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Bacteriocinogenic Activity of Lactobacilli Isolated from Cheese and Baby Faeces

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Summary

Lactobacillus strains, isolated from cheese (30 strains) and from baby faeces (41 strains) were examined for bacteriocin production, by deferred agar spot (DAS) test and by agar well diffusion (AWD) assay, using 29 Gram-positive test strains from different genera. Eight cheese isolates and 2 human isolates showed antimicrobial activity against at least one test strain. The proteinaceous nature of the inhibitor was confirmed in four cheese isolates and in both human isolates (LF221 and K7). Human isolates also inhibited some non-LAB bacteria. Strain Lactobacillus acidophilus LF221 was additionally tested by AWD assay against a wide range of bacteria, including pathogens. Besides some lactic acid bacteria test strains, particular strains of the following species were also inhibited: Listeria innocua, Enterococcus faecalis, Bacillus cereus, Staphylococcus aureus and Clostridium sp. The inhibitor was found to be a heat stable protein, as it was inactivated by trypsin, proteinase K, pronase and was resistant to heat (100 °C). Strain L. acidophilus LF221 was bactericidal, but not bacteriolytic to Lactobacillus helveticus ATCC 11509. Bacteriocin molecules were present in the supernatant in the form of aggregates with relative molecular masses exceeding 150 kDa.

Key words: bacteriocin, Lactobacillus acidophilus LF 221

Introduction

Lactic acid bacteria (LAB) produce a variety of antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins. By definition, bacteriocins are antimicrobial proteins, active against bacteria, usually closely related to the producer organism and are commonly produced by Gram-positive bacteria *(1,2)*. Several novel bacteriocins of LAB were reported in recent years *(3–5)*. LAB bacteriocins with wide inhibition spectra are less common and some of them are also active against food borne pathogens, such as *Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus* and *Clostridium sporogenes*. The producers of such bacteriocins, termed pediocin- and nisin-like bacteriocins, are commonly isolated from fermented meat, silage or vegetables and less often from fermented milk *(6–11)*. The possible application as natural food preservatives, bacteriocinogenic LAB strains or LAB bacteriocins active against food-borne pathogens and food-spoilage bacteria are gaining research interest *(12,13)*.

Lactobacilli are also believed to play an important role in the balance of microflora in the intestinal tract of humans and animals and are therefore likely candidates for probiotics. Many of them also produce bacteriocins, which may confer to their colonizing and competitive ability *(3,14,15)*. Human faeces is a common source of bacteriocinogenic probiotic bacteria *(16–21)*.

The aim of this study was the screening of *Lactobacillus* strains isolated from human faeces and cheese, for their antibacterial activity, especially bacteriocin–like activity, against a wide range of Gram-positive bacteria. The presence of bacteriocins was confirmed by their sensitivity to proteolytic enzymes. Potential bacteriocin producers were selected for additional studies and characterization of their bacteriocins.

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Material and Methods

Bacterial strains and growth media

Thirty isolates of lactobacilli from cheese and 15 isolates from faeces of a breast-fed baby were obtained from the culture collection of Istituto di Microbiologia, Facolta di Agraria, Universita Cattolica Sacro Cuore di Piacenza. They were stored at –70 °C in MRS with 20 % glycerol and propagated in MRS broth (Difco) at 37 °C, in microaerophilic atmosphere (GasPack, Oxoid). Additional 26 isolates were isolated at Institute for Dairying, Ljubljana, from faeces of a breast-fed baby. Strains used as test microorganisms in DAS tests, their origin and culturing conditions are listed in Table 1. The origin of additional test microorganisms used for determination of the inhibition spectrum of isolate *L. acidophilus* LF221, by AWD assay, is summarized in Table 3. *Streptococcus thermophilus* strains were propagated in M17 broth (Difco) at 42 °C and *Lactococcus* sp. strains in M17 broth at 30 °C. *Campylobacter* and *Clostridium* strains were incubated in anaerobic atmosphere obtained by GasPack system (Oxoid) at 37 °C. Reinforced clostridium medium (RCM, Difco) was used for *Clostridium* sp. and brain heart infusion medium (BHI, Oxoid) for *Campylobacter* sp. All other bacteria were grown aerobically in BHI (Torlak) broth at 37 °C.

