shown that monounsaturated acids are characterized by dominated content of monounsaturated oleic acid, while polyunsaturated acids are characterized by dominated linoleic acid. When cultivating bifidobacteria in a nutrient solution with cedar oil, we observed increase in the content of linoleic acid in biomass, and decrease in concentration of linolenoic and oleic acids, that might be associated with their metabolism. In the biomass of bifidobacteria with cedar oil we detected γ [gamma]- linolenoic acid of omega-6 family, which was characterized by high physiological activity. Optimization of the nutrient solution by polyunsaturated fatty acids (PUFAs) increases the number of viable bifidobacteria cells that contributes to more intense destruction of cholesterol. Based on the obtained experimental data, we have developed production technology of dietary supplements, enriched with polyunsaturated fatty acids with high cholesterindegrading activity.

Key words: bifidobacteria, polyunsaturated fatty acids, cedar oil, cholesterindegrading activity, bacterial concentrate.

INTRODUCTION

At present, diseases of the cardiovascular system represent the main danger to public health and a major problem to medical care. Atherosclerosis is one of the most important problems of contemporary medicine, and its manifestations play an important role in occurrence and subsequent course of coronary heart disease, which, in turn, takes the first place in the general structure of the cardiovascular system diseases. In pathogenesis of atherosclerosis, an important role is given to disorders of cholesterol metabolism. In this regard, over the past 30 years, numerous attempts have been made to create hypocholesterolemic drugs [1-4].

Currently used anti-atherosclerotic agents possess the ability to reduce the level of lipids and lipoproteins in the blood by 17-40%. However, the experience of their practical application shows that the widely used anti-atherosclerotic drugs are not deprived of the ability to cause serious complications, and even the most contemporary and effective drugs from the group of statins have side effects.

In this regard, the preparations of natural origin, which are characterized by safety and the possibility of their long-term application without complications, are of great interest [5-7].

It is known that the intestinal flora, mainly bifidobacteria and lactobacilli, provides the most intense metabolism of bile acids and cholesterol entering the intestine that results in removal of decomposition products of the latter from the body. On the contrary, in gnotobionts, i.e. experimental animals deprived of the intestinal microflora, when entering the intestine, bile acids and cholesterol are not exposed to any metabolic changes and are almost completely absorbed back into the blood that leads to a slowdown in the metabolism of cholesterol and development of hypercholesterolemia [8-12].

Involvement of intestinal microflora in the metabolism of cholesterol is confirmed by the clinical data. So, when studying microflora in patients with atherosclerosis, 6.7% of the strains, very actively metabolizing cholesterol, were found, while 51.3% were inactive strains. In the group of healthy people, these figures were 22 and 19%, respectively [13-16].

In previous studies we have revealed that lactobacilli have a cholesterol-metabolizing activity, which depends on the origin of species and strains. We have revealed that the vegetable oils, such as cedar oil and flaxseed oil have genetic properties of bifidobacteria and increase their biochemical activity [17-18].

The need to enrich the human diet with biologically active compounds such as PUFA (polyunsaturated fatty acid), which are known to actively participate in many physiological reactions of the body, is currently not in doubt. The value of PUFA in the human body is very high. On the one hand, they prevent the development of atherosclerosis and reduce the level of cholesterol in the blood, and on the other hand, they have an anti-inflammatory effect. In addition to binding cholesterol, PUFAs have a choleretic effect. The more bile acids are excreted from the liver, the more cholesterol is spent for these purposes [19-20].

There are evidences concerning ability of PUFAs to reduce the risk of development of most medical conditions, such as cardiovascular diseases, diabetes, fatty degeneration, and certain neurological diseases, as well as their usefulness at treatment of these diseases [1]. The physiological role of PUFAs is manifested primarily in

their involvement in fat metabolism, in the transfer of cholesterol from the insoluble esters of saturated fatty acids into the soluble forms, which are oxidized in metabolic processes into easily utilizable low molecular weight compounds [21].

The level of PUFAs in the human body depends directly on the food consumed. In this regard, the development of dietary supplements, which contain the probiotic microorganisms and polyunsaturated fatty acids possessing high curative and preventive properties, is of great interest.

