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ISOLATION AND CHARACTERISTICS OF ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA FROM WHITE CHEESE SAMPLES

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Abstract

Lactic acid bacteria (LAB), which have GRAS status, have been used since ancient times by representatives of different civilizations as starter or co-cultures for the production of various fermentation products. As part of these products, they transform carbohydrate components to fermentation and synthesize a large amount of lactic acid, which, creating an acidic environment, first - gives the products pleasant taste and organoleptic properties, the second - prevents the development of accompanying microbial populations and protects them from spoilage and contamination pathogens. Along with lactic acid, LAB produce many metabolites with antimicrobial properties. Important of them are BLIS (Bacteriocin – like inhibitory substances), active peptides - synthesized on ribosomes and post-translationally modified. With selective antimicrobial activity, these peptides usually affect closely related species, but some have strong activity against pathogenic and opportunistic microbes. Thus, interest in them is increasing everyyear.

In the present work, two previously isolated from three samples of Apsheron white cheese, were unidentified as bacteriocinogenic strains. Primary screening was carried out using "direct plating" method.The antimicrobial activity of isolated strains was determinedby the variants of agar diffusion method. The strains were active against gram-positive and gram-negative bacteria, including *L. monocytogenes, S. aureus, L. brevis* and *E. coli*. The maximum titer of the BLIS produced was found in the cultivation medium at the end of the exponential growth phase of the LAB cultures, which defines them as the primary metabolites. Phenotypic identification of active strains revealed their belonging to two species of the genus *Enterococcus*: *E. faecium* P1-3 and *E. faecalis* P3-2.

Keywords: white cheese, lactic acid bacteria, antimicrobial activity, bacteriocin.

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, catalase- and oxidase-negative, non-motile, non-spore-forming spherical and/or rod-shaped bacteria. They are anaerobic, but are also capable of growing under

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microaerophilic conditions. The source of energy for the life of LAB is carbohydrates, which are converted into lactic acid through fermentation (9).The accumulation of lactic acid leads to increased acidity in the medium and inhibits the growth of accompanying bacterial populations. In addition to lactic acid, the arsenal of antimicrobial metabolites of LAB also includes bacteriocins, hydrogen peroxide, diacetyl and short-chain fatty acids. These bacteria have GRAS (Generally Recognized as Safe) status. All these properties allow LAB to be widely used in the production of a wide variety of fermented milk and other fermented food products [17].

Bacteriocins are antimicrobial substances of peptide origin, which are synthesized in ribosomes [10]. They are colorless, odorless and tasteless, stable at high temperatures, low pH and a wide range of salt concentrations [5, 3].These antimicrobialagents are active against a wide range of food pathogenic and food spoilage bacteria [3]. In addition, bacteriocins produced by lactic acid bacteria are sensitive to digestive enzymes such as pancreatin complex, trypsin and chymotrypsin, and for this reason do not have a negative effect on the intestinal microbiota [6]. The above properties of bacteriocins contribute to their potential applications in the food industry and medicine [11].

Bacteriocins or BLIS inhibit the growth of microorganisms by various mechanisms. They can suppress cell membrane components (peptidoglycans), influence the expression of various genes [15], form pores in membranes, thereby leading to changes in the membrane potential of the cytoplasm and, ultimately, to cell death [5].

The aim of this study was to isolate bacteriocinogenic LAB from traditional Azerbaijani white cheese samples and to partially characterize their inhibitory activity. Considering the important diversity of climatic and ecological niches of Azerbaijan, as well as the ancient pastoral traditions of different ethnic groups living in this region, research in this direction is of great interest.

2. Materials and methods

The sources of active LAB strains were 3 different samples of ready-to-eat white semi-hard cheese, which were prepared using traditional methods at home from fresh cow's milk. They were purchased from the suburbs of Baku. Samples (250 g) were placed in previously prepared sterile jars and stored at -20^{0}C until use.