Detection of antimicrobial activity by deferred agar spot test (DAS)

Antibacterial activity of *Lactobacillus* isolates was first determined by deferred agar spot test *(1)*. Five microliters of overnight culture of lactobacilli was spot inoculated on the surface of M17 agar and grown for 48 h at 37 °C. Soft overlay MRS agar (3 mL, 0.75 % agar), inoculated with approximately 10^7 cells from stationary-phase test culture (10–100 μL of inoculum/3 mL soft agar) was poured over the surface of spot inoculated M17 agar. Inhibition zones were observed after 24 to 48 h of incubation under appropriate conditions for each test strain (Table 1). Clear zones of inhibition with sharp

Table 1. Test strains used in DAS tests and the culture (= test) conditions

Test strain	Origin[a]	Culture (= test) conditions[b]
Lactobacillus casei ATCC 334	ATCC	MRS, 37 °C, microaerophilic
L. fermentum ATCC 9338	ATCC	MRS, 37 °C, microaerophilic
L. salivarius NCDO 2747	NCDO	MRS, 37 °C, microaerophilic
L. delbrueckii subsp. *bulgaricus* ATCC 11842	ATCC	MRS, 42 °C, microaerophilic
L. acidophilus ATCC 4356	ATCC	MRS, 37 °C, microaerophilic
L. helveticus ATCC 15009	ATCC	MRS, 42 °C, microaerophilic
L. reuteri DSM 20016	DSM	MRS, 37 °C, microaerophilic
L. reuteri ZIMBI 40	ZIM	MRS, 37 °C, microaerophilic
L. curvatus NCDO 2739	NCDO	MRS, 30 °C, microaerophilic
L. sake NCDO 2714	NCDO	MRS, 30 °C, microaerophilic
L. salivarius 1	IML	MRS, 30 °C, microaerophilic
L. plantarum NCDO 1193	NCDO	MRS, 37 °C, microaerophilic
L. plantarum ZIMBI 42	ZIM	MRS, 37 °C, microaerophilic
Clostridium sporogenes CS 22/10	TNO	RCM, 37 °C, anaerobic
C. tyrobutyricum CT 3.5	TNO	RCM, 30 °C, anaerobic
C. tyrobutyricum NCDO 1754	NCDO	RCM, 30 °C, anaerobic
Pediococcus pentosaceus FBB 63	TNO	M17, 30 °C, microaerophilic
Ped. pentosaceus PC 1	TNO	M17, 30 °C, microaerophilic
Propionibacterium acidipropionici NCDO 563	NCDO	M17, 30 °C, microaerophilic
Leuc. cremoris DB 1275	TNO	M17, 30 °C, microaerophilic
Lactococcus lactis subsp. *lactis CAa 130, ZIMBI44*	ZIM	M17, 30 °C, microaerophilic
Lact. lactis subsp. *cremoris* CNRZ 117	CNRZ	M17, 30 °C, microaerophilic
Streptococcus thermophilus ST112	TNO	M17, 42 °C, microaerophilic
Strep. thermophilus ST20	TNO	M17, 42 °C, microaerophilic
Enterococcus faecalis EF	TNO	BHI, 37 °C, aerobic
Staphylococcus aureus SA 113	TNO	BHI, 37 °C, aerobic
Staph. carnosus MC 1	TNO	BHI, 37 °C, aerobic
Bacillus cereus ATCC 9139	ATCC	BHI, 37 °C, aerobic
B. cereus ATCC 11778	ATCC	BHI, 37 °C, aerobic
Listeria innocua BL 86/26	TNO	BHI, 37 °C, aerobic

[a] ATCC: American Type Culture Collection, Rockville, USA
NCDO: National Collection of Dairy Organisms, National Institute for Dairying, Reading, England
TNO: Nutrition and Food Research, Zeist, The Netherlands
ZIM: Collection of Industrial Microorganisms, Ljubljana, Slovenia
IML: Institute for Dairying, Zootechnical Department, Biotechnical Faculty, University of Ljubljana, Slovenia
CNRZ: Centre Nationale de Recherche Zootechniques, Jouy-en-Josas, France

[b] Microaerophilic and anaerobic atmosphere was obtained by GasPack system (Oxoid)

edges around spots of LAB were considered as positive results and only *Lactobacillus* strains producing such inhibition zones were considered as potential bacteriocin producers.

