

New Antifungal Bacteriocin-Synthesizing Strains of *Lactococcus lactis* ssp. *lactis* as the Perspective Biopreservatives for Protection of Raw Smoked Sausages

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Abstract: Problem statement: Screening for the effective bacteriocin-synthesizing strains of *Lactococcus lactis* as the perspective biopreservatives was performed. We used a raw milk and dairy products from different climatic regions as well as from powerful drinks of mixed lactic acid and alcoholic fermentation: kurunga, kumiss and Iranian "Dough", that were widely used by local population to prevent diseases. **Approach:** The special interest was paid to isolates of lactococci with antagonistic activity. According to their morphological, cultural, physiological, biochemical properties and sequence of *16S rRNA* gene they were identified as *Lactococcus lactis* ssp. *lactis*. Only nine from the selected 94 strains expressed a broad spectrum of activity against Gram-positive and Gram-negative bacteria including pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella gallinarum*), moulds (*Aspergillus*, *Fusarium*, *Penicillium* genera), as well as yeasts (*Rhodotorula*, *Candida*). **Results:** It reveals the unique biological properties for isolated natural strains of *Lactococcus lactis* species. Most effective new bacteriocin-synthesizing strains 194 and K-205 were isolated from raw cow milk and kurunga from Buryatia. These strains had high antibiotic activity up to 3600 and 2700 IU mL⁻¹ as compared to nisin and up to 2500-1700 IU mL⁻¹ as compared to fungicidal antibiotic nistatin. In our experiments we used raw smoked sausages that were infected with fungi. The identification of this infection showed the presence of *Eurotium repens* de Bary on the sausages. Treatment of the raw smoked sausages with cultural broth of *L. lactis* ssp. *lactis* 194 and K-205 inhibited growth of these microorganisms. After treatment the sausages had longer shelf-life and was in accordance with basal production data (Russian State Standard Specification 16131-86). **Conclusion:** The results of this study indicated that the treatment with lactococci strains can prevent a contamination of raw smoked sausages by potential food-borne pathogens.

Key words: Anti-fungal bacteriocins, biopreservatives, lactococci, smoked sausages

INTRODUCTION

Bacterial and fungal spoilage of foodstuffs is a common and global-wide phenomenon. In addition to economic losses, the potential production by toxinogenic bacteria and fungal toxins is a particular health concern. It is true that the growth of toxinogenic bacteria and moulds can now be prevented in almost all foodstuffs by means of a large number of chemical additives or by physical treatments (heat, ultra-violet rays etc). Chemical preservatives and antibiotics that increase shelf-life of foodstuffs cause concern. In last

year preservative «Delvocide» (Gist-Brocade Co., The Netherlands) made on basis of fungicidal antibiotic Natamicin is used in sausage production (Gost 16131-86). The reasons for these are to be found in the allergenic potential of many additives. Besides, the possibility cannot be excluded that the preservatives are metabolized *in vivo* to produce a toxic substances. One example of this is the formation of carcinogenic nitrosamines from nitrite which is used for meat storage.

In turn lactic acid bacteria, due to their completely harmless to health and antagonistic activity against a large variety of microorganisms, are widely used as

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biopreservatives. The traditional use of dairy lactococci in various food fermentations, their “Generally Recognized As Safe” (GRAS) status, non-pathogenicity and presence of antagonistic properties provide the basis for its safely use for food protection as a food biopreservatives. Antagonistic activity of lactococci provided by synthesis of organic acids (acetic and lactic acids), diacetyl, bacteriocins and also low-molecular substances with antifungal activity (Roy *et al.*, 1996; Lowe *et al.*, 2004).

Bacteriocins are protein substances which exhibit antibacterial activity against closely related species (Jack *et al.*, 1995). Bacteriocins can inhibit very restricted spectrum of bacteria, mostly limited to Gram-positive bacteria. Different bacteriocins differ from each other by chemical structure and antagonistic activity. *Lactococcus lactis* produces such bacteriocins as lacticin 481, lacticin 3147, several forms of nisin and lactococcins (Morgan *et al.*, 2005; Fujita *et al.*, 2007). Nisin is the most studied bacteriocin which is allowed for application as food preservative by (European Parliament and Council, 1997) (code E234). Since 1997 nisin has status «GRAS» and it has been recognized as absolutely safe. Additions of bacteriocins decrease sterilization time and increase shelf-life of foodstuff (Drider *et al.*, 2006). As a result power inputs decrease by 15-25% as well as increase of safe food value. Nisin is the low-molecular mass protein so it is easy to apart it into amino acids during digestion and do not influence microbiota of gastrointestinal tract (Nete *et al.*, 2006). Several types of nisin (A, B, C, D, E, Z, R, Q) differ from each other by some physicochemical properties, amino acid composition and antimicrobial activity spectrum. All these bacteriocins are produced by different strains of *Lactococcus lactis* ssp. *lactis* (Jensen *et al.*, 2001, Zendo *et al.*, 2003; Yildirim *et al.*, 2004). But use of bacteriocins as biopreservatives is problematic because bacteriocins, including nisin, frequently lack stability in the foodstuffs and strains resistant to nisin have occur (Cutter and Siragusa, 1994; Mantovani *et al.*, 2001; Drider *et al.*, 2006; Galvez *et al.*, 2007). So now nisin is considered as not effective for storage of meat and other foodstuffs. At present, the ways of targeted synthesis of antibiotic substances by lactococci are studied in order to obtain new ones with more valuable properties and safe for use as biopreservatives (Drider *et al.*, 2006; Stoyanova *et al.*, 2006).