Screening of active strains of LAB. This procedure was carried out according to a previously described method [7]. To detect the antimicrobial activity of the isolated strains, the direct plating method was used [4]. Samples of each type of cheese were homogenized in saline and then serially diluted 10-fold with saline. Aliquots (1 ml) were placed in soft MRS agar medium (0.8%, w/v) and incubated at 37°C for 48 hours. Serial dilution plates were coated with an indicator strain (*Lactobacillus delbrueckii subsp. bulgaricus* 340) inoculated (5%, v/v) in 10 ml of soft MRS agar medium (0.8% agar) and incubated for 24 h at 37^{0}C.Inhibition was assessed as positive if there was a noticeable zone of clearing around the colony of the producer strain. Positive colonies were selected at random and removed using a sterile Pasteur pipette. An agar plug from a Pasteur pipette was inoculated into liquid MRS medium for 24 hours at 37 ^{0}C. LAB isolated from cheese were stored at -80°C in MRS broth containing 20% glycerol.

Determination of antimicrobial activity. This procedure was carried out according to a previously described method [10]. Antimicrobial activity of LAB was determined using the well-diffusion method [16] using 20 ml of soft agar medium (0.8% agar) containing 100 µl of an indicator strain. After this, wells (9 mm in diameter) were cut out in the agar, and 100 µl of cell-free supernatant (centrifugation at 10,000 g for 15 min at 4^0 C) of potential producer strains was placed into each well. To eliminate the inhibitory effect of lactic acid on test organisms, the supernatants were adjusted to pH 6.5 with 1N NaOH, followed by filtration through a 0.22 µm filter. A clear zone of inhibition with a diameter of at least 2 mm was recorded as positive.

Determination of the dynamics of bacteriocin production. To determine the dynamics of bacteriocin production, 10 ml of an overnight culture of LAB strains was inoculated into 100 ml of MRS broth, then incubated at 37 ^{0}C. At appropriate intervals, cell growth was monitored by measuring absorbance at 600 nm) and pH. Samples were removed at 2-hour intervals, and neutralized cell-free supernatants treated with catalase (1 mg/ml) for an hour were tested for antimicrobial activity as measured by clear zona diameter.

Determination of the spectrum of antimicrobial activity. For this purpose, the method of well-diffusion analysis was used. Microorganisms, the list of which is given in Table 1, were used as indicator strains.

Identification of active strains. The process of identifying active strains included the gram test, catalase test, microscopic examination, growth at 10 ^{0}C, 15 ^{0}C, 37 0C and 45 ^{0}C, in the presence of 6.5% sodium chloride and at pH 9.6. Species-level identification of these strains was carried out according to the scheme of Murray et al. [13].

Statistical analysis of results. All experiments were performed in triplicate. Statistical analysis was performed using Microsoft Office Excel 2007 and Sigma Plot-12. Results were expressed as the mean ± SD (standard deviation) of three replicates, assuming the p value was <0.05.

3. Results

Isolation of BLIS producers. The sources of active strains were 3 different samples of white semi-hard cheese, which were made in Apsheron region, at home using traditional methods from fresh cow's milk and ready for consumption. Samples (250 g) were placed in previously prepared sterile jars and stored at -20^{0}C until use.

From three cheese samples, 48 single colonies were isolated, based on different morphology on MRS agar. Obtained 48 pure cultures were pre-cultured twice in MRS broth (pH 6.5). Collected spent - cultures (CFS -cells-free supernatants) were initially tested by the direct plating method against *L. bulgaricus* 340. The results are shown in Fig. 1, from which it is clear that around 8 colonies the growth of the indicator strain is suppressed, thus, they have antimicrobial activity. Active colonies were numbered according to the cheese samples and designated P1-1, P1-2, P1-3, P2-1, P2-2, P2-3, P3-1 and P3-2.

