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Studying the physiological and biochemical properties of lactic bacteria cultures in the creation of sourdough for bread

The article presents the results of screening lactic acid bacteria cultures of the genus *Lactobacillus* and *Pediococcus* from the collection of Kazakh Scientific Research Institute of Processing and Food Industry, previously isolated from wheat grain, flour, rye starter cultures, as well as kumis and shubat. For a comprehensive assessment of probiotic properties of the selected 7 strains of lactic acid bacteria, their physiological and biochemical properties were studied: acid-forming, antagonistic activity, saccharolytic profile, resistance to various concentrations of bile, sodium chloride, growth at different pH values, antibiotic resistance. As a result of strains screening 5 most active cultures of lactic acid bacteria were selected for inclusion in the sourdough for bread: *Limosilactobacillus pontis 9K3, Limosilactobacillus fermentum 3Ш1, Lb. paracasei 82, Lb. paracasei 114, Lacticaseibacillus paracasei 126.*The selection criteria were high enzymatic, acid-forming, proteolytic and antagonistic activity of the strains against opportunistic and pathogenic microflora (*B. subtilis* ATCC 6633, *Escherichia coli*-1257, *Staphylococcus sp.209-P*, *Salmonella Typhimurium*). We show that this result of the analysis indicates the prospects of using a consortium of these cultures to obtain a probiotic starter culture for bakery products. This, in turn, will increase production efficiency, ensure the quality and safety of food products in the baking industry.

Keywords: starter culture, lactic acid bacteria, bread, pure culture, *Limosilactobacillus*, *Pediococcus*, probiotic properties, antibiotic resistance.

Introduction

In modern conditions, bakery products in human life have a special place in nutrition. According to the Bureau of National Statistics of the Agency for Strategic Planning and Reforms of the Republic of Kazakhstan, Kazakhstan annually produces about 640 thousand tons of bread and bakery products. However, the microbiological quality of grain and flour is deteriorating more and more every year [1–6]. To prevent the spoilage of bread, physical, chemical, and biological methods of suppression of alien microflora are used. Biological methods are more attractive and widespread, in particular, the use of starter cultures with antimicrobial properties [7–9]. Sourdough is a semi-finished product made from water and flour, which contains lactic acid bacteria and yeast. Mankind has been using sourdough to make bread for over 4,000 years. Starter cultures containing pure cultures of yeast and lactic acid bacteria introduced in sufficient quantities provide fast, reliable stabilization of the dominant microflora, good fermentation, and guarantee production against accidents. With the help of pure cultures, it is possible to intelligently control the work of microbes and use their activity in a given direction [10–13]. The use of starter cultures for bread can reduce the risk of contamination of not only spoilage microorganisms, but also pathogenic microorganisms, and also reduce the risk of the formation of mycotoxins, which is important for obtaining microbiologically safe products [14–21].

However, for bread starters to bring significant benefits, it is necessary to correctly select species for one or another technological scheme, constant control over the purity and activity of the culture, strict adherence to technology, and, finally, proper microbiological control, which allows monitoring the development of introduced microorganisms. Bakeries are constantly in need of effective starter cultures that can fight the ever-changing spontaneous microflora of flour, as well as ensure the full quality of bread and bakery products.

The purpose of the work is to isolate new active domestic cultures of yeasts and lactic acid bacteria that have probiotic properties to create on their basis new consortia and starter cultures of domestic origin for the production of bakery products.

Experimental

The object of research was cultures of lactic acid bacteria of the genus *Lactobacillus* and *Pediococcus* from the collection of Kazakh Research Institute of Processing and Food Industry (KazRIPFI), previously isolated from a grain of wheat, flour, rye sourdoughs, as well as kumis and shubat. LAB cultures were maintained on an MRS medium (cultivation was carried out at a temperature (of 37 ± 1 °C) for 48 hours.

The maintenance and study of industrially valuable cultures of microorganisms were carried out according to the standard research protocol [21] and generally accepted methods [23–25].

