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Ability of Chosen Lactic Acid Bacteria to Produce Antibacterial Substances

Sposobnost odabranih bakterija mliječne kiseline da proizvedu antibakterijske supstancije

*Blaženka Brkić, Jagoda Šušković, S. Matošić and K. Gjuračić**

Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia

* Pliva Research Institute, Laboratory of Molecular Biology, Prilaz baruna Filipovića 89, 10000 Zagreb, Croatia

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Summary

Six strains of lactic acid bacteria isolated from silage, fermented milk and vegetables, were examined for antagonistic activities against entheropathogenic microorganisms and lactic acid bacteria. The two screening methods used were the agar spot test and the disc assay method. Cell-free supernatants of lactic acid bacteria inhibited the growth of ***Staphylococcus aureus*** *3048,* ***Staphylococcus aureus*** *K-144,* ***Salmonella mumum, Escherichia coli, Bacillus cereus*** *and* ***Bacillus subtilis.***

*However, concentrated and neutralized supernatants from four of the six strains (****Lactobacillus acidophilus, Lactobacillus plantarum*** *L4,* ***Lactobacillus plantarum*** *Z88,* **Enterococcus faecium***) showed antibacterial activity against Gram-negative and Gram-positive target strains, as well as some strains of lactic acid bacteria. These results confirmed the presence of antibacterial substances other than lactic acid. Furthermore, plasmids had been isolated from these four lactic acid bacteria which exhibited higher antibacterial activity.*

Sažetak

Antibakterijska aktivnost šest sojeva bakterija mliječne kiseline, izolirana iz silaže, fermentiranog mlijeka i povrća, ispitana je prema enteropatogenim mikroorganizmima i bakterijama mliječne kiseline. Testiranje antibakterijske aktivnosti provedeno je metodama difuzije u agar s filtar-diskovima i s dvostrukim slojem agara (»agar spot test«). Supernatanti kultura inhibiraju rast test-mikroorganizama: ***Staphylococcus aureus*** *3048,* ***Staphylococcus aureus*** *K-144,* ***Salmonella mumum, Escherichia coli, Bacillus cereus*** *i* ***Bacillus subtilis.***

*Koncentrirani i neutralizirani supernatanti četiri ispitivana soja bakterija mliječne kiseline (****Lactobacillus acidophilus, Lactobacillus plantarum*** *L4,* ***Lactobacillus plantarum*** *Z88,* ***Enterococcus faecium****) pokazuju antibakterijsko djelovanje prema gram-negativnim i gram-pozitivnim mikroorganizmima, obuhvaćajući i neke sojeve bakterija mliječne kiseline. Rezultat upućuje na aktivnost antibakterijske supstancije u supernatantu. Plazmidi su također izolirani iz bakterija mliječne kiseline koje su pokazale jače antibakterijsko djelovanje.*

Introduction

Lactic acid bacteria are essential in the manufacture of fermented foods and beverages, silage and probiotics. They are used as starter cultures to ensure the right properties of the fermented products such as taste, rheological properties, shelf-life and nutritional quality *(1–3)*. Their prime functions include also a strong antimicrobial activity against many spoilage bacteria and food-born pathogens mainly by the production of lactic acid and the resulting pH decrease. However, lactic acid bacteria can also produce other inhibitory compounds such as hydrogen peroxide, acetaldehyde, diacetyl and bacteriocins *(2,3)*.

Bacteriocins are biologically active proteins which exhibit bactericidal effect against closely related bacteria. On the basis of biochemical and genetic studies, bacteriocins from lactic acid bacteria were divided into four major classes *(4)*. Class I bacteriocins are lantibiotics which contain unusual amino acids, lanthionin and β-methyllanthionine. The best characterized lantibiotic is nisin, which is produced by *L. lactis* subsp. *lactis*. Class

