Lactobacilli Isolated from Lump Sheep's Cheeses and their Antimicrobial Properties

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Abstract

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A total of 34 strains of lactobacilli were isolated from the lump sheep's cheeses produced from raw sheep milk. The strains were identified by MALDI-TOF MS, and 20 of them demonstrating the best fermentation and sensoric properties in milk were chosen and tested for their antimicrobial activity. All selected strains were active against the indicator bacteria and moulds. The highest inhibitory effect was observed with the strains *Lactobacillus paracasei* 314, *L. paracasei* 316, *L. plantarum* K816, *L. plantarum* L718, and *L. plantarum* 2L2. The subsequent research was focused on the metabolites causing this inhibition. The production of lactic and acetic acids was studied under different cultivation conditions (0, 2, 4, and 6.5% NaCl addition; cultivation at 15, 30, 37, and 45°C; and pH value of the broth before sterilisation 5 and 9). *L. plantarum* L718 produced the highest concentration of lactic and acetic acids under most of the cultivation conditions. Antimicrobial substances such as phenyllactic acid (62.54–101.62 mg/dm^3), H_2O_2 (0.78–2.30 μg/cm^3), and diacetyl (produced by *L. plantarum* K816 and L718) were studied as well.

Keywords: *Lactobacillus plantarum*; *Lactobacillus paracasei*; antimicrobial potential

It is known that the natural microflora, present in the traditional biotechnological processes during manufacturing, participates in the production of the characteristic aroma, taste, and texture of different cheeses. At first, only artisanal starters were used for the dairy although fermentations. Although they are still used in certain cases, their microbiological instability promoted the evolution of more precisely defined mixtures of lactic acid bacteria (LAB). As cheesemaking became more industrialised, pasteurised milk and standardised bacterial and fungal inocula were introduced with the aim of obtaining a more stable acidifying activity and consistent quality of the products (Carminati *et al*. 2010). In spite of this trend, the production of raw milk cheeses is still significant, as consumers seek organic foods or traditional sensory characteristics. However, the diversity of microbial populations that have developed in the original cheesemaking environments is largely undefined and is a valuable source of new strains for commercial uses (Laurenčík *et al*. 2008). For example, non-starter LABs with the potential to inhibit the undesirable microflora are used in the biopreservation of foods (Rodríguez *et al*. 2000).

As far as the technology is concerned, several characteristic properties of the bacterial strains of LAB (acid production in different media and at different temperatures, proteinase and peptidase activities, autolysis, production of volatile compounds, resistance to bacteriophages, production of inhibitory compounds) are important for their use as starters or adjuncts and have been evaluated in the screening and characterisation studies (Piraino *et al*. 2008).

The main task of this work was to observe the antimicrobial activity of 20 newly isolated strains of lactobacilli and to study the technological potential

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of 5 selected strains (2 strains of *L. paracasei* and 3 strains of *L. plantarum*), which showed very good antimicrobial activities.

MATERIAL AND METHODS

Microorganisms studied. 34 strains of lactobacilli were isolated from the lump sheep's cheeses produced from raw sheep milk in different farms of the Slovak Republic. The isolates were identified by MALDI-TOF MS. 20 strains of lactobacilli (10 *L. paracasei* and 10 *L. plantarum*) were chosen for the subsequent testing in view of the fermentative and sensoric properties in milk.

Identification. For MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) bacterial analysis, the cells from a single colony of fresh overnight culture were used for each isolate to prepare samples according to the microorganism profiling ethanol-formic acid extraction procedure, as recommended by the manufacturer. Each sample spot was overlaid with 2 µl of the matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) and air-dried again for 15 min (Bruker Daltonik GmbH, Bremen, Germany). To identify the microorganisms, the raw spectra obtained with each isolate were imported into BioTyper software, Version 2.0 (Bruker Daltonik GmbH, Bremen, Germany), and analysed without any user intervention (Kmeť & Drugdová 2012).