Determination of acid formation activity. The energy of acid formation was determined by the quantity of lactic acid which is accumulated by lactic acid bacteria with minimal contamination of skimmed milk (or whey) for 17 hours [21]. To determine the energy of acid formation in the studied cultures, they were sown in the amount of 0.1 ml in 10 ml of milk. The culture tubes were placed in a thermostat at a temperature of 37 ± 1°. After 17 hours, the energy of acid formation was determined according to the Turner method. To do this, 10 ml of the sample was diluted with 20 ml of distilled water, followed by the addition of 1-2 drops of the phenolphthalein indicator. Titration was carried out with 0.1 N NaOH until a stable pink color was obtained. The results were expressed in Turner degrees according to the formula: K = X x 10 (where K is the energy of acid formation, X is the amount of NaOH used for titration in ml, 10 is the conversion factor of ml to Turner degrees).

Determination of resistance to antibiotics. Resistance to antibacterial drugs was determined by the disco-diffusion method in accordance with the method specified in MUC 4.2.1890-04 “Determination of the sensitivity of microorganisms to antibacterial drugs”. MRS-agar was used as a nutrient medium, which was poured into sterile Petri dishes so that the layer thickness was 4 = 0.5 mm. 1 cm 3 inoculates of the studied strain were applied to the surface of MRS- agar and evenly distributed over the surface of the nutrient medium, then discs impregnated with standard solutions of rifampicin, kanamycin, oleandomycin, pefloxacin, lincomycin, furazolidone, ampicillin, benzylpenicillin, erythromycin, vancomycin, gentamicin, tetracycline were applied using sterile tweezers. After, the Petri dish was thermostated at 37 ± 1 °C for 18–24 hours. Resistance to antibacterial drugs was assessed by growth retardation zones around the discs. All studies were carried out in 3-5-fold repetition.

Determination of lactic acid cultures’ proteolytic activity was carried out on milk agar, which was inoculated with a culture of lactic acid bacteria to obtain isolated colonies. 1 ml of culture or appropriate dilution was pipetted into Petri dishes and poured into 10-15 ml of molten and cooled to 40–45 °C milk agar. The inoculum was thoroughly mixed with milk agar. After the agar solidified, the Petri dishes were turned upside down and kept in a thermostat at a temperature of 30 °C for 48 h. After incubation, colonies were counted, around which clearing zones formed by proteolytic microorganisms were observed, and the diameter of clearing zones was measured.

Determination of antagonistic activity. The use of cultures of lactic acid bacteria-antagonists to *B. subtilis* and mold fungi in the form of wheat sourdoughs in various methods of preparing wheat bread guarantees its microbiological safety and prevents potato disease. The primary study of antagonism in lactic acid bacteria was carried out using the method of perpendicular strokes, as well as the method of diffusion in agar concerning indicator cultures *B. subtilis* ATCC 6633 (test culture for determining antibiotic activity), *Escherichia coli-1257, Staphylococcus sp.209-P, Salmonella typhimurium* with slight modification. Nutrient Agar medium (Hi Media Laboratories Pvt. Ltd. Mumbai — 400086, India) was used for this. Test cultures in the form of a cell suspension in an amount of 1 billion/ml (according to the bacterial turbidity standard) were applied to the surface of a dense medium in Petri dishes, after which 5 mm wells were cut out in the medium with a sterile spout, 3 wells for each studied strain of lactic acid bacteria. 40 µl of supernatant was added to each well. LAB supernatants were obtained as follows: 1 mL of LAB culture was added to 20 ml of MRS liquid medium, incubated for 24 hours at 37 °C. The cells were then removed by centrifugation at 8000xg rpm for 5 min. The supernatant was added to the first well. To eliminate the inhibitory activity due to organic acids, the pH of the supernatant was adjusted to pH 6.0 by adding 1 MNaOH and then added in a volume of 35 µl to the second well. The supernatant with pH 6.0 was added to the third well, and, in addition, catalase at a final concentration of 1 mg per 1 ml was added to it to eliminate hydrogen peroxide. The cups were placed in a thermostat for a day. A positive result for the presence of bacteriocin in the supernatant was the presence of a zone of growth inhibition of test cultures around the third well.

Determination of bile resistance was carried out as follows: in the MRS medium containing bile at a concentration of 20, 30, 40 % (pH 6.8-7.0), the test culture was inoculated (one loop per 8-10 ml of the medium) and thermostated for 48 hours at a temperature of 37°C. Culture growth or its absence was noted after shaking the tube by the presence or absence of turbidity and by microscopy.

Definition of resistance to NaCl was carried out in an MRS medium containing various concentrations of NaCl (2, 4, 6 %). The studied culture was inoculated in the amount of 1 loop per 8-10 ml of hydrolyzed milk (pH 6.8-7.0). The inoculations were kept in a thermostat for 48 hours at a temperature 37°C. Culture

growth or its absence was noted visually by the presence or absence of turbidity after shaking. The tubes were also controlled by a microscopic preparation.