Detection of antimicrobial activity by agar well diffusion assay (AWD)

Preparation of concentrated supernatant for AWD assay

Cells from 500 mL of 18 h MRS culture of strains tested for antibacterial activity were removed by centrifugation at 3500 g for 10 min. The supernatant was concentrated 10-fold by ultrafiltration with Millipore 500 mL unit with a cellulose membrane Spectra/por 10 kDa MWCO (relating molecular mass cut-off) and sterilized by microfiltration (0.22 μm, Sigma). One half of the concentrated supernatant was neutralized to a final pH = 6.8 with 5 M NaOH and then filter-sterilized.

Preparation of crude bacteriocin(s) of LF221

Solid ammonium sulphate (313 g/L) was added to the cell free supernatant of *L. acidophilus* LF 221 to achieve 50 % (w/v) saturation. Precipitate was removed by centrifugation, resuspended in 0.1 M potassium phosphate buffer (pH = 7) and dialysed against the same buffer. Filter-sterilized catalase from bovine liver (Sigma), dissolved in 50 mM Na-phosphate buffer was added to dialysate to a final concentration of 100 E/mL. pH was adjusted to 6.8 with 1 M NaOH and dialysate was sterilized through a 0.22 μm filter (Sigma).

Agar well diffusion assay *(3)*

The overlay agar inoculated with test microorganisms, as described for DAS assay, was poured onto M17 agar plates. Wells 3 mm in diameter were cut into the agar and filled with 20 μL of crude bacteriocin(s) preparation. Plates were preincubated at room temperature for 4 hours to allow diffusion of any inhibitory metabolites into the surrounding agar, and then incubated at the optimum growth temperature of the test microorganism. The plates were examined for clear zone in the agar surrounding the well.

Determination of bacteriocin titer

Lactobacillus helveticus ATCC 15009 was routinely used as test microorganism for determination of bacteriocin titer. M17 agar plates overlayed with test microorganisms (10^7 cells/3 mL soft agar) were prepared as described for the AWD assay. Wells cut into agar were filled with 20 μL aliquots of serial two-fold dilutions. Bacteriocin activity was expressed in bacteriocin activity units (BA) per milliliter. Units represent reciprocal of the highest dilution still causing inhibition zone on the test microorganism lawn.

Sensitivity of bacteriocin preparations to proteolytic enzymes

The sensitivity of crude bacteriocin(s) of potential bacteriocin producers to trypsin was tested by a modified AWD assay. The plate was prepared and bacteriocin preparation applied into the well cut in the agar as described for AWD assay. A second well was cut into agar near the well filled with bacteriocin preparation, at the distance where the edge of inhibition zone was expected, and filled with 10 μL of trypsin solution containing 5 mg/mL trypsin – 40 E/mg (Boehringer Mannheim) in 50 mM Na-phosphate buffer (pH = 7). Reduced zones of inhibition at the side where trypsin was applied, indicated the sensitivity of bacteriocins to trypsin. Trypsin at a concentration of 5 mg/mL did not inhibit any of the test microorganism.

For strain LF221, additional tests were performed with three proteolytic enzymes: trypsin – 40 E/mg (Boehringer Mannheim), pronase (Boehringer Mannheim) and proteinase K – 20 E/mg (BRL, Gaithersburg MD, USA). Stock solutions of all three enzymes were prepared by disolving the enzymes in 50 mM Na-phosphate buffer (pH = 7), to a concentration of 50 mg/mL, and then filter-sterilized. Fifty microliters of stock solution was added to 450 μL of bacteriocin preparation, incubated for 2 h at 37 °C and the bacteriocin titer determined as described above.

Sensitivity of bacteriocin(s) of strain LF221 to heat

Samples (500 μL) of 10-fold concentrated bacteriocin preparation were heated at 100 °C for 5, 10, 20 or 30 min, and at 121 °C for 15 min. After cooling in ice-water, bacteriocin activity was determined in AWD assays, by measuring the diameter of inhibition zone relative to non-heated control.