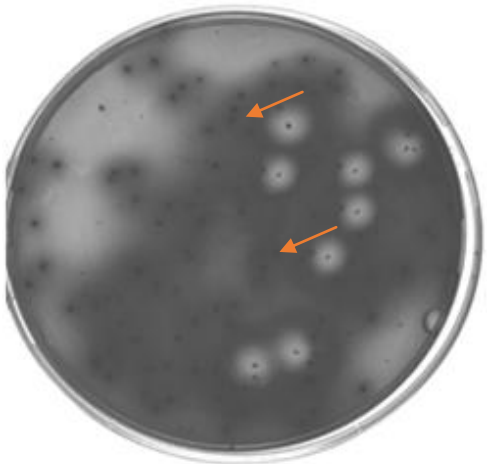

Fig.1. Screening of LAB for antimicrobial activity in cheese samples using the “direct plating” method: arrows indicate BLIS-producing strains

As already noted, the antagonistic activity of LAB is carried out by the secretion into the medium of organic acids, hydrogen peroxide and bacteriocins. The biochemical nature of the

active component of each strain was tested using the agar diffusion method, as described in the methodological part of the article. The results of these studies are summarized in table. 1. From the table it follows that after neutralization of the culture liquid, the antimicrobial activity of 4 strains (P1-1, P1-2, P2-1 and P3-1) did not appear. This fact suggests that the antimicrobial activity of 4 strains was due to the secretion of lactic acid into the medium, as a result of which the medium was strongly acidified and the indicator strain did not grow under such conditions.

Treatment of the CFS of exponential cultures of active strains with catalase (1 mg/ml) for 2 hours led to the loss of antimicrobial activity of strains P2-2 and P2-3. This is a character that the active component of these strains is hydrogen peroxide, which was cleaved by catalase and the strains were deprived of antimicrobial activity (Table 1).

Table 1. Influence of pH, catalase and proteinase K enzymes on the antimicrobial activity of lactic acid bacteria isolated from cheese samples

	P1-1	P1-2	P1-3	P2-1	P2-2	P2-3	P3-1	P3-2
pH6,5	-	-	+	-	+	+	-	+
Catalase	+	+	+	+	-	-	+	+
Protease	+	+	-	+	+	+	+	-

Next, the CFS of the active strains was treated with proteinase K (1 mg/ml) for 2 hours. The results of these experiments are shown in Table 1 and Fig. 2. Judging by the results, the spent cultures of 6 strains in MRS broth retained their antimicrobial activity after treatment with a proteolytic enzyme. However, activity in the CFS of strains P1-3 and P3-2 did not appear. This fact suggests that the antimicrobial activity of these 2 strains was due to a substance of peptide origin - bacteriocine. Apparently, under the action of proteinase K, the polypeptide chain of this metabolite is cleaved and loses its activity.

Fig. 2. Effect of protoeinase K on antimicrobial metabolites of active strains of lactic acid bacteria. (Indicator organism - *L. bulgaricus* 340)

Thus, from 3 cheese samples, 8 strains with antimicrobial properties were isolated. The activity of 4 strains was associated with lactic and other organic acids, 2 strains with hydrogen peroxide and 2 strains with the production of BLIS.

Dynamics of growth and production of bacteriocin. In the next series of experiments, the dynamics of growth, synthesis of organic acids and production of BLIS of strains P1-3 and P3-2 were studied. The results obtained are shown in Fig. 3.From this figure it follows that the growth

dynamics curves of both strains corresponded to the classical growth pattern of microorganisms and included lag phases, phases of slow and exponential growth, stabilization and death. The starting optical density of the suspension at a wavelength of OD-600 nm was 0.37. Maximum growth of both strains was detected after 10 hours. At the same time, the OD of strain P1-3 reached a value of 5.6, and that of strain P3-2 reached a value of 6.5.

From these graphs it is also clear that in the variant with P1-3 at the very beginning of fermentation, the pH of the MRS medium was equal to 6.2. During the first 10 hours, this indicator decreased almost linearly to pH 5.0.This pH level coincided with the end of the exponential phase and the maximum growth value of this strain. Further, the increase in the acidity of the suspension continued for another 4 hours and reached a pH level of 4.8, then stabilized at this level until the end of the fermentation process. A similar picture was observed with strain P3-2, with the only difference being that the final pH level in this case was 4.4, that significantly lower than in the suspension of the first strain (Fig. 3).

