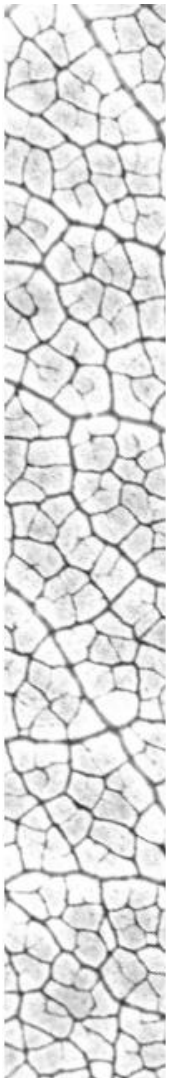

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Consortium of starter cultures with lactose-utilizing and probiotic properties technology of production of delactosed sould - milk products

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Abstract
The main purpose of the study was to develop technologies for the production of lactose-free fermented milk products based on active strains of lactose-utilizing microorganisms. As a result, 12 consortiums were created, which include lactic acid bacteria and yeast. To determine the starter properties of the created compositions, the biocompatibility of microorganism cultures was evaluated using the perpendicular streak technique on a dense nutrient medium. The viability score was checked by the Miles&Misra method, the antagonism score with the block, hole, prick, and stroke methods. All cultures of the consortium members: C136, C198, C196, C190, DK26, C237, LB4, LB24, LC59, LR 12, L98, LB24, LC70, LC90, LR 12, LC 58, LB4, L98 - are biocompatible, antagonists to opportunistic microorganisms such as: *E. coli, S. marc., S. typh.* With high viability, having the ability to utilize milk sugar - lactose, which was confirmed by us in previous scientific studies.

Keywords: lactose intolerance, lactose-free dairy products, lactic acid bacteria, probiotic properties

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INTRODUCTION

Today, many people suffer from intolerance to milk sugar lactose and cannot eat products containing milk.

Everyone knows that milk sugar is necessary for newborns to restore normal intestinal microflora, improve the absorption of calcium, magnesium, and manganese. In turn, they are necessary for the development of the nervous system, the synthesis of vitamins of B group, as well as for the occurrence of anemia and rickets. For adults, dairy products are a supplier of vitamins, trace elements, such as calcium, and are also necessary for the normalization of the intestinal microflora (Kaishev 2002).

Currently, lactase intolerance is quite common around the world. According to statistics, about 75 % of the world population is lactose intolerant. In this regard, the production of lactose-free fermented milk products is perspective in the modern market (Pokrovsky 2013). This pathology is caused by insufficient production of the lactase enzyme or its complete absence. At the same time, the degree of its severity may be different.

One of the methods of treatment is the use of lactose-free or low-lactose dairy products.

When receiving lactose-free dairy products, various methods are used. In order to separate the lactose enzyme from fermented milk products, a membrane filtration process is used (Doronin and Shenderov 2013). As a result, lactose is hydrolyzed using a functional carrier polymer that separates and binds lactose. This method has been used by milk producers for 40 years (Patent EP № 1803359 от 7.04.2007).

In addition, lactose-fermenting cultures of microorganisms are used, such as: lactic acid bacteria, yeast, and mold fungi (Collado et al. 2016).

Products that include consortiums of LAB and yeast play an important role in the nutrition of people, especially children, elderly and sick persons. The dietary properties of such products consist primarily in the fact that they improve metabolism, stimulate the secretion of gastric juice and the appetite (Polyanskaya et al. 2014).

It is known that lactic acid bacteria are common in natural and industrial substrates. In this regard, they are the main components used in the food and biotechnology industry (Lipatov 2014). Since the main advantages of lactic acid bacteria are a wide range of biological effects: adhesion, antagonism to pathogenic

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and opportunistic microorganisms, etc. In this regard, it is used as a probiotic drug in diseases associated with lactose intolerance. The directing action of lactic acid bacteria used in ferments is aimed at splitting milk sugar lactose for people suffering from milk protein intolerance (Mikhailova et al. 2013).

