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Probiotic Properties of *Lactobacillus plantarum* L4

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Summary

Lactobacillus plantarum is used as starter culture for vegetable fermentation, olives conservation, ensiling and probiotic preparations for humans and animals. For the administration of probiotics to humans or animals, strains of lactic acid bacteria should be resistant to the specific conditions of the gastrointestinal tract. The strain Lactobacillus plantarum L4 was examined for probiotic activities in experiments in vitro. The culture supernatant of L. plantarum L4 showed antimicrobial activity against enteropathogenic, spore-forming and fungal test microorganisms. That activity was higher than the antimicrobial activity of the corresponding concentration of lactic acid alone. At low pH values of the medium and in the presence of lysozyme and substances such as phenol and bile salts, bacterium L. plantarum L4 showed a satisfactory degree of survival. Its resistance against different antibiotics that are often used in therapy was confirmed by determination of the minimum inhibitory concentration.

Keywords: probiotic effect, lactic acid bacteria, *Lactobacillus plantarum*

Introduction

Several health or nutritional benefits may be derived from certain species of lactic acid bacteria. The lactic acid bacteria comprising the genera *Lactobacillus, Streptococcus, Pediococcus, Lactococcus, Leuconostoc, Bifidobacterium, Carnobacterium, Enterococcus* and *Sporolactobacillus* – not only are involved in the preservation of certain foods but are responsible for unique identity and sensory attributes unattainable by other food processing methods *(1–3)*.

In recent years an increased emphasis has been placed on studies of the probiotic activity of lactic bacteria which may play an important role improving the intestinal microflora balance of the host (man and animals) *(4–8)*.

Lactobacillus plantarum strains are important as starter cultures in the production of fermented meats and vegetables as well as in the fermentation of grass to produce silage (*9*).

Beyond its fermentative abilities this bacterium has been attributed an additional role – a beneficial effect on the microflora balance of the host (humans and animals). The roles of *L. plantarum* in the intestinal tracts of humans and animals are not well defined. In the selection of strains for use in defining these roles, it is important that the bacterium survives and establishes itself under the conditions encountered in the intestinal environment.

This work was part of the study of selection for the probiotic use of bacterium *L. plantarum* L4, performed *in vitro* simulating conditions in the gastrointestinal tract. These experiments are a prerequisite for probiotic activity investigations to be carried out *in vivo*.

Materials and Methods

Bacterial Strains

The bacterial strain *Lactobacillus plantarum* L4 was examined for probiotic properties. It was used as starter culture in the fermentation of vegetables and silage. The antimicrobial activity of this strain was investigated against the test microorganisms: *Staphylococcus aureus* 3048, *Staphylococcus aureus* K-144, *Escherichia coli* 3014, *Salmonella mumum, Bacillus cereus, Bacillus subtilis* ATCC 6633, *Enterococcus faecium, Candida tropicalis* and *Candida pseudotropicalis*. All microorganisms used in this study were from the Culture Collection of Microorganisms of the Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia. *L. plantarum* L4 was propagated and maintained in the MRS medium *(10)*. An overnight culture (16 hours at 37 °C) was used as inoculum for survival assay and for cell-free supernatant preparations.

The test bacteria were propagated and maintained on nutrient agar, and test yeasts on malt agar (Difco Laboratories, Detroit, Michigan, USA).

Antimicrobial Activity of L. plantarum L4

The antimicrobial activity of the *L. plantarum* L4 cell-free supernatant was calculated from the absorbance (at 645 nm) of the nutrient broth measured during the growth of test microorganisms and from the viable count number read from the standard curve (*11*).

The cell-free supernatant was prepared from broth culture by centrifugation and filtration through a 0.2 μm Millipore filter, and added to the nutrient media (10 and 50%) for the detection of the growth or inhibition of the test microorganisms. A decreased specific growth rate of the test microorganisms was a result of the antimicrobial activity of *L. plantarum* L4. The results were expressed as percentages of growth inhibition, calculated as follows:

$$\text{growth inhibition / \%} = 100 \cdot (\mu_K - \mu_S) / \mu_K$$

μ_K – specific growth rate of test microorganisms in control medium,

μ_S – specific growth rate of test microorganisms in media with the cell-free supernatant added.