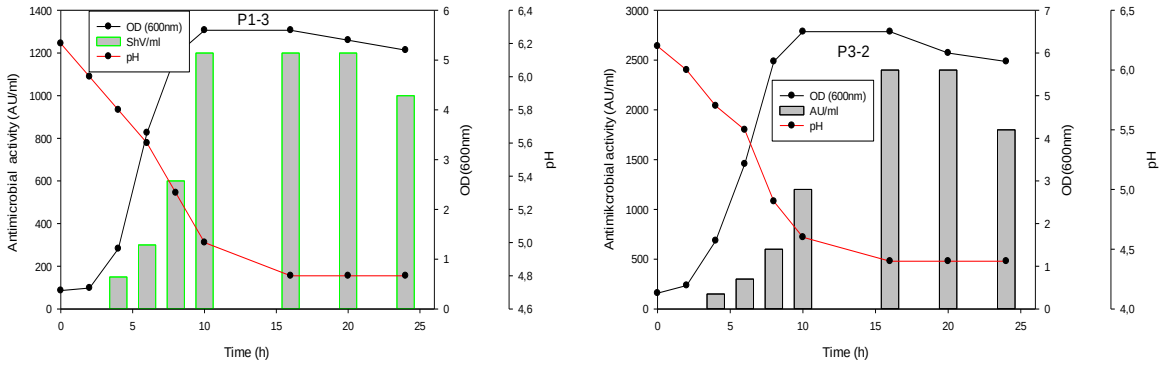

Fig. 3. Dynamics of growth, medium acidification and bacteriocin production of strains P1-3 and P3-2 isolated from cheese samples

As for the graphs of bacteriocin synthesis and secretion, both similarities and significant differences were found between the performance of these two strains (Fig. 3). Thus, in both strains, the first signs of bacteriocin secretion into the medium appeared in the early phase of slow growth of the suspension. However, if the maximum bacteriocin titer in suspension P1-3 was detected at the end of the exponential growth phase of the strain (1200 AU/ml), then in the suspension of strain P3-2 a similar indicator was achieved at the end of the stabilization phase of strain growth at a level of 2400 AU/ml.

Spectrum of antimicrobial activity of strains. The next series of experiments was devoted to determining the spectrum of antimicrobial activity of these two strains. For this purpose, 11 different indicator strains were tested. The results obtained are summarized in Table 2.

Table 2. Spectrum of *in vitro* antimicrobial activity of strains P1-3 and P3-2 (zones of inhibition of indicator strains are indicated in mm; bacteriocin titer 1200 AU/ml)

Indicator strains	Activestrains	
	P1-3	P3-2
L. bulgaricus 340	24±1	20±0,8
Levilactobacillus brevis F145	16±0,5	10±0,2
Listeria monocytogenes 302	10±0,2	-
Listeria innocua CIP 80.11	-	-
Staphylococus aureus CIP 9973	12±0,2	-
Escherichia coli BAS 23355	-	8±0,1

*E. coli*ATCC 25922	-	10±0,1
*E. coli*CIP 104368	-	8±0,1
Candida pseudotropicales	-	-
Sacharomyces cerevisiae DSH213.83	-	-
Fusarium culmorum 302	-	-

It can be seen that the both strains have a broad spectrum of activity against various indicator strains. However, they differ from each other in the spectrum of antimicrobial activity. Thus, strain P1-3 suppressed the growth of the following four strains: *L.bulgaricus* 340, *L. brevis* F145, *L. monocytogenes* 302 and *Staphylococcus aureus* CIP 9973. Strain P3-2 demonstrated a different spectrum of antimicrobial activity and inhibited the growth of the following five indicator strains: *L. bulgaricus* 340, *L. brevis* F145, as well as all three tested *E. coli* strains. Microscopic fungi and molds turned out to be resistant to the effects of bacteriocin of active strains.

Identification of active strains. Our studies showed that both of these strains were Gram-positive, catalase-negative cocci. They grew at temperatures of 10^0C, 30^0C, 37^0Cand 45^0C, in a medium of 6.5% (w/v) sodium chloride and at pH 9.6. These properties are characteristic of representatives of the genus *Enterococcus*.

Species-level identification of these strains was carried out according to the scheme of Murray et al. [13]. It turned out that strain P1-3 was capable of fermenting mannose, arabinose, raffinose and sucrose, as well as arginine. Tellurite, pyruvate, sorbitol, sorbose and methyl-D-glucopyranose were not utilized by this strain.The bacteria were immobile and did not produce pigment. Based on these characteristics, this strain was identified as *Enterococcus faecium*.