For people who are forced to abandon the use of dairy products, a lactose-free “milk” is offered on the modern market. The production of such products is a complex process. From the main product - milk - through special processing, filtration and pasteurization, lactose is completely removed. To taste, lactose-free products are not inferior to the “milk” familiar to everyone, and sometimes even surpass it (Prosekov 2015).

Global consumption of dairy products will increase by about 2.3% annually over the next ten years, while their production will grow by 1.8% each year over the same period (Kovaleva and Khrenova 2016).

The success of a food company when launching new products on the market depends on many factors, but first of all on the degree of compliance of product characteristics with consumer needs. In the world, the trend of a healthy lifestyle is increasingly developing and, as a result, the range of dairy products that meet this demand is expanding, including lactose-free products.

According to the World Health Organization, LI affects between 10 and 80% of people in various population groups, so this market seems very perspective (Semenova).

The implementation of countries’ priorities in the field of healthy nutrition provides for the development of new types of food products using the achievements of biotechnology. Fermenting microorganisms with new functional properties and enzyme preparations that form a high digestibility and biological value, a variety of taste and consistency of fermented milk products are widely used (Zyuzina et al. 2016).

The use of two types of bio-products simultaneously is implemented in the development of dairy products for people with lactose intolerance. Fermented milk beverages partially correspond to the physiology of nutrition in secondary lactose intolerance, but in primary - special nutrition is required using products that are partially or completely devoid of lactose. To obtain such products provides for the enzymatic hydrolysis of lactose due to the filtering (removal) of lactose, the implementation of the lactase enzyme or lactose-utilizing microorganisms.

MATERIALS AND METHODS

Evaluation of the Biocompatibility of Microorganism Cultures in a Consortium of Starter Cultures

To identify the biocompatibility of Lactobacillus and yeast cultures in the consortium, the perpendicular streak technique on a dense nutrient medium was used (Irkitova et al. 2015). To do this, two-day broth cultures along the diameter of the plate were sown in a Petri dish on the agar medium MRS-4, incubated for 48 hours at 37°C. Then, perpendicular to its stroke, the rest of the studied lactobacilli and yeast were sown in strokes. Thick suspensions of cultures were used for plating. The plates were kept in a thermostat at 37°C for 48 hours. If cultures grow close to each other, then cultures do not show antagonism to each other.

Method for Determining the Maximum Indicator of Viability of Microorganism Cultures

The indicator of viability of microorganism cultures was estimated using the Miles&Misra method (Skorodumov et al. 2016).

Preparation of dilutions: the culture was washed off in a separate tube with a nutrient broth suitable for culture; for the preparation of dilutions, sterile tap water or saline solution was poured in 9 ml; 1 ml of the initial suspension was added into the first tube - this is the 1st dilution (10^{-1}); titration was carried out to 10^{-12} degrees.

For the preparation of each dilution, it is necessary to use a new pipette. Ignoring this rule leads to an erroneous result.

Plating on Medium and Registering Results

The outer back of the Petri dish was divided into eight equal sectors. A precisely measured volume of bacterial suspension (20 µl) was applied to the surface of the medium using a sterile pipette (tip). Sowing on a dense medium was carried out, as a rule, from the last eight dilutions. Plating was made with different pipettes (tips). The pipette is held vertically. Drops do not rub, their area can be increased by slightly swaying the Petri dish. The cultures are incubated and then the number of colonies grown is determined. After plating, the Petri dishes are placed in the thermostat with the lids down. Incubation.

Cell counting. The number of cells in 1 ml of the test substrate is calculated using the formula 1:

$$M = a \times 10^{n} \times V \times 50 \quad (1)$$

where M - the number of cells in 1 ml;

a - the average number of colonies when sowing the dilution from which the sowing is made;

10^{n} - the dilution factor;

V - volume of the suspension taken for plating, in ml;

- the conversion factor from µl to ml.

