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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

INVESTIGATION OF *IN SITU* EFFECTIVENESS OF BACTERIOCINS IN MILK AND MODEL DAIRY PRODUCTS

Speciality: Biotechnology (including bionanotechnologies)

Field of science: Biology

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INTRODUCTION

Relevance of the topic and degree of development. In modern times, due to the gradual disruption of the ecological balance, the provision of safe food products to the world's population, which is increasing by approximately 80 million people a year and reached yet 8 billion, is characterized as a global challenge. The most obvious example of this problem is "*the uneven distribution of food products among the world's population, as a result of which nearly 2 billion people suffer from malnutrition"*[1]. Therefore, scientific research works in the direction of increasing the shelf life of food products and protecting their biological safety are in the focus of modern laboratories.

It is known that currently various synthetic preservatives are widely used to prevent spoilage of food products. Most of them are chemically synthesized and have a harmful effect on human health. For this reason, consumers' demand for food products without the use of chemical additives, or in small quantities, is increasing day by day. As a result, there is a need to explore natural, harmless alternative preservatives for product preservation. Bacteriocins are particularly interesting in the research conducted in this direction. They consist of a limited number of amino acid residues and are synthesized in bacterial cells with the participation of ribosomes. In the process of biological evolution, the formation of genes and expression products of this group of active peptides plays an important role in the formation of ontogonistic regularities existing among living things.

Bacteriocins have the potential to inhibit the growth of microorganisms that cause food spoilage. For this purpose, there are wide prospects of using STB bacteriocins, which are considered harmless and safe for consumption.

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[1] Fataliyev, H. Biotechnology / H. Fataliyev, Sh. Aliyeva, T. Musayev, - Baku; Ekoprint, - 2019. - 360 p.

"*Bacteriocins are active in a wide range of pH and temperature"*[2]. Unlike classical antibiotics, "*they do not need any specific receptors on the membrane or wall of the target cells, so they can connect to any point of the cell wall and exert their antimicrobial effect*"[3]. For this, a very small amount of bacteriocins is enough compared to antibiotics. Bacteriocins *"can effectively fight against harmful, opportunistic and pathogenic microbes* that are resistant to a wide range of antibiotics, *and there is no danger of the emergence of resistant microbes*"[4].

Some of the bacteriocins have a synergistic effect with classical antibiotics, enabling the use of the latter in lower doses. As a result, "*the side effects of antibiotics are prevented and the development of drug-resistant forms of strains is prevented*"[5-6].

It should be noted that the first researchers of bacteriocins in Azerbaijan were the employees of the former Department of Biochemistry and Biotechnology of Baku State University, where I was a doctoral student, and as a result of their work, "*many active*

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[2]Ramu, R. Bacteriocins and their applications in food preservation / R.Ramu, P. S. Shirahatti, A.T. Devi [et al.] // Critical Reviews in Food Science and Nutrition, - 2015. 60 (18), https://doi.org/10.1080/10408398.2015.1020918. 00–00

[3]Bonhi, K.L.R. Role of bacteriocin in tackling the global problem of multi-drug resistance: An updated review / K.L.R. Bonhi, S.Imran // Bioscience Biotechnology Research Communication, - 2019. 12, - p. 601–608.

[4]Cotter, P. D. Bacteriocins - Aviable alternative to antibiotics? / P.D.Cotter, R.P.Ross, C.Hill // Nature Reviews Genetics, - 2013. (11), - p. 95–105.

[5]Cavera, V.L. Bacteriocins and their position in the next wave of conventional antibiotics / V.L. Cavera, T.D. Arthur, D. Kashtanov [et al.] // International Journal of Antimicrobial Agents, - 2015. 46, - p. 494–501.

[6] Huseynova, N.F. Partial cleaning and characterization of the bacteriocin strain of Enterococcus faecium S5, isolated from Azerbaijan cheese / N.F. Guseynova, S.G. Gulahmadov, A.F. Akhmedova [et. al.] // Reports HAH of Azerbaijan, - Baku: - 2009, T. LXV, No. 5, - p. 95-103.

strains were obtained"[7-8]. Both the probiotic properties of those strains and the antimicrobial and molecular properties of their bacteriocins were studied in detail. and kept as a collection8. However, the activities of those strains in in-situ conditions, in other words, the properties of protecting food products from pathogenic microbes, have not been studied.

The object and subject of the research. Breast milk samples, bacteriocinogenic STB strains isolated from them and in the department's collection and their bacteriocin preparations, cow's milk, artificial baby food, model cheese samples, as well as pathogenic and conditionally pathogenic bacterial strains were used as research objects.

Research goals and objectives. The aim of the research work was to isolate a potentially bacteriocinogenic STB strains from human resident microbiota, to study the *in situ* effectiveness of his bacteriocins and other bacteriocinogenic strains in milk and model sour milk products.

To achieve the goal of the research work, the solution of the following tasks is envisaged:

1. Isolating bacteriocinogenic lactic acid bacteria from breast milk samples and characterizing its antimicrobial activity;
2. To study the characteristic technological properties of isolated active lactic acid bacteria;
3. To study the biochemical and molecular properties of their antimicrobial metabolites;
4. To determine the potential of the isolated strain(s) and other bacteriocinogenic strains from our collection to control the development of pathogenic and conditionally pathogenic microorganisms in various dairy products;

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[7]Gulahmadov, S.G. Antimicrobial properties of strain Lactobacillus paracasei BN ATS 8W / С.Г. Гюльахмедов, N.F. Abdullaeva, A.A. Kuliev // News of Baku University. Natural sciences series, - Baku: -2016, No.4, - p. 62-71.

[8]Gulahmadov S.G. Metabolites of lactic acid bacteria of Azerbaijan with antimicrobial properties and their practical significance: / Dis. doc. sciens. in biology. / - Baku, 2016. - 376 p

5. To study the *in situ* effectiveness of active strains during the preparation of model cheese samples;
6. To determine the bioprotection potential of osins for the purpose of long-term protection of milk and model sour milk products (such as cheese samples).

Research methods. Researches were carried out by screening, replica, diffusion, fermentation, API 50 test, microscopy, centrifugation, adsorption-desorption, electrophoresis, statistical data processing and computer methods.