To examine the antimicrobial activity of lactic acid alone, the concentration of lactic acid in the L4 overnight culture supernatant was determined by titration with NaOH (0.1 mol/L). The amount of lactic acid corresponding to this concentration in the supernatant (10 and 50%), previously used for the detection of growth inhibition, was added to the nutrient medium which was then inoculated with the test microorganisms.

Survival Assay

The sensitivity of *L. plantarum* L4 was investigated in respect to pH = 4.0 (pH adjusted with pure lactic acid), and to the presence of: lysozyme (100 μg/mL), phenol (w = 0.1, 0.2, 0.3, 0.4 and 0.5%), conjugated bile salts, sodium desoxycholate (w = 0.1, 0.2, 0.3 and 0.5%), and different antibiotics (5, 10, 15 and 30 μg/mL) in the MRS medium.The survival of *L. plantarum* L4 was determined by the plate method using the MRS medium (*10*) at 37 °C. The Soxhlet-Henkel degree – titratable acidity (°SH) was estimated by titration with NaOH solution (0.1 mol/L) (*12*). Lysozyme, conjugated bile salts and sodium desoxycholate were products of Difco Laboratories, Detroit, Michigan, USA.

In a sodium phosphate buffer (0.1 mol/L, pH= 6.5) and pH = 3.0 (pH adjusted with 0.001 mol/L HCl) at 37 °C cell lysis was determined by measuring absorbance (A) at 645 nm. The presence of nucleic acids and proteins in the cell-free supernatant was measured at A_{260} and A_{280}, respectively, by means of a UV/Vis spectrophotometer Carry 3 Varian. The protein concentration was calculated as follows:

γ(protein)/(mg/mL) = $1.45 \cdot A_{280} - 0.74 \cdot A_{260}$ (*13*).

Antibiotic Resistance

The antibiotic sensitivity of *L. plantarum* L4 was tested by the disc assay method (*14*). The antibiotics were products of Pliva, Zagreb, Croatia, with the exception of nisin which was from Dorset, UK, nystatin from Sanofi Pharma, Bruxelles, Belgium and gentamycin from Novate, Milano, Italy. The minimum inhibitory concentration (MIC) was determined as proposed by Curragh and Collins (*15*).

Results and Discussion

Table 1 shows antimicrobial effect of *Lactobacillus plantarum* L4 against the enteropathogenic (*Staphylococcus aureus* 3048, *Staphylococcus aureus* K-144, *Escherichia coli*, *Salmonella mumum*), spore-forming (*Bacillus cereus* and *Bacillus subtilis*), fungal (*Candida tropicalis* and *Candida pseudotropicalis*) and lactic acid bacterium (*Enterococcus faecium*) test microorganisms. The results illustrate a decrease in specific growth rate of the test microorganisms as a consequence of the antimicrobial activity of *L. plantarum* L4. Comparison of the antimicrobial activities of the supernatant and the corresponding concentrations of lactic acid suggests that the inhibitory effects were not identical. Namely, the antibacterial and antifungal activities of *L. plantarum* L4 were higher than the antimicrobial activity of pure lactic acid. A more pronounced antimicrobial effect of the culture supernatants than of pure lactic acid solution may implicate the presence of other substances with antimicrobial activity (*16*). The antimicrobial activity of *L. plantarum* L4 against other gut microorganisms could influence its ability to survive in the gut and operate as an effective probiotic by suppressing pathogens and other harmful bacteria such as those responsible for depressing the growth of the host animals.