Methods for Evaluating Antagonism (Irkitova et al. 2015)

Block method. The test culture of bacteria is sown in depth in a nutrient agar, in a Petri dish, and incubated in optimal, strictly observed conditions for the formation and accumulation of inhibitory compounds in the agar. Plating on the surface of the agar plate can be done. Then, with a sterile cork drill, cut out an agar disk (block) with the grown culture of bacteria and place it in another Petri dish on the surface of the agar medium that has just been sown with the culture of pathogen. The plate is

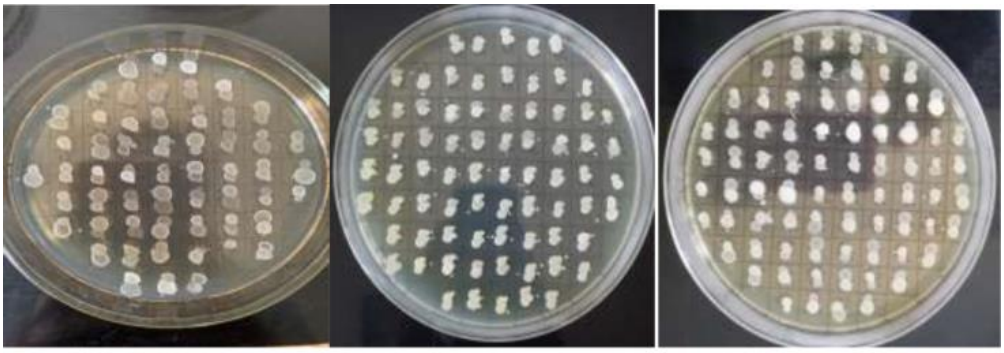

Fig. 1. Study of the biocompatibility of LAB (a), yeast (b), and LAB with yeast (c)

kept for 30 minutes in the refrigerator (to avoid premature growth of the pathogen) to diffuse inhibitory compounds from the block into the agar thickness with the pathogen, then incubated under certain conditions optimal for the latter. The degree of antagonistic activity of the test bacterium is judged by the size of inhibition zone of the pathogen growth around the agar block (Bannikova et al. 2015).

Well method. In principle, similar to the above method of agar blocks, but in this case, instead of installing the unit in a layer of agar containing the pathogen, by the cork drill cut well with a diameter of 5-7 mm and in it is placed a certain number (for example, 0.2 cm3) of liquid or semi-liquid medium with grown culture of the test antagonist. Do not make wells until the end, so that the culture fluid does not flow under the medium, which distorts the result. The plate is kept in the refrigerator for diffusion of inhibitory substances from the well into the agar thickness, then - in the thermostat for growth of the pathogen, after which the inhibition zone of the antagonist around the well is measured.

Stabbing method. Plating one loop of daily culture in the thickness of the agar plate containing the pathogen. Then incubation and the result reading (Kiselev et al. 2014).

Streak method: plating perpendicularly. Then incubation and the result reading (Gudkova 2014).

Consortium

To create different variants of the consortium, selected strains of lactobacilli and yeast were looped into the liquid nutrient medium MRS broth. To prepare MRS broth, 52 g of powder (which is a homogeneous loose yellow powder) was dissolved in 1 liter of distilled water. Boiled until the particles were completely dissolved, then poured into test tubes. The resulting medium has a density of 1.2% and an amber color. Incubation was carried out for 48-72 hours at a temperature of 37°C (ARIBCM 2016).

Then, for 3 days, pasteurization and sterilization of 3.2% of “Zenchenko” milk was carried out at a temperature of 98°C, with the simultaneous removal of the formed membranes, cooling was carried out at a temperature of +2; +4°C in the refrigerator.

After completing the process of sterilization and pasteurization of milk and the formation of grown colonies on the liquid nutrient medium MRS broth, create a consortium of 12 starter cultures for the production of lactose-free products.

Grown consortiums of 12 starter cultures in a concentration of 2 drops are added into a test tube with milk using a disposable pipetter. Then put it in the thermostat at 37°C (Rozhkova 2015).