The main provisions defended:

- Among the bacteria isolated from breast milk, there are lactic acid bacteria with promising probiotic properties;
- -*L. delbrueckii* spp. *lactis* A7 strain bacteriocin synthesis is an inductive process and depends on the type of carbon and nitrogen sources in the environment and other factors;
- -A7 strain *L. delbrueckii* spp. *lactis* is a rare representative of the species that synthesizes lantibiotics;
- When applying the adsorption-desorption method to various bacteriocins, a serious methodical modification is required for obtaining a partially purified preparation;
- Bacteriocinogenic lactic acid bacteria and their ocins are safe and reliable alternatives to artificial chemical preservatives that prevent the development of pathogenic microflora in milk and dairy products;
- In order to protect the long-term safety of food products, it is more appropriate to use its producer then the bacteriocin preparation.

Scientific novelty of the research. For the first time, the search for bacteriocinogenic LAB in breast milk samples was carried out and 6 representatives with appropriate properties were found. The promising A7 strain, which has the broadest spectrum of antimicrobial action, and its metabolite of antimicrobial nature have been characterized in detail. The bacteriocin synthesis ability of *L. paracasei* spp. *paracasei* BN ATC 8w and *E. faecium* S5 strains stored in the collection of our department for more than 10 years,

was restored (resuscitated) to the initial level, and together with the A7 strain, the *in situ* effectiveness of all three against dangerous pathogens was studied separately in model dairy products, which was carried out in the Republic of Azerbaijan is the first such research work.

Theoretical and practical significance of the research. *L. delbrueckii* spp *lactis* A7 strain isolated from breast milk is a representative of the normal resident microbiota of the human and taking into account the wide range of probiotic properties proven by our experiments, its use in milk, infant food, sour milk products, fermented vegetable types, etc. can be added to valuable food products and achieve a significant positive effect on people's health.

The 3 different bacteriocinogenic strains we studied and their natural antimicrobial peptides, unlike chemical preservatives, can be used for short and long-term protection of food products from pathogenic microbes such as *Staphylococcus aureus, Listeria monocytogenes* and *Esherichia coli* without any harm to human health.

It is possible to use the inexpensive and effective adsorption-desorption method, which we have seriously modified, in laboratory conditions for the purpose of partial purification of the bacteriocin preparation of the A7 strain.

Publication, **dissertation approval and application.** 20 scientific works related to the topic of the dissertation have been published. The main results of the dissertation were reported at: the International Scientific Conference "Ecology: problems of nature and society" (Baku, 2017), the International Scientific Conference "Innovative Approaches in Modern Biology" (Baku, 2018), the International Scientific Conference "Innovative Approaches in Modern Biology" (Baku, 2019), the IX Scientific Conference "Scientific achievements and challenges in Biology" (Baku, 2021), the X International Scientific Conference "1st International Congress on Natural Sciences" (ICNAS-2021) (Turkie, Erzurum, 2021), the XI International Scientific Conference “Scientific Advances And Challenges In Biology”, (Baku, 2022) and the LXXVI International

Scientific-practical Conference "Natural sciences and medicine: theory and practice" (Russia F., Novosibirsk, 2024).

The institution where the dissertation was carried out. The dissertation work was first started at the Department of Biochemistry and Biotechnology of the Faculty of Biology of Baku State University, and after 2019, the work was completed at the Department of Molecular Biology and Biotechnologies.

The structure and scope of the dissertation. The dissertation work consists of an introduction, a literature review, research materials and methods, and the results of personal research and their discussion, a conclusion, results, practical recommendations, and a list of references, which total 235,298 characters.

CHAPTER I

LACTIC ACID BACTERIA, CHARACTERISTICS AND APPLICATION SIGNIFICANCE OF THEIR BACTERIOCINS

In this chapter, information published mainly in the last 10 years about LAB, their characteristics and importance, bacteriocins, their nature, mechanism of action and classification, as well as the practical and application potential of bacteriocins, has been collected and analyzed and summarized in 3 paragraphs.

CHAPTER II

MATERIALS AND METHODS

Using active STB dilution method from breast milk samples, their antimicrobial activity was carried out by "*agar-diffusion*"[9], the effect of antibiotics on the growth of wholes by "*disk-*

[9]Parente, E. A comparison of methods for the measurement of bacteriocin activity / E. Parente, C. Brienza, M. Moles [et al.] // J. Microbiol Methods, - 1995. 22 (1) – p. 95–108

diffusion"[10], antioxidant properties of producer strains by "*Son"*[11], partial purification of bacteriocin preparation was carried out by severely modified "*Yang"*[12], and its electrophoretic analysis was carried out by "*Schägger"*[13] method.

The A7 strain "*was identified"*[14] using the scheme of Sharpe et al. (1996) and the API 50 CH (L) system (bioMerieux, Lyon, France).

CHAPTER III
RESULTS OBTAINED AND THEIR DISCUSSION

3.1. Isolation of antimicrobial strains from breast milk samples and their characteristics

16 different breast milk samples were used as a source of active bacteriocinogenic strain. After those samples were planted in MRS-agar medium, a total of 148 colonies were formed in Petri dishes. Their antimicrobial activity was determined by the replica method and such activity against L. bulgaricus 340 strain was detected in 64 colonies.

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[10]Charteris, W. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species / W.Charteris, P.Kelly, L. Morelli [et al.] // J. Food Prot., - 1998. 61, - p.1636–1643.

[11]Son, S. Free radical scavenging and anti-oxidative activity of caffeic acid amide and ester analogues: Structure-activity relationship / S.Son, B.A.Lewis // J. Agric. Food Chem., - 2002. 50, - p. 468-472.

[12]Yang, R. Novel method to extract la rge amounts of bacteriocins from lactic acid bacteria / R.Yang, M.C.Johnson, B.Ray // Appl. Environ. Microbiol., - 1992. 58, - p. 3355-3359.

[13]Schägger, H. Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range from 1 to 100 kDa / H.Schägger, G. von Jagow // Anal.Biochem., - 1987. v.166, - p. 368-379

[14]Sharpe, M. In "Identification methods for microbiologists" / M.Sharpe, T.Fryer, D.Smith - New-York: Acad. Press. – 1996. - 419 p.