The aim of this study was to isolate and identify the new antifungal lactococci and evaluate their potential use for preventing fungal spoilage.

MATERIALS AND METHODS

Isolation of lactococci strains: The probes from raw milk and dairy products from different climatic regions

and also powerful drinks of mixed lactic acid and alcoholic fermentation: kurunga, kumiss and Iranian “Dough” were taken to isolate the cultures. Raw cow’s milk probes were obtained from the milk farm of the Timiryazev Agricultural University (Moscow, Russia), milk-processing plants of Klin (Moscow region, Russia) and Ulan-Ude (Buryatia, Russia) and also were used in this work. Mare’s milk and kumiss were obtained from Ufa (Bashkiria, Russia), kurunga – from Ulan-Ude, drink “Dough” – from Teheran (Iran). The tubes with milk probes were incubated during 17 h for self-fermentation at 28°C. In each tubes with sterile skim milk 0.5 vol. % of probe was added. Then bacteria from the tubes with dense milk clot underwent to several passages in sterile milk and cultivated for 10–17 h. To isolate a homolactic lactococci the cultures were transferred on the agar milk hydrolysate with bromocresol purple as indicator. Individual acid-forming colonies from the surface of the agar medium were transferred in Petri dish with a lawn of test culture *Bacillus coagulans* using a sterile replicator and, in parallel, to the MRS medium without the test culture. The clones that formed largest growth inhibition zones of the test microorganism were selected. To obtain a lawn of the test culture, a suspension of 24-h-old culture of the thermophilic spore-forming bacterium *B. coagulans* 429, preliminary grown at 55°C on the solid organic medium (10.0 g/l glucose, 5.0 g/l peptone, 5.0 g/l NaCl, 20.0 g/l agar with an addition of Hottinger’s broth at a concentration of 0.028 % of amine nitrogen; pH 7.0) was transferred in Petri dishes.

The morphology of the isolated strains was examined with an MBI-15 microscope equipped with a KF-4 phase contrast condenser (LOMO, MicMed, St.Petersburgh, Russia). Cultural properties of the isolated strains were examined by classical microbiological methods for identification and determination of the culture (Hoelt *et al.*, 1994).

Subculture (OD₅₄₀ 0.14-0.19) was inoculated in an amount of 5% (v/v) into the base fermentation medium, which contained 20 g L⁻¹ KH₂PO₄, 10 g L⁻¹ glucose, yeast autolysate (0.035 % ammonium nitrogen), 1 g L⁻¹ NaCl and 0.2 g L⁻¹ MgSO₄ (pH 6.8-7.0; pH was adjusted by 10% NaOH).

To study the effect of carbon source on biomass growth, pH kinetics and the level of antibiotic activity, a series of carbohydrates was used: D-arabinose, D-xylose, D-ribose, D-glucose, L-rhamnose, D-maltose, D-sucrose, D-mannose, D-lactose, D-galactose, raffinose, D-fructose, D-sorbitol, dulcitol, mannitol, dextrin, glycerol and starch. Each carbohydrate was added to the base fermentation medium in the amount of 1.0% each (Hoelt *et al.*, 1994; Stoyanova and Levina, 2006).

The effect of amino acids on bacterial growth and synthesis of antibiotic substances was studied using DL-valine, DL-threonine, DL-leucine, L-serine, L-lysine, DL-cystine, L-glutamate, L-aspartate, L-isoleucine, L-methionine and L-cysteine. Each amino acid was added into the fermentation medium in the amount of 0.01% (Stoyanova *et al.*, 2008).

The antibiotic activity of lactic acid bacteria was determined by agar diffusion method using *Bacillus coagulans* as test microorganism (Stoyanova *et al.*, 2006).

Spectrum of antimicrobial action evaluation: The spectrum of antibiotic activity of the strains was studied by culturing strains under stationary conditions in the basic fermentation medium for 17-20 h. The microorganisms used in these experiments were from the Culture Collection of the Microbiology Department of Moscow State University: six strains of Gram-positive bacteria: *Micrococcus luteus* 128, *Bacillus mycoides* 32, *B. subtilis* 2, *B. coagulans* 429, *B. cereus* 112 and *Staphylococcus aureus* 144; five strains of Gram-negative bacteria: *Alcaligenes faecalis* 82, *Escherichia coli* 52, *Proteus vulgaris* 206, *Pseudomonas aeruginosa* 54, *Salmonella gallinarum*; five strains of fungi: *Fusarium oxysporum* 61, *Penicillium chrysogenum* 37, *Aspergillus niger* 369, included two yeast strains: *Candida albicans* 217 and *Rhodotorula aurantiaca* 226.

The bacilli were grown on an organic medium containing (g L⁻¹): glucose (10.0), peptone (5.0), NaCl (5.0) and agar (25.0); the medium was supplemented by Hottinger's broth (0.025 % amine nitrogen) and had a pH of 7.0. The yeasts were grown on a wort (6-8°B) medium supplemented with 2.5% agar (pH 6.8). The medium for the fungi was similar (wort 3-4°B with 2.0% agar; pH 6.0). In the case of bacilli, staphylococci, *E. coli* and micrococci the temperature of cultivation was 37°C; *B. coagulans* 55°C and yeasts and fungi- 28°C. Petri dishes were inoculated with 1 day cultures of test microorganisms (0.1 mL cell suspension in physiological saline per dish; the concentrations were adjusted to 10⁹ CFU mL using a bacterial turbidity standard).