II bacteriocins are characterized as small, heat stable, hydrophobic peptides with a high isoelectric point. A number of class II bacteriocins have been identified and purified from *Lactobacillus* sp., e.g. sakacin A, produced by *L. sake (5)*, lactacin B *(6)* and lactacin F *(7)*, produced by *L. acidophilus* and *L. johnsonii*, respectively. The Class III bacteriocins, which have to date only been found in *Lactobacillus* sp., include heat-labile proteins of large molecular mass (e.g. acidophilucin A *(8)*, lacticin *(9)*, helveticin V-1829 *(10)*. Class IV bacteriocins consist of protein complexes associated with lipid or carbohydrate moieties required for activity. Plantaricin S, bacteriocin produced by *L. plantarum*, is a glycolipoprotein that is inactivated by treatments with α-amylase, lipase and protease *(11)*. Leuconocin S and pediocin SJ-1 are also inactivated by α-amylase *(12,13)*. Some of bacteriocins, produced by lactic acid bacteria, have a broad spectrum of antimicrobial activity which include other genera, such as *Listeria, Staphylococcus, Clostridium* and *Bacillus (6,14)*. In view of the interesting range of antagonistic activity, these bacteriocins could be used as natural food preservatives. Until recently, nisin was the only bacteriocin that has been exploited commercially in the food industry *(3)*. The antibacterial properties of lactic acid bacteria are of special interest in developing strongly competitive starter culture for milk and vegetables fermentations, silage and probiotic preparations. Homofermentative lactic acid bacteria, such as *L. plantarum* L4 and *E. faecium*, can be added to the silage in order to compensate for the low number of these bacteria present on the crops. Increase of the fermentation rate supported by addition of a silage inoculants has been shown *(15)*, but there are no reports on their bacteriocin production. Importance of bacteriocin production in fermented vegetables, such as cabbages, olives and cucumbers, has been addressed by Daeschel *et al. (14)*. Bacteriocins are believed to be important also in the ability of lactic acid bacteria to compete in non-fermentative ecosystems, such as the gastrointestinal tract. *L. acidophilus* and *E. faecium* are cited most often in this connection. Indicated therapeutic values of these and other lactic acid bacteria include anticholesteremic properties, tumor suppression, an control over growth and colonization of potentially pathogenic microorganisms *(3)*.

The aim of the present paper was to investigate the potentiality of lactic acid bacteria to produce antibacterial substances active against different food spoilage bacteria and lactic acid bacteria.

Materials and Methods

Bacterial strains and cultivation conditions

The bacterial strains tested for antibacterial activity were: *Lactobacillus acidophilus, Lactobacillus plantarum* L4, *Lactobacillus plantarum* Z88, *Enterococcus faecium, Lactobacillus* sp. J91 and *Lactobacillus* sp. E. These lactic acid bacteria as well as the enteropathogenic microorganisms: *Staphylococcus aureus* 3048, *Staphylococcus aureus* K-144, *Salmonella mumum, Escherichia coli* and spore forming bacteria *Bacillus cereus* and *Bacillus subtilis* were used as the target organisms. All bacterial strains were from Culture Collection of the Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia.

All lactic acid bacteria were propagated and maintained in MRS medium *(16)*. Overnight cultures (16 h at 37 °C) were used as inocula for growth inhibition studies and for cell-free supernatant preparations. Enteropathogenic microorganisms and spore forming bacteria were propagated and maintained on nutrient agar.

Cell-free supernatant preparations

The bacterial strains to be tested for antibacterial activity were grown with agitation, in 500 mL Erlenmeyer flasks containing 200 mL of the MRS broth, with controlled pH = 6 and without pH control, for 24 h at 37 °C. The samples were taken after 8, 12 and 24 h of cultivation. The cells were removed by centrifugation and the supernatants were filtered through a 0.2 μm Millipore filter. Cell-free supernatants were concentrated 5-fold by rotary evaporation at 40 °C. In order to avoid any pH effect, the supernatants were tested for inhibitory activity with the pH unadjusted and adjusted to 6 with NaOH solution (1 mol/L). Furthermore, catalase was added at a final concentration of 1 μg/mL, to provide against the possible presence of hydrogen peroxide. The free lactic acid concentration in supernatants was estimated by titration with NaOH solution (0.1 mol/L). The antibacterial activity of the respective concentration of lactic acid was also examined.

Examination of antibacterial activity

Sensitivity of lactic acid bacteria to inhibitory substances of producer microorganisms was determined by using the agar spot test method *(17)*. Overnight cultures of the strains were spot-inoculated with needle on the surface of agar plates of MRS and incubated at 37 °C for 24 h to allow colonies to develop. Ten mL of MRS soft agar (0.7 % agar) seeded with 75 μL of the culture to be tested for sensitivity were poured over the plates on which the potential producer was grown. After incubation for 24 h at 37 °C a clear zone around the colonies was measured.