There is a large arsenal of metabolites that ensure the antagonistic activity of bacteria with co-populations. Therefore, the biochemical nature of antimicrobial metabolites of active bacteria obtained from milk samples was studied. The obtained results are reflected in table 1. Different factors had different effects on the antimicrobial metabolites of the isolated bacteria. Thus, as a result of the neutralization of the active acidity (actual pH values) of the environment, only 24 of the 64 colony cells included in the study were able to maintain their antimicrobial activity, while the remaining 40 colony cells became passive. This result demonstrates that the antimicrobial activity of those bacterial cells is related to organic acids.

Table 1

Effect of some environmental factors on antimicrobial metabolites of active bacteria isolated from breast milk samples

Variants of influence on antimicrobial components	Retaining antimicrobial activity number of strains
Control (sterile culture fluid)	64 (64)•
Neutral pH	24 (64)
Catalase (1mg/ml)	46 (64)
Proteolytic enzymes (1 mg/ml)	58 (64)

• – total number of colonies included in the study

After incubation of the antimicrobial components of the culture liquid with catalase enzyme at a concentration of 1 mg/ml for 2 s, the number of bacterial colonies that maintained their antimicrobial activity was 46. From here it can be concluded that the corresponding activity of the remaining 18 colony cells is related to the hydrogen peroxide they secrete into the environment.

The effect of proteinase K and trypsin VIII enzymes on the active components of the studied cultures was also studied. After incubation of 64 culture fluids with the presence of these proteolytic enzymes, only 6 of them lost their antimicrobial activity. This is a

sign that the antimicrobial metabolites of those cultures are peptide in nature, in other words, they are bacteriocin-like substances.

At the next stage, the spectrum of antimicrobial effect of those strains was studied. The spectrum of antimicrobial activity of 6 bacteriocinogenic strains isolated from different breast milk samples was different from each other. Thus, the spectrum of antimicrobial action of L2 and T8 strains was the same, and their bacteriocin-like metabolite inhibited the development of 2 gram-positive strains (*L. bulgaricus* 340 and *L. lactis* sub. *lactis* DF04). Active metabolites of strains E5 and R1 could not affect any other passive microorganisms except *L. bulgaricus* 340. Bacteriocin-like metabolite of strain G9 inhibited the growth of 3 passive strains, 2 gram-positive (*L. bulgaricus* 340 and *L. brevis* F145) and 1 gram-negative (*E. coli* ATCC 25922) bacteria. Bacteriocins of the isolated strains had not any effect on fungi.

The richest activity spectrum of the isolated bacteriocinogenic strains was observed in the A7 strain. The bacteriocin of this strain inhibited the growth of 6 out of 22 passive microorganism samples tested.

At the next stage, the initial phenotypic identification of the A7 strain was carried out using the method proposed by Sharpe et al. (1996) and the API 50 CH (L) system (bioMerieux, Lyon, France) and it was concluded that it belongs to *Lactobacillus delbrueckii* spp. *lactis*.

3.2. *Lactobacillus delbrueckii* spp. *lactis* A7 strain probiotic properties

"*When* probiotic bacteria *pass through the organs of the human gastrointestinal system, they are exposed to the effects of acid*

stress in the stomach and bile acids in the duodenum"[15]. "*It is dangerous that they are resistant to antibiotics"*[16].

The growth dynamics of A7 strain differed under different acidity stress conditions. Thus, at pH 2.5, the number of cells partially decreased in the first hour, but the number of cells recovered in the following hours. At this time, no noticeable increase was observed. However, a significant increase in the bacterial population was observed in the environment with pH 3.0 and above (Fig. 1).

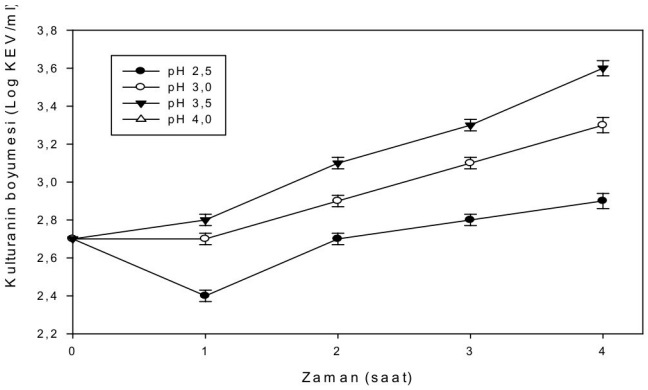

Figure. 1. Effect of environmental pH on the growth of L.delbrueckii spp. lactis A7 strain

In the next step, the effect of Na salt of taurodeoxycholic acid (NaTDHT) on the growth of strain A7 was investigated (Fig. 2). As the concentration of bile acid increased, the optical density of the studied strain decreased. Thus, the 0.1% concentration of NaTDHT weakened the growth of the strain very slightly and practically did

[15]Maryam, T.E. Traditional Iranian dairy products: A source of potential probiotic Lactobacilli / T.E. Maryam, C.O. Arthur, A.H. Mohamad [et al.] // Afr. J. Microbiol. Res., - 2011. 5, - p. 20-27.

[16]Hassan, M. Natural antimicrobial peptides from bacteria: Characteristics and potential applications to fight against antibiotic resistance / M.Hassan, M.Kjos, I. F. Nes, [et al.] // Journal of Applied Microbiology, - 2012. 113, - p. 723–736.

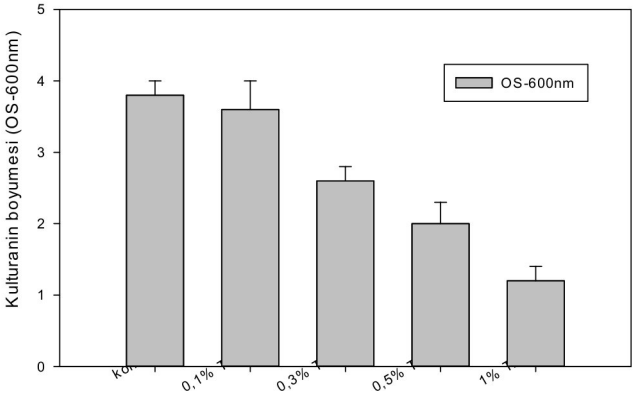

Figure 2. Effect of Na salt of taurodeoxycholic acid at different concentrations on the growth of L. delbrueckii spp.l actis A7 strain:

- Incubation period - 12 s;

-The optical density of the primary inoculum is 0.3

not affect the population density. Considering that the concentration of bile acid in human small intestine is 0.3%, when its concentration is increased to physiological concentration, i.e. 0.3%, it was determined that the optical density of the population of strain A7 was 30% lower compared to the control variant (MRS variant). However, extensive growth of A7 strain was observed as a result of adaptation to the environment starting from the 5th hour of experimental exposure.