This work aims at studying the effects of polyunsaturated fatty acids of cedar oil on cholesterindegrading activity of bifidobacteria.

MATERIALS AND METHODS

The research objects were strains of *Bifidobacterium longum DK-100*, *B379M Bifidobacterium longum*, *Bifidobacterium bifidum* 8_3 bifidobacteria obtained from the Russian National Collection of Industrial Microorganisms (VKPM) of FGUP "GosNIIGenetika", activated by biotechnological method, which was developed at the East Siberia State University of Technology and Management (ESSUTM) [22].

The cultivation of probiotic microorganisms was carried out with the use of nutrient solution based on the brightening serum with the introduction of the growth components, developed at ESSUTM [24]. Extra virgin cedar oil was used as a functional ingredient (TU 914-001-73225681-25). The stationary growth phase of activated bifidobacteria culture of the stationary growth phase in an amount of 5% of the volume of the nutrient solution was used as the inoculum. The cultivation was carried out in flasks at a temperature of (37±1) ^{o}C.

The viability of bifidobacteria was assessed by the number of colony forming units (CFU) at inoculation of cell suspensions from the appropriate dilutions in the maleic hydrazide medium [17].

The concentration of cholesterol in the nutrient solution was determined by enzymatic method [23]. Purified blood serum was used as the source of cholesterol.

The principle of the method lies in the fact that under the action of the enzyme of cholesterol esterase, cholesterol esters are transformed into cholesterol and fatty acids. Further, cholesterol under the influence of cholesterol oxide dismutase gives a stained compound and hydrogen peroxide. The staining intensity in the reaction mixture is directly proportional to the cholesterol concentration in the sample. Next, we measured the optical density of the experimental sample (E) and that of the calibration sample (E_k) against the working reagent, consisting of enzymes mixture, at a wavelength of 450 nm. The cholesterol concentration in a sample was determined by the computational method. When conducting measurements in samples, having the intense green color, it was allowed diluting samples two times with physiological solution. In the calculation, the data obtained were divided by 2.

$$C = \frac{E}{E_к} \cdot 4,65 \; ; \quad (1),$$

where C – was the cholesterol concentration in the sample (mmol/l);

E – was the optical density of test samples;

E_k – was the optical density of the calibration samples;

4.65 – was the cholesterol concentration in calibrator (mmol/l).

Fatty-acid composition was determined by the content of methyl esters of fatty acids according to GOST R 51483-99 with the use of Kristall 2000M gas chromatograph with proportional-integral differential flame detector, HP-FFFAP (USA) capillary column 50 m, 0.32 mm, 0.52 μm, and nitrogen as carrier gas. The experimental conditions were as follows: the definition was conducted in programming mode at a rate of 4°C/min, the column temperature varied from 180 to 220°C, the evaporator temperature was 250°C, and the detector temperature was 250°C [24]. The samples were prepared according to GOST 30418-96, GOST 30623-98.

The chloroform (10 ml) was added to the biomass (10 ml), and the mixture was gently shaken for 3-5 min (vigorous shaking results in formation of inseparable emulsion). After 5 min, the obtained mixture was centrifuged. The chloroform extract was separated and filtered through the funnel filled with drying agent. The chloroform was removed by means of a rotary evaporator, while the residue was dissolved in hexane (1.9 ml). The resulting solution was mixed with a solution of sodium methoxide in methanol (0.1 ml). The reaction mixture was intensively mixed for 2 min, then kept without stirring for 5 min, and filtered through paper filter. The resulting solution was analyzed (while storing for 24 hours the composition of the sample did not change). For all samples, two parallel syntheses were made. In a control experiment with addition of sodium methylate, no precipitate was observed in the reaction mixture.

All experiments were carried out in 3-5 replicates. The obtained data were processed based on the statistical software package Excel using the Mann-Whitney U test. Below are considered only statistically significant differences at $p<0.05$.

RESULTS AND DISCUSSION

At the first stage we studied the effect of different doses of cedar oil on cholesterol-metabolizing activity of bifidobacteria. The research results are shown in Table 1.