Antibacterial activity of cell free supernatants against indicator strains, including enteropathogenic microorganisms and spore forming bacteria, was determined by the disc assay method (18). The culture supernatants (80 μL) were applied to sterile filter discs (12 mm) placed on the surface of solidified nutrient agar or MRS agar (for lactic acid bacteria as indicator strains) seeded with overnight cultures of indicator strains. The plates were kept at 4 °C for 3 h to permit diffusion on the assay material, then incubated at 37 °C for 16 h. The diameters of clear inhibition zones were then measured.

Isolation of plasmid DNA

Plasmid isolation was achieved with the generally accepted method for plasmid isolation from *E. coli (19)* with a few modifications. Cells were harvested from 40 mL of culture by centrifugation, suspended in 700 μL of cold solution I (glucose, 50 mmol/L, Tris HCl, 25 mmol/L, pH = 8.0, EDTA, 10 mmol/L) and after 5 min at room temperature 250 μL lysozyme (10 mg/mL in Tris HCl,

25 mmol/L, pH = 8.0) was added and samples were incubated at 37 °C for 1 h. Incubation was followed by the addition of 1.4 mL of solution II (NaOH, 0.2 mol/L, SDS, 1 %). After incubation at 0 °C for 30 min, 1.05 mL of solution III (potassium acetate, 5 mol/L, pH = 4.8) was added and incubated another 5 min. The tube was then centrifuged at 10000 rpm for 20 min. Supernatant was transferred and proteins were extracted with 450 μL of a mixture of 25:24:1 (volume ratio) phenol-chloroform-isoamylic alcohol at least twice. The aqueous phase above the white protein interface was removed and the plasmid DNA precipitated with one volume of 8 mol/L ammonium acetate and cold ethanol at –20 °C overnight. The DNA pellets were dried and resuspended in 20 μL of TE (Tris HCl, 10 mmol/L, pH = 7.5, EDTA, 1 mmol/L). Aliquots of each sample (5 μL) were subjected to electrophoresis on 0.6 % agarose gels run at 50 V for 2 h.

Results and Discussion

Six strains of lactic acid bacteria were examined for antagonistic activity against a set of target microorganisms. Four of the six tested strains (*L. acidophilus*, *L. plantarum* L4, *L. plantarum* Z88 and *E. faecium*) produced antimicrobial compounds which were active against the other lactic acid bacteria (Table 1) and exhibited inhibitory activity against food-borne pathogens (Fig. 1–4). For this reasons they were chosen for further investigations.

The lactic acid bacteria produced their antagonistic compounds extracellulary and the production of bacteriocin has been reported to occur at various stages in the cell growth cycle *(1,2,14)*. The level of inhibition by cell free supernatants of *L. acidophilus*, *L. plantarum* L4, *L. plantarum* Z88 and *E. faecium* was higher at the end (*i.e.* after 12 h of cultivation) than in the middle of exponential growth phase (Fig. 1–4). There was no or little reduction in antibacterial activity of *L. acidophilus* during stationary phase (12–24 h of cultivation). However, prolonged incubation (from 12 to 24 h) of *L. plantarum* L4, *L. plantarum* Z88 and *E. faecium* cause partial loss of their antibacterial activity as it could be seen from inhibition zone for target microorganisms: *E. coli*, *S. mumum* and *B. cereus* (Fig. 2–4). The comparison of antibacterial activities of the supernatants and the corresponding concentration of lactic acid suggests that the inhibitory effects were not identical. Namely, antibacterial activities of the supernatants of lactic acid bacteria were mostly higher than the antibacterial activity of the pure lactic acid itself (Fig. 1–4). The highest difference in diameters of inhibition zones between the supernatants and respective concentrations of lactic acid has been obtained with the supernatant of *E. faecium*. All target microorganisms

