Development of a Dietary Ferrous Milk Product based on Local Probiotic Crops

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ABSTRACT

The paper presents the results of research on the selection of probiotic cultures of the genus *Lactobacillus* for the design of dietary fermented milk products. Cultures of lactobacilli were identified to the species level, characterized by the results of biochemical tests, and their antiradical activity and sensitivity to antibiotics were studied. Selected strains are included in 2 types of fermented dairy products. HPLC analysis of the spectrum of free amino acids in these products showed that the products contain a high amount of essential amino acids. Product B contains 8 essential amino acids, due to which it can be recommended as a possible nutraceutical in the food and medical industries.

KEYWORDS
Lactobacillus, Culture, HPLC analysis, Product B, Probiotic crops, Strains of lactobacilli

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INTRODUCTION

Functional dairy products are products that can provide health benefits and are a key opportunity for the dairy sector. The use of milk for the production of functional food products is associated with the composition of milk, which is a good food matrix for probiotic bacteria, such as lactic acid bacteria and bifidobacteria.[1] Fermentation processes typically increase the nutritional value of many foods and increase the bioavailability of nutrients. The enzymatic action of certain strains of Lactic Acid Bacteria (LAB) can result in the removal of toxic or ant nutritional factors such as lactose and galactose from fermented milk to prevent lactose intolerance and galactose accumulation.[2] the conversion of lactose to lactic acid is the most important fact, apart from other bioactive components. In addition, bacterial enzymes convert milk carbohydrates into oligosaccharides, some of which have prebiotic properties.[3] Fermented dairy products are rich in many vitamins and minerals with high bioavailability. They represent an important contribution of vitamins A, B_1, B_2, B_6, B_{12}, niacin, pantothenic acid and folic acid, as well as vitamin D, calcium, phosphorus, potassium, magnesium, zinc and potassium iodide.[4,5] Many of these micronutrients have a higher bioavailability in fermented milk products than in raw milk due to the process of acid formation and fermentation, which mainly affects the content of vitamins.[6] According to the guidelines for screening candidate microorganisms for probiotic activity developed by the joint FAO / WHO working group, it is necessary to screen candidate microorganisms for the following criteria: (1) tolerance to conditions in the gastrointestinal tract, (2) safety and (3) the possession of probiotics useful properties.[7]

Purpose of the research

The purpose of this study was to select probiotic strains of starter cultures of lactic acid bacteria and to assess the degree of enrichment with useful substances of a fermented milk product developed on their basis.

MATERIAL AND METHODS

The objects of research were 6 wild strains of lactobacilli: *Lactobacillus* delbrueckii P_2, *Lactobacillus S. plantarum* K_7 / 3, *Lactobacillus* fermentum SXT - 2, *Lactobacillus* rhamnosus TC - 1 and *Lactobacillus* rhamnosus bazilik, *Lactobacillus* brevis CO_2 isolated from fermented milk products, cheeses, fermented vegetables and plants. All strains were stored frozen (- 80˚C) in the collection of the laboratory "Microbiology and biotechnology of probiotics" of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan. Biochemical activity of strains of lactobacilli. The enzymatic activity of lactobacilli strains was judged by the spectrum of carbohydrates fermented by them and by their total titratable acidity.[8] for genetic identification of the *L. plantarum* K 7 / 3 strains, universal primers of the 16_S rRNA gene of the genus *Lactobacillus* were used. BLAST analysis of the obtained sequence of the 16_S rRNA gene of *L. plantarum* K 7 / 3 was performed using the NCBI database using the MegaX program, using the clustalw algorithm, a phylogenetic tree was compiled using the mega X program using the Neighbor joining algorithm.[9] Antiradical activity of strains of lactobacilli. The antiradical activity of lactobacilli strains was judged by the activity of binding free oxide radicals of 2, 2 – Diphenyl - 1 - picrylhydrazyl (DPPH) according to the method described in LiS. etal.[10] The tests were carried out in triplicate. Antibiotic sensitivity of strains of lactobacilli. The sensitivity of strains of lactobacilli to antibiotics was determined by the disk diffusion method according to the guidelines of MUK 4.2. 1890-04. The following antibiotic disks were used: Ofloxacin (5 µg / disk), rifampicin (5 µg / disk), chloramphenicol (30 µg / disk), co -trimoxazole (25 µg / disk), cefotaxime (30 µg / disk), erythromycin (15 mcg / disk), gentamicin (10 mcg / disk), amikacin (30 mcg / disk), lincomycin (15 mcg / disk), tetracycline (30 mcg / disk), cefazolin (30 mcg / disk), oxacillin (1 mcg /ml), ampicillin (10 µg / disk) (HI - Media, India). Preparation of fermented milk products using isolated strains. Using the selected strains, 2 fermented milk products were prepared: product A, product B. A mixture of cultures in a volume of 3 ml was added to 100 ml of sterile skimmed cow's milk. Incubation was carried out in a thermostat at 37˚C. The fermentation time was recorded. The total titratable acidity of the finished product was determined by titration. High Performance Liquid Chromatography (HPLC) analysis of amino acids in the composition of the developed fermented milk products. The synthesis of FTC (phenylthiocarbomayl) derivatives of free amino acids of the developed fermented milk products was carried out according to the method of Steven A, Cohen Daviel.[12] Identification of FTC - amino acids is carried out on an Agilent Technologies 1200 chromatograph on a 75 x 4.6 mm Discovery HS C 18 column. Solution A: 0.14 M CH_3COONa + 0.05 % tea pH 6.4, B: CH_3CN. Flow rate 1.2 ml / min, absorbance 269 nm. Gradient % B / min: 1 – 6 % / 0 - 2.5 min, 6 – 30 % / 2.51 – 40 min, 30 -60 % / 40.1 - 45 min, 60 - 60 % / 45.1 - 50 min; 60 – 0 % / 50.1 - 55 min.