Ultrafiltration studies

Cells of *Lactobacillus acidophilus strain* LF221 were removed from 2 L of a 24 h culture by centrifugation at 3.500 g for 10 min. The supernatant was filtered through 0.22 μm membrane in a Minitan S unit (Millipore). Four aliquots (500 mL) of filtered supernatant were concentrated 20-fold by ultrafiltration using Minitan S unit (Millipore), fitted with: 5 (PLCC), 10 (PTGC), 30 (PTTK) or 100 (PTHK) kDa molecular mass cut-off membranes. The concentrated supernatant was adjusted to pH = 6.8 with 5 M NaOH and sterilized through a 0.22 μm filter (Sigma). Catalase from bovine liver (Sigma), dissolved in 50 mM Na-phosphate buffer and filter-sterilized, was added to the concentrated supernatant to final concentration of 100 U/mL. The bacteriocin titer in the concentrated supernatant was determined as described above.

Effect of bacteriocin(s) from LF221 on L. helveticus ATCC 15009

Cells from 100 mL of a log-phase culture of *Lactobacillus helveticus* ATCC 15009 grown for 6 h at 42 °C, were centrifuged (3500 g for 10 min) and washed with 0.9 % saline, then resuspended in 100 mL 0.9 % saline. Aliquotes (9 mL) of cell suspension containing $7.5 \cdot 10^5$ cells/mL were added to 1 mL samples of bacteriocin with titer ranging from 100 to 3200 BA/mL. The mixture was then incubated at 7 °C for 30, 90 and 360 min. The inhibitory effect of LF221 bacteriocin(s) on *L. helveticus* ATCC 15009 was measured by following absorbance at 600 nm of the incubation mixture and by reduction of CFU/mL as determined by plate counting on MRS agar, relative to control without bacteriocin.

Results

Eight of 30 *Lactobacillus* isolates from cheese and two of the 41 isolates from baby faeces inhibited at least one test strain in DAS assay (Table 2). Only 10 strains producing inhibition zones with sharp edge were considered as potentially bacteriocinogenic. Since bacteriocins are degraded by proteolytic enzymes, concentrated supernatants of those 10 strains were tested for the effect of trypsin and catalase on the inhibition zones in AWD assays. If bacteriocins are produced, the zone of inhibition is reduced at the site of enzyme application. Based on the inhibition assays and apparent nature of inhibitory substances, 10 potentially bacteriocinogenic isolates could be grouped into four distinct groups.

1) Four isolates from cheese (1Mn340, 6Me221, 6Me-234, 5Mne373) inhibited at least one test strain in DAS assay (indicated by letter A in Table 2). In most cases, the concentrated (10-fold) supernatants of those strains were also inhibitory in AWD assays (indicated by letters AC in Table 2) but neutralized supernatants were not. Therefore, low pH (3.9–4.2) of the native or concentrated supernatant could have been responsible for inhibition.

Table 2. Antibacterial activity of *Lactobacillus* isolates (10 strains) against test microorganisms (32 strains)

