VERSITA

Antimicrobial activity of selected lactic acid cocci and production of organic acids

Zuzana Hladíková, Jana Smetanková, Gabriel Greif, Mária Greifová

Institute of Biotechnology and Food Science, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic
zuzana.hladikova@stuba.sk

Abstract: Antimicrobial activity and production of organic acids by selected lactic acid bacteria were monitored in this study. The largest antimicrobial activity against indicator microorganisms showed *Pediococcus* sp. G5, whereas *Streptococcus thermophilus* had no inhibitory effect. The inhibitory effect of *Pediococcus* sp. G5 was strongest against *Bacillus subtilis* (17.78 %). Lactococci inhibited the growth of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (% of inhibition ≤ 5.25). The growth of *Asperglillus flavus*, *Penicillium funiculosum* and *Rhizopus oryzae* was not inhibited by all of tested cocci. Cocci produced varying quantities of organic acids (lactic acid, acetic acid, succinic acid, etc.). Lactic acid was in large amounts and phenyllactic acid was produced only by *Pediococcus* sp. G5 (49.65 mg/L).

Keywords: antimicrobial activity, *Lactococcus lactis*, organic acids, *Pediococcus* sp., *Streptococcus thermophilus*

Introduction

Lactic acid bakteria (LAB) are a group of gram-positive, non-spore forming cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. They consisted of many genera including *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Rattanachaikunsopon and Phumkhachorn, 2010).

Lactococcus, Lactobacillus, Leuconostoc, Streptococcus and *Pediococcus* are genera most commonly used as starter cultures in fermentation processes of milk, meat and vegetable products (Nieto-Lozano et al., 2010). *Lactococcus lactis* is used as a mesophilic starter for its capacity to acidify milk leading to coagulation and to generate aroma during ripening (Jeanson et al., 2009).

Lactic acid bacteria isolated from dairy products have received increased attention as a potential food preservative due to their antagonistic activity against many food-borne pathogens such as *Listeria monocytogenes* (Mezaini et al., 2009; Jamuna and Jeevaratnam, 2004) and other pathogens, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus,* or *Escherichia coli*.

Inhibition of pathogenic microorganisms by lactic acid bacteria is a complex phenomenon involving number of inhibitory factors. Organic acids (lactic acid, acetic acid, formic acid, etc.) are major metabolites in dairy fermentation and have been demonstrated to be one of the inhibitory factors (Wong and Chen, 1988). LAB also produce various compounds such as diacetyl, acetoin, acetaldehyde, hydrogen peroxide, ethanol and bacteriocins during lactic fermentations. Phenyllactic and hydroxy-phenyllactic acids have also been found as metabolites involved in the formation of cheese flavour and produced by lactic acid bacteria strains through phenylalanine (Phe) and tyrosine (Tyr) degradation, respectively (Valerio et al., 2004; Kieronczyk et al., 2003; Yvon et al. 1998; Yvon et al. 1997).

LAB are the most commonly used microorganisms in fermented foods. Their crucial importance is associated mainly with their physiological features, such as substrate utilization, metabolic capabilities and probiotic properties. Their common occurrence in foods coupled with their long historical use contributes to their acceptance as GRAS (Generally Recognized As Safe) for human consumption (Liu et al., 2011; Silva et al., 2002).

The aim of study

The aim of this study was to screen the antibacterial and antifungal activity of selected lactic acid cocci (*Lactococcus lactis* ZS25, *Lactococcus lactis* LM25, *Streptococcus thermophilus* M37 and *Pediococcus* sp. G5) isolated from Slovak traditional sheep's cheeses against *Aspergillus flavus* CCM F-108, *Bacillus cereus* DFST, *Bacillus subtilis* CCM 2216, *Escherichia coli* CCM 3988, *Fusarium nivale* DBM 1/89, *Listeria monocytogenes* NCTC 4886, *Mucor racemosus* DBM 1/90, *Penicillium funiculosum* CCM F-161, *Pseudomonas aeruginosa* CCM 3955, *Rhizopus oryzae* DBM 1/90, *Staphylococcus aureus* CCM 3953 using the diffusion method. The next step was monitoring the production of phenyllactic acid from phenylalanin as precursor and the production of organic acids in broth using HPLC method.

 Acta Chimica Slovaca, Vol. 5, No. 1, 2012, pp. 80—85, DOI: 10.2478/v10188-012-0013-3

Materials and methods

Microorganisms

Four strains of lactic acid bacteria isolated from sheep's cheeses (*Lactococcus lactis* ZS25, *Lactococcus lactis* LM25, *Streptococcus thermophilus* M37, *Pediococcus* sp. G5) were used in this work, as illustrated in Table 1..