The antimicrobial activity was studied by the dualculture overlay diffusion method (Magnusson 2003). Lactobacilli were inoculated (0.1 ml of overnight culture) into sterile disks (Rotilabo®-test flakes, ø 12 mm; Carl Roth GmbH+Co. KG, Karlsruhe, Germany) on MRS agar plates (Merk, Darmstadt, Germany) and allowed to grow at 37°C for 48 h in aerobic conditions. The plates were then overlaid with soft BHI agar (broth + 0.8% agar; Merk, Darmstadt, Germany) containing the inoculum of the indicator bacteria (inoculum 1%) or with soft Sabouraud agar (broth + 0.8% agar; Imuna, Šarišské Michaľany, Slovakia) containing the inoculum of the indicator mould (10^4 per ml mould spores). After 24 h (bacteria) and 48 h (moulds) of aerobic incubation at 37°C (bacteria) and 25°C (moulds), the respective zone of inhibition was measured and % of inhibition of the total area of the Petri dish (PD) was calculated. The inhibition tests were done in duplicates. The antimicrobial activity of lactobacilli was observed against 6 indicator bacteria (*Bacillus cereus* OPT, *Bacillus subtilis* CCM 2216, *Escherichia coli* CCM 3988, *Listeria monocytogenes* NCTC 4886, *Pseudomonas aeruginosa* CCM 3955 and *Staphylococcus aureus* CCM 3953) and 5 indicator moulds (*Alternaria alternata* CCM F-128, *Aspergillus flavus* CCM F-171, *Mucor rouxii* CCM F-220, *Penicillium chrysogenum* CCM F-432 and *Rhizopus oryzae* CCM F-8284) (CCM – Czech Collection of Microorganisms, Czech Republic; OPT – Collection of Department of Food Science and Technology, Slovakia; NCTC – National Collection of Type Cultures, UK).

Subsequently, the lactobacilli with the best antimicrobial activity were investigated for their production of antimicrobial substances.

Antimicrobial substances. *Organic acids production*. The ability of lactobacilli to produce lactic and acetic acids was studied in MRS broth (Merk, Darmstadt, Germany) with different additions of NaCl (no addition, 2, 4, and 6.5% addition) at 37°C; in MRS broth at different temperatures (15, 30, 37, and 45°C) and in MRS broth with pH value adjusted before sterilisation (pH 5 and 9) at 37°C. The inoculum was 1%. The samples were analysed using HPLC after 50 h of fermentation.

Phenyllactic acid production was observed in MRS broth with the addition of 0.1% of L-phenylalanin (Merk, Darmstadt, Germany). Lactobacilli were incubated at 37°C for 72 h in anaerobic conditions (inoculum 1%). The concentrations of phenyllactic acid were determined with HPLC (Greif *et al*. 2008).

Hydrogen peroxide production. Lactobacilli were grown in MRS broth at 37°C for 16 h, harvested by centrifugation (4000 *g*, 5°C, 20 min), washed twice in cold physiological saline, and resuspended in sodium fosfate buffer (pH 6.5). Following the incubation (24 h at 5°C), the cells were removed by centrifugation and the supernatant was assayed for H_2O_2. 2.5 ml of the cell free supernatant was placed into tubes containing 0.5 ml of 0.1% aqueous solution of peroxidase (Boehringer, Mannheim, Germany) and 0.05 ml of 1% aqueous solution of *o*-diasinidine (SERVA, Heidelberg, Germany). The blank was prepared using 2.5 ml of sodium phosphate buffer instead of the sample supernatant. The tubes were icubated at 37°C, for 10 minutes. The reaction was stopped by adding 0.1 ml of 4N HCl. The absorbance (λ = 400 nm) of each sample was read and peroxide content was determined by comparison with the standard curve (Villegas & Gillialand 1998).