Table 1. Growth inhibition of the test microorganisms cultivated in the media containing 10 and 50% of the *L. plantarum* L4 culture supernatant (calculated with respect to the specific growth rate; means of three replicates)

Test microorganisms	Growth inhibition / %			
	w (supernatant of *L. plantarum* L4) / %		w (lactic acid) / %	
	10	50	0.1*	0.5*
S. aureus 3048	29	65	8	46
S. aureus K-144	9	96	0	91
E. coli	10	90	9	88
S. mumum	6	98	2	96
B. cereus	15	98	2	95
B. subtilis	22	100	16	94
E. faecium	50	97	24	92
C. tropicalis	14	52	8	50
C. pseudotropicalis	14	43	7	40

* The mass fraction of added lactic acid corresponds to the fraction of lactic acid in the media containing 10 and 50% of the supernatant

The destruction of ingested lactic acid bacteria in the gut is mainly due to the presence of the acid in the stomach. *In vitro* experiments could provide information on the sensitivity of potential probiotic strains to low pH values. The survival of *L. plantarum* L4 in low pH media (pH = 4.0) was higher than of other disadvantageous microorganisms (Fig. 1). Spectrophotometric monitoring of

absorbance at 645 nm, protein and nucleic acids concentrations, as well as of the cell content in case of lysis, assayed at pH = 3.0 (pH adjusted with HCl), suggests high acid tolerance of *L. plantarum* L4 (Table 2). Lactic acid bacteria are able to maintain a cytoplasmic pH more alkaline than the growth medium. Lactobacilli generally tolerate a significantly more acidic cytoplasmic pH of 4.4 (external pH = 3.5) *(17)*. The maintenance of alkaline cytoplasm has been shown to occur primarily by means of a proton-translocating ATP-ase (H^+-ATP-ase), which expels protons from the cytoplasm to the external medium, utilizing the energy of ATP hydrolysis *(18)*.

There are certain enzymes in the gastrointestinal tract that are deleterious to microorganisms. One of these is lysozyme which cleaves the N-acetylmuramic acid-N-acetylglucosamine bond in peptidoglycan of the bacterial cell wall. At the lysozyme concentration of 100 μg/mL, the viable cell count slowly decreased and then remained stable (Fig. 2). The lysozyme concentration used in this assay was much higher than the physiological intestinal lysozyme concentration. This concentration is usually used in experiments involving the cell wall lysis *(19)*. Researchers who isolated DNA from lactobacilli encountered great difficulty in lysing the lactobacilli by classical methods which involved the use of lysozyme *(20)*. Gilliland *(21)* showed that mainly Gram-positive bacteria were sensitive to lysozyme, but that the genera *Lactobacillus* and *Streptococcus* were more resistant than any other Gram-positive bacteria. These results (Fig. 2) indicate that *L. plantarum* L4 is resistant to lysozyme and thus likely to have no problem in overcoming this barrier in the digestive tract.

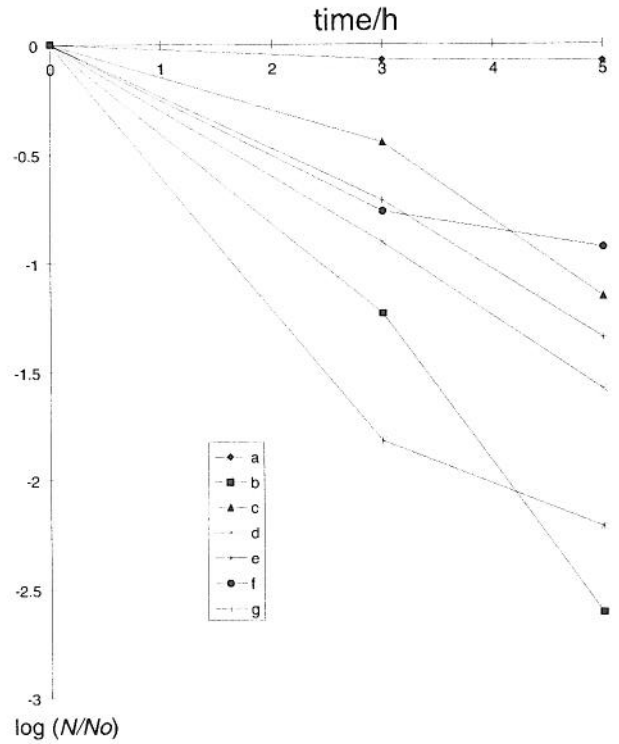

Fig 1. Survival of *L. plantarum* L4 and other microorganisms in the nutrient media at pH = 4.0 (pH adjusted with pure lactic acid; *N* = CFU/mL)
a – *L. plantarum* L4; b – *S. aureus* 3048; c – *S. aureus* K-144; d – *E. coli*; e – *S. mumum*; f – *B. cereus*; g – *B. subtilis*