	Strains tested for antibacterial activity									
Test microorganisms	2So70	1Mn340	1Mn336	1Me1	6Me221	6Me234	4Me145	5Mne373	LF221	K7
Lactobacillus casei ATCC 334	A B C D	–	A B C D	A B C D	–	–	A B	–	–	–
L. fermentum ATCC 9338	–	–	–	–	–	–	–	–	–	n.d.
L. salivarius NCFB 2747	–	–	–	–	–	–	–	–	–	–
L. bulgaricus ATCC 11842	–	–	–	–	–	–	–	–	A B C D	n.d.
L. acidophilus ATCC 4356	–	–	–	–	–	–	–	–	A B C D	A B C D
L. helveticus ATCC 15009	–	–	–	–	–	–	–	–	A B C D	A B C D
L. reuteri DSM 20016	–	–	–	–	–	–	–	–	–	–
L. reuteri ZIMBI 40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	A B C D	A B C D
L. curvatus NCFB 2739	–	–	–	–	–	–	–	–	–	–
L. sake NCFB 2714	A B C D	–	A B C D	A B C D	–	–	–	–	A B C D	A B C D
L. salivarius 1	–	–	–	–	–	–	–	–	A B C D	A B C D
L. plantarum NCDO 1193	–	–	–	–	–	–	–	–	–	–
L. plantarum ZIMBI 42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	A B C D	A B C D
Clostridium sporogenes CS 22/10	–	A C	–	–	–	–	A C	–	A B C D	–
C. tyrobutyricum CT 3.5	–	A C	–	–	–	–	A C	–	A B C D	–
C. tyrobutyricum CT 1754	–	A C	–	–	–	–	A C	–	A B C D	–
Pediococcus pentosaceus FBB 63	–	–	A	–	–	–	–	–	C D	–
Ped. pentosaceus PC 1	–	–	A	–	–	–	–	–	A B C D	A B C D
Leuconostoc cremoris DB 1275	A	–	A	A	–	–	A	A	–	–
Lactococcus lactis subsp. *cremoris* CNRZ 117	–	–	–	–	–	–	–	–	C D	–
Lact. lactis subsp. *lactis CAa 130, ZIMBI44*	–	–	–	–	–	–	–	–	–	–
Propionibacterium acidipropionici NCDO 563	–	–	–	–	–	–	–	–	–	–
Streptococcus thermophilus ST112	–	–	–	–	–	–	–	–	A B C D	–
Strep. thermophilus ST20	–	–	–	–	–	–	–	–	A B C D	–
Staphylococcus aureus SA 113	–	A C	–	–	A C	A C	A C	A C	C D	–
Enterococcus faecalis EF	–	–	–	–	–	–	–	–	A B C D	A B C D
Staph. carnosus MC 1	–	–	–	–	–	–	–	–	–	–
Bacillus cereus ATCC 9139	–	–	–	–	–	–	–	A	A B C D	–
B. cereus ATCC 11778	–	–	–	–	–	–	–	–	–	–
Listeria innocua BL 86/26	–	–	–	–	–	–	–	–	A B C D	A B C D

A: inhibition of test microorganisms determined in DAS tests; only clear zones of inhibition with sharp edges were considered positive results

B: zone of inhibition affected by trypsin (DAS+trypsin assay);

C: inhibition of test microorganisms by 10x concentrated supernatant (pH = 4–4.2) in agar well diffusion assay (AWD);

D: inhibition of test microorganisms by 10x concentrated and neutralized supernatant (pH = 6.8) in agar well diffusion assay (AWD);

–: no inhibition; n.d.: not determined

2) Another three strains (2So70, 1Mn336, 1Me1) showed identical antibacterial activity against *L. casei* and *L. sake* but differred in activity against other test strains. The inhibition of *L. casei* and *L. sake* was reduced by trypsin (indicated by letter B in Table 2) but the neutralized supernatants were also inhibitory (indicated by letter D in Table 2) to *L. casei* and *L. sake*. Therefore, these three strains could be considered as true bacteriocin producers. The observation that inhibitory activity of those strains was not affected by catalase indicates that hydrogen peroxide was not responsible for inhibition. The three strains also inhibited *Leuc. cremoris* and *Ped. pentosaceus* but, in this case, inhibition was not affected by trypsin.

3) Strain 4Me145 produced an inhibitor active against *L. casei*, that was affected by trypsin, but the concentrated supernatant of 4Me145, however, was not active. Since supernatants were concentrated by ultrafiltration through the membrane with MWCO 10 kDa, it is possible, that bacteriocin molecules were smaller and might have passed through the membrane. The activity of 4Me145 against clostridia, *Staph. aureus* and *Leuc. cremoris* was probably due to low pH, because the neutralizing of the supernatant eliminated the inhibition and because the zone of inhibition was not affected by trypsin.