We inoculated 10 ml of broth with 1 mL of frozen tested strain (*Lactococcus lactis* ZS25, *Lactococcus lactis* LM25) and incubated at 25 °C (*Lactococcus lactis* ZS25, *Lactococcus lactis* LM25) and at 37 °C (*Streptococcus thermophilus, Pediococcus* sp.) for 16 hours. *Lactococcus lactis* ZS25, *Lactococcus lactis* LM25 and *Streptococcus thermophilus* M37 were cultivated in M17 broth and for the cultivation of *Pediococcus* sp. was used MRS broth.

Indicator strains

Aspergillus flavus CCM F-108, *Bacillus cereus* DFST, *Bacillus subtilis* CCM 2216, *Escherichia coli* CCM 3988, *Fusarium nivale* DBM 1/89, *Listeria monocytogenes* NCTC 4886, *Mucor racemosus* DBM 1/90, *Penicillium funiculosum* CCM F-161, *Pseudomonas aeruginosa* CCM 3955, *Rhizopus oryzae* DBM 1/90, *Staphylococcus aureus* CCM 3953

CCM — Czech Collection of Microorganisms (Brno, Czech Republic)

DFST — Department of Food and Science Technology (FCHPT, STU, Bratislava, Slovakia)

DBM — Department of Biochemistry and Microbiology (FCHPT, STU, Bratislava, Slovakia)

NCTC — National Collection of Type Cultures (UK laboratory)

Strains of pathogens were kept in BHI broth (MERCK, Darmstadt, Germany) and moulds on the Sabouraud agar (Imuna, Šarišské Michaľany, Slovakia) at 5 ± 1 °C.

Evaluation of antimicrobial activity

Cocci were screened for antimicrobial activity using the diffusion method described by Magnusson et al. (2003).

Cocci were inoculated in 2.5 — cm lines on M17 agar plates (*Lactococcus lactis* ZS25, *Lactococcus lactis* LM25, *Streptococcus thermophilus* M37) and on MRS agar plates (*Pediococcus* sp.) and allowed to grow at 25 °C (*Lactococcus lactis* ZS25, *Lactococcus lactis* LM25) and at 37 °C (*Streptococcus thermophilus* M37, *Pediococcus* sp. G5) for 48 h under aerobic conditions.

Antibacterial activity

After growing cocci on plates, these were overlaid with 10 ml of BHI soft agar (0.8 %; MERCK, Darmstadt, Germany) containing 10^7 CFU/mL indicator pathogens. After 24 h of aerobic incubation at 37 °C, the zone of inhibition was measured.

The inhibition was graded by relating the inhibited growth area per inoculation streak to the total area of the Petri dish (%).

Antifungal activity

After growing cocci on plates, plates were overlaid with 10 mL of Sabouraud soft agar (0.8 %; Imuna, Šarišské Michaľany, Slovakia) containing 10^4 mould spores/mL. After 48 h of aerobic incubation at 24 °C, the zone of inhibition was measured.

The inhibition was graded by relating the inhibited growth area per inoculation streak to the total area of the Petri dish (%).

Inhibition tests were done in duplicates.

Production of organic acids in broth

Cocci were grown in broth 72 h (*Lactococcus lactis* ZS25 and *Lactococcus lactis* LM25 at 25 °C; *Streptococcus thermophilus* M37 and *Pediococcus* sp. at 37 °C) and then were centrifuged to obtain a cell-free supernatant. Cell — free supernatant was then applied onto the HPLC column.

HPLC analysis

Organic acids were analysed using an HPLC-apparatus consisting of a DeltaChrom™ SDS 030 pump (Watrex, Bratislava, Slovakia), a manual injector Rheodyne 7725i, a Polymer IEX H^+ (250 × 8 mm) column (Watrex, Bratislava, Slovakia), a column heater DeltaChrom™ Temperature Control Unit (50 ± 0.1 °C). One mmol/L sulphuric acid was used as the mobile phase at a flow rate of 1 mL/min. For detection of organic acids a refractometric detector RI K-2301 (Knauer, Berlin, Germany) was used. For detection of phenyllactic acid an UV detector Applied Biosystems 759A was used. Recordings

Tab. 1. Strains of tested cocci.

Sample	Classification	Origin
ZS25	*Lactococcus lactis*	lump sheep's cheese (Dairy Research Institute, Žilina, Slovakia)
LM25	*Lactococcus lactis*	lump sheep's cheese (Dairy Research Institute, Žilina, Slovakia)
M37	*Streptococcus thermophilus*	lump sheep's cheese (Dairy Research Institute, Žilina, Slovakia)
G5	*Pediococcus sp.*	sheep's cheese "bryndza" (Food Research Institute, Bratislava, Slovakia)

were made on Clarity (DataApex, Praha, Czech Republic).