The bacteriocin-synthesizing activity was assessed as nisin production (judged by the suppression of growth of the indicator culture *B. coagulans*, a thermophilic spore-forming acid-fast bacterium). A 1-day culture was introduced into the agar medium as a suspension with a density of 10⁹ cell mL⁻¹. Quantitative determination of the antibiotic activity was performed by measuring the zones of growth suppression with

subsequent calculation involving a calibration plot for standard nisin solutions. Solutions of the preparation Nisaplin (Applin and Barrett, United Kingdom, 1000 IU mg⁻¹) and Nistatin (Sigma, Germany, 4670 units mg⁻¹) used as the standards (Stoyanova *et al.*, 2006).

Isolation of antibiotic substances: Methods for isolation and purification of antibiotic substances have been developed to obtain individual components of antibiotic complex as chromatographically pure preparations. In order to separate the antibiotic substances into fractions and obtain chromatographically pure components, the aqueous concentrate was extracted with a water-immiscible organic solvent, after which the extracts were pooled and the solvent was evaporated on a rotary evaporator to yield an oily residue. This oily residue was precipitated with methanol. The precipitate was separated via glass filter, dried under vacuum and then kept in a desiccator (Stoyanova *et al.*, 2007).

Chromatographic analysis: Fractions of the antibiotic substances were separated by TLC on Silufol (Czech Republic) or DC-Alufolien Kieselgel 60 (Merck, Germany) plates, using the developing system methanol-water (96:4). Preparative chromatography (to obtain individual components of the fractions) was performed under the same conditions.

Electrophoresis was performed on a Filtrak F-14 paper (Germany), using a V-shaped Durum instrument and two electrolytes: E1, pH 2.4 (2 N acetic acid; 550 V, 2 h) and E2, pH 1.1 (28:20:52 mixture of formic acid, acetic acid and water; 270 V, 2-3 h).

Bioautography was performed with *B. coagulans* 429, *B. subtilis* ATCC 6633 (RIA 445) and *Aspergillus niger* 369 were served as test microorganisms.

The fungicidal activity was tested with indicator culture *Eurotium repens*. Solutions of the fungicidal antibiotic nistatin (activity 4670 IU mg⁻¹) were served as the standards.

Testing the lactococci strains as preservatives: Experiments to show a perspective of the strains of *L. lactis* ssp. *lactis* 194 or K-205 as biopreservatives were performed. Two samples of raw smoked sausages, prepared accordingly with Russian State Standard (Gost 16131-86,) and became infected with moulds, were used as objects of investigation. Samples from the surface of raw smoked sausages with visible spots of spoilage were collected and placed on the surface of

two different media: Chapek agar (CA, pH 5.5), containing (g L⁻¹): sucrose-30.0, NaNO₃-1.0, KH₂PO₄-1.0, MgSO₄×7H₂O-0.5, KCl-0.5, FeSO₄-0.01, agar-15.0; and malt agar (MA, pH 6.5) containing 3.5% of malt extract (Maltax 10 standard, Finland). Plates were incubated at 25°C until fungal colonies were observed. Isolated colonies were transferred periodically into plates at 25°C until pure cultures were obtained. Fungal colonies were identified on the basis of morphological and physiological characteristics using common guides (Klich *et al.*, 2002).

The surfaces of all samples of infected raw smoked sausages were disinfected with 70% solution of ethanol, dried and then treated by cultural broth of strains 194 or K-205 (OD₅₄₀ 1.2, pH 4.2). The samples of raw smoked sausages without treatment of cultural broth were served as control. The control and experimental samples of raw smoked sausages were stored for 1 month at 10°C.

In second series of experiments before syringing with sausage stuffing's and further output, the sausage casing were dipped into cultural broth of strains of *L. lactis* ssp. *lactis* 194 or K-205 (OD₅₄₀ 1.2, pH 4.2) and exposed there for 10 and 20 min. The technological and microbiological indexes of the samples of raw smoked sausages were studied accordingly to Russian State Standard (Gost 16131-86).

The experiments were comprised in three series of triplicate measurements each. The results were processed statistically with program OriginPro v.8.1 for Windows (OriginLab, Data Analysis Graphing Software, USA), Statistica for Windows, v.5.0 (StatSoft Inc., USA).

RESULTS

520 lactococcal strains were isolated from raw milk probes and dairy products and also from powerful drinks: kurunga, kumiss and Iranian "Dough". Overall, 155 colonies of mesophylic acid-producing bacteria were selected. Overall, 94 isolated colonies inhibited the growth of test-microorganism *B. coagulans*, twelve of them displayed the highest activity (Table 1).

Strains designated as 115, 119, 119x were isolated from Timiryazev Agricultural University (Moscow, Russia) and strain 229-from the milk-processing plants of Klin (Moscow Region, Russia), IR1 and IR4-from Iranian milk products, strain 805-from mare's milk (Bashkiria, Russia) and 194, K-205-from cow milk of Ulan-Ude (Buryatia, Russia), were chosen for further study.