Then, the effect of 12 different antibiotics on the growth of strain A7 was tested. It was determined that the A7 strain showed sensitivity to 8 antibiotics widely used in modern medicine, such as ampicillin, penicillin, vancomycin, erythromycin, gentomycin, kanamycin, rifamycin and metrodinozole. However, the A7 strain was resistant to streptomycin, cefotaxime, chloramphenicol and ofloxacin.

Antioxidant properties of A7 strain and other strains included in our *in situ* studies – BN ATS 8w and S5 strains – were studied. All three strains inhibited the activity of DPPH. However, their antioxidant activity was different from each other. Thus, the highest activity (31.2%) of *Lactobacillus paracasei* BN ATS 8w strain, 21.9% of *Lactobacillus delbrueckii* A7 strain, and 11.2% of *Enterococcus faecium* S5 strain were observed in the suspension cultivated in MRS-bros.

The next task before us was to characterize the antimicrobial activity of the isolated active LAB.

Since bacteriocinogenesis is an inductive process, we determined the inductive environmental factors and tried to determine their optimal parameters. The study of the effect of carbon dioxide on the growth of the strain and bacteriocin titer in the medium showed that the most effective carbon dioxide was glucose (Fig.3).

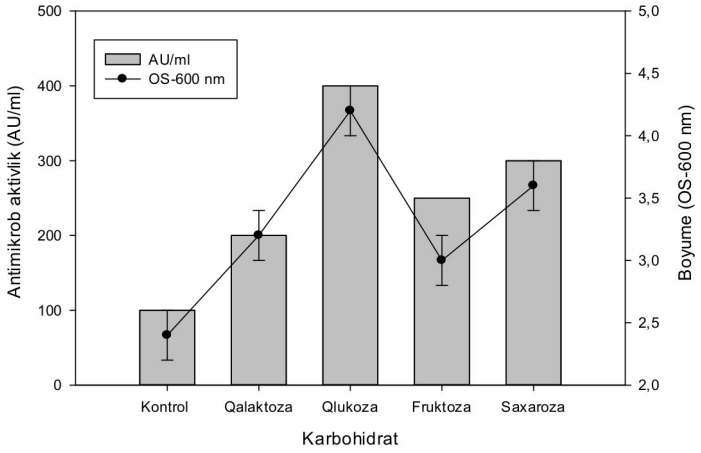

Figure 3. Effect of different carbohydrates on growth and bacteriocin synthesis of *Lactobacillus delbrueckii* spp. *lactis* A7 strain

The optimal concentration of glucose was 0.6 g/l, and at that time the bacteriocin titer was 1800 CFU/ml.

The most effective nitrogen source for strain growth and bacteriocin synthesis was yeast extract (Fig. 4). Its optimal concentration was 3%, and at that time the bacteriocin titer was 2400 CFU/ml.

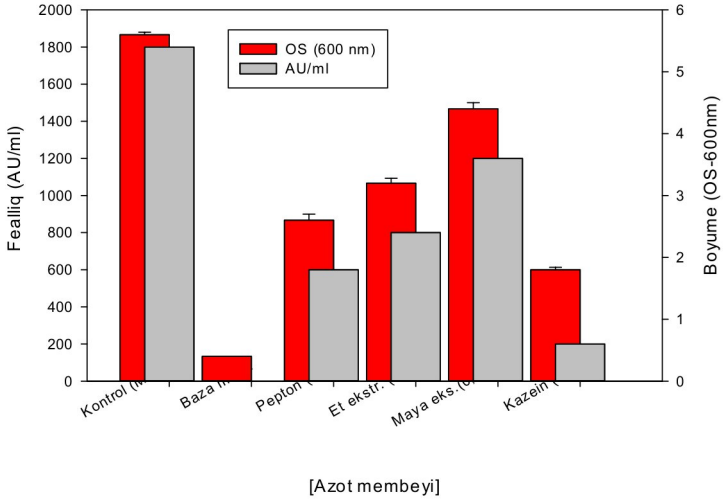

Figure 4. Effect of organic nitrogen sources on the growth and synthesis of bacteriocins of *L. delbrueckii* spp. *lactis* A7 strain

In the next part of our experiments, the influence of mineral salts, vitamins and glycerol on the growth and antimicrobial activity of *L. delbrueckii* spp.*lactis* A7 strain was studied. At this time, the inductive effect of manganese sulfate attracted the most attention.

When a small concentration of MnSO4·H2O salt (0.005 g/l) was added to the medium, the bacteriocin titer increased significantly and the optical density of the culture increased significantly compared to the control variant. Different concentrations of MnSO4 salt *in vitro* were used to induce *L. delbrueckii spp. lactis* A7 strain. The effect on growth and bacteriocin synthesis of was studied.

In the absence of Mn ions in the medium, growth was sharply reduced, bacteriocin was not synthesized (base medium). However, when its concentration was increased 4 times, the density of the suspension increased by 11%, and the bacteriocin titer increased by approximately 28%. The subsequent increase in the concentration of the inductor did not affect both indicators.

The stimulating effect of Mn ion is due to several reasons. Thus, this ion is important in meeting energy needs by being included in the enzymes that break down glucose. In addition, Mn ion protects the cell from oxidative stress in the superoxide-dismutase enzyme. A similar stimulating effect of this ion was observed in "*L. sakei*"[17], "*Pediococcus acidilactici*"[18] and "*L.acidophilus*"[19].