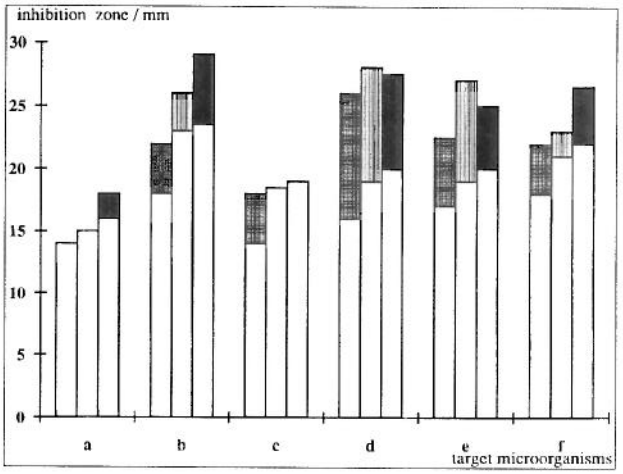

Fig. 1. Inhibition of target strains by culture supernatants from *L. acidophilus*, prepared after 8, 12 and 24 h of cultivation (whole bars). The darkened parts of the bars represent the additional inhibitory action provided by antibacterial substances produced in the cells. Empty parts of the bars represent inhibition with the respective concentration of pure lactic acid, corresponding to the amount of lactic acid estimated in the supernatants by titration with NaOH, 0.1 mol/L (□). Disc assay method. Means of triplicate determinations are presented.
(a) *Staphylococcus aureus* 3048, (b) *Staphylococcus aureus* K-144, (c) *Escherichia coli*, (d) *Salmonella mumum*, (e) *Bacillus cereus*, (f) *Bacillus subtilis*.

Slika 1. Inhibicija rasta test-mikroorganizama supernatantom kulture *L. acidophilus*, prirađenim nakon 8, 12 i 24 sata uzgoja (cijeli stupci). Tamni odsječci predstavljaju dodatnu inhibiciju kao posljedicu nastanka antibakterijskih supstancija u stanicama. Neispunjeni odsječci predstavljaju inhibicijsko djelovanje mliječne kiseline u koncentracijama koje odgovaraju koncentracijama mliječne kiseline u supernatantima, određenim titracijom s NaOH, 0,1 mol/L (□). Metoda difuzije u agar s filtar-diskovima. Prikazana je srednja vrijednost rezultata triju ponovljenih pokusa.
(a) *Staphylococcus aureus* 3048, (b) *Staphylococcus aureus* K-144, (c) *Escherichia coli*, (d) *Salmonella mumum*, (e) *Bacillus cereus*, (f) *Bacillus subtilis*.

Table 1. Growth inhibition of six lactic acid bacteria by the colonies of producer organism (agar spot test method)
Tablica 1. Inhibicija rasta šest sojeva bakterija mliječne kiseline kolonijama proizvodnog mikroorganizma (metoda difuzije s dvostrukim slojem agara)

Producer strain	Inhibition zone / mm					
	Lactobacillus acidophilus	*Lactobacillus plantarum* L4	*Lactobacillus plantarum* Z88	*Enterococcus faecium*	*Lactobacillus* sp. J91	*Lactobacillus* sp. E
Lactobacillus acidophilus	n.d.	3	3	11	0	0
Lactobacillus plantarum L4	10	n.d.	5	7	3	3
Lactobacillus plantarum Z88	6	3	n.d.	14	0	0
Enterococcus faecium	0	0	0	n.d.	0	3
Lactobacillus sp. J91	0	0	0	0	n.d.	0
Lactobacillus sp. E	0	0	0	0	0	n.d.

n.d. – not determined

have shown significant sensitivity to antibacterial substance of *E. faecium*, other than lactic acid (Fig. 4). *S. aureus* and *E. coli* were inhibited mostly with lactic acid from the supernatants of *L. acidophilus* and *L. plantarum* strains (Fig. 1–3).

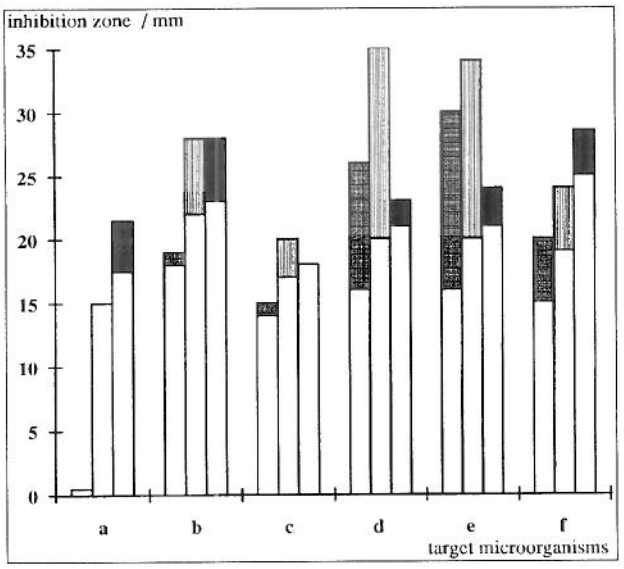

Fig. 2. Inhibition of target strains by culture supernatants from *L. plantarum* L4, prepared after 8, 12 and 24 h of cultivation. Disc assay method. For the legend see Fig. 1.
Slika 2. Inhibicija rasta test-mikroorganizama supernatantom kulture *L. plantarum* L4, priređenim nakon 8, 12 i 24 sata uzgoja. Metoda difuzije u agar s filtar-diskovima. Vidi legendu uz sliku 1.