The morphology of the isolated strains demonstrated that the cultures were represented by cocci assembled in pairs or short chains of various lengths (four-seven cells). Strain 115, with morphology being reflecting of *L. lactis* ssp. *cremoris*, had the longest chains. All the bacteria isolated appeared to be Gram-positive. They did not differ by their cultural features from the bacteria belonging to the *Lactococcus* genus.

All these strains grew equally well in the liquid media with milk hydrolysate, a glucose-yeast medium and a meat-peptone broth with 2.0-4.0% NaCl. Bacterial growth was indicated by an increasing of turbidity of the cultures (0.24-1.56, OD₅₄₀) and the decrease pH from 6.8-4.1. Growth was absent in the meat-peptone broth containing 6.5% NaCl and at pH 9.6. The pH from 6.6-7.2 was optimal for the growth and development of these strains. The optimal incubation temperature was 28°C; at 10°C the growth was minimal and was completely absent at 45°C.

Table 1: Distinctive phenotypic properties of isolated antagonistic strains of *Lactococcus lactis*

Number of Products	Bacteriocins produced strains isolated	Perspective strains	Strains' main by strains, IU mL ⁻¹	distinctive properties
Kurunga (Buryatia, Russia)	36	K-205	2500	Ala ⁺ ; Ara ⁺ ; Mtl ⁺ ; Dext ⁺
«Doogh» (Iran)	25	IR 3	3400	Ala ⁻ ; Mal ⁺ ; Mtl ⁺ ; Dext ⁺
Curdled milk (Moscow, Russia)	40	119	2700	Pro ⁺ ; Ura ⁺ ; Ara ⁺ ; Mal ⁺ ; Dext ⁺
Cow milk (Moscow, Russia)	197	729	300	Ala ⁻ ; Ura ⁺ ; Mal ⁺
	119x	3700	Ala ⁻ ; Ara ⁻ ; Mtl ⁻ ; Dext ⁻	
	115	2500	Ala ⁺ ; Suc ⁻ ; Mal ⁺ ; Dext ⁺	
Cow milk (Klin, Russia)	54	229	2200	Ala ⁺ ; Ara ⁻ ; Mtl ⁻ ; Dext ⁺
Cow milk (Buryatia, Russia)	78	194	3600	Ala ⁺ ; Ara ⁺ ; Xyl ⁺ ; Dext ⁺
Cow milk (Iran)	35	IR 1	3100	Ara ⁺ ; Mtl ⁻ ; Dext ⁺
	IR 2	3000	Asn ⁺ ; Mal ⁺ ; Suc ⁻	
	IR 4	2900	Ala ⁺ ; Mtl ⁻ ; Dext ⁺	
Mare's milk (Bashkiria, Russia)	55	805	2500	Ala ⁻ ; Mal ⁺ ; Mtl ⁻ ; Suc ⁺

Table 2: Antimicrobial action of isolated strains of *Lactococcus lactis* ssp. *lactis* and Nisaplin

Strains isolated										
Test organisms	119	119x	729	229	194	K-205	IR3	IR4	MSU	Nisaplin (3000 IU mL ⁻¹)
Diameter of growth inhibition zone, mm										
<i>Bacillus mycoides</i>	20	16	10	16	19.5	16.0	11.0	24	13	17
<i>Bacillus subtilis</i>	12	18	10	15	22	20.0	11.0	24	16	18
<i>Bacillus coagulans</i>	17	23	8	15	23	18.0	20.0	17	14	21
<i>Bacillus cereus</i>	18.5	16	10	14	21	19.0	15.0	12	16	18
<i>Micrococcus luteus</i>	21.5	20	13	20	22.5	16.5	21.5	19	19	25
<i>Staphylococcus aureus</i>	16	12	0	16	20	17.0	17.0	16	12	15
<i>Alcaligenes faecalis</i>	0	0	9	10	12.5	12.5	15.0	12	0	0
<i>Escherichia coli</i>	0	0	11	0	15	14.0	19.0	12	0	0
<i>Salmonella gallinarum</i>	0	0	0	0	16	14.0	10.5	12	0	0
<i>Proteus vulgaris</i>	0	0	9	0	16.5	16.0	14.0	16	0	0
<i>Pseudomonas aeruginosa</i>	0	0	0	0	16.5	15.5	12.0	12	0	0
<i>Fusarium oxysporum</i>	0	0	0	0	16.5	12.0	12.0	10	0	0
<i>Penicillium chrysogenum</i>	0	0	0	0	17.5	10.5	12.0	16	0	0
<i>Aspergillus niger</i>	0	0	0	0	21	16.0	14.0	15	0	0
<i>Rhodotorula aurantiaca</i>	0	0	0	0	19.5	10.0	13.0	14	0	0
<i>Candida albicans</i>	0	0	0	0	11	12.0	10.0	12	0	0