After studying the effect of inductive factors on the antimicrobial activity of A7 strain, using the optimal values of environmental factors that stimulate bacteriocin synthesis, its daily cultivation was carried out and the studied parameters were compared with those observed in the usual MRS buffer. The results are shown in figures 5 and 6.

As can be seen from the pictures, the bacteriocin titer in the modified MRS medium was 3600 CFU/ml, which is 2 times more

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[17]Aasen I., Moretro T., Katla T., et al. Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687 / Appl Microbiol Biotechnol., 2000, v.53, pp.159–166

[18]Abo-Amer, A. E. Optimization of bacteriocin production by *Lactobacillus acidophilus* AA11, a strain isolated from Egyptian cheese // Annals of Microbiology, - 2011. v.61, - p. 445–452.

[1919]Anastasiadou S., Papagianni M., Ambrosiadis I., Koidis P. Rapid quantifiable assessment of nutritional parameters influencing pediocin production by *Pediococcus acidilactici* NRRL B5627 // Bioresour Technol., 2008, v.99, p.6646–6650

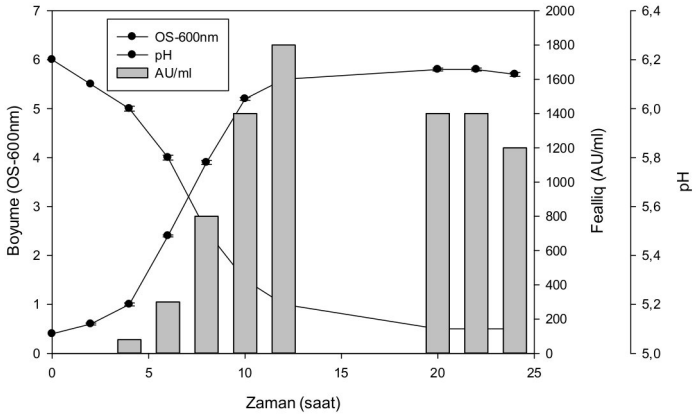

Figure 5. Dynamics of growth, organic acids and bacteriocin synthesis processes of *Lactobacillus delbrueckii* spp *lactis* A7 in MRS medium: Passive culture – *L. bulgaricus* 340

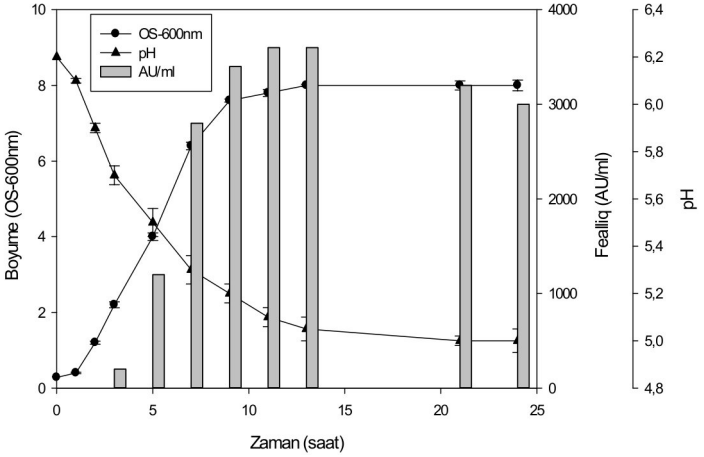

Figure 6. Dynamics of growth, organic acids and bacteriocin synthesis processes of *Lactobacillus delbrueckii* spp. *lactis* A7 strain in modified MRS medium: Passive culture – *L. bulgaricus* 340, cultivation temperature – 37^0 C.

than the similar indicator determined when cultured in the standard MRS medium.

3.3. Partial purification and electrophoretic analysis of A7 strain bacteriocin

The adsorption-desorption method we used to obtain a partially purified preparation of the bacteriocin-like substance of strain A7 was described by Yang, et al. (1992)[12] did not give positive results in the form he proposed. For this reason, various parameters of that method have been subjected to quite serious modification. The obtained results are reflected in table 2. When the concentration of primary producer titer was 3 log/ml (2 times lower than specified in the method), bacteriocin titer in the medium increased by 24% during fermentation. Cultivation time of the producer strain has been reduced from 20 hours to 16 hours. Because, the maximum bacteriocin synthesis of *L. delbruecki* spp. *lactis* A7 strain occurs in the middle of the stable phase of culture development, and this phase is reached by the strain in 14-16 h. The obtained results showed that after stirring the culture for 10 h (the time specified in the method) in a cold environment, the bacteriocin titer in the supernatant is quite high. For this reason, by further extending this period, the optimal time was determined to be 16 h. At this time, the initial bacteriocin titer in the supernatant was reduced by 86% and the producer adsorbed on the surface of the cells.

One of the modified parameters was the replacement of 0.5M NaCl salt with 1M KCl salt used to create ionic strength in the suspension medium. As a result, the amount of desorbed peptides increased by 64% compared to NaCl (Table 3). Only after this substitution, a band of the studied bacteriocin was visible in the PAA gel during electrophoretic analysis. The obtained result is reflected in figure 7. As can be seen from the electrophoregram, the molecular kD. weight of the polypeptide chain of antimicrobial

Table 2

Effect of modified parameters of adsorption-desorption method (Yang, et al. 1992) on bacteriocin titer change dynamics (BTC) in medium

Producents initial titer (log/l)			Cultivation period (hour)			Mix duration the suspension at 4^0 C (hours)			Ionic strength of salt (mol/l)		
Yung method	Modi-fikation	BTC	Yung method	Modi-fikation	BTC	Yung method	Modi-fikation	BTC	Yung method	Modi-fikation	BTC
6 log/ml	2 log/ml	10%<	20s	12h	0	10s	12h	11%>	NaCl - 0,5M	KCl – 0,5M	13%>
	3 log/ml	24%>		14h	33%>		14h	28%>		KCl – 1M	64%>
	4 log/ml	18%>		16h	33%>		16h	46%>		KCl – 1,5M	58%>

Table 3

Growth rates of S. aureus CIP 9973 and E. coli BAS23355 cells after 23 s in different media (log CFU/ml)