Determination of resistance to the alkaline reaction of the environment (pH 8.3; 9.2; 9.6) was carried out by seeding one loop of the studied culture per 10 ml of medium (meat-peptone broth with 2 % yeast autolysate) with different pH values. The crops were kept at 37°C for 48 hours. Growth was determined similarly to culture growth in a medium with bile [21].

Results and Discussion

For a comprehensive assessment of the probiotic properties of lactic acid bacteria cultures that are promising for inclusion in the composition of starter cultures for bread, we studied their physiological and biochemical properties: resistance to various concentrations of bile, sodium chloride, growth at various pH values, acid-forming, antagonistic activity, and antibiotic resistance.

Active growth at a bile concentration in the medium of 20 and 30 % was shown by all the studied strains. At a content of 40 % bile in the medium, strains grow *Limosilactobacillus pontis 9K3, Lb. fermentum 3Sh1, Lb. paracasei 82, Lb. paracasei 114.* These strains are considered to be highly resistant to bile and can be used for the production of probiotic preparations.

All 7 strains that were studied grew well in a nutrient medium containing 2 and 4 % NaCl. However, in the nutrient medium that had a concentration of 6 % NaCl, only two of the strains showed growth: *Limosilactobacillus pontis 9K3* and *Lb. paracasei 82,* these strains grew well along the entire length of the culture liquid column.

Table 1 presents the results of determining the activity of acid formation, primary antagonistic, and proteolytic activity in 7 strains of lactic acid bacteria.

Table 1

Characterization of physiological and biochemical parameters of lactic acid bacteria cultures from the KazRIPFI collection of microorganisms

Strain	pH	Energy of acid formation	Antagonistic activity (to Bacillus subtilis ATCC-6633), zone diameter, mm	Proteolytic activity, zone diameter, mm
Lactobacillus plantarum Smg-1	4.3	164±5.1	10±0.2	8±0.3
Lb. paracasei 82	4.0	360.4±0.6	23±1.2	5±0.2
Lacticaseibacillus paracasei 126	4.1	310.2±2.7	26±2.0	9±0.3
Lb. paracasei114	4.1	280.4±2.0	23±0.6	9±0.2
Pediococcus acidilactici P2-6	4.2	165±3.2	21±2.2	10±0.5
Limosilactobacillus pontis 9K3	4.1	103±1.2	16.0±1.5	9±0.2
Lb. fermentum 3SH1	4.0	120±2.1	13.1±0.2	10±0.5

As can be seen from Table 1, the energy of acid formation in the studied cultures had limits from 93°T to 360°T.

Table 2 demonstrates the results of determining the sensitivity of lactic acid bacteria to antibiotics.

Table 2

Sensitivity of lactic acid bacteria to antibiotics

Strain	Zone of stunting (mm)											
	tetracycline	rifampicin	kanamycin	oleandomycin	ampicillin	gentamicin	pefloxacin	vancomycin	lincomycin	furazolidone	benzylpenicillin	erythromycin
Lactobacillus plantarum Smg-1	14	29	11	16	30	7	29	0	36	8	31	29
Lb. paracasei 82	20	15	12	11	35	0	23	19	24	27	34	26
Lacticaseibacillus paracasei 126	22	19	17	0	19	21	17	21	18	18	18	20

Продолжение таблицы 2

Strain	Zone of stunting (mm)											
	tetracycline	rifampicin	kanamycin	oleandomycin	ampicillin	gentamicin	pefloxacin	vancomycin	lincomycin	furazolidone	benzylpenicillin	erythromycin
Lb. paracasei 114	19	31	0	0	15	10	19	14	0	0	15	0
Pediococcus acidilactici P2-6	17	24	0	21	17	17	0	0	23	0	14	21
Limosilactobacillus pontis 9K3	28	32	14	33	31	26	20	19	37	28	33	30
Lb. fermentum 3SH1	24	26	10	20	35	19	15	7	39	20	15	28

Further, in all cultures, antagonistic activity against opportunistic and pathogenic microorganisms was more widely studied: *B. subtilis, Escherichiacoli, Staphylococcus sp., Salmonellatyphimurium.* Antagonism data are presented in Table 3.