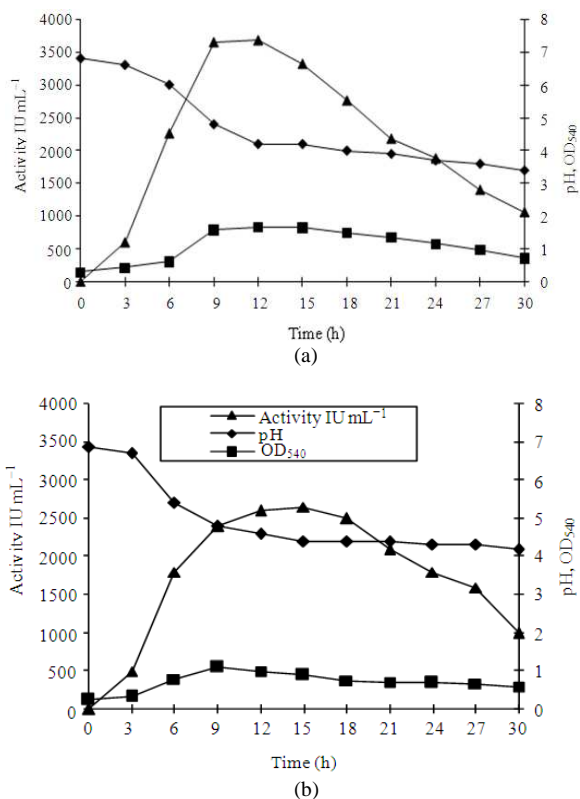


Fig. 1: Dynamic of growth and bacteriocins' synthesis by strains of *Lactococcus lactis* ssp. *lactis* 194(a) and K-205 (b) in mineral medium with glucose (20 g L⁻¹ KH₂PO₄, 10 g L⁻¹ glucose, yeast autolysate)

The distinctive features of these strains with respect to the fermentation of pentoses and sugar alcohols and their requirements in growth factors, asparagine, aspartic acid, proline and uracil were determined. The strains K-205 and IR3 that have been isolated from powerful drinks had possibility to utilize mannitol (Table 1).

According to the morphological, cultural, physiological, biochemical properties and gene sequence of 16S rRNA these strains were identified as *Lactococcus lactis* ssp. *lactis*.

The studies have revealed differences in antimicrobial activity between strains against different groups of microorganisms. It was demonstrated that the bacteriocins they produced varied in antagonistic activity. Some strains, isolated from milk and dairy products of Moscow region, were nisin-synthesizing (inhibit Gram-positive bacteria only). Only nine of the isolated 94 strains expressed a broad spectrum of activity against potential food pathogens: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella gallinarum* and fungi of *Aspergillus*, *Fusarium*, *Penicillium* genera, as well as against *Rhodotorula aurantiaca* and *Candida albicans* (Table 2). Strains 229 and 115 produced bacteriocins that were similar to nisin A in their activity spectrum. Most effective new bacteriocin-synthesizing strains 194 and K-205 were isolated from raw cow milk and kurunga drink from Buryatia. These strains had high antibiotic activity up to 3600-2700 IU mL⁻¹ respectively, as compared with nisin. It was important that bacteriocins produced by these strains

Table 3: Physico-chemical properties of individual antibiotic substances, produced by *Lactococcus lactis* ssp. *lactis* 194

Properties	Active components			
	194-A	194-B	194-C	Nisin A
Molecular mass, (M+H) ⁺ , m/z, (MALDI-MS)	607.5	619.6	1995	3353
UV-VIS spectrum, λ _{max} , nm, (solvent)	206.8 (C ₂ H ₅ OH)	224.8 (C ₂ H ₅ OH)	257 (C ₂ H ₅ OH)	215 (H ₂ O)
TLC (SiO ₂), R _f in system: MeOH-H ₂ O (96:4)	0.6	0.42	0	0
Paper electrophoresis in electrolytes 30% AcOH: pH = 2.4, 550 B, 2 h, cm	0	0	7.6	9.5
Antimicrobial spectrum	Gram + and Gram- bacteria, fungi	Gram+ bacteria	Gram+ bacteria	Gram+ bacteria

had a wide range of activity that made them perspective for further studies.

A study of the growth dynamics of these strains in the fermentation medium with glucose and yeast autolysate demonstrated that their growth was characteristic of mesophylic lactococci (Fig. 3a). Growth in the medium containing glucose caused a pH lowering from 6.8-4.1. The pH of the medium decreased to 4.8 over 9 h of the growth of strain 194, which accompanied by biomass increase (OD₅₄₀, from 0.30-1.58). During cultivation antibiotic activity level increased to 3700 IU mL⁻¹. The biomass accumulated gradually to 12 h and nisin accumulated in parallel with biomass. It was confirmed by increase of the antibiotic activity of the culture broth: after 12 h the pH dropped to 4.1, biomass increased to 1.66 and the level of antibiotic activity elevated by 1.2-fold (to 4500 IU mL⁻¹). After 12 h of incubation, the cells passed from the exponential growth phase to the stationary phase, which lasted for 3 h. After 15 h, a further decrease of pH, antibiotic activity and optical density were observed due to cell lysis. The antibiotic synthesis by *L.lactis* ssp. *lactis* 194 as strain K-205 was in parallel to growth of producer with maximum of antibiotic activity at 10-12 h of cultivation (Fig. 1).

