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INFLUENCE OF L. PARACASEI SPP. PARACASEI BN ATC 8W ON THE GROWTH OF E. COLI IN SKIMMED MILK

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ABSTRACT

The bacteriocinogenic strain Lactobacillus paracasei spp. paracasei BN ATC 8w was isolated from traditional «Motal» cheese and has strong antimicrobial activity against some Gram-positive and Gram-negative bacteria. The in situ activity of the strain was studied in skimmed milk against E. coli ATCC 25922 for 24 hours. Cooperative cultivation of the active strain L. paracasei spp. paracasei BN ATC 8w with E. coli ATCC 25922 cells in skimmed milk at a concentration of OD 0.28 after 24 hours (300C) led to a decrease up to almost 50% of the concentration of E. coli, which was found in the absence of an active strain in the medium. This indicates the in situ efficiency of the L. paracasei spp. paracasei BN ATC 8w in skim milk for the biological protection of dairy products from opportunistic pathogens.

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Introduction.

Lactic acid bacteria (LAB) belong to the Clostridium branch of Gram positive bacteria. They are catalase negative, non-sporing, aero tolerant anaerobes that lack respiratory chain. According to their morphology LAB are divided to rods and cocci. In the human and animal bodies, LABs are part of the normal microflora, the ecosystem that naturally inhabits the gastrointestinal and other tracts [1].

LABs are traditionally used as source of probiotics, vitamins, enzymes and exopolysaccharides, that can satisfy the increasing consumers demands for natural products and functional foods in relation with human health. Besides LABs are used as natural starters in the manufacture of cheeses and fermented milk products because of their functions of preservation and for their contribution to flavor and aroma. In fermented food products, LAB perform both acidification, due to the production of lactic and acetic acids, flavor-compounds production, as well as protection of the food from spoilage and pathogenic microorganisms by producing organic acids, hydrogen-peroxide, diacetyl, antifungal compounds such as fatty acids or phenyl lactic acid, and bacteriocins. Therefore they are of a great economic importance[1, 2, 3].

Bacteriocins are antimicrobial peptides or small proteins that inhibitmicroorganisms that are usually closely related to the producer strain. A bacteriocin producer protects itself against its own antimicrobial compounds by defense system, which is expressed concomitantly with the antimicrobial peptide(s) [2].

The detection of bacteriocins and other antimicrobial substances from LAB has involved the use of variety of methodologies [4, 5].All methods developed to date for determining the antagonistic activity of microorganisms are traditionally divided into two groups: in vitro methods (in vitro) and in

vivo methods (in a living organism). The term in vivo refers only to those cases when the host organism is the external environment for the interaction of microorganisms. However, examples of the practical use of the phenomenon of microbial antagonism are much wider. Therefore, it is more fair to divide these methods into in vitro methods and in situ methods. The term "in situ" in relation to biology means that a phenomenon is studied where it naturally occurs (for example, directly in cheese or in a fermenter), that is, without moving it into an artificial environment. Thus, in vivo methods can be considered a special case of in situ methods. Methods for studying the antagonistic activity of microorganisms in situ are the most time-consuming, but they are also recognized as the most objective. As a rule, these methods are switched after the antagonistic activity of a particular strain of LAB has been experimentally proven in vitro[6].

In situ methods can be carried out by conducting experimental developments, for example, cheeses or fermented milk products with exact observance of all parameters of the corresponding technological process, using the test antagonist strain in the starter culture at a given initial level of infection with a test microbe and taking into account the subsequent abundance of this test microbe in semi-finished products and finished products; they can also consist in setting up experimental production of a product using an antagonist strain and statistical accounting of the number of natural sanitary-indicative microflora of this product in comparison with control production. In turn, in vivo methods involve feeding an antagonist strain to experimental animals infected with a test microbe, followed by the detection of a test microbe in feces or taking probiotic drugs by people in various dysbiotic conditions, followed by an analysis of qualitative and quantitative changes in the intestinal microflora [6].

The aim of this study was to progress in the identification of bacteriocin-producing LAB isolated from native Azerbaijani cheese and to partially characterize their inhibitory activities, as well as in-situ activity in skimmed milk.

Material and Methods.