When 12 variants of the consortium of starter cultures are sown in the prepared MRS broth, they work on the burner flame. The selection of each of the consortium use disposable tips for pipetters. 1 variant of the consortium: LB4 + C136 + LC70 in a concentration of 5 ml, pull out of the MRS broth, transfer to a milk substrate. The above method is used for all variants of the consortium (Hamagayeva et al. 2014).

RESULTS

Evaluation of the Biocompatibility of Microorganism Cultures in a Consortium of Starter Cultures

Based on the results of the study of inter-strain relationships (**Fig. 1**), the studied strains showed biocompatible and bio-incompatible relationships, i.e. antagonistic. The antagonism was strong and weak.

Fig. 1 shows all compatibility variants. As can be seen, there is a complete “fusion” of spots in the variation and an increase in the growth of the studied strains in the zone of co-culture in the other variations. This is typical in the LAB group, and the LAB group with yeast. Yeast showed 100% compatibility (**Fig. 1b**).

Data on interspecific relationships obtained by us allowed to identify a group of bacteria and yeast that are most perspective for co-culture.

Determination of the Maximum Indicator of Viability of Microorganism Cultures

Then we studied the maximum indicator of viability (**Table 1**, **Fig. 2**), as well as antagonistic activity (**Table 1**, **Fig. 2**) in relation to opportunistic microorganisms.

In the study for antagonism, it was found that associations #1-6 are good at suppressing Salmonella

Table 1. Results of the evaluation of viability and the antagonism of consortium variants

Variant No.	Cultures included in the consortium	Viability, CFU/ml	Test-strains (mm)		
			E. coli	*S. marc.*	*S. typh.*
1	LB4 + C136 + LC70	23×10^{10}	-	10	11
2	LB24 + C198 + L90	9×10^{10}	-	12	10
3	LC59 + C196 + LR12	10^{9}	-	12	4
4	LR12 + C190 + LC58	9×10^{10}	-	10	4
5	L98 + DK26 + LB4	10^{10}	-	10	6
6	LB24 + C237 + L98	10^{10}	-	10	13
7	LB4 + C136 + LC70 + 10LR + LB17	42×10^{10}	4	-	-
8	LB24 + C198 + L90 + 7LG	10^{10}	3	4	5
9	LC59 + C196 + LR12 + 3LP	10^{10}	10	1	-
10	LR12 + C190 + LC58 + 8LG	45×10^{10}	1	1	-
11	L98 + DK26 + LB4 + L108	10^{10}	6	7	-
12	LB24 + C237 + L98 + L91	10^{10}	8	11	7

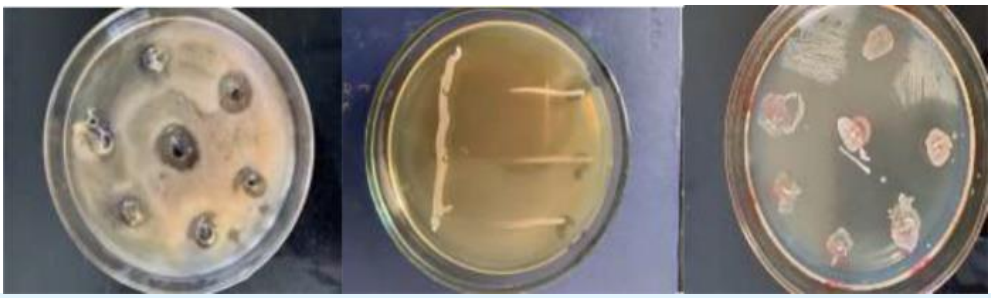

Fig. 2. Study of the biocompatibility of LAB (a), yeast (b), and LAB with yeast (c)

and serratia. In relation to serratia, the indicators are from low to high. In the case of Salmonella, 100% suppression, the numbers are high.

The viability of all the studied variants showed a high result.

Evaluation of Antagonism (7)

An important factor in screening for probiotic drugs is antagonism to various pathogenic and opportunistic microorganisms.