It has been revealed, that the both strains *Lactococcus lactis* ssp. *lactis* 194 and K-205 produced antibiotic complexes that are consisted of three components (Fig. 2). Biological and physicochemical distinctions of the bacteriocins formed by these strain were determined. Two of these components are uncharged and hydrophobic (A, B), one (C) component was positively charged. It has been elucidated that the C-component was a peptide; like nisin; accordingly to its electrophoresis migration and biological activity. Analytical HPLC, TLC, FAB-MS and FD-MS methods were carried out to determine of physico-chemical properties of new antibiotic complex components. Individual antibiotic substances differ from each other by molecular mass, R_f values and biological properties (Table 3). Component C had a molecular mass 1995 Da

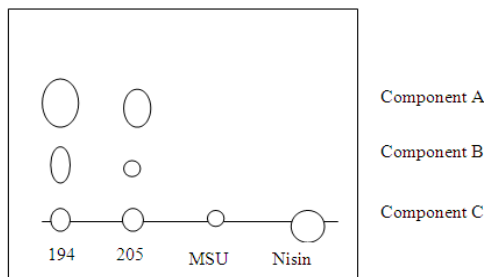


Fig. 2: TLC of antibiotic substances produced by *Lactococcus lactis* ssp. *lactis* strains 194, K-205 and MSU as compared to nisin. TLC (SiO₂), R_f in system: MeOH-H₂O (96:4), bio-autography, test-microorganism was *B. coagulans*

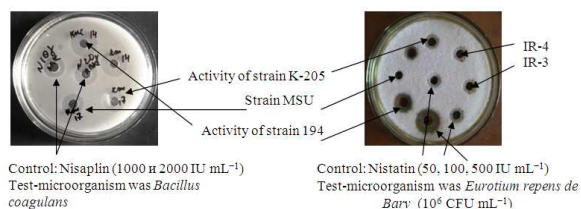


Fig. 3: Antibiotic activity of cultural broth of *Lactococcus lactis* ssp. *lactis* 194, K-205 and strain MSU against bacilli and fungi

and was a bacteriocin like nisin. Component B was a minor substance and its biological activity was insignificant.

Component A was hydrophobic with ketone group with double unsaturated bonds in the molecule (alkyl aromatic ketone) and had a low-molecular mass (M_r = 607.5 Da). Study of antimicrobial activities of the isolated components showed that component A exhibited a broad-spectrum activity against the majority of Gram-positive, Gram-negative bacteria and fungi.

The strains 194 and K-205 had high antibiotic activity of 3600 and 2700 IU mL⁻¹ respectively in comparison with nisin and up to 2500-1700 IU mL⁻¹ as compared with fungicidal antibiotic Nistatin, but nisin-synthesizing strain MSU was not effective against *Eurotium repens* (Fig. 3).

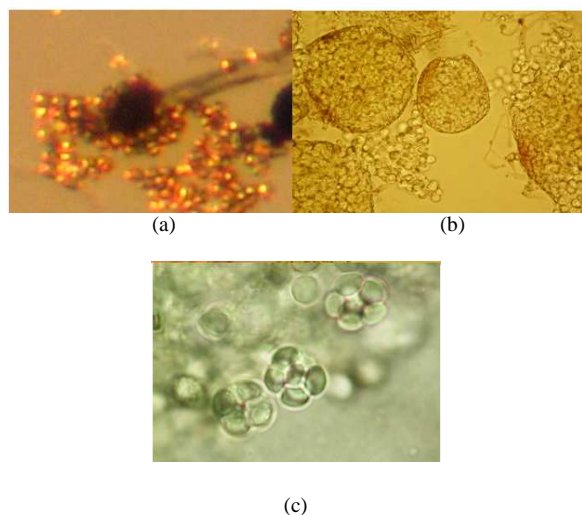


Fig. 4: Morphology of fungus *Eurotium repens* de Bary (anam. *Aspergillus repens* Samson and Gams), isolated from smoked raw sausages, as revealed by light microscopy (phase contrast, $\times 1260$) (a) conidia; (b) ascospores; (c) asci and ascospores



Fig. 5: Effect of treatment by *Lactococcus lactis* subsp. *lactis* 194 cultural broth on infection developing on the surface of raw smoked sausages (1-control; 2-after the treatment).

In our experiment we used raw smoked sausages that were infected with mould. The identification of this infection showed the presence of *Eurotium repens* on the sausages (Fig. 4).

In the experiment on the sausages protection the two samples of raw smoked sausages were infected with *Eurotium repens* (4.3×10^7 and 3.6×10^4 CFU g^{-1}). After treatment the surfaces of two samples of infected raw smoked sausages with 70% ethanol solution, they were washed by cultural broth of strains 194 (OD_{540} 1.2, pH 4.2) and then stored for one month at $10^\circ C$. The spoilage of sample 1 decreased to 1.5×10 KFU g^{-1} and sample 2 had no mould at all. The samples

of raw smoked sausages without treatment of cultural broth which were stored for one month at $10^\circ C$ too, were served as control. The spoilage of sample 1 was 2.3×10^6 CFU g^{-1} and sample 2- 1.1×10^3 CFU g^{-1} .

So it was shown that the treatment of the infected by *Eurotium repens* raw smoked sausages with cultural broth of strains of *L. lactis* subsp. *lactis* inhibited the spoilage. (Fig. 5).

The use of the cultural broth of *L. lactis* ssp. *lactis* 194 in the technology of production of raw smoked sausages prevented contamination of raw smoked sausages with mould. We established the longer storage of raw smoked sausages without mould developing. The integrated products corresponded to technological and microbiological indexes: food pathogens (*Clostridia* spp., *Staphylococcus aureus*, *E. coli*, *Salmonella* sp.) were absent.