Fermentation environment	*S. aureus* CIP 9973					
	8 h			23 h		
	S.aur. (along)	*S.aur*+*L.p.* 8w	*S.aur.*+ Bac. 8 w	*S.aur.*	*S.aur.*+*L.p.* 8 w	*S.aur.*+ Bac. 8 w
BHI - medium	4,4±0,012	4,4±0,011	3,2±0,021	5,4±0,023	3,6±0,019	3,0±0,011
Skim milk	3,3±0,011	3,1±0,012	2,5±0,021	4,5±0,021	3,8±0,018	2,2±0,011
Natural cow's milk	3,2±0,012	3,1±0,011	2,6±0,021	4,2±0,019	3,2±0,014	2,0±0,013
"Kabrita1Gold" baby food	3,8±0,013	3,4±0,012	3±0,021	5,1±0,023	3,6±0,012	3,1±0,012

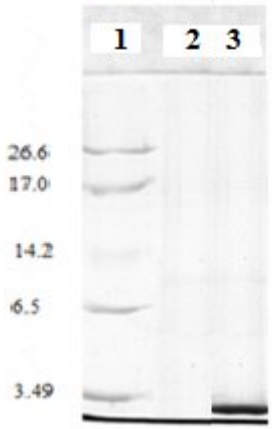

Figure 7. Electrophoregram of the bacteriocin-like substance of *L. delbrueckii* spp. *lactys* A7 strain:

1. **Marker proteins;**
2. **Desorption fraction in the presence of 0.5M NaCl;**
3. **Desorption fraction in the presence of 1M KCl.**

nature is lower than 3.49.

Such small-molecule bacteriocins belong to class I bacteriocins and are called lantibiotics. "*Their molecular weights are usually in the range of 2-4 kDa"*[20]. Bacteriocins belonging to this class are synthesized by some types of STBs such as *Lactococcus lactis, Lactobacillus sakei, Lactobacillus plantarum, Lactobacillus acidophilus* and "*prevent the development of mainly gram-negative and gram-positive bacteria"*[21]. However, information about the

[20]Bari M. L. Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population of inoculated fresh-cut produce./ M.L.Bari, D.O.Ukuku, T.Kawasaki [et al.]// J Food Prot, 2005, 68(7):1381–1387

[21]Carr FJ, Chill D, Maida N (2002) The lactic acid bacteria: a literature survey. Crit Rev Microbiol 28(4):281–370

synthesis of lantibiotics by *L. delbruecki* spp. *lactis* species was not found in literature sources.

3.4. Effect of active strains and their ocins on daily development of pathogenic microflora in various dairy products

The next goal of our dissertation work was to determine the potential of bacteriocinogenic strains to control the development of pathogenic and conditionally pathogenic microorganisms in milk and model dairy products *in situ*. First, in MRS media and listed dairy products the growth and bacteriocin synthesis dynamics of *L.paracasei* spp *paracasei* BN ATC 8w strain were studied. Both the growth of the producer strain and the intensity of its bacteriocin synthesis in the dairy products involved in the experiments as an experimental object were significantly weaker compared to the MRS medium.

According to the literature data, growth and development processes in bacterial populations can usually "*vary depending on a number of endogenous and exogenous factors"*[17-18]. The amount, quality, pH and temperature of the environment necessary for the development of bacteria in the environment are the basis of those factors. Although the producer strain in the studied environment is cultivated in optimal temperature conditions for it in all environments, even a slight difference between the constituent components of the cultivation environments must necessarily manifest itself in their studied parameters, and the obtained results confirm it once again.

In the next part of our experiments, free growth of pathogenic and conditional pathogenic strains involved in *in situ* experiments in the media listed above (as a control variant), growth in the presence of cells of an active producer strain, and also the nature of the growth of that strain in the medium with the addition of a partially purified bacteriocin preparation were monitored.

First, those experiments were performed with *S. aureus* CIP 9973 strain and the obtained results are summarized in table 3. As it can be seen here, in BHI nutrient medium, which is usually used for representatives of the genus *Staphylococcus, S. aureus* CIP 9973 strain developed and grew only during the first 8 s of cultivation, and the amount of initial culture material added to the medium at 3 Log CFU/ml increased approximately 1.5 times to 4 It has risen to the level of 4,4 Log CFU /ml. It is interesting that the same density of Co-cultivation with cells of the active strain *L. paracasei spp. paracasei* BN ATC 8w practically did not affect the development of the pathogen. However, by adding bacteriocin 8w to the medium, a significant (1.2 log units) decrease in population density was observed during its cultivation. This means a slight growth of the pathogen *in vitro* in the presence of bacteriocin. During the study of the same parameters after 23 h of cultivation, it was determined that the development of the pathogen was significantly delayed both during co-cultivation with the producer strain and during cultivation in the presence of bacteriocin of the active strain. During this period, the number of passive strain cells remained at 5.4 Log CFU/ml in BHI medium, 4.6 Log CFU/ml in combination with active strain, and 3 Log CFU/ml in combination with bacteriocin 8w.

The fact that the *S. aureus* CIP 9973 strain was practically prevented from growing during the co-cultivation of the active strain with the bacteriocin preparation indicates the effective bacteriostatic effect of bacteriocin 8w against this strain.

After the completion of our experiments with *S. aureus* CIP 9973 strain, similar experiments were conducted with *E. coli* BAS23355 strain and similar results were obtained. It was determined that bacteriocin 8w has a bacteriostatic effect on the growth of this strain in *in situ* experiments.