Strain P3-2, in addition to mannose and sucrose, was unable to ferment carbohydrates. In return, tellurite, pyruvate, as well as arginine and sorbitol were utilized. Bacteria of this strain were also immobile and did not form pigment. Based on these characteristics, this strain was identified as *Enterococcus faecalis*.

4. Discussion

Geographically, the territory of Azerbaijan lies at the crossroads between Europe and Asia. A number of traditional fermented milk and fermented food products of plant origin are cultivated in this country. This is due to the fact that the country has historically been inhabited by people of different nationalities with their own cultural characteristics and national cuisine. The large variety of fermented foods makes them very interesting sources of LAB with probiotic properties.One such fermentation product is a wide variety of cheese products, especially white cheese. More than half of the population of Azerbaijan uses this product [7].

In this study, 8 LAB with antimicrobial activity were isolated from 3 cheese samples. Biochemical analysis of the antimicrobial metabolites of these strains showed that two of them had the ability to produce BLIS. Primary phenotypic identification of these strains was carried out and it was found that both of them belong to the genus *Enterococcus*: strain P1-3 was identified as *E. faecium*, and P3-2 - as *E. faecalis*.

The literature contains numerous examples of the search and detection of bacteriocinogenic LAB strains in various cheese samples. At the same time, different numbers of such strains were found in different samples of cheese products. For example, 5 strains of LAB were isolated from Carpathian cheese, including three strains of the genus *Enterococcus* [11]. Another 5 LAB strains were isolated from different types (Minas, Manteiga and Caipira) of cheese [6]. *E. faecalis* KT11, which also produces a bacteriocin, was isolated from traditional Kargi Tulum cheese [1]. Bacteriocinogenic strain *L. plantarum* KLDS 1, isolated from Mongolian cheese [12]. Mohammed

and Chon [10] isolated five LAB strains with similar activity from white cheese samples, three of which (S1113, S092, S1121) were also members of the genus *Enterococcus*.

Thus, cheese samples are rich in bacteriocinogenic strains of various types of LAB. Among these strains there are many species belonging to the genus *Enterococcus*. The difference in the species composition of active strains is the result of the cultivation of cheese samples in different ecological niches.

A broad spectrum of antimicrobial activity of the studied strains with antagonistic effect on different bacteria was shown. At the same time, bacteriocin P1-3 suppressed only the growth of Gram-positive bacteria (*L. bulgaricus* 340, *L. brevis* F145, *L. monocytogenes* 302 and *S. aureus* CIP 9973), whereas, strain P3-2 affected the growth of gram-positive and gram-negative bacteria such as *L. bulgaricus* 340, *L. brevis* F145, as well as all three tested *E. coli* strains - BAS 23355, ATCC 25922 and CIP 104368.

According to the literature, *E. durans* FMA8, a strain from Carpathian cheese, had antimicrobial activity against *S. typhimurium*, *L. monocytogenes*, *E. coli* and *S. aureus* [14]. The bacteriocin *E. faecalis* KT11 from Karga Tulum had inhibitory activity against *S. aureus, M. luteus*, and *L. monocytogenes* [8].

These data show that representatives of the genus *Enterococcus* in different cheese samples have different spectra of antimicrobial activity, but are able to inhibit the growth of both Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative (*E. coli*) opportunistic bacteria. In this respect, they are not particularly different from the active strains we isolated.

Conclusion

1. White semi-hard cheese samples used as a source of LAB with antimicrobial activity were not very rich in this aspect;
2. A total of 8 active previously unidentified LAB strains were isolated from three sources, two of which had the ability to synthesize an antimicrobial substance of peptide origin. Primary phenotypic identification of two bacteriocin-producing strains showed that strain P1-3 belongs to the *E. faecium* species, and P3-2 belongs to the *E. faecalis* species;
3. The isolated BLIS producers had broad spectrum antimicrobial activity against gram-positive and gram-negative opportunistic microorganisms

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