RESULTS AND DISCUSSION

Morphological, cultural and physiological properties of the strains used. The strains used in the work were isolated by us from various natural substrates suitable for the isolation of lactobacilli and identified to the genus by morphological, cultural, physiological properties and biochemical tests (Table 1). Also, their species affiliation was established using the MALDI - TOFMC technique (Brucker, Germany).

strains	Selection source	Gram paint	Titratable acidity	Fermentable carbohydrates	Cell morphology
L casei K_7 / 3	sauerkraut	Gram+	90 °T	glucose, fructose, galactose, cellobiose, maltose, sucrose, sorbitol, lactose	Short thin sticks with rounded ends, arranged mainly in 2 and in short chains
L brevis CO_2	Salted cucumbers	Gram+	60 °T	raffinose, maltose, galactose, sucrose, fructose	Thin sticks, connected in pairs, with rounded ends, form short, and sometimes long chains.
L delbrueckii P_2	curdled milk	Gram+	270 °T	lactose	Long sticks arranged in chains
L fermentum SXT - 2	cheese	Gram+	95 °T		thin, straight sticks with rounded ends, arranged in chains
L rhamnosus TC – 1	cheese	Gram+	150 °T	Cellobiose, sorbitol, rhamnose, maltose, galactose, sucrose, lactose, fructose	Straight sticks collected in short or long chains
L rhamnosusbazilik	basil flowers	Gram+	85 °T	Arabinose, rhamnose, cellobiose, galactose, fructose	Thin, short sticks with rounded ends

Table 1. Morphological, Cultural and Physiological Properties of the Studied Strains.

Phylogenetic analysis revealed that the sequence product from *L. plantarum* K 7 / 3 is very close to the strain *L. plantarum* HBUA53012. Analysis of the obtained phylogenetic tree showed that *L. plantarum* strains can be grouped into three separate branches, of which one branch is divided into two branches. The *L. plantarum* K 7 / 3 strain was formed as one of the off shoots along with another offshoot of the *L. plantarum* HBUA53012 strain (Figure 1).

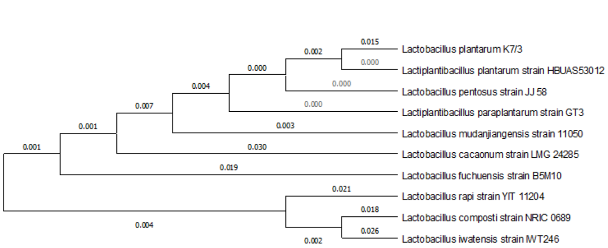

Figure 1. Original Phylogenetic Tree of Strain K 7/3.

Antiradical activity of strains of lactobacilli. The antioxidant activity of the studied strains differed between strains, and the degree of radical removal activity of culture supernatants was 96.4% and 74.6%. The *L brevis* CO_2 culture supernatant showed the highest activity (96.4 ± 0.40 %), while the *L. delbrueckii* P_2 culture supernatant (74.6 ± 0.60 %) showed the least activity (Figure 2).

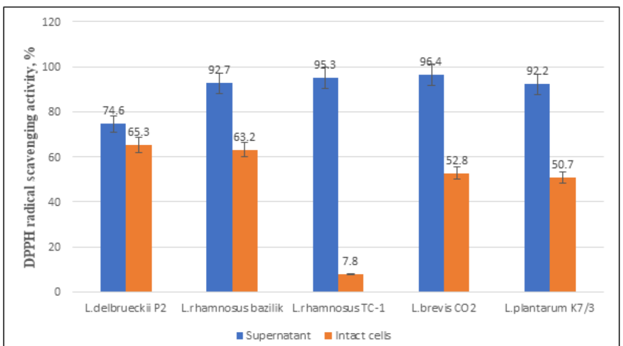

Figure 2. Antiradical Activity of Lactobacilli Strains.