Diacetyl production. The revitalised strains were inoculated in 9 ml of UHT milk and allowed to grow at 30°C for 24 h in aerobic conditions (inoculum 1%). 1 ml of each cell suspension was combined with

0.5 ml of 1% ethanolic solution of α-naphthol (Merck, Darmstadt, Germany) and 0.5 ml of 16% aqueous solution of KOH (Mikrochem, Pezinok, Slovakia), and incubated at 30°C for 10 minutes. Diacetyl production was indicated by the formation of a red ring at the top of the test tubes (Dal Bello *et al*. 2012).

RESULTS AND DISCUSSION

Antimicrobial activity of 20 new-isolated strains of lactobacilli (10 *L. paracasei* and 10 *L. plantarum*) was observed in this study (Table 1 and Figure 1). All monitored strains showed considerable inhibition of the indicator bacteria *B. cereus*, *B. subtilis*, *E. coli*, *L. monocytogenes*, *P. aeruginosa*, and *S. aureus* (the average more than 20% of the total area of the PD).

Antifungal activity of *Lactobacillus* strains varied. The inhibition zones were in the interval of 4.1–14.3% of the total area of the PD. The activities of the strains *L. plantarum* and *L. paracasei* were comparable. The most inhibited were *P. chrysogenum* and *R. oryzae* (an average of 10.4% and 9.1% of the total area of PD). *A. alternata*, *A. flavus,* and *M. rouxii* were inhibited at an average of 7.2, 7.2, and 8.4% of the total area of PD.

Two strains of *L. paracasei* (marked 314 and 316) and 3 strains of *L. plantarum* (marked K816, 2L2, and L718) were chosen for the subsequent testing due to their best antimicrobial activity of all the lactobacilli studied. Their average antibacterial activity was higher than 25% and their average antifungal activity was higher than 8.5% of the total area of PD.

The ability of *L. paracasei* and *L. plantarum* to possess antimicrobial activity had been previously published as well. The antibacterial activities of 17 strains of *L. plantarum*, isolated from a Tunisian traditional salted meat, were studied by Essid *et al*. (2009). All strains displayed inhibition zones against *S. aureus*, 94% against *E. coli*, and 88% against *P. aeuroginosa*. Ren *et al*. (2012) observed the effects

Table 1. Antimicrobial activity (% of inhibition of the total area of the PD)

		Indicator bacteria						Indicator moulds				
	Isolated strains	*B. cereus*	*B. subtilis*	*E. coli*	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*	*A. alternata*	*A. flavus*	*M. rouxii*	*P. chrysogenum*	*R. oryzae*
L. paracasei	113	26.5	20.2	11.9	16.5	34.2	24.8	8.8	6.7	2.5	13.3	9.3
	119	18.3	19.2	26.5	19.4	27.7	38.8	7.6	5.0	6.3	7.5	9.8
	211	21.9	28.4	20.2	22.5	19.9	20.8	6.3	11.1	6.4	12.1	7.1
	314	19.5	30.1	27.6	22.7	29.3	34.6	8.0	9.0	6.3	12.7	13.7
	316	20.8	34.0	18.3	23.6	32.4	32.4	7.2	7.2	14.3	12.9	9.2
	11L5	13.8	33.2	14.1	21.9	26.1	30.7	5.9	6.8	8.4	12.2	11.4
	11L7	30.3	30.7	12.0	13.0	28.8	21.2	8.8	7.6	4.9	7.4	6.0
	12L1	23.1	34.5	16.5	29.3	35.3	21.5	6.6	7.0	5.7	12.3	4.1
	12L3	15.9	30.7	10.4	26.5	25.0	27.2	6.3	8.0	13.9	12.2	6.8
	12L6	19.2	31.1	14.2	29.7	25.0	36.2	6.6	6.8	7.0	6.6	6.9
L. plantarum	115	16.5	24.6	25.9	29.1	20.7	26.8	7.2	5.3	11.5	12.2	7.7
	219	19.2	22.5	13.6	21.0	37.1	38.4	7.8	7.6	5.8	7.6	7.6
	L 717	17.7	28.6	23.6	19.5	12.3	22.9	8.4	10.9	5.1	11.4	13.5
	L 718	28.2	28.2	32.4	29.1	28.8	24.8	7.8	1.3	14.3	9.3	9.8
	812	41.6	25.7	22.2	25.2	10.3	30.7	5.0	7.4	5.0	9.9	14.1
	K 816	16.2	19.5	33.2	19.5	35.3	29.1	6.0	6.5	7.3	14.1	13.8
	K 817	23.9	19.9	13.3	19.2	30.3	37.9	12.6	7.7	7.2	7.5	6.8
	1L5	19.4	28.0	11.1	23.9	29.5	44.3	5.4	5.0	6.0	8.0	7.1
	2L2	23.8	45.4	18.3	17.1	32.0	26.1	5.4	7.7	13.3	12.2	8.7
	1L10	24.3	37.1	20.5	17.7	23.9	19.7	6.4	9.3	16.7	7.4	9.2