In our research, antagonistic activity was studied for such test strains as *S. typhimurium, S. marcescens,* and *E. coli.* The research was carried out by diffusion into agar using methods such as plating with blocks, wells, streak and stabbing to refine the method and select a more effective one. Yeast does not have antagonistic activity in our studies. LAB are antagonistically active. The wells were mostly of zero degree of activity - in 90% of cases, 2 cultures gave a medium degree - 5 mm to *S. marcescens* - LB2 and L95. Cultures suppress basically all test strains. Streak plating has a high degree of indicators - within 10-30 mm, however, this is a less informative method. According to it, LB6, LB38, LB42, LB46, LC76, L85, L86, L98, 2LP, 3LP, 8LG are active for all test strains. The stabbing method is more informative. Active microorganisms - LB8, L91, 3LR, 8LG, 11LR. 24 cultures are active to two microorganisms in different combinations. The method of sowing in blocks did not yield results.

Therefore, different methods of evaluating antagonism give different results, which depends on the diffusing ability of microorganisms, the density of nutrient media, and the skills of the researcher.

Table 2. Antagonistic activity of LAB

Test strain	Activity level (%) and absolute number							
	«Zero»		Low		Medium		High	
	abs.	%	abs.	%	abs.	%	abs.	%
E. coli	77	75.4	6	5.8	16	15.6	3	2.9
S. marcescens	83	81.3	5	4.9	9	8.8	5	4.9
S. typhimurium	37	36.2	7	6.8	35	34.3	23	22.5

Fig. 2 shows the antagonistic activity of Lactobacillus isolates.

The results of digital manifestations of antagonistic activity of the studied objects are provided in **Table 2**, low activity - 1.0-4.9 mm, medium - 5.0-8.9, high - more than 9 mm. In this case, one culture is active to 2-3 test microorganisms.

In relation to antagonists, the main volume consists of cultures with an antagonism zone up to 1 mm: *E. coli* - 77, *S. typhimurium* - 37 and *S. marcescens* - 83 LAB.

S. typhimurium is the most active, and there are high- and medium-level cultures.

Isolates with a suppression zone of 10 to 15 mm make up a high degree.

In general, the isolated isolates of LAB showed effective antagonistic activity ($p<0.001$), the main mass is made up of isolates with a medium and high degree of activity.

It is necessary to constantly conduct research on new production-value strains of microorganism cultures.

So, we investigated the antagonistic activity of strains of lactobacilli and yeast by diffusion method in relation to test cultures of microorganisms *Escherichia coli, S. typhimirium, S. marcescens.*

Table 3. Results of organoleptic evaluations of starter cultures (points)

Variant No.	Consistency	Evaluation	Taste and smell	Evaluation	Color	Evaluation
1	Thick	4	Sour-milk	5	Milky white	5
2	Thick	5	Sour-milk, soft	5	Milky white	5
3	Loose	3	Sour-alcohol	4	Milky white	5
4	Thick	4	Sour-alcohol	4	Milky white	5
5	Thick	4	Sour-milk	5	Milky white	5
6	Thick, homogeneous	5	Milky	5	Milky white	5
7	Thick	5	Sour-milk, without foreign tastes	5	Milky white	5
8	Thick	5	Sweet and sour, pleasant smell	5	Milky white	5
9	Loose	4	Sour-milk, without foreign tastes	5	Milky white	5
10	Soft	5	Sour, without foreign tastes	5	Milky white	5
11	Soft	5	Sour, smell of alcohol	4	Milky white	5
12	Soft	5	Sour, smell of kurt	3	Milky white	5

Fig. 3. Consortium #2 and #6

As a result of processing the obtained data of probiotic properties, we selected the following cultures for further work: LB2, LB3, LB6, LB7, LB8, LB38, LB42, LB46, LC76, LC78, LC84, L85, L86, L91, L95, L98, L104, L105, 2LP, 3LP, 8LG, 11LR, 18LH.

In all categories, LB8, L91, 3LR, 8LG, 11LR are active.