Intact cells of the studied cures were characterized by relatively low antiradical activity. Intact *L. delbrueckii* P_2 cells demonstrated high antiradical ability (65.3 ± 0.45 %). Intact cells of *L. rhamnosus* TC - 1 culture showed the lowest antiradical activity (7.8 ± 0.23 %). as *lactococcus* lactis subsp. lactis 12 and *Bifidobacterium* animalis RH and *lipoteichoic* acid on *bifidobacteria*.[13-15] (2019) report that *Lactobacillus spp.* Isolated from kefir, DPPH showed radical scavenging activity associated with Total Phenol Content (TPC) and Total Flavonoid Content (TFC) In the present study, it was found that the studied microorganisms have a high antioxidant activity against DPPH radicals.[16] Antibiotic resistance profile of *lactobacillus* strains. The antibiotic resistance profile of the 2 strains of *L. rhamnosus* TC - 1 and bazilik did not agree that *L. rhamnosus* bazilik has absolute resistance to ciprofloxacin, while *L. rhamnosus* TC - 1 is moderately resistant (Figure 3).

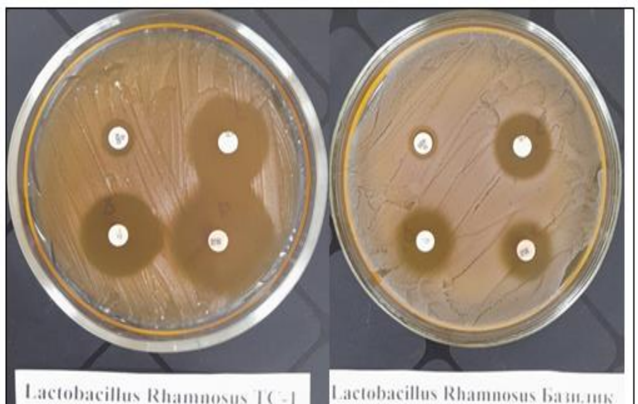

Figure 3. Antibiotic Resistance of *L.rhamnosus* Cultures.

L. plantarum K7 / 3 is sensitive to rifampicin, erythromycin, cefotaxime, azithromycin, tetracycline; moderate resistance to ofloxacin, gentamicin, lincomycin, cefazolin, ciprofloxacin and ampicillin; resistant to amikacin (Table 2).

NO	Cultures	Content, µg / disk	*L. delbrueckii* P_2	*L. plantarum* $K_7/3$	*L. rhamnosus* TC - 1	*L. rhamnosusbazilik*	*L. fermentum* SXT - 2	*L. brevis* CO_2
1	Cefazolin	30	0 / S	14 / MR	0 / S	0 / S		0 / S
2	Amikacin	30	0 / S	9 / S	11 / MR	14 / MR		8 / S
3	Erythromycin	15	45 / Sen	37 / Sen	35 / Sen	23 / Sen		30 / Sen
4	Ampicillin	10	12 / MR	18 / MR	0 / S	0 / Sen		9/S
5	Azithromycin	15	40 / Sen	26 / Sen	26 / Sen	19 / Sen		22 / Sen
6	Gentamicin	10	14 / MR	18 / MR	11 / MR	9 / S		10 / MR
7	Tetracycline	30	32 / Sen	28 / Sen	33 / Sen	17 / MR		18 / MR
8	Lincomycin	15	40 / Sen	15 / MR	24 / Sen	19 / Sen		33 / Sen
9	Ofloxacin	5	0 / S	14 / MR	14 / MR	9 / S		9 / S
10	Ciprofloxacin	5	0 / S	15 / MR	16 / MR	0 / S		0 / S
11	Cefotaxime	30	30 / Sen	30 / Sen	0 / S	0 / S		18 / MR
12	Rifampicin	5	40 / Sen	30 / Sen	30 / Sen	22 / Sen		23 / Sen

Table 2. Antibiotic Resistance Profile of *Lactobacillus* Strains.