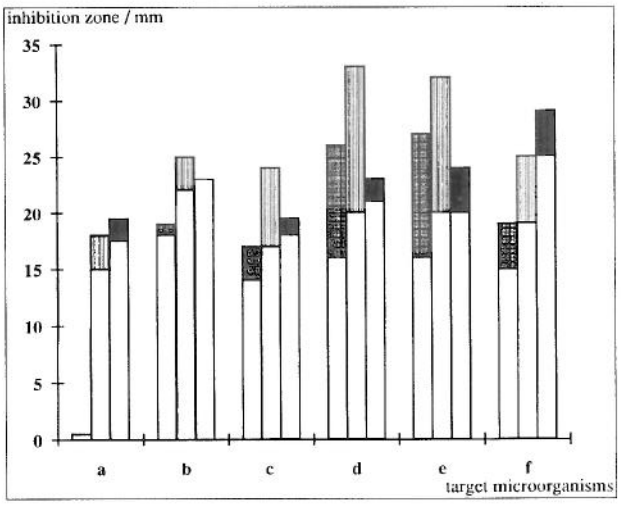

Fig. 3. Inhibition of target strains by culture supernatants from *L. plantarum* Z88, prepared after 8, 12 and 24 h of cultivation. Disc assay method. For the legend see Fig. 1.
Slika 3. Inhibicija rasta test-mikroorganizama supernatantom kulture *L. plantarum* Z88, priređenim nakon 8, 12 i 24 sata uzgoja. Metoda difuzije u agar s filtar-diskovima. Vidi legendu uz sliku 1.

Table 2. Growth inhibition of target strains: a) food-borne pathogens b) lactic acid bacteria, by concentrated supernatants of lactic acid bacteria with the pH unadjusted and adjusted to pH = 6.0. (disc assay method)

Tablica 2. Inhibicija rasta test-mikroorganizama: a) kontaminanata prehrambenih proizvoda i b) bakterija mliječne kiseline, koncentriranim supernatantima odabranih bakterija mliječne kiseline, prije i nakon podešavanja pH na 6,0 (metoda difuzije u agar s filtar-diskovima)

a)

Producer strain	pH	Inhibition zone / mm					
		S. aureus 3048	*S. aureus* K-144	*E. coli*	*S. mumum*	*B. cereus*	*B. subtilis*
Lactobacillus acidophilus	6.00	0	0	0	0	0	0
	4.10	42	46	37	30	41	43
Lactobacillus plantarum L4	6.00	0	0	0	0	22	22
	3.68	32	37	33	42	41	37
Lactobacillus plantarum Z88	6.00	0	0	0	0	13	0
	3.68	34	39	36	42	33	39
Enterococcus faecium	6.00	30	32	28	27	33	25
	4.38	31	37	29	29	30	33

b)

Producer strain	pH	Inhibition zone / mm				
		L. acidophilus	*L. plantarum* L4	*L. plantarum* Z88	*E. faecium*	*Lactobacillus* sp. E
Lactobacillus acidophilus	6.00	n.d.	15	15	14	0
	4.10	n.d.	16	15	27	34
Lactobacillus plantarum L4	6.00	14	n.d.	0	0	16
	3.68	21	n.d.	16	21	39
Lactobacillus plantarum Z88	6.00	0	0	n.d.	0	0
	3.68	18	17	n.d.	27	34
Enterococcus faecium	6.00	0	0	0	n.d.	18
	4.38	0	13	0	n.d.	15

n.d. – not determined

Several lactic acid bacteria have demonstrated enhanced production of antibacterial substances including bacteriocins when grown in solid media *(18)*. For this reason lactic acid bacteria were investigated for production of antagonistic metabolites using agar spot test method, which allows secretion of antimicrobial compounds into the surrounding solid media during the growth of producing strains. *E. faecium* has expressed significant sensitivity to producer strains *L. acidophilus*, *L. plantarum* L4 and *L. plantarum* Z88. The highest antibacterial activity has been detected by the colonies of *L. plantarum* L4 (Table 1). However, if secretion of an antibacterial substance (bacteriocin) was advantageous to an organism, this strain could become the dominant lactic acid bacteria in the mixed culture fermentation *(12)*.