4) In the last group, two isolates from faeces (LF221, K7) could be placed. Their antibacterial activity was affected by trypsin and was not reduced by neutralizing the supernatant. Both strains inhibited some *Lactobacillus* strains, as well as members of other genera, such as *Enterococcus faecalis* and *Listeria innocua*. Strain LF221 was particularly interesting because of its inhibition of clostridia (*C. sporogenes, C. tyrobutyricum*), *Staphylococcus aureus* and *Bacillus cereus* and was therefore selected for additional experiments. The concentrated and neutralized (to exclude the effect of low pH) supernatant of *L. acidophilus* LF221 was tested on another set of test microorganisms isolated from fermented milk. Filter-sterilized catalase was added to concentrated and neutralized supernatant in order to exclude possible inhibition by hydrogen peroxide. All four strains of *Streptococcus thermophilus* (out of four tested) and three strains of *Lactococcus lactis* subsp. *lactis* (out of eight tested) were inhibited, whereas none of Gram-negative test bacteria was affected. Among other Gram-positive strains tested, strains of *C. difficile, C. septicum* and *C. feseri* were sensitive to bacteriocins present in *L. acidophilus* LF221 supernatant (Table 3). To confirm the proteinaceous nature of the inhibitor, the supernatant of LF221 was treated with proteolytic enzymes trypsin, proteinase K and pronase as described in Methods. Incubation of the supernatant with proteolytic enzymes resulted in a complete loss of activity, confirming that the inhibitor was a protein. Antibacterial activity was not affected by heating the supernatant for 5 min at 100 °C, was only partially reduced when supernatant was left at 100 °C for 10 to 30 min, but was completely lost when the supernatant was autoclaved.

Bacteriocin concentration of 40 BA/mL was sufficient to reduce viability of cells of *Lactobacillus helveticus* ATCC 15009 by 99 % in 6 h at 7 °C (Table 4). Observation that absorbance remained unchanged during incubation, indicates that a complete cell lysis did not occur.

The titers of four bacteriocin preparations obtained by ultrafiltration using membranes with MWCO 5, 10, 30 and 100 kDa were practically identical (data not

Table 3. Inhibition spectrum of *Lactobacillus* sp. strain LF221, as determined by agar well diffusion assay (AWD)

Test microorganism *	Inhibition **
A: isolates from fermented milks	
Streptococcus thermophilus – STC 17, STC 5, STC 112, STC 21/22 [a]	+
Lactococcus lactis subsp. *lactis* – LL2, LL4, LL23 [a]	+
Lactococcus lactis subsp. *lactis* ZIM BI04-08 [a] (5 strains)	–
Lactococcus lactis subsp. *cremoris* ZIM BI45-47 [a] (3 strains)	–
Bacillus cereus (ZIM BI15-BI29 [a] strains)	–
B: humane isolates	
Salmonella enteritidis (2 strains) [c]	–
Salmonella typhimurium (2 strains) [c]	–
Shigella sonnei (2 strains) [c]	–
Shigella flexneri (3 strains) [c]	–
Yersinia enterocolitica 0/3 (2 strains) [c]	–
Escherichia coli (11 strains) [c]	–
Campylobacter jejuni (3 strains) [c]	–
Listeria monocytogenes (5 strains) [c]	–
Streptococcus agalactiae (5 strains) [c]	–
S.pyogenes (5 strains) [c]	–
Aeromonas hydrophila (6 strains) [b]	–
Aeromonas sobria (6 strains) [b]	–
Aeromonas caviae (2 strains) [b]	–
C. difficile [e] (7 strains)	+
C: isolates from food and water	
B. cereus (10 strains) [d]	–
enterotoxic *Staphylococcus* [d]	
A (5 strains)	–
B (5 strains)	–
C (5 strains)	–
D (5 strains)	–
C: isolates from sheep and pigs	
Clostridium perfringens (5 strains) [f]	–
C. septicum [f]	+
C. feseri [f]	+

*The origin of test strains:

[a] Institute for Dairying, Zootechnical Department, Biotechnical Faculty, University of Ljubljana

[b] Institute of Microbiology, Medical Faculty, University of Ljubljana

[c] Institute of Public Health, Department for human microbiology, Ljubljana

[d] Institute of Public Health, Department for Food Sanity, Ljubljana

[e] Department of Biology, Biotechnical Faculty, University of Ljubljana

[f] Veterinary Faculty, University of Ljubljana

** Inhibition criteria:

+ : the distance between the edge of the well and the edge of the inhibition zone > 1mm

– : the distance between the edge of the well and the edge of the inhibition zone < 1mm

Table 4. Effect of LF221 bacteriocin(s) on survival of *Lactobacillus helveticus* ATCC 15009 [b] after incubation for 90 and 360 min at 7 °C