Table 2. Changes in absorbance of the cells and concentration of proteins and nucleic acids in the cell- free supernatant during incubation of *L. plantarum* L4 at pH = 6.5 and 3.0 (37 °C).

Time /h	A_{645}		γ (proteins)/ (mg/mL)*		Nucleic acids A_{260}	
	pH = 6.5	pH = 3.0	pH = 6.5	pH = 3.0	pH = 6.5	pH = 3.0
0	1.80	1.73	0.24	0.25	0.55	0.63
1	1.73	1.67	0.24	0.25	0.69	0.87
2	1.71	1.65	0.26	0.45	0.71	0.90
3	1.69	1.64	0.41	0.52	0.71	0.90

* γ (proteins)/(mg/mL) = 1.45 · A_{280} – 0.74 · A_{260}

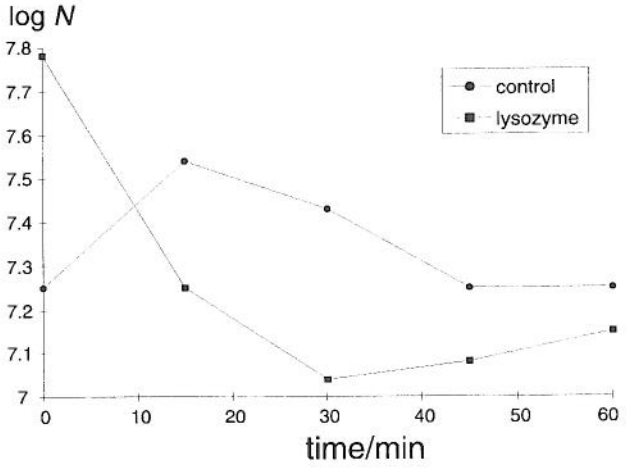

Fig. 2. Effect of lysozyme (100 μg/mL) on the viable count of *L. plantarum* L4 (*N* = CFU/mL)

Metabolic reactions occurring in the gut can have consequences both local – for example on the gut mucosa – or systematic. An example of a remote consequence of a metabolite produced by the gut bacteria is the generation of phenols and cresols from aromatic amino acids which have been found to possess tumour-promoting and convulsant activity *(22)*. One of the most important ways in which a probiotic organism may exert a beneficial effect on its host is to modify metabolic processes which result in the generation of toxic or carcinogenic metabolites. Fig. 3 shows the survival of the examined strain at different phenol concentrations in the growth media. According to Rašić and Kurman *(12)* the lactobacilli tolerance against phenol could be defined by measuring the Soxhlet-Henkel degree of medium acidification. They established that the lactic culture tolerant to 0.3% of phenol can acidify medium to 25 °SH in eight hours. Results from Fig. 3 show correlation between log *N* of *L. plantarum* L4 and the Soxhlet-Henkel degree of acidification at examined phenol concentrations, thus confirming the high tolerance of *L. plantarum* L4 towards phenol, which is very significant for probiotic strains. The tolerance of *L. plantarum* L4 against phenol may be explained by the formation of the »heat shock proteins«. It is well known that proteins exert a protective effect on microorganisms. The mechanism of protein protection of microbial cells remains unexplained, but it has