The preliminary production of substances with antimicrobial activity by isolated LAB was screened by the antagonism method using various indicator strains. The bacterial strains used in this study as indicator microorganisms are listed below. All media were supplied by Difco. Other chemicals were provided by Sigma-Aldrich. LAB was propagated in MRS broth at 30 ◦C. *Listeria innocua* and *Staphylococcus aureus* were propagated in BH medium at 37 ◦C. Other indicator strains were grown in LB medium (*Escherichia coli*) and YPD medium (*Saccharomyces cerevisiae*, *Candida pseudotropicalis*).Before use, the strains were propagated twice in broth from18 to 24h. As source of LAB with antimicrobial activities Motal cheeseoriginally made of goat's milkwas used. Isolation was performed by direct plating method [4]. Samples from cheesewere homogenized in saline solution then 10-fold serially diluted with saline solution. Aliquotes (1 mL) were plated in soft MRS agar (0.8%, w/v) medium and incubated at 37 ◦C for 48 h. Multiple plates of serial dilutions were overlaid with indicator strain (*L. bulgaricus* 340) inoculated (5%, v/v) into 10 mL of soft agar MRS medium (0.8% agar) and incubated for another 24 h period at 37 ◦C. Inhibition was scored positive in presence of a detectable clearing zone around the colony of the producer strain. Positive colonies were randomly selected and removed using sterile Pasteur pipette. The agar plug from Pasteur pipette was inoculated in MRS liquid medium for 24 h at 37 ◦C. LAB isolated from cheese were stored at − 80 ◦C in MRS broth containing 20% glycerol.

The antimicrobial activity of LAB was detected by well diffusion assay [5] using 20 mL soft agar medium (0.8% agar) containing 100 µL indicator strain. Thereafter, wells (9 mm in diameter) were cut into the agar and 100 µL of the cell-free supernatant (centrifugation at 10,000 × *g* for 15 min at 4 ◦C) of the potential producer strains was placed into each well. In order to eliminate the inhibitory effect of lactic acid on the test organisms, the supernatants were adjusted to pH 6.5 with 1 N NaOH followed by filtration through a 0.22 µm pore size filter. Prior to incubation for 24 h at the optimal growth temperature for the indicator strains, plates were refrigerated (4 ◦C) for 4 h to allow the radial diffusion of the compounds contained in the supernatant. A clear zone of inhibition of at least 2 mm diameter was recorded as positive.

LABs showing positive results after the well diffusion assay were identified by API 50 CHL System.

The protein nature of the antimicrobial agents produced by lactobacilli was checked by enzyme treatment with the following enzymes (Sigma): pronase (11.4 U/mg), proteinase K (45 U/mg), and trypsin (10.6 U/mg) at a final concentration of 1 mg/mL in 20 mM phosphate buffer (pH 7.0). The supernatants were incubated with these enzymes at 37 ◦C for 2 h. Each solution was further tested against *Lactobacillus bulgaricus* 340 in the presence of negative controls such as the initial antimicrobial supernatant and the buffered enzyme solutions of each enzyme. The remaining activity was determined by a good diffusion assay. Untreated samples were used as controls.

The *in situ* activity of the strain was studied in skimmed milk against *E. coli ATCC 25922* for 24 hours. The optical density of both microorganisms introduced into the medium was OD 0.28 (at 600 nm).

Results and discussion.

Cheeses simple was initially screened for antimicrobial compound production against *L. bulgaricus* 340, as an indicator strain, by means of the direct plating method. In this step, the possible inhibitory effect of the organic acids and hydrogen peroxide was not excluded. The cell-free supernatants from the active strains were treated with catalase, neutralized, and tested by well diffusion assay against *L. bulgaricus* 340.

Four unidentified LAB strains (BN ATS 5w, BN ATS 7w, BN ATS 8w, and FAZ 16m) were found to maintain an antimicrobial activity against the indicator, showing a measurable clear zone around the well (Fig.1).

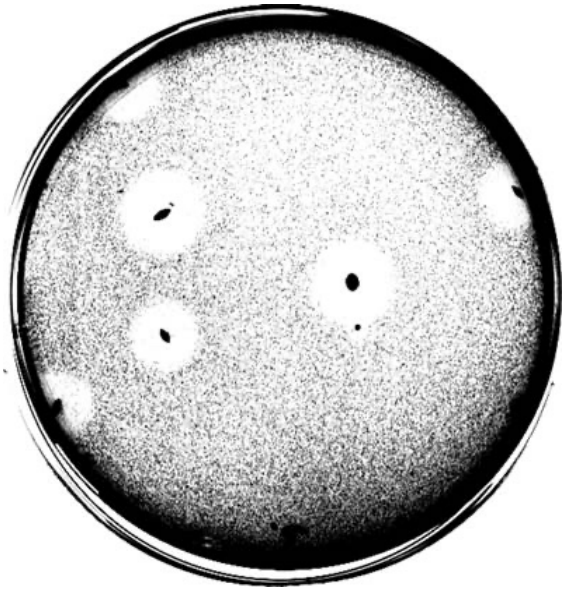

Fig. 1. Determination of antimicrobial activity of the selected strains against L. bulgaricus 340 by direct plating method

The four LAB strains, not identified previously, were subjected to phenotypic identification (Fig. 1). The strains BN ATS 5w, BN ATS 7w, BN ATS 8w, and FAZ 16m are catalase-negative, Gram-positive, rod-shaped organisms.