In our next experiments, the *in situ* activities of *L. delbrueckii* spp. *lactis* A7 and *E. faecium* S5 strains and their extracts against *L. monocytogenes* 302 and *E. coli* CIP 104368 strains were

Table 4

***L. delbrueckii spp. lactis* A7 and *E. faecium* S5 strains *in situ* activities of and their ocins against *L. monocytogenes* 302 and *E. coli* CIP 104368 strains in different milk samples**⬩

Variants \ Milk samples	Natural cow's milk	"Kabrita1Gold" baby food
L. monocytogenes 302 (single culture)	3,6±0,017	3,8±0,024
L. monocytogenes 302+ *L.del.* A7	2,4±0,011	2,5±0,012
L. monocytogenes 302+ Bac. A7	1,2±0,005	1,4±0,004
L. monocytogenes 302+ *E. faec.* S5	2,0±0,010	2,0±0,010
L. monocytogenes 302+ Enteros. S5	-•	-
E. coli CIP 104368 (single culture)	4,3±0,012	4,1±0,024
E. coli CIP 104368+ *L.del.* A7	3,2±0,015	3,1±0,013
E. coli CIP 104368+ Bac. A7	1,6±0,006	1,5±0,005
E. coli CIP 104368+ *E. faec.* S5	2,2±0,015	2,0±0,009
E. coli CIP 104368+ Enteroc. S5	1,4±0,005	1,2±0,005

⬩The amount of initial planting material is 3 Log CFU /ml. Bacteriocin titer – 1200 AU /ml. Cultivation period 14 h.
•Not observed.

studied. The obtained results are summarized in table 4. As can be seen from the table, the growth rate of *E. coli* CIP 104368 strain in both studied milk samples exceeded the growth rate of another pathogenic strain - *L. monocytogenes* 302. Thus, while the *L. monocytogenes* 302 population increased its density by 0.6 log units and 0.8 log units, respectively, in natural milk and "Kabrita1Gold" baby food, the *E. coli* CIP 104368 strain increased that indicator by 1, It increased by 3 log units and 1.1 log units. However, co-cultivation with both active strains was accompanied by a significant reduction in the amount of pathogens in the studied milk samples. In cow's milk and baby food with *L. delbrueckii* spp. *lactis* A7 strain, the number of listeria cells decreased by 1.25 log units and the

number of *E. coli* cells by 1.05 log units compared to the control. At this time, the initial population density of listeria cells decreased by 0.55 log units, while the number of *E. coli* cells decreased relative to the control and approached the maximum initial density. When its bacteriocin (bac. A7) was added to the culture medium instead of the producer strain, a sharp reduction in pathogenic cells was observed in both milk samples. Thus, the listeria culture grown with the presence of bac. A7 was diluted by 1.7 log units on average, and the *E. coli* culture by 1.45 log units.

Interesting results were obtained during co-cultivation of pathogenic cultures with *E. faecium* S5 and its enterocin (table 4). Active strain cells and *Listeria* population were co-cultivated in both milk samples, the amount of passive cells decreased by 1 log unit to 2 Log CFU/ml. However, when culturing was performed by adding its enterocin to the medium rather than the strain itself, no viable *Listeria* cells were observed in the culture after 14 h. This situation has shown itself in both cow's milk and baby food.

Regarding the *in situ* effectiveness of *E. faecium S5* strain against *E coli* cells, as a result of co-cultivation of the strain with the conventional pathogen, the number of cells of the latter decreased by an average of 0.9 Log CFU/ml. In the presence of enterocin S5 in the medium, the amount of *E. coli* cells further decreased to 1.3 CFU/ml.

Thus, summing up this part of our experiments, we can note that all three bacteriocinogenic strains and their ocens, whose *in situ* effectiveness was studied, inhibited the development of passive pathogenic and conditionally pathogenic strains that were tested in different milk product samples and included in the antimicrobial spectrum of those strains. At this time, *L. paracasei* spp. *paracasei* BN ATC 8w strain and its bacteriocin were bacteriostatic to *S. aureus* CIP 9973 and *E. coli* BAS23355 strains in vitro and in situ, *L. delbrueckii* spp. *lactis* A7 and *E. faecium* S5 strains and their osins had a bactericidal effect against *L. monocytogenes* 302 and *E. coli* CIP 104368 strains.

3.5. Daily *in situ* efficacy of bacteriocinogenic strains against harmful bacteria in model cheese samples

In these experiments the effect of *L. delbrueckii* spp. *lactis* A7 strain on the growth of *L. monocytogenes* 302 strain in model cheese samples was studied *in situ*. The obtained results are shown in figure 8.

In the control version, the growth of *L. monocytogenes* for 302 days was 0.46 CFU/g.

The second portion of *L. delbrueckii* spp. *lactis* A7 was added and pathogen development was monitored in mixed culture. During the first 3 h, there was no change in the amount of the pathogen in this portion.

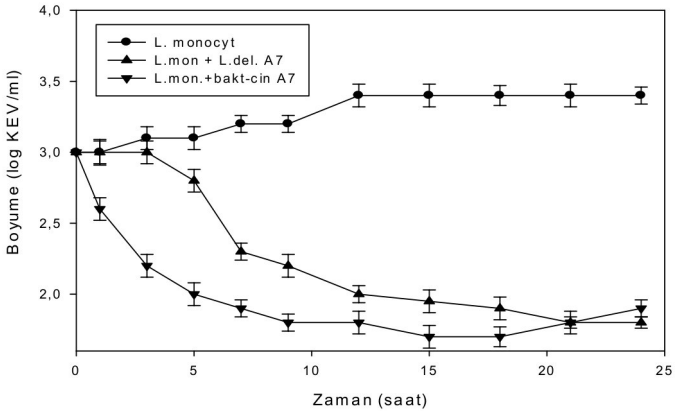

Figure 8. The dynamics of changes in the amount of *L. monocytogenes* 302 strain during the preparation of cheese samples to which *L. delbrueckii spp. lactis* A7 and its bacteriocin was added

However, after 12 h, the amount of pathogen decreased to 2.2 log CFU/g. In the subsequent period, the bactericidal effect of the active polypeptide continued, and at the end of the experiment, the density of the *Listeria* population remained at 1.3 log CFU/g.

Preparation of A7 bacteriocin with activity of 1600 IU/ml was added to the prepared 3rd portion at the same time as *L. monocytogenes* 302 cells. At the end of the first hour of incubation, the number of Listeria cells decreased by 0.4 log units, and by 0.8 log units in the 3rd hour. In the following hours, this trend continued, and between the 15th and 18th hours, its amount decreased to the minimum level and remained only 1.2 log units. But after that, the bactericidal effect of bacteriocin weakened and, as a result, the density of the pathogen began to increase. At the end of 24 seconds, the concentration of the pathogen in the medium has reached 2 log units.