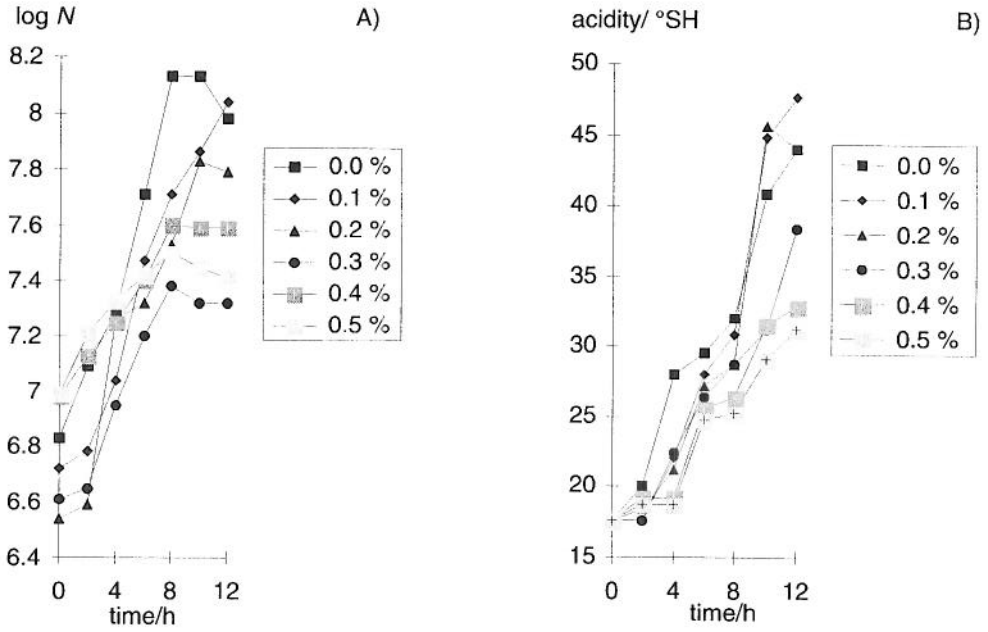

Fig. 3. Effect of different phenol concentrations on growth (A) and change of °SH value (B) during cultivation of *L. plantarum* L4 in MRS medium (*N* = CFU/mL)

been known for many years that proteins may increase the stability of enzymes and other proteins of the microbial cell *(23, 24)*.

The metabolic activity, multiplication or colonization in the small intestine are required for optimal activity of the probiotic strains; a tolerance for bile is an essential criterion in the selection of microbial strains. The bile salts, which are endogenous metabolites necessary for normal lipid metabolism, are extensively transformed by intestinal bacteria during their enterohepatic circulation *(25)*. In the human intestinal tract, bile salts appear as conjugated and deconjugated forms in the physiological concentration range (from 0.03 to 0.3%). The bacterium *L. plantarum* L4 failed to exhibit growth in the presence of different concentrations of conjugated bile salts, but survived satisfactorily after eight hours of cultivation. The results in Fig. 4 show a slow decrease in the viable count of the examined strain at all concentrations of conjugated bile salts. However, the viable cell count remained higher than 10^6/mL. In the presence of sodium-desoxycholate (Fig. 5), deconjugated bile salt, the examined strain survived better than in the presence of conjugated bile salts (Fig. 4). These results might implicate that the examined strain did not have the hydrolytic activity of the bile salts. The bile salt hydrolysis is a qualitatively and quantitatively important modification in the intestinal tract, which is mediated by various Gram-positive genera including some species from the genus *Lactobacillus*. Bile salt hydrolase catalyses cleavage of the amino acid moiety from the steroid nucleus of conjugated bile acids (deconjugation). Although the bile salt hydrolysis activity is a commonly observed phenomenon, its significance is far from being understood *(25)*. Savage *(26)* suggests that deconjugation might be a detoxification mechanism to lactobacilli. However, bacterial bile salt hydrolase in the host intestinal tract could adversely affect the host, causing decrease in conjugated bile salts concentrations below the level necessary for