Phenyllactic acid

Phenyllactic acid production was monitored in M17/MRS medium enriched by 0.1 % phenylalanine as precursor after 72 h of anaerobic cultivation at 37 °C. Initial concentration of cocci was 10^7 CFU/mL.

Results and Discussion

Lactic acid bacteria produce substances that inhibit pathogenic and spoilage microorganisms in food products. The antagonistic property is attributed to the lowered pH, the undissociated organic acids and production of antimicrobial metabolites, such as diacetyl, acetoin, acetaldehyde, hydrogen peroxide, ethanol and bacteriocins.

In the present study, the antibacterial and antifungal effect of four lactic acid bacteria isolates from sheep's cheeses was investigated.

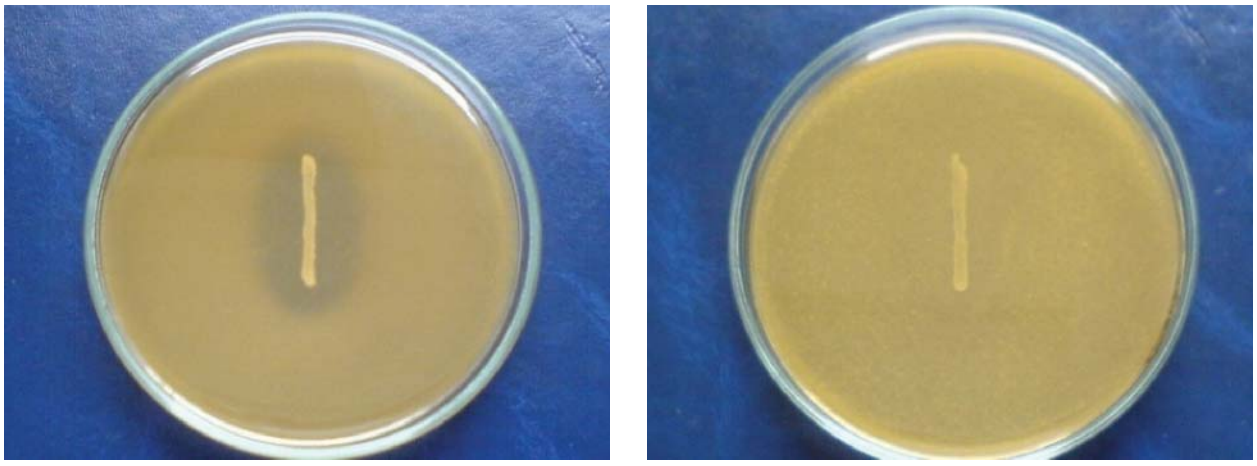
The highest antibacterial activity displayed *Pediococcus* sp. G5 against all indicator strains of pathogens (*Bacillus cereus, Bacillus subtilis, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa and Staphylococcus aureus*). The results of these experiments are illustrated in the Fig. 1. One can see a zone of inhibition around *Pediococcus* sp. G5 against E. coli. The negative result was found with *Lactococcus lactis* LM25, which did not form inhibitory zone against *E. coli* (Fig. 1). The most sensitive bacteria against attack of LAB were *Bacillus subtilis* (17.78 %), followed by *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*, respectively.

Results shown in the Table 2 demonstrate that the inhibition zones of *Pediococcus* sp. against pathogens were varied in the range between 4.33 % and 17.78 %. *Lactococcus lactis* ZS25 inhibited only the growth of *Escherichia coli* (1.75 %) and *Staphylococcus aureus* (5.25 %), and *Lactococcus lactis* LM25 inhibited the growth of *Pseudomonas aeruginosa* (3.06 %) and *Staphylococcus aureus* (3.75 %). *Streptococcus thermophilus* M37 did not show any inhibitory activity against pathogenic bacteria used in this study.

Similar study was carried out by Tadesse et al. (2005) who studied the antimicrobial activ-

Tab. 2. Antibacterial activity of cocci against indicator bacteria in broth (aerobic conditions).