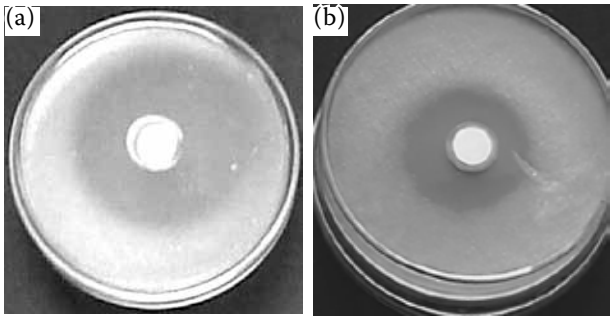

Figure 1. Inhibitory activity of (a) *L. paracasei* 314 against *E. coli* and (b) *L. plantarum* L718 against *M. rouxii*

of the selected strains of lactobacilli (*L. salivarius* subsp. *salicinius* CICC 23174, *L. plantarum* CGMCC 1.557, *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 4356) on the undesirable *S. aureus*. *L. paracasei* susp. *paracasei* that was isolated from breast-fed newborns' faeces, showed anti-listerial activity due to the ablility to produce a bacteriocin-like substance (Bendali *et al*. 2008).

Antifungal activity of *L. paracasei* (isolated from Edam cheese) was observed against *Fusarium proliferatum* M 5689, followed by *Penicillium* sp. DMF 0006 and *Aspergillus niger* DMF 0801 (Tůma *et al*. 2007). *L. plantarum* 01, *L. paracasei* 05, *L. paracasei* SF1 and *L. plantarum* 2142 (strains from collections) inhibited the growth of *A. flavus* DMF 0802 (Hudáček *et al*. 2007).

LABs have traditionally been used as natural biopreservatives in food and animal feed, sauerkraut, and silage. Their preserving effect relates mainly to the formation of organic acids and H_2O_2, competition for nutrients and production of antimicrobial substances (Voulgari *et al*. 2010). The acidic condition and the reduced pH of the fermented dairy products, as well as the antimicrobial activity of the undissociated lactic acid molecules, prevent the growth or survival of many spoilage and pathogenic bacteria and moulds (Carminati *et al*. 2010; León Peláez *et al*. 2012).

The produced concentrations of lactic and acetic acids are given in Table 2. Generally, the growth and organic acids production were supported by the addition of 2% and 4% of NaCl. The temperatures of 30 and 37°C and pH value 5 had positive effects on the lactic acid production. Acetic acid was produced in higher quantities at the temperatures of 37 and 45°C and pH value of 9. Out of all the strains analysed, *L. plantarum* L718 produced the highest concentration of lactic and acetic acids in most of the cultivation conditions.