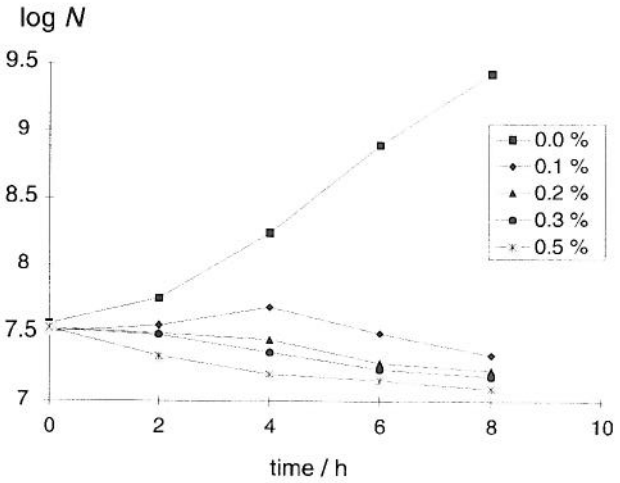

Fig. 4. Survival of *L. plantarum* L4 in the presence of different concentrations of conjugated bile salts in MRS medium (*N* = CFU/mL)

optimal digestion and absorption of lipids *(27)*. Therefore, the bile salt hydrolase activity is an undesirable property of a bacterial strain for probiotic use. Studies by Klaenhammer and Kleeman *(28)* showed that lactobacilli growing as smooth colonies possessed a higher bile resistance than rough colonies. *L. plantarum* L4 showed differences in bile resistance dependent on colonial morphology (unpublished results) which is in agreement with the findings of Klaenhammer and Kleeman *(28)*. In the presence of bile the colonial morphology changed to rhizoid and cell morphology showed protrusions of cytoplasmic membrane induced by gaps in the cell wall. This could mean that the colonial and cellular morphologies are valuable parameters in the selection of *Lactobacillus* strains for probiotic use.

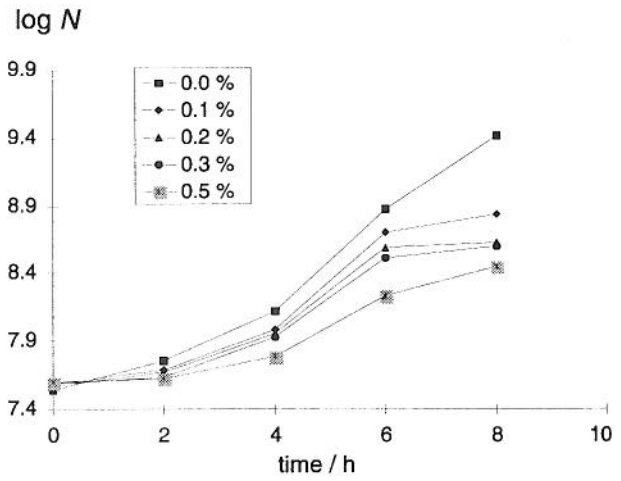

Fig. 5. Growth of *L. plantarum* L4 at different concentrations of sodium desoxycholate in MRS medium (*N* = CFU/mL)

It is now recognized that the indigenous microflora of humans and animals provides protection against infections with pathogenic microorganisms. The oral administration of high doses of antibiotics decreases the resistance of humans and animals to colonization with non-indigenous organisms, presumably by disrupting the intestinal flora. Thus, one of the criteria for the selection of the probiotic strain, along with other desirable properties, might be the antibiotic resistance (*29*). *L. plantarum* L4 was examined for susceptibility to most often prescribed antibiotics (Table 3). Each antibiotic was added to the filter disc at the concentration commonly used for the sensitivity test procedure *(30)*. The absence of the inhibition zone was expressed as resistance (R). *L. plantarum* L4 was resistant to most of the antibiotics tested (Table 3). Comparison of the experimental minimum inhibitory concentrations (MICs) with MIC-values of the resistance factor (R-factor) carrying strains indicated that the strain *L. plantarum* L4 did not carry determinants for the high level resistance to any of these antimicrobial agents (Table 4) *(31,32)*. The results of the antibiotic resistance of *L. plantarum* L4 indicate that this bacterium can effectively protect the natural balance of intestinal microflora during therapy with some commonly used antibiotics and after it. *Lactobacillus plantarum* is also an important bacterium in the fermentation of plants, vegetables, dairy products, cereal foods and probiotic preparations and may be exposed to antibiotic agents present in the fermenting food products. The use of this bacterium, resistant to some antibiotics, could be efficient in growth promotion and treatment of farm animals. The residues of antibiotics, used for growth promotion and/or disease prevention, in animal tissues may affect the lactic acid bacteria used in the fermentation of meat products, leading to abnormal fermentation and starter failure. On the other hand, antibiotic resistant lactic acid bacteria could act as a source of resistant genes, giving resistance to undesirable bacteria and pathogens during fermentation processes and in the intestine, especially if the antibiotic resistance is plasmid born. The plasmid profile of *L. plantarum* L4 showed one plasmid band *(16)*. The function of the plasmid isolated from the tested bacterium has not yet been identified.