In relation to the high content of proteins and peptides in the growth medium, lactic acid bacteria produced antibacterial substances in very low concentrations *(6,13)*. Culture supernatants, 5-fold concentrated, gave the most intense antibacterial activity (Table 2). The antagonistic effect of lactic acid in 5-fold concentrated culture supernatants was restricted with pH = 6 adjustment. Concentrated and neutralized supernatant of *L. acidophilus* showed inhibitory activity against lactic acid bacteria (Table 2b), while no inhibition of other target strains (e.g. tested food-borne pathogens, Table 2a) was observed. In contrast, the antimicrobial compound produced by *E. faecium* inhibited only one of tested lactobacilli, while it was more active against food-borne pathogens (Table 2). Table 3 also confirms the presence of

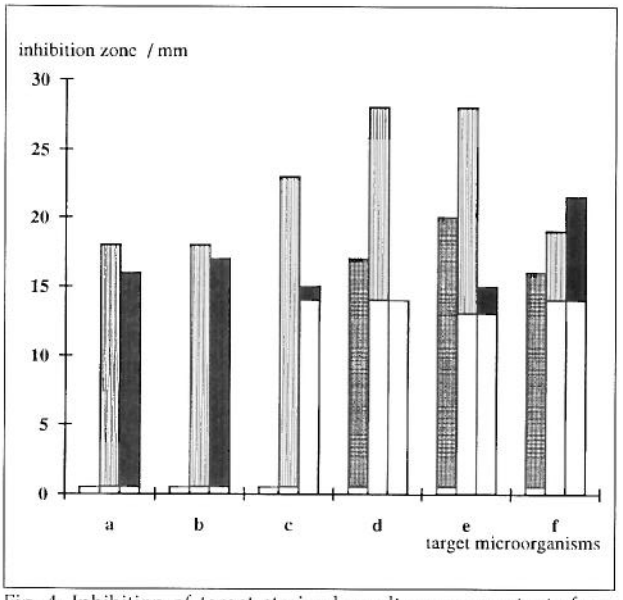

Fig. 4. Inhibition of target strains by culture supernatants from *E. faecium*, prepared after 8, 12 and 24 h of cultivation. Disc assay method. For the legend see Fig. 1.

Slika 4. Inhibicija rasta test-mikroorganizama supernatantom kulture *E. faecium*, priređenim nakon 8, 12 i 24 sata uzgoja. Metoda difuzije u agar s filtar-diskovima. Vidi legendu uz sliku 1.

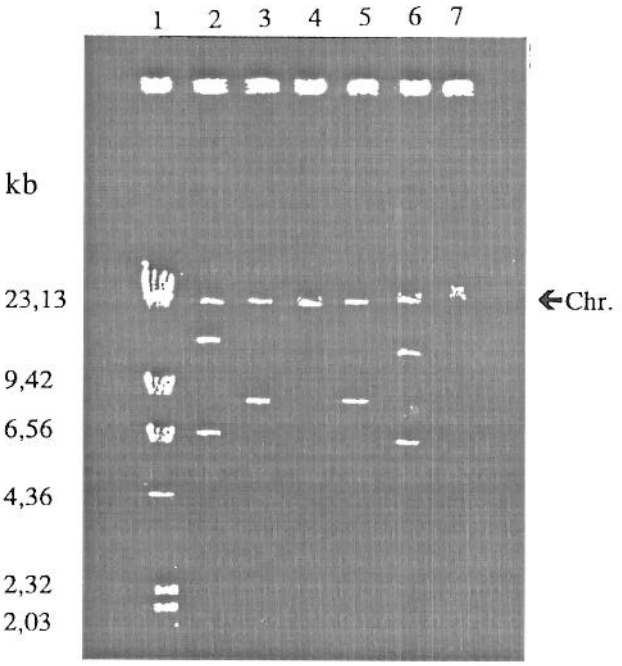

Fig. 5. Agarose gel electrophoresis of chromosomal and plasmid DNA isolated from lactic acid bacteria. **1** – Standard – digestion of λ DNA with *Hind* III; **2** – *L. acidophilus*; **3** – *L. plantarum* L4; **4** – *Lactobacillus* sp. J91; **5** – *L. plantarum* Z88; **6** – *E. faecium*; **7** – *Lactobacillus* sp. E. **Chr.** – chromosomal DNA.