Incubation time / min	30	90	360
Bacteriocin activity (BA/mL)		(Reduction of CFU/mL)/ %[a]	
0	–	–	0
10	–	–	63
20	–	–	85
40	–	21	>99
80	–	15	>99
160	–	20	>99
320	–	22	>99

[a] Percent reduction was calculated as:
(CFU/mL control without bacteriocin – CFU/mL sample with bacteriocin)/CFU/mL control x 100

[b] Initial concentration of cells of *Lactobacillus helveticus* ATCC 15009 was $6.8 \cdot 10^5$ CFU/mL

– : CFU/mL was not reduced

shown), indicating that bacteriocin molecules of LF221 are present in the supernatant in the form of aggregates with molecular masses exceeding 100 kDa.

Discussion

Agar diffusion method was usually used for detection of antimicrobial activity of lactic acid bacteria and also bacteriocin activity. Other methods, such as liquid assay(s) in microtiter plates have also been used *(1,3,18, 22)*. Although testing in microtiter plates is less time consuming, additional steps are required to confirm the proteinaceous nature of the inhibitor. In our study, trypsin was used to confirm the proteinaceous properties of antibacterial compounds present in the supernatants of strains tested for antibacterial activity. Almost all known LAB bacteriocins are sensitive to trypsin but sensitivity to other enzymes may vary and can be used to distinguish between bacteriocins *(2–4)*.

In our study, 30 strains isolated from cheese and 41 strains isolated from baby faeces, were tested for bacteriocin-like activity and eight cheese isolates and two human isolates were potential bacteriocin producers. Numerous studies report isolation and screening of LAB for antibacterial activity. Barefoot and Klaenhammer *(16)*, examined 53 strains *of L. acidophilus* for inhibition of lactobacilli and *E. faecalis* under conditions eliminating the effects of hydrogen peroxide and organic acids, and found that 63 % strains were inhibitory to at least one test strain. Similarly, Kawai *et al. (18)* found that 62 (63 %) out of 98 isolates of *L. acidophilus* and related species (*L. crispatus, L. amylovorus, L. gallinarium, L. gasseri, L. johnsonii*), from human baby or adult faeces, produced bacteriocins active against at least one of four test strains: *L. delbrueckii* subsp. *bulgaricus* and three food borne pathogens (*L. monocytogenes, Bacillus cereus, Staphylococcus aureus*). Isolates from fermented sausages also produce bacteriocins *(23)*. Gariga *et al.* found that 56 strains of 254 lactobacilli examined, produced bacteriocins affecting other lactobacilli whereas two isolates of *L. sake* and one isolate of *L. plantarum*, also inhibited *L. monocytogenes (23)*.

The estimates of number of bacteriocinogenic strains in the normal population of lactobacilli, depend on the choice of test strains and on the screening or test method. Closely related strains or strains from the same ecological niche are often chosen as test microorganisms. The choice of test strains may also depend on potential application. Usually, different tests based on diffusion of antimicrobial substances in agar are used in the first stage of screening. Pre-concentrating of supernatants of potential bacteriocin producers is often required because of the generally low level of bacteriocins in a medium, rich with other polypeptides and limited diffusion of bacteriocins in agar. Aggregation and inactivation by protease(s) may also lead to false negative results *(22)*.

In case of our strain LF221, potential bacteriocin producer, some test bacteria were inhibited by concentrated supernatant (*Ped. pentosaceus* FBB63, *L. lactic* CNRZ117, *S. aureus* SA113), although inhibition zones in the DAS test were not observed (Table 2). It is therefore possible to miss some interesting strains, when antibacterial substances are not concentrated. The 10 kDa membrane was used for concentration because it partially removes the low molecular mass antimicrobials. In most cases, bacteriocins of LAB are in aggregates, so they are retained in the concentrate, although individual subunits are smaller than 10 kDa. In the present study, strain 4Me145 showed inhibition in DAS assays, the zone of inhibition was affected by trypsin, but the concentrated supernatant, however, was not inhibitory. It was, therefore, assumed that the inhibitor passed through the ultrafiltration (UF) membrane, because its molecules or aggregates were smaller than 10 kDa.