Table 3. Antibiotic sensitivity of *L. plantarum* L4 tested by the disc assay method

Antibiotic	γ / (μg/mL)	Inhibition zone/mm
Penicillin G	10.0*	R
Ampicillin	0.5	16
Oxytetracycline	1.0	R
Cephalexin	30.0	17
Streptomycin-sulphate	25.0	R
Sulphonamide	25.0	R
Kanamycin	15.0	16
Erythromycin	10.0	33
Bacitracin	5.0*	18
Nystatin	100.0*	R
Nisin	128.0	R
Sulphamethoxazole-trimethoprim	25.0	21
Gentamycin	10.0	R
Chloramphenicol	30.0	R
Nitrofuranthoin	50.0	R

R – antibiotic resistance;
* international units

Table 4. Minimum inhibitory concentration (MIC) of antibiotics towards *L. plantarum* L4

Antibiotic	MIC/(μg/mL)
Penicillin G	20*
Ampicillin	5
Oxytetracycline	100
Cephalexin	150
Streptomycin-sulphate	500
Nisin	1000
Sulphamethoxazole-trimethoprim	600
Gentamycin	500
Nitrofuranthoin	600
Chloramphenicol	500

* international units/mL

»Curing experiments« with ethidium bromide and acriflavine did not cause a loss of plasmid nor was a loss of resistance detected in the colonies of the treated strain (data not presented).

In conclusion, this study demonstrated that the bacterium *L. plantarum* L4 exhibited antimicrobial activity against enteropathogenic, spore-forming and fungal test microorganisms. In a low pH medium, in exposure to lysozyme and in the presence of different concentrations of substances like phenol and bile salts, bacterium *L. plantarum* L4 showed a satisfactory degree of survival. Likewise, *L. plantarum* L4 did not prove to be sensitive to different antibiotics that are often used for therapeutic purposes, which gives it a big chance of survival *in vivo*.

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Probiotička svojstva bakterije *Lactobacillus plantarum* L4

Sažetak

Lactobacillus plantarum primjenjuje se kao starter-kultura za fermentiranje povrća i maslina, te pripravu silaže i probiotika za ljude i životinje. Pri uporabi probiotika za ljude i životinje, sojevi bakterija mliječne kiseline trebaju biti otporni prema specifičnim uvjetima u gastrointestinalnom traktu domaćina. Probiotičke aktivnosti soja Lactobacillus plantarum L4 ispitane su u in vitro pokusima. Supernatant kulture L. plantarum L4 pokazao je antimikrobnu aktivnost prema enteropatogenim, sporotvornim i fungalnim test-mikroorganizmima. Antimikrobna je aktivnost supernatanta kulture veća od antimikrobne aktivnosti odgovarajuće koncentracije mliječne kiseline u supernatantu. U uvjetima niske pH-vrijednosti podloge, u prisutnosti lizozima, te pri različitim koncentracijama supstancija kao što su fenol i žučne soli, bakterija L. plantarum L4 pokazala je zadovoljavajući stupanj preživljavanja. L. plantarum L4 otporan je prema različitim antibioticima koji se primjenjuju u terapiji što je potvrđeno određivanjem minimalnih inhibicijskih koncentracija.