3.5.Effect of active strains and their ocins on the development of pathogenic microbiota during ripening of cheese samples

During the ripening period (1 month) of model cheese samples, the effect of *L. delbrueckii* spp. *lactis* A7 and *E. faecium* S5 strains and their osins on the growth of *L. monocytogenes* 302 strain was studied (Figure 9). During this period, in the control variant, *L. monocytogenes* 302 grew rapidly and its amount increased 2.5 times to 7.6 log units.

In the second portion of the samples, those dynamics were studied with the participation of osin producers, and in the third portion, bacteriocin and enterocin themselves. In all variants, a reduction in the number of pathogenic cells was observed and an obvious inhibition of growth was observed. At this time, the inhibitory effect of *E. faecium* S5 and enterocin on *L. delbrueckii* spp. *lactis* A7 and its bacteriocin showed a more pronounced effect than the similar effect. Thus, in the sample to which the A7 strain was added, the amount of the pathogen was 2.1 log units lower than the control, and in the sample with S5, it was 2.8 log units lower.

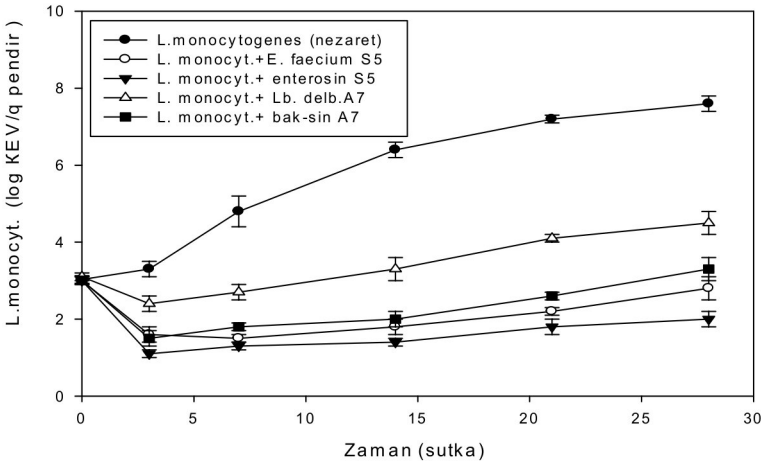

Figure 9. The effect of *L. delbrueckii spp. lactis* A7 and *E. faecium* S5 strains and their ocins on the growth of *L. monocytogenes* 302 during the ripening period of cheeses (correspondence of the curves is shown inside the graphs)

As for the osins themselves, both active polypeptides showed a similar effect – they significantly inhibited the growth of *L. monocytogenes* cells during cheese ripening. At this time, the inhibitory effect of enterocin S5 was stronger than the similar effect of bacteriocin A7. At the end of ripening, the population density of the variant with bacteriocin A7 was 2.5 times lower than the control variant, and 3.8 times lower in the variant with enterocin S5.

Thus, *L. delbrueckii* spp. *lactis* A7 and *E. faecium* S5 strains are both able to control the cell density in the population of *L. monocytogenes*, one of the dangerous pathogens in the cheese product. This ability of theirs is related to the synthesis and secretion of peptide antimicrobial metabolites called ocins *in situ*. The control of pathogen growth in cheeses can be carried out both by the

producers of the ocins and by the strains themselves. Enterocin of *E. faecium* S5 was the most active of the tested ocins.

RESULTS

1. Lactic acid bacteria with antimicrobial activity were isolated from breast milk samples and the biochemical nature of their active metabolites was studied. Among the bacteriocinogenic strains, *L. delbrueckii* spp. *lactis* A7, which have promising probiotic properties such as resistance to strong acidity stress and physiological concentration of bile acid, antioxidant activity, as well as sensitivity to a wide range of antibiotics, inhibited the growth of 6 different bacterial strains in vitro [1,2,3,12,15].
2. It was determined that organic carbon and nitrogen compounds of the nutrient medium, as well as Mn ions, have an inductive stimulating effect on the bacteriocinogenesis of A7 strain. *L. delbrueckii* spp. *lactis* A7 strain grown in MRS medium in the presence of glucose (6 g/l) as a carbon source, yeast extract (3%) as a nitrogen source and 200 mg/l Mn ions bacteriocin titer was doubled [4,5,6,7,8,9,17].
3. *L. delbrueckii* spp. *lactis* A7 strain bacteriocin was partially purified by a highly modified adsorption-desorption method, electrophoretically analyzed and its molecular mass was shown to be less than 3.9 kDa. Bacteriocin was concluded to belong to the group of lantibiotics, and thus, a rare strain lantibiotic-synthesizing *L. delbrueckii* of subspecies *lactis* was discovered [18].
4. For the first time in Azerbaijan, *L. delbrueckii* spp. *lactis* A7, *L. paracasei* spp. *paracasei* BN ATC 8w and *E. faecium* S5 strains and their ocins were studied in situ against dangerous pathogens in milk and model cheese samples. It was determined that the in situ efficiency of the studied producer strains in milk and model sour milk products is significantly

lower than the in vitro efficiency in the nutrient environment characteristic of them [10,11,13,14,16,20].

5. It has been shown that *L. paracasei* spp. *paracasei* BN ATC 8w strain and its bacteriocin have a bacteriostatic effect on the development of *S. aureus* CIP 9973 and *E. coli* ATCC 25922 strains in the studied products and inhibit their growth by 50% on average [14].
6. In milk and model cheese samples, *L. delbrueckii* spp. *lactis* A7 and *E. faecium* S5 strains and their ocins have a bactericidal effect on the grows of *Listeria monocytogenes* 302 population. Bacteriocin A7 reduces the number of cells in the pathogen population by 2.5 times, and enterocin S5 - by 3.8 times [19].

PRACTICAL RECOMMENDATIONS

1. Taking into account the known probiotic properties of *L.delbrueckii* spp. *lactis* A7 strain isolated from breast milk, and its safety as a representative of resistant microbiota, it is possible to help the formation of the normal microflora of the intestines by giving it to newborns who are deprived of the opportunity to be breastfed for various reasons.
2. In order to protect the biological safety of food products, it is possible to use both the purified preparations of bacteriocins themselves and the producer strains. It is more effective to use bacteriocin preparations during the protection of products with a short shelf life, and to use producer strains during the protection of products with a longer shelf life. In the second case, the absence of virulence factors in the genome of the producer strain is an important condition.

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