Gerez *et al*. (2013) observed very good antifungal activities in 4 strains of *L. plantarum*, isolated from different food sources, and analysed the antifungal substances. After 24 h of cultivation in MRS broth, lactobacilli produced 19.52–24.13 g/dm^3 of lactic acid; 0.77–1.20 g/dm^3 of acetic acid, and 33.23–581.60 mg/dm^3 of phenyllactic acid. The antifungal activity, however, is not related only to lactic acid, phenyllactic acid, or any other single inhibitory substance, but rather to the combination of such substances (Wang *et al*. 2013).

The bactericidal effect of H_2O_2 has been attributed to its strong oxidising effect on the bacterial cells and to the destruction of basic molecular structures of the cell proteins. Zalán *et al*. (2005) showed that *L. plantarum* 2142 produced enough H_2O_2 (2.45 µg/ml)

Table 2. Concentration of lactic acid and acetic acid (g/dm^3) after 50 h cultivation in different growth conditions

Isolated strains	NaCl addition (%)				Temperature of cultivation (°C)				pH of broth	
	0	2	4	6.5	15	30	37	45	5	9
Lactic acid										
314	9.061	9.350	11.932	1.593	0.247	9.419	9.102	8.107	11.336	8.931
316	15.264	16.880	11.837	1.185	1.694	14.279	14.469	0.427	21.371	8.138
K816	9.745	10.650	16.039	4.146	1.796	8.210	8.835	7.156	11.207	10.158
L718	17.373	17.347	16.474	11.289	8.286	16.087	16.505	4.990	18.259	2.261
2L2	8.147	9.730	14.329	7.707	0.859	9.790	9.813	9.390	13.593	8.395
Acetic acid										
314	0.805	0.697	0.572	0.250	nd	0.978	0.881	0.846	1.084	1.558
316	nd	0.138	0.208	0.088	0.091	0.089	0.137	0.125	nd	0.181
K816	1.378	1.546	1.065	0.214	nd	0.360	0.674	0.854	0.923	1.319
L718	0.897	0.863	1.275	0.590	0.745	1.453	1.464	0.824	0.551	ND
2L2	0.761	1.341	1.002	0.373	nd	0.515	0.900	1.070	1.314	1.480

nd – not detected

Table 3. Production of other important antimicrobial substances

Isolated strains	Antimicrobial substances		
	phenyllactic acid (mg/dm^3)	H_2O_2 (μg/cm^3)	diacetyl[1]
314	85.13	1.88	–
316	62.54	0.78	–
K816	101.62	2.30	+
L718	65.70	1.73	+
2L2	94.98	2.27	–

[1]clasification scale: – no production; + production

after 24 h at 5°C in phosphate buffer to inhibit the growth of *L. monocytogenes* and *B. cereus*.

In this study, antimicrobial substances such as phenyllactic acid (62.54–101.62 mg/dm^3), H_2O_2 (0.78–2.30 μg/cm^3), and diacetyl were also studied (Table 3). Diacetyl was produced only by the strains *L. plantarum* K816 and L718, but Nikolic *et al.* (2008) demonstrated its production also by the strains of *L. paracasei* subsp. *paracasei* (isolate from Bukuljac cheese). Antimicrobial activity of diacetyl is known; for example, the activity against *E. coli*, *L. monocytogenes*, and *S. aureus* was proved by Lanciotti *et al.* (2003).

CONCLUSIONS

Five lactobacilli strains were selected due to their marked antimicrobial activities, and their antimicrobial metabolites were analysed. The inhibitory activity of lactobacilli is dependent on the producer, cultivated medium, and also on the strains inhibited, especially in the case of moulds. The determined concentrations of lactic and acetic acids confirmed the ablility to inhibit the undesirable microflora. Phenyllactic acid, H_2O_2 and diacetyl were also involved in the synergic inhibitory effect. Therefore these lactobacilli could be used as adjunct cultures. However, in the first place other studies on their safe application in foods are needed.

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