Our isolates from cheese were mesophilic homofermentative lactobacilli and most of them belong to *L. casei* (data not shown). The only bacteriocin of *L. casei* reported to date, caseicin 80, has a narrow range of activity *(24)*. The bacteriocin-like activity of four isolates from cheese (2So70, 1Mn336, 1Me1 and 4Me145) was confirmed only in the case when the test strain was *L. casei* or *L. sake*, therefore the range of activity was narrow as well.

Strains LF221 and K7 are the members of *L. acidophilus* group, as previously determined by DNA/DNA homology *(25)*. Bacteriocins of *L. acidophilus* group (*L. acidophilus, L. gasseri, L. johnsonii*) usually inhibit only closely related lactobacilli. The exceptions are acidocin B, which inhibits also *L. monocytogenes, C. sporogenes, Brochotrix thermosphacta*, and acidocin A, which inhibits

also *E. faecalis, L. monocytogenes* and *Streptococcus bovis (21,26).* Common probiotic strains, belonging to *L. acidophilus* group, *L. brevis, L. plantarum, L. casei* and others often produce bacteriocins with narrow inhibition spectra (*4,5,17–20,21,24*). The inhibition spectrum of strain LF221 is therefore not typical, since it includes some non-LAB bacteria: *Clostridium* sp., *Bacillus cereus, Staphylococcus aureus, Listeria innocua.* Therefore, strain LF221 is particularly interesting as a probiotic strain with a wide spectrum of activity. Further studies are necessary to purify and characterize bacteriocin(s) from this strain.

Conclusions

By screening of 71 lactobacilli, isolated from cheese and baby faeces, 10 strains with antimicrobial activity were selected. The proteinaceus nature of the inhibitor(s) was confirmed by sensitivity of »bacteriocin(s)« to trypsin in four (out of eight) cheese isolates and two human isolates. Whereas the bacteriocin(s) of four cheese isolates were active against three lactobacilli test bacteria, the inhibition spectrum of concentrated bacteriocin preparation of two human isolates was much wider, and included some LAB, clostridia and individual strains of *Staphylococcus aureus* and *Bacillus cereus.* In strain LF221 of *Lactobacillus acidophilus,* bacteriocins were present in the supernatant in aggregates exceeding 100 kDa. Additional evidence for the presence of bacteriocin(s) in the supernatant was provided by inactivation of the inhibitory substance by trypsin, pronase and proteinase K, by its thermostability and by bactericidal activity against *L. helveticus* ATCC 15009.

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Detekcija bakteriocinske aktivnosti laktobacila izoliranih iz sira i fecesa novorođenog djeteta

Sažetak

Sojevi laktobacila, izolirani iz sira (30 sojeva) i fecesa dva novorođena djeteta (41 soj) ispitani su u svezi s njihovom sposobnosti proizvodnje bakteriocina sljedećim mikrobiološkim postupcima: modificiranim postupkom difuzije s dvostrukim slojem agara i postupkom difuzije u agar iz rupica koristeći 29 Gram-pozitivnih testnih sojeva iz različitih rodova. Osam izolata iz sira i 2 humana izolata pokazali su protumikrobnu aktivnost na barem jedan testni mikroorganizam. Proteinska svojstva inhibitora potvrđena su za četiri izolata iz sira i dva humana izolata (LF221 i K7). Posljedna su dva soja inhibirala i neke druge bakterije osim bakterija mliječne kiseline. Soj Lactobacillus acidophilus LF221 dodatno je ispitan postupkom difuzije u agar iz rupica, prema širokom rasponu bakterija, uključujući i patogene sojeve. Osim nekih testnih bakterija mliječne kiseline, određeni sojevi

sljedećih vrsta također su bili inhibirani: Listeria innocua, Enterococcus faecalis, Bacillus cereus, Staphylococcus aureus i Clostridium sp. Ustanovilo se da je inhibitor protein jer su ga inaktivirali tripsin, proteinaza K i pronaza, a bio je i postojan pri visokim temperaturama (100 °C). Soj LF221 bio je baktericidan, ali ne bakteriolitički za testni mikroorganizam Lactobacillus helveticus ATCC 11509. U supernatantu su bakteriocinske molekule bile u obliku agregata s relativnom molekularnom masom preko 150 kDa.