Table 3

Evaluation of the antagonistic activity of lactic acid bacteria (at pH 6.0 and in the presence of catalase in LAB supernatants)

Strain	Zones of inhibition of growth of indicator crops (mm)			
	Escherichia coli-1257	*Staphylococcus sp.209-P*	*B. subtilis ATCC 6633*	*Salmonella typhimurium*
Lactobacillus plantarum Smg-1	11+0.4	10+0.1	10±0.2	0
Lb. paracasei 82	11+0.5	14+0.6	24±1.2	10±0.1
Lacticaseibacillus paracasei 126	11+0.5	10±0.1	26±2.0	21+0.7
Lb. paracasei 114	13+0.5	12+0.4	23±0.6	18+0.6
Pediococcus acidilactici P2-6	18+0.6	14+0.5	21±2.2	12+0.6
Limosilactobacillus pontis 9K3	14+ 0.6	12+ 0.3	16.0±1.5	12+0.5
Lb. fermentum 3SH1	19+0.5	22+0.7	13.1±0.2	15+0.7

When comparing the acid formation energy of the studied cultures with their antagonistic activity against *Bacillus subtilis* ATCC 6633 (reference strain for determining antibiotic activity), shown in Table 1, we can establish that the antagonism of lactobacilli cultures, in this case, may be related not only with the formation of acids, but also with the synthesis of bacteriocins. Thus, the *Pediococcus acidilactici P2-6* culture, accumulating less acid (up to 103°T), exhibits a rather high antagonistic activity comparable to the activity of *Lactobacillus paracasei 114* and *Lactobacillus paracasei 82* cultures, which are strong acid formers (respectively 280.4 ± 2.0 and 360, 4±0.6°T). In the resistance study of 7 of our lactic acid bacteria cultures to 12 antibiotics, we dedicated the presence among them of both strains, generally not possessing antibiotic resistance genes, and strains resistant to certain antibiotics (cultures of the genera *Lactobacillus* and *Pediococcus*) (Tab. 2).

Antagonistic relationships are widespread in the world of microorganisms and are one of the factors in the formation of microbial communities. They are characterized by the fact that one type of microorganisms somehow inhibits or delays the growth of others. Lactic acid bacteria can form antibiotic substances and thereby have a bacteriostatic and bactericidal effect on adverse microflora. This ability has found wide application in medicine, veterinary medicine, agriculture, and the food industry. The use of cultures of lactic acid bacteria-antagonists to B. subtilis and mold fungi in the form of wheat sourdough in various ways of making wheat bread guarantees its microbiological safety and prevents potato disease and mold.

Evaluation of the antagonistic activity of 7 mono-cultures of lactic acid bacteria by the method of delayed antagonism showed that most of our strains have a strong antimicrobial effect.

Almost all isolated strains, except for one, showed antagonistic activity against all indicator cultures. In strains *Lb. paracasei 82, Lacticaseibacillus paracasei 126* and *Lb. paracasei 114* and *Lb. pediococcus* antagonistic activity against all test cultures was the highest. The manifestation of antagonism by lactic acid

bacteria in the presence of catalase and the neutralization of acid exclude the action of hydrogen peroxide and organic acids but indicate the possible synthesis of bacteriocinogens by these cultures.

Conclusions

As a result of lactic acid bacteria strains screening included in the Kazakh Research Institute of Processing and Food Industry cultures collection, we selected the 5 most active lactic acid bacteria cultures with probiotic properties for inclusion in the consortium: *Limosilactobacillus pontis 9K3, Lb. fermentum 3Sh1, Lb. paracasei 82, Lb. paracasei 114, Lacticaseibacillus paracasei 126.* The selection criteria were resistance to high concentrations of salt, bile, high enzymatic, acid-forming, proteolytic, and antagonistic activity of strains against opportunistic and pathogenic microflora (*B. subtilis ATCC 6633, Escherichia coli-1257, Staphylococcus sp. 209-P, Salmonella typhimurium*). Antagonistic activity to the test culture *Bacillus subtilis* ATCC 6633 was stable in all strains, was not inhibited at pH 6.0, and in the presence of catalase, i.e., was due to the production and secretion of bacteriocins by the studied strains. This study was to create and characterize a new consortium of domestic strains of lactic acid bacteria for starter cultures and probiotics. The new consortium of microorganisms will serve as the basis for the creation of new competitive domestic biological products to protect the bread from microbiological spoilage. In the future, the biocompatibility of selected strains of lactobacilli in the consortium will be determined to be included in the sourdough for bread.