It is seen from Table 1 that increasing the dose of added cedar oil from 0.5 to 1.5% leads to more active degradation of cholesterol in the course of cultivation of all strains of probiotic microorganisms as compared to control. Most pronounced destructive activity with regard to cholesterol is peculiar to *B. longum DK-100* strain, which upon addition into a nutrient solution of cedar oil in amount of 1.5% at the end of the cultivation destroys 68.09% of cholesterol. At that, cholesterol-metabolizing activity of bifidobacteria increases by 28%.

Table 1. The effect of different doses of cedar oil on cholesterol-metabolizing activity of bifidobacteria

Stain of microorganisms	Added component dose, %	Content of cholesterol in the nutrient solution, kmol/l							The level of cholesterol decomposition, %
		Duration of cultivation, h							
		0	4	8	12	16	20	24	
B. bifidum 8_3	control	4.92	4.92	4.9	4.76	4.42	3.72	3.12	36.59±0.02
	0.5	4.92	4.92	4.83	4.52	3.97	3.21	2.43	50.61±0.04
	1	4.92	4.91	4.79	4.31	3.84	3.07	2.01	59.15±0.03
	1.5	4.92	4.9	4.76	4.26	3.68	2.83	1.85	62.39±0.02*
B. longum DK-100	control	4.92	4.92	4.87	4.61	4.31	3.63	2.95	40.04±0.02
	0.5	4.92	4.91	4.81	4.47	3.82	2.71	2.37	51.83±0.04
	1	4.92	4.9	4.73	4.26	3.64	2.57	1.85	62.02±0.03
	1.5	4.92	4.89	4.68	4.1	3.35	2.25	1.57	68.09±0.03*
B. longum B379M	control	4.92	4.92	4.91	4.81	4.54	3.84	3.21	34.76±0.02
	0.5	4.92	4.92	4.89	4.62	4.19	3.56	2.57	47.76±0.04
	1	4.92	4.91	4.85	4.45	4.02	3.32	2.21	55.08±0.02
	1.5	4.92	4.91	4.81	4.31	3.87	3.14	1.96	60.16±0.03*

Note: * - statistically significant differences compared to control ($p<0.05$).

Table 2. Fatty-acid composition of the bifidobacteria biomass in a nutrient solution with cedar oil

Name of the indicator		Cedar oil, control	Name of the test sample, biomass		
			B. longum DK-100 **with cedar oil**	**B. *longum B379M* with cedar oil**	***B. bifidum*** 8_3 **with cedar oil**
Content of fatty acids, %	Saturated acids:	7.67	8.35	9.15	11.32
	$C_{10:0}$ caprylic acid	-	-	-	0.15
	$C_{12:0}$ lauric acid	-	-	-	0.19
	$C_{14:0}$ myristic acid	-	-	0.08	0.55
	$C_{16:0}$ palmitic acid	4.48	6.22	5.37	6.36
	$C_{18:0}$ stearic acid	2.79	2.13	3.40	3.79
	$C_{20:0}$ arachidic acid	0.40	-	0.30	0.28
	Monounsaturated acids:	26.53	19.48	22.53	22.69
	$C_{18:1}$ oleic acid	25.25	19.48	21.89	22.06
	$C_{20:1}$ gondoinic acid	1.28	-	0.64	0.63
	Polyunsaturated acids:	63.28	69.12	66.17	64.56
	$C_{18:2}$ linoleic acid	43.59	54.14	56.02	54.83
	$C_{18:3}$ linolenoic acid	19.57	14.98	10.01	9.61
	$C_{20:2}$ eicosadienoic acid	0.12	-	0.14	0.12
	Total:	97.48	96.95	97.85	98.57
Content of polyunsaturated fatty acids, %	ω-6 linoleic acid	43.59	54.14	56.02	54.83
	γ- linolenoic acid	19.57	14.98	10.01	9.61

Further research concerned the fatty-acid composition of bifidobacteria biomass. The research results are presented in Table 2.