DISCUSSION

Screening of effective bacteriocin-synthesizing strains of *L. lactis* as the perspective biopreservatives was performed. Altogether 520 lactococcal strains were isolated from raw milk probes and dairy products from various climatic regions and also from powerful drinks of mixed lactic acid and alcoholic fermentation: kurunga, kumiss and Iranian "Dough" which are widely used by local populations to prevent diseases. The lactococcal colonies were selected on the media with the indicator bromocresol purple, which changed the color of the medium from violet to yellow indicating the acidification degree. It is known that the major product of homolactic fermentation is lactic acid. Uniform growth of bacteria along the entire inoculum's length in a stab of an agar medium is characteristic of facultative anaerobes (Hoelt *et al.*, 1994).

Physiological and biochemical features of isolated strains were studied and compared to the nisin-producing strain *Lactococcus lactis* ssp. *lactis* MSU. The strains K-205 and IR3 that have been isolated from powerful drinks of mixed lactic acid and alcoholic fermentation kurunga and Iranian "Dough" and therefore were adapted to alcohol substrate, had possibility to utilize mannitol. This characteristic is the property of *L. lactis* ssp. *cremoris*, but not *L. lactis* ssp. *lactis* newly established. According to the morphological, cultural, physiological, biochemical properties and gene sequence of *16S rRNA* these strains were identified as *Lactococcus lactis* ssp. *lactis*.

The special interest was paid to isolates of *L. lactis* with antifungal activity. It was demonstrated that the bacteriocins they produced varied in antagonistic activity. Some strains, isolated from milk and dairy products of Moscow region, were nisin-synthesizing (inhibit Gram-positive bacteria only). Only nine of the isolated 94 strains expressed a broad spectrum of activity. This property is unique for isolated natural strains of *Lactococcus lactis* species.

Synthesis of bacteriocin as a protein proceeds concurrently with growth of producer and DNA, RNA synthesis (Diep and Nes, 2002). A study of the growth dynamics of these strains in the fermentation medium with glucose and yeast autolysate demonstrated that their growth was characteristic of mesophylic lactococci. The studies have revealed differences in antimicrobial activity between strains against different groups of microorganisms. Screening of effective antifungal strains from milk and dairy products from different climatic regions and also powerful drinks of mixed lactic acid and alcoholic fermentation: kurunga, kumiss and Iranian "Dough" was performed. These strains were identified as *L. lactis* ssp. *lactis* that have "GRAS" status. The most perspective strains were *L. lactis* ssp. *lactis* 194 and K-205, which synthesis antibiotic complex with broad activity spectrum against food-borne pathogens included fungi. Antimicrobial substance A, in concordance with UV-spectrum, molecular mass and spectrum of antimicrobial activity was identified as novel and was absent in J. Berdy database BNPD. Component C was a peptide with molecular mass 1995 Da smaller than bacteriocin nisin.

Treatment of the raw smoked sausages with cultural broth of these strains of *L. lactis* ssp. *lactis* inhibited the spoilage by *E. repens*. After treatment the sausages had longer shelf-life and integrated products correspond to technological and microbiological indexes. The results of this study indicated that the treatment with these lactococci strains can prevent a contamination of raw smoked sausages with mould.

CONCLUSION

Screening of effective antifungal strains from milk and dairy products from different climatic regions and also powerful drinks of mixed lactic acid and alcoholic fermentation: kurunga, kumiss and Iranian "Dough" was performed. These strains were identified as *L. lactis* ssp. *lactis* that have "GRAS" status. The nucleotide sequences of the *16S rRNA* genes of novel strains were deposited to the GenBank database under following accession numbers: DQ255952 (strain MGU),

DQ255951 (115), DQ255953 (229), EF114309 (805), EF1143308 (K-205), DQ255954 (194).

The *Lactococcus* strains enriched the collection of efficient producers of nisin and other bacteriocins. Most effective new bacteriocin-synthesizing strains 194 and K-205 were isolated from raw cow milk and kurunga drink from Buryatia. These strains had high antibiotic activity up to 3600-2700 IU mL⁻¹ respectively, as compared with nisin and up to 2500-1700 IU mL⁻¹ as compared to fungicidal antibiotic nistatin. Antifungal activity among natural strains of *L. lactis* is unique property. This strains produced novel antibiotic complex with broad activity spectrum against food-borne pathogens included fungi that was absent in J. Berdy database BNPD.

Treatment of the raw smoked sausages with cultural broth of these strains of *L. lactis* ssp. *lactis* inhibited the spoilage by *E. repens*. After treatment the sausages had longer shelf-life and products after storage were correspond to technological and microbiological indexes stated by Russian standards.

So, potential applications of cultural broth of these strains in various food fermentations allow to recommend it as potential perspective biopreservatives for preventing fungal spoilage of foodstuffs and edible raw materials.