Most of these characteristics suggested that these isolates could belong to the genus *Lactobacillus*. The carbohydrate fermentation pattern (fig.2) of the strains determined by API 50 CHL System and the properties listed in table 1, suggested that three of these strains (BN ATS 5w, BN ATS 7w, and BN ATS 8w) could be identified as *Lactobacillus paracasei* subsp. *paracasei* and one (strain FAZ 16m) as *Lactobacillus rhamnosus*.

Table 1. Properties tested for the identification of active LAB strains obtained from Azerbaijani cheeses

Strains	Gram	Shape	Cat.test	pH	Temp.	of	growth	
					10 ◦C	30◦C	37◦C	45◦C
BNATS5w	+	bacilli	-	4.03	+	+	+	+
BNATS7w	+	bacilli	-	4.02	+	+	+	+
BNATS8w	+	bacilli	-	4.03	+	+	+	+
FAZ 16m	+	bacilli	-	4.12	+	+	+	+

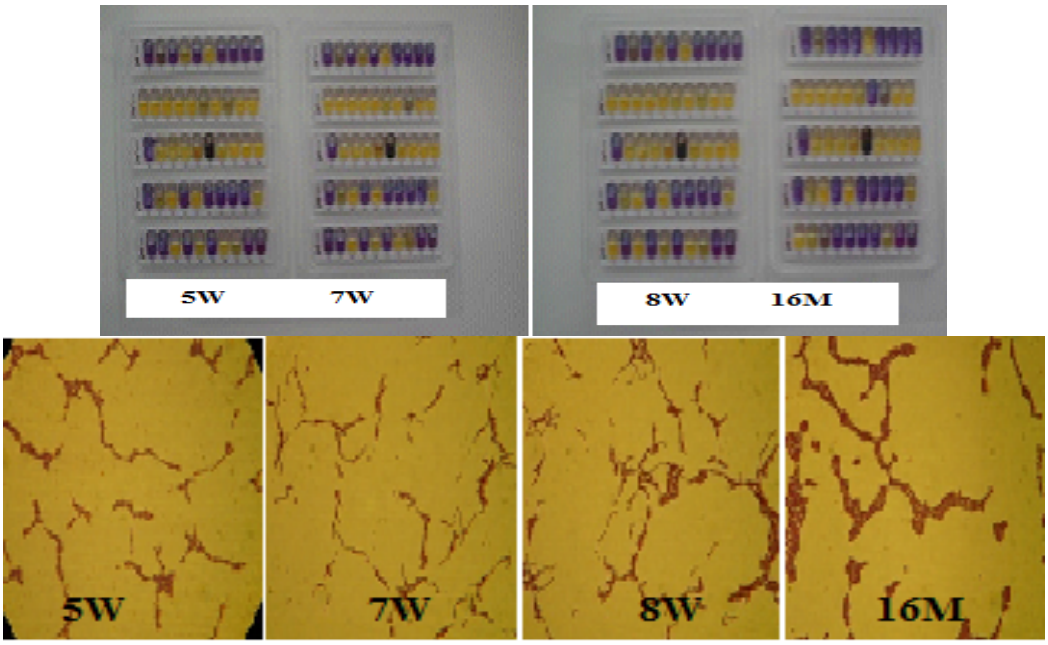

Fig. 2. Carbohydrate fermentation pattern and a light microscope photographs of the active strains determined by API 50 CHL System

The results of studies for identification of antimicrobial activities of selected strains showed, that test strains such as *L. bulgaricus* 340 and *S. cerevisiae* were inhibited by the four isolated strains. Antibacterial activity against *E. coli* ATCC 25922was detected in *L. paracasei* BN ATS 8w and *L. rhamnosus* FAZ 16m. *L. paracasei* BN ATS 5w and 8w, and *L. rhamnosus* FAZ 16m showed inhibitory effect on *Staphylococcus aureus* Cip 9973. The inhibition of *Candida pseudotropicalis* was detected only when using *L. paracasei* species BN ATS 5w and 7w. Culture of *Listeria innocua* was insensitive to the antimicrobial substances of all the studied strains.