Consortium

To study the obtained fermentation properties of the created consortia, an analysis was performed on organoleptic indicators. As a result of organoleptic studies of starter cultures (**Table 3**), the #2 and #6 variants were rated 5 points, they had a sour-milk taste, without foreign tastes, and had a thick and homogeneous consistency. 6 variant had a milky white color, uniform throughout the mass (**Fig. 3**). The other options had a good rating, with the exception of variant 3. This variant had a loose consistency, which was estimated at 3 points

Thus, we have studied probiotic characteristics, created consortia of starter cultures, which will be further developed.

DISCUSSIONS

As a result of our previous research, the objects used for lactic acid bacteria and yeast were isolated from factory-made (35) and home-made (50) lactic acid products, as well as human biotopes (12 samples). Dairy products are presented: koumiss - 8, cottage cheese - 13, kefir - 5, yogurt - 5, sour milk - 16, probiotic kvass - 1, butter - 7, kurt - 7, sour cream - 12, colostrum - 1, shubat - 4, cheese - 6 samples. Human biotopes are represented by secretions such as the urine and feces of children (Bannikova et al. 2015).

The following microorganisms were selected by the applicants for use as starter cultures based on antagonism: C136, C198, C196, C190, DK26, C237, LB4, LB24, LC59, LR 12, L98, LB24, LC70, LC90, LR 12, LC 58, LB4, L98.

In addition, our laboratory studies were based on determining the resistance of microorganism cultures to the secrets of the gastrointestinal tract. The results of an earlier study showed that they are resistant to 40% of bile (lactobacilli - 86.3%, yeast - 94%), 2, 4, 6% of table salt (100%), the sequential action of acid and bile (lactobacilli - 63.7%, yeast - 93.9%), and gastric juice (lactobacilli - 99.02%, yeast - 63.9%) (Gorbatova 2014).

Strains C136, C198, C196, C190, DK26, C237, LB4, LB24, LC59, LR 12, L98, LB24, LC70, LC90, LR 12, LC 58, LB4, L98 are resistant to GI secretory fluids, which is one of the main indicators of probiotic quality, due to the fact that most lactobacilli are inactivated when exposed to bile and low pH of the stomach. It is known that probiotics must survive passing through low pH of gastric juice and bile acids in order to reach the intestinal tract, colonize the host epithelium and have a beneficial effect (Kornienko 2016).

In addition, probiotics selected for commercial use must survive in industrial production and storage to ensure long-term activity. The obtained results of the resistance of isolated isolates to stress factors *in vitro* indicate the ability of cultures that are promising as bio-drugs and ferments to survive under unfavorable conditions in the upper gastrointestinal tract (Akusu et al. 2017, Grinevich 2014).

In this regard, in our study, based on the technological properties of cultures of lactic acid bacteria and yeast, ferments were designed to create lactose-free fermented milk products.

In conclusion, an important factor in screening in the production of lactose-free fermented milk products is the

biocompatibility and viability of microbial compositions. The viability of candidates for the drug meets the requirement for microorganisms: 10^7 CFU/ml or more: in LAB is 10^9-10^{10} CFU/ml; in yeast - 10^{10} CFU/ml. As a result, based on the results of biocompatibility, consortia of starter cultures were developed.

CONCLUSIONS

The research was carried out within the framework of the project of program-targeted financing of the Ministry of Education of the Republic of Kazakhstan on the topic: Technology of production of lactose-free fermented milk products. The field of scientific research, which is Microbiology. The base for the research is Astana branch «Kazakh research institute of processing and food industry», the laboratory of «Microbiology and biotechnology». As a result of scientific research, a patent was obtained for «A method for obtaining a starter culture for the manufacture of lactose-free products». The present invention relates to the food, biotechnological, medical and microbiological industries. The use of a probiotic product containing lactose-utilizing microorganisms will correct the dysbiotic processes that occurred against the background of the main disease or deficiency of milk sugar. The technical result obtained from the use of the invention is the possibility of developing and creating new highly effective probiotic drugs and consortia in Kazakhstan, based on bacterial strains isolated mainly from local sources. Today, it is an actual approach to search for new strains of lactic acid microorganisms that are promising as ferment cultures and probiotics.

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