REFERENCES

- Cutter, C. and G. Siragusa, 1994. Decontamination of beef carcass tissue with nisin using a pilot scale model carcass washer. *Food Microbiol.*, 11: 481-489. DOI: 10.1006/fmic.1994.1054
- Drider, D., Y. Fimland, H. McMullen and H. Prevost, 2006. The continuing story of class IIa bacteriocins. *Microbiol. Molec. Biol. Revs.*, 70: 564-582. DOI: 10.1128/MMBR.00016-05
- European Parliament and Council, 1997. Regulation (EC) № 258/97 of the European Parliament and of the Council of 27 Jan. 1997 concerning novel foods and novel food ingredients. *Official J.*, 14: 1-7. http://www.biosafety.be/GB/Dir.Eur.GB/FF/258_97/258_97.html
- Fujita, K., S. Ichimasa, T. Zendo, S. Koga and F. Yoneyama, 2007. Structural analysis and characterization of lacticin Q, a novel bacteriocin belonging to a new family unmodified bacteriocins of gram-positive bacteria. *Applied Environ. Microbiol.*, 73: 2871-2877. DOI: 10.1128/AEM.02286-06
- Galvez, A., H. Abriouel, R. Lopez and N. Omar, 2007. Bacteriocin-based strategies for food biopreservation. *J. Food Microbiol.*, 120: 51-70. DOI: 10.1016/j.ijfoodmicro.2007.06.001

- Gost, 2001. 16131-86 *Dry sausages. Specifications*, Sb. Gostov M.:IPK Izdatelstvo standartov, Russia. <http://docs.cntd.ru/document/1200016987/0>
- Hoelt, J., N.R. Kried, P.H. Sneath, J.T. Staley and S.T. Williams, 1994. *Bergey's Manual of Determinative Bacteriology*. 9th Edn., Springer-Verlag, New-York, ISBN: 978-0-683-00603-2
- Jack, R., J. Tagg and B. Ray, 1995. Bacteriocins of gram-positive bacteria. *Microbiol. Rev.*, 59: 171-200. PMID: PMC239359
- Jensen, N., C. Melchiorsen, K. Jokumsen and J. Villadsen, 2001. Metabolic behavior of *Lactococcus lactis* MG1363 in microaerobic continuous cultivation at a low dilution rate. *Applied Environ. Microbiol.*, 67: 2677-2682. DOI: 10.1128/AEM.67.6.2677-2682.2001
- Klich, M.A., U. States, A.R. Service, S.R.R. Center, 2002. *Identification of Common Aspergillus Species*. Centraalbureau Voor Schimmelcultures. The Netherlands, pp: 116. ISBN: 90-70351-46-3
- Lowe, D. and E. Arendt, 2004. Lactic acid bacteria in malting and brewing with their relationships to antifungal activity, mycotoxins and gushing: A review. *J. Inst. Brew*, 110: 163-180. <http://www.scientificsocieties.com/jib/papers/2004/G-2004-0812-237>
- Mantovani, H. and J. Russell, 2001. Nisin resistance of *Streptococcus bovis*. *Applied Environ. Microbiol.*, 67: 808-813. DOI: 10.1128/AEM.67.2.808-813.2001
- Morgan, S., P. O'Connor, P. Cotter, R. Ross and C. Hill, 2005. Sequential actions of the two component peptides of the lantibiotic lactacin 3147 explain its antimicrobial activity at nanomolar concentrations. *Applied Environ. Microbiol.*, 49: 2606-2611. DOI: 10.1128/AAC.49.7.2606-2611.2005
- Nete, B., T. Licht, C. Brogren, B. Jelle and A.H. Johansen, 2006. Effects of *Lactococcus lactis* on composition of intestinal microbiota: Role of nisin. *Applied Environ. Microbiol.*, 72: 239-244. DOI: 10.1128/AEM.72.1.239-244.2006
- Roy, U., V.K. Batish, S. Grover and S. Neelakantan, 1996. Production of antifungal substance by *Lactococcus lactis* subsp. *lactis* CHD-28.3. *Int. J. Food Microbiol.*, 32: 27-34. DOI: 10.1016/0168-1605(96)01101-4
- Stoyanova, L., N. Egorov, G. Fyodorova, G. Katrukha and A. Netrusov, 2007. A Comparison of the properties of bacteriocins formed by *Lactococcus lactis* subsp. *lactis* strains of diverse origin. *Applied Biochim. Mikrobiol.*, 43: 604-610. DOI: 10.1134/S0003683807060063
- Stoyanova, L. and N. Levina, 2006. Components of fermentation medium regulate bacteriocin synthesis by the recombinant strain *Lactococcus lactis* subsp. *lactis* F-116. *Microbiol*, 75: 342-348. DOI: 10.1134/S0026261706030088
- Stoyanova, L., T. Sul'timova, S. Botina and A. Netrusov, 2006. Isolation and identification of new nisin-producing *Lactococcus lactis* subsp. *lactis* from milk. *Applied Biochem. Microbiol.*, 42: 492-499. DOI: 10.1134/S0003683806050085
- Stoyanova, L., T. Sul'timova and A. Netrusov, 2008. Establishment of taxonomic status of new prospective bacteriocin-synthesizing *Lactococci* strains of various origins. *Moscow Univ. Biol. Sci. Bull.*, 42: 156-162. DOI: 10.3103/S0096392508040044
- Yildirim, Z., M. Yildirim and M.G. Johnson, 2004. Mode of action of lactococcin R produced by *Lactococcus lactis* R. *Nahrung*, 48: 145-148. DOI: 10.1002/food.200300404
- Zendo, T., M. Fukao, T. Higuchi, J. Nakayama and K. Sonomoto, 2003. Identification of the lantibiotic nisin Q, a new natural nisin variant produced by *Lactococcus lactis* 61-14 isolated from a river in Japan. *Biosci. Biotechnol. Biochem.*, 67: 1616-1619. DOI: PMC1892864