It is known that plasmids exist in lactobacilli and *bifidobacteria*, especially in strains isolated from the intestine, which have genes encoding antibiotic resistance. When selecting probiotic strains, it is recommended that probiotic bacteria should not contain transmissible drug resistance genes encoding resistance to clinically used drugs. According to M. Danielsen and A. Wind, genes for resistance to chloramphenicol, erythromycin, and tetracycline are susceptible to horizontal transmission.[17] Bacteria of the genus *Lactobacillus*, as a rule, are sensitive to these antibiotics and to most antibiotics that inhibit protein biosynthesis, with the exception of aminoglycosides.[18,19] The reduced sensitivity of bacteria of the genus *Lactobacillus* to aminoglycosides is also considered natural and this property is associated with the low permeability of antibacterial drugs of this group through the membranes of lactobacilli.[20]

Thus, the studied strains of lactobacilli revealed a resistance profile typical of this genus of lactobacilli. Designing a dietary fermented milk product and studying their amino acid composition. The composition of Product A includes cultures: *L. rhamnosus* TC - 1*, L. plantarum* K_7 / 3*, L. fermentum* SXT - 2; Cultures fermented milk for 8 hours with the formation of a dense clot. The finished product has a delicate sour - milk taste and a moderately dense clot, without gas bubbles and without released whey. The total titratable acidity is 160 oT. Cultures included in Product B: *L. delbrueckii* P_2, *L. brevis* CO_2, *L. rhamnosus bazilik*. The finished product has a slightly viscous texture, the taste of the product is sour milk, the formation of gas bubbles is not observed and whey is not released. The total titratable acidity is 260 oT. Using the HPLC technique, a quantitative analysis of 20 free amino acids in the composition of milk and 2 developed dietary fermented milk products was carried out. Milk was used as a control. So, in Product A, the content of free amino acids was 2.894919 mg / ml. It was noted that an increase in the amount of essential 7 amino acids: cysteine, aspartic acid, threonine, arginine, methionine, histidine, tryptophan, phenylalanine, lysine than in milk (Table 3).

Amino acids	milk	Product A	Product B
	Concentration mg / g		
Asparagine acid	0,006886	0,036149	0,26589
Glutamine acid	0,577735	0,111389	15,79,545
Serene	0,086538	0,042355	0,529495
Glycine	0,060934	0,085564	0,02221
Asparagine	0,061051	0,085218	0,022894
Glutamine	0,300688	0,137417	15,32,731
Cysteine	0,011018	0,302432	1,18,693
Threonine	0,085633	0,254503	0,303167
Arginine	0,014827	0,041315	12,70,004
Alanine	0,027076	0,050396	0,068969
Proline	0,03509	0,0458	0,163613
Tyrosine	0,23372	0,022642	14,03,443
Valine	0,175993	0,22133	0,252504
Methionine	0,002015	0,046096	0,065239
Isoleucine	0,019289	0,012206	0,679777
Leucine	0,023589	0,022523	12,27,596
Histidine	0	0,122486	0,211566
Tryptophan	0,011969	0,075703	23,37,373
Phenylalanine	0	11,62,816	1,07,393
Lysine HCl	0	0,01658	0,487755
Total	17,34,052	28,94,919	14,68,463

Table 3. Quantitative Content of Free Amino Acids in the Studied Dietary Fermented Milk Products.

Thus, product B differs greatly in the content of free amino acids, the total value of which was 14.68463 mg / ml. It is important to note that this product contains 8 essential amino acids: cysteine, aspartic acid, threonine, arginine, methionine, histidine, tryptophan, phenylalanine, and lysine. Especially this product contains a high amount of arginine equal to 1.270004 mg / ml (milk 0.014827 mg / ml). Arginine is an essential amino acid for children and adolescents, the elderly and sick people, in whom the level of arginine synthesis is often insufficient.[21] Basically all cultures of lactic acid bacteria accumulate glutamic acid and proline during the fermentation of milk. In addition to these dominant amino acids, some species and strains accumulate aspartic acid, alanine, leucine, lysine, etc. Therefore, by appropriate selection of lactic acid bacteria strains, a certain set of free amino acids can be provided in the finished product, which is especially important for the taste properties of cheeses and fermented milk products. Amino acids formed as a result of the breakdown of milk proteins and not used by microorganisms for the synthesis of cellular proteins and other nitrogen - containing substances undergo further enzymatic transformations. Microorganisms break down amino acids with the formation of numerous intermediate products (organic acids, aldehydes, etc.) accumulated in the nutrient medium.[22]

CONCLUSION

According to the results of screening the profile of antibiotic resistance and the ability to bind oxide radicals, strains of lactobacilli were selected and 2 fermented milk products were designed on their basis. Studies have shown that product B contains a high amount of essential amino acids, which are of functional importance in the prevention and treatment of a number of diseases of the endocrine and cardiovascular systems. The data obtained indicate that product B, enriched with valuable amino acids, has not only nutritional value, but also high biological value and functional properties.

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