Slika 5. Gel-elektroforeza kromosomske i plazmidne DNA bakterija mliječne kiseline. **1** – Standard – λ DNA pocijepana s enzimom *Hind* III; **2** – *L. acidophilus*; **3** – *L. plantarum* L4; **4** – *Lactobacillus* sp. J91; **5** – *L. plantarum* Z88; **6** – *E. faecium*; **7**– *Lactobacillus* sp. E. **Chr.** – kromosomska DNA.

Table 3. Antibacterial activity of lactic acid bacteria supernatants, prepared after cultivation in MRS broth with pH control at 6.0 (disc assay method)
Tablica 3. Antibakterijska aktivnost supernatanata ispitivanih bakterija mliječne kiseline, priređenih nakon uzgoja u MRS-podlozi pri kontroliranoj pH-vrijednosti (pH = 6,0) (metoda difuzije u agar s filtar-diskovima)

Producer strain	Time of cultivation of producer strain / h	Inhibition zone / mm					
		S. aureus 3048	*S. aureus* K-144	*E. coli*	*S. mumum*	*B. cereus*	*B. subtilis*
Lactobacillus acidophilus	12	0	0	0	15	0	0
	24	0	15	0	15	0	0
Lactobacillus plantarum L4	12	15	15	15	23	18	0
	24	0	0	0	18	17	0
Lactobacillus plantarum Z88	12	16	0	16	20	18	0
	24	16	15	16	21	20	0
Enterococcus faecium	12	16	17	17	22	21	17
	24	20	22	21	22	22	18

inhibitory substances other than lactic acid in the supernatants. When the fermentation was carried out at constant pH (6.0), the antibacterial activity of *E. faecium* was higher than the activity obtained after growth without pH control (Fig. 4). It was reported that the growth at constant pH might prolong the logarithmic growth phase and increase bacteriocin production *(2,6,14)*.

The potential of various spoilage and pathogenic microorganisms to grow in fermented products is reported by several authors *(6,14,20–22)*. The results of antibacterial activity of chosen lactic acid bacteria (Fig. 1–4, Tables 1–3) indicate that these bacteria can be effective to control the growth of undesirable bacteria, ensuring the microbiological safety of food.

Plasmids had been isolated from lactic acid bacteria *L. acidophilus*, *L. plantarum* L4, *L. plantarum* Z88 and *E. faecium*, which exhibited high antibacterial activity (Fig. 5). The bacteriocin production might be linked to plasmid DNA. The plasmid profiles showed one (*L. plantarum* L4, *L. plantarum* Z88) or two plasmid bands (*L. acidophilus*, *E. faecium)* (Fig. 5). The occurrence of plasmid DNA in lactic acid bacteria was first reported by Klaenhammer and Sutherland *(23)*. The potential developments in this field were hampered due to paucity of genetic transfer systems and information on native plasmid-encoded traits. Efforts to accumulate genetic information about lactic acid bacteria, are opening possibilities for the improvement of lactic acid bacteria strains. Production of β-galactosidase, proteolytic activity and antibiotic resistance may be plasmid-borne in lactic acid bacteria *(24–26)*. Plasmid linkage of bacteriocin production has been reported for some strains of *L. acidophilus*, *E. faecium*, *Lactobacillus sake*, *Lactobacillus brevis* and *Pediococcus acidilactici (6,7,12)*. The function of the plasmids isolated from *L. acidophilus*, *L. plantarum* L4, *L. plantarum* Z88 and *E. faecium* have not yet been identified.

The production of an antibacterial substances (bacteriocins) with a broad spectrum of antibacterial activity against other genera of lactic acid bacteria and foodborne pathogens can be an important property for starter cultures and is of special interest in controlled lactic acid fermentation, which naturally contain competitive bacterial flora. Also, the genetic determinants for bacteriocin production have a great potential as genetic markers in recombinant DNA technology for application in the future production of food additives or supplements from microorganisms.

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