As shown in Table 2, cedar oil is a source of omega-6 linoleic and γ- linolenoic acids. It should be noted that γ- linolenoic acid is the physiologic antagonist of the arachidonic acid and is included competitively in the molecules of phospholipids of various specialization, creating a reserve of membrane polyunsaturated fatty acids, necessary to maintain an adequate level of appropriate eicosanoids, i.e. prostagladins, thromboxanes, and leukotrienes. They have versatile physiological activity, and are important for the normal functioning of the cardiovascular system [23].

The results obtained indicate that the biomass of bifidobacteria contains in the right amounts essential fatty acids that are adjustable stimulants of numerous biochemical processes in the body.

Thus, in consequence of the conducted research it is revealed that polyunsaturated fatty acids of the cedar oil not only stimulate the growth of bifidobacteria, but also increase their cholesterol-metabolizing activity.

The obtained experimental data allowed developing the production technology of dietary supplements containing bifidobacteria and polyunsaturated fatty acids. The feature of the proposed technology is the addition of cedar oil in an amount of 1.5% into cooled nutrient solution due to the instability of fatty acids at their sterilization. In this process *Bifidobacterium longum DK-100* strain with high cholesterindegrading activity is applied as the inoculum. Qualitative characteristics of the dietary supplements are presented in Table 3.

Table 3. Qualitative characteristics of probiotic dietary supplements enriched with polyunsaturated fatty acids

Indicator name	Indicator value
Consistency and external appearance	Homogeneous. Serum may separate
Color	From white to light yellow
Taste and smell	Pure, sour-milk, with a taste of pine nuts
Limit value of pH, units	5.3-7.5
Cholesterol-metabolizing activity, %	68.09
Temperature at release from the factory, at most, °C	4-6
The amount of bifidobacteria, CFU/cm^3, not less than	$2 \cdot 10^{11}$
The volume of the product (cm^3), which should not content:	
Coliform bacteria (coliforms)	10
S. aureus	10
Pathogenic microorganisms (including salmonella)	50
Yeast and mold, CFU/cm^3, at most	10

Analysis of the data presented in Table 3 shows that the dietary supplements have a high cholesterol-metabolizing activity and can be recommended for the prevention of diseases caused by accumulated elevated levels of cholesterol in the blood.

Optimization of the nutrient solution by the cedar oil increases the amount of viable cells of bifidobacteria, which carry out intensive metabolism of cholesterol. The increase in the titer of bifidobacteria is facilitated not only by PUFA, but also by biologically active compounds (vitamins, trace elements, etc.) contained in them in significant amounts. It should be noted that polyunsaturated fatty acids are essential factors not only for the microorganisms, but also affect their various biological functions.

The present work shows for the first time the relationship between the content of polyunsaturated fatty acids and cholesterindegrading activity of bifidobacteria. It is revealed that introduction of cedar oil enhances the biotransformation and destruction of cholesterol in the course of increasing the biomass of bifidobacteria. The obtained results are consistent with the literature data on the influence of fatty acids with chain length from C_{14} to C_{20} on biochemical activity and biological functions of microorganisms. Cedar oil is a unique natural substance, which in the form of a derived lipid is a structural component of cell membranes and performs energetic and regulatory functions [25].

The mechanism of interaction of unsaturated fatty acids with the cells of bifidobacteria can be explained by their adsorption and incorporation into the composition of the outer membrane, change of the physical and chemical properties of cell surface, as well as properties of the lipid bilayer [26]. In this context it is important to note that in certain concentrations they act as growth factors for bifidobacteria, and can be attributed to the probiotics, while the developed dietary supplements are synbiotic products.

Thus, when developing biological products using unsaturated fatty acids, it is necessary to consider the complex mechanism of their biological impact on the properties of the microorganisms. The obtained results show that cedar oil is characterized by significant biological potential, which can be used for the production of functional nutrition products.

CONCLUSION

In consequence of conducted research, we have developed a dietary supplement, which normalizes the level of cholesterol in the blood. It is revealed that polyunsaturated fatty acids of the cedar oil enhance cholesterol-reducing activity of bifidobacteria. The greatest hypocholesterolemic effects are peculiar to bifidobacteria strain of *Bifidobacterium longum DK-100*, which destroys up to 68% of the cholesterol in the nutrient solution containing cedar oil.

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