Activity was preserved under conditions that eliminate the possible effect of organic acids by adjusting the pH of cell-free supernatant to 6.5, and of hydrogen peroxide by catalase treatment. Complete inactivation or significant reduction in antibacterial activity of the agents produced by all the selected strains was observed after treatment of cell-free supernatants with pronase E and proteinase K

but not with trypsin (except for *L. rhamnosus* FAZ 16m), which indicated the protein nature of the active agents (Data not shown). As inhibitory protein compounds of closely related bacteria can be included in the category of the bacteriocins.

The *in situ* activity of the strain was studied in skimmed milk against *E. coli ATCC 25922* for 24 hours. The optical density of both microorganisms introduced into the medium was OD 0.28 (at 600 nm). Under optimal temperature conditions, the growth of the producer strain (37^0C) in MRS medium at the end of cultivation was OD 5.4, in skimmed milk - OD 3.1 (Fig 3.A).

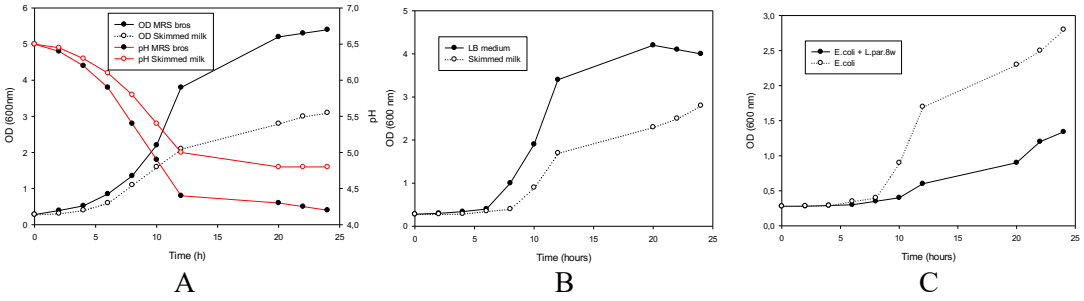

Fig. 3. The main results of in situ investigation of Lactobacillus paracasei spp. paracasei BN ATC 8w efficiency against E. coli ATCC 25922:

A. Kinetics of growth and medium acidification of the *Lactobacillus paracasei spp. paracasei BN ATC 8w* cultivated in different nutrient media (37^0C);

B. Growth kinetics of *E. coli* ATCC 25922 strain grown on different nutrient media (30^0C);

C. Grows of E.coli*ATCC 25922* in the presence or absence *Lactobacillus paracasei spp. paracasei BN ATC 8w* cultivated in skimmed milk

Similar indicators of the passive strain (30^0C) in LB medium were OD 4.2, and in skimmed milk - OD 2.8 (Fig.3 B). The inhibition of bacterial growth is probably due to the lack of necessary elements for the growth of bacterial cells in the composition of skimmed milk [2]. Simultaneously adding *L.paracasei*spp. *paracasei BN ATC 8w*and *E. coli ATCC 25922*cells to skimmed milk at a concentration of OD 0.28 after 24 hours of cultivation at 30^0C led to the fact that the growth of the latter increased only to OD 1.34. This is almost 50% less than the concentration of *E. coli*, which was found in the absence of an active strain in the medium (Fig.3 C). Inhibition of the growth of passive culture cells is associated with the release of antimicrobial metabolites (lactic acid, bacteriocin, etc.) into the medium by the cells of the active strain. This indicates the *in situ* efficacy of the *L.paracasei spp. paracasei BN ATC 8w* in skim milk for the biological protection of dairy products from opportunistic pathogens, such as *E. coli*.

Conclusions.

For LAB strains were isolated from traditional Azerbaijani cheese "MOTAL".Three of them (BN ATS 5w, BN ATS 7w, BN ATS 8w) were identified as *Lactobacillus paracasei* ssp. *paracasei* and one (FAZ 16m) as *Lactobacillus rhamnosus*. All of strains had strong antimicrobial activity against same G+ and G-, as well as, against microscopic fungi. The most active was BN ATS 8w, which inhibited the growth of 5 out of 6 passive strains.The *in situ*efficiency of the most active strain BN ATS 8w in skim milk against *E. coli* was studied. It has been shown that in the presence of an active strain, the growth of the conditional pathogen slows down almost twofold.

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