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Нанға ашытқы жасау кезінде сүт қышқылы бактериялары дақылдарының физиологиялық және биохимиялық қасиеттерін зерттеу

Мақалада бұрын бидай, ұн, қара бидай ашытқысынан, сондай-ақ қымыз және шұбат дәндерінен бөлінген Қазақ қайта өңдеу және тамақ өнеркәсібі ғылыми-зерттеу институтының коллекциясынан *Lactobacillus* және *Pediococcus* тұқымдас сүт қышқылы бактериялары дақылдарының скрининг нәтижелері ұсынылған. Сүт қышқылы бактерияларының іріктелген 7 штамының пробиотикалық қасиеттерін кешенді бағалау үшін олардың физиологиялық және биохимиялық қасиеттері зерттелді: қышқыл түзуші, антагонистік белсенділігі, сахаролитикалық бейіні, әртүрлі өт концентрациясына, ас тұзына, түрлі pH көрсеткіштерінде өсуі, антибиотикке төзімділігі. Штамдарды скрининг нәтижесінде нанға арналған ұйытқы құрамына енгізу үшін сүт қышқылды бактериялардың белсенді 5 дақылдары іріктеліп таңдалды*: Limosilactobacillus pontis 9K3, Limosilactobacillus fermentum 3Ш1, Lb. paracasei 82, Lb. paracasei 114, Lacticaseibacillus paracasei 126.* Іріктеу критерийлеріне негіз болған, ол шартты-патогенді және патогенді микрофлораға (*B. subtilis ATCC 6633, Escherichia coli-1257, Staphylococcus sp.209-P, Salmonella typhimurum*) қатысты штамдардың жоғары ферментативтік, қышқыл түзуші, протеолитикалық және антагонистік белсенділік көрсетуі. Алынған талдаудың нәтижесіне қарап, іріктелген дақылдардың консорциумын пайдалану алдағы уақытта нан-тоқаш өнімдеріне арналған отандық пробиотикалық ашытқы алуда көптеген себебі бар. Бұл өз кезегінде өндірістің тиімділігін арттыруға, нан пісіру өнеркәсібінде тамақ өнімдерінің сапасы мен қауіпсіздігін қамтамасыз етуге мүмкіндік береді.

Кілт сөздер: ашытқы, сүт қышқылы бактериялары, нан, таза дақыл, *Limosilactobacillus, Pediococcus*, пробиотикалық қасиеттері, антибиотикке төзімділігі.

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Изучение физиолого-биохимических свойств культур молочнокислых бактерий при создании заквасок для хлеба

В статье представлены результаты скрининга культур молочнокислых бактерий рода *Lactobacillus* и *Pediococcus* из коллекции Казахского научно-исследовательского института перерабатывающей и пищевой промышленности, выделенных ранее из зерна пшеницы, муки, ржаных заквасок, а также кумыса и шубата. Для всесторонней оценки пробиотических свойств отобранных 7 штаммов молочнокислых бактерий были изучены их физиолого-биохимические свойства: кислотообразующая, антагонистическая активность, сахаролитический профиль, устойчивость к различным концентрациям желчи, поваренной соли, рост при различных показателях pH, антибиотикорезистентность. В результате скрининга штаммов для включения в состав закваски для хлеба отобраны 5 наиболее активных культур молочнокислых бактерий: *Limosilactobacillus pontis 9K3, Limosilactobacillus fermentum 3Ш1, Lb. paracasei 82, Lb. paracasei 114, Limosilactobacillus paracasei 126*. Критериями отбора служила высокая ферментативная, кислотообразующая, протеолитическая и антагонистическая активность штаммов в отношении условно-патогенной и патогенной микрофлоры (*B. subtilis ATCC 6633, Escherichia coli-1257, Staphylococcus sp.209-P, Salmonella typhimurum).* Нами показано что этот результат получения анализа свидетельствует о перспективности использования консорциума этих культур для получения пробиотической закваски для хлебобулочных изделий. Это, в свою очередь, позволит повысить эффективность производства, обеспечить качество и безопасность пищевых продуктов в хлебопекарной промышленности.

Ключевые слова: закваска, молочнокислые бактерии, хлеб, чистая культура, *Limosilactobacillus, Pediococcus*, пробиотические свойства, устойчивость к антибиотикам.

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