Indicator strain	*Lactococcus lactis* ZS25	*Lactococcus lactis* LM25	*Streptococcus thermophilus* M37	*Pediococcus* sp. G5
	% of inhibition			
Bacillus cereus	0	0	0	8.11
Bacillus subtilis	0	0	0	17.78
Escherichia coli	1.75	0	0	14.07
Listeria monocytogenes	0	0	0	13.38
Pseudomonas aeruginosa	0	3.06	0	13.16
Staphylococcus aureus	5.25	3.75	0	4.33



G5 LM25

Fig. 1. A zone of inhibition around *Pediococcus* sp. G5 and *Lactococcus lactis* LM25 (no zone) against *Escherichia coli.*

ity of 118 LAB strains isolated from Borde and Shamita, traditional Ethiopian fermented beverages, against some pathogens. Lactic acid bacteria were grouped in to *Lactobacillus* sp. (20 homofermentors and 40 heterofermentors), *Leuconostoc* sp. (15 isolates), *Pediococcus* sp. (18 isolates) and *Streptococcu*s sp. (25 isolates). *Lactobacillus* isolates resulted in the highest diameter of inhibition zone than other LAB isolates on the test strains (*Salmonella* spp., *Shigella flexneri, Staphylococcus aureus, Escherichia coli* O157:H7). *Leuconostoc* isolates also showed some inhibitory activities against the test strains and *Pediococcus* isolates showed a relatively larger zone of inhibition on the test strains next to *Lactobacillus* isolates. *Staphylococcus aureus* was the most sensitive to inhibitory activity of *Pediococcus* isolates. *Streptococcus* isolates also showed some degree of inhibition against the test strains and they were the most effective against *Salmonella* spp.

Our results showed antifungal activity of *Pediococcus* sp. against *Alternaria alternata, Mucor racemosus* and *Fusarium nivale* (Table 3). The indicator mould *Alternaria alternata* was the most sensitive fungi tested (16.84 %). *Lactococcus lactis* ZS25, *Lactococcus lacis* LM25 and *Streptococcus thermophilus* M37 did not show inhibitory activity (no zones of inhibition). Antifungal activity of *Pediococcus* sp.G5 is probably associated with phenyllactic acid production (0.049 mg/mL). Production of phenyllactic acid by *Lactococcus lactis* ZS25, *Lactococcus lactis* LM 25 and *Streptococcus thermophilus* M37 was undetectable (Table 4.)

Lavermicocca et al. (2003) studied antifungal activity of phenyllactic acid (PLA) against a variety of fungal species isolated from bakery products and flours and two ochratoxin A-producing strains isolated from cereals. For each strain, the minimal fungicidal or inhibitory PLA concentration was determined together with the behaviour at pH conditions more similar to those in real food systems with respect to the ability to inhibit and delay mold growth. The effect of PLA in combination with the main organic acids produced in culture by *L. plantarum* 21B was also investigated. PLA showed a broad spectrum of activity by inhibiting all fungal strains, with MIC_{90} (minimum inhibitory concentration, 90 %) ranging from 3.75 to 7.5 mg/mL PLA showed fungicidal activity at levels of ≤ 10 mg/mL against 19 strains (of the 23 strains tested) belonging to 13 different species (*Fusarium sp., Penicillium verrucosum, Penicillium chrysogenum, Penicillium solitum, Penicillium roqueforti, Penicillium commune, Penicillium polonicum, Aspergillus ochraceus, Penicillium sp., Aspergillus niger, Aspergillus flavus, Aspergillus terreus, Penicillium brevicompactum, Penicillium citrinum*).

Organic acids are relevant in dairy products for nutritional reasons and because they contribute to the flavor and aroma. They are major products of carbohydrate catabolism of lactic acid bacteria and non-starter bacteria associated with milk (Izco et al., 2002).

Non-dissociated forms of weak organic acids diffuse through the pathogenic bacterial cell membrane. These diffused acids dissociate inside

Tab. 3. Antifungal activity of cocci against indicator moulds in broth (aerobic conditions).

Indicator strain	*Lactococcus lactis* ZS25	*Lactococcus lactis* LM25	*Streptococcus thermophilus* M37	*Pediococcus* sp. G5
	% of inhibition			
Alternaria alternata	0	0	0	16.84
Aspergillus flavus	0	0	0	0
Fusarium nivale	0	0	0	3.15
Mucor racemosus	0	0	0	7.89
Penicillium funiculosum	0	0	0	0
Rhizopus oryzae	0	0	0	0

Tab. 4. Production of organic acids and ethanol (aerobic cultivation) and phenyllactic acid (anaerobic cultivation) in broth.

Sample	Lactic acid	Acetic acid	Succinic acid	Ethanol	Phenyllactic acid
	g/L				mg/L
ZS25	2.877	0.748	0.421	12.286	UD
LM25	2.902	0.835	0.385	11.224	UD
M37	3.649	0.696	0.187	8.462	UD
G5	15.282	0.945	0.190	UD	49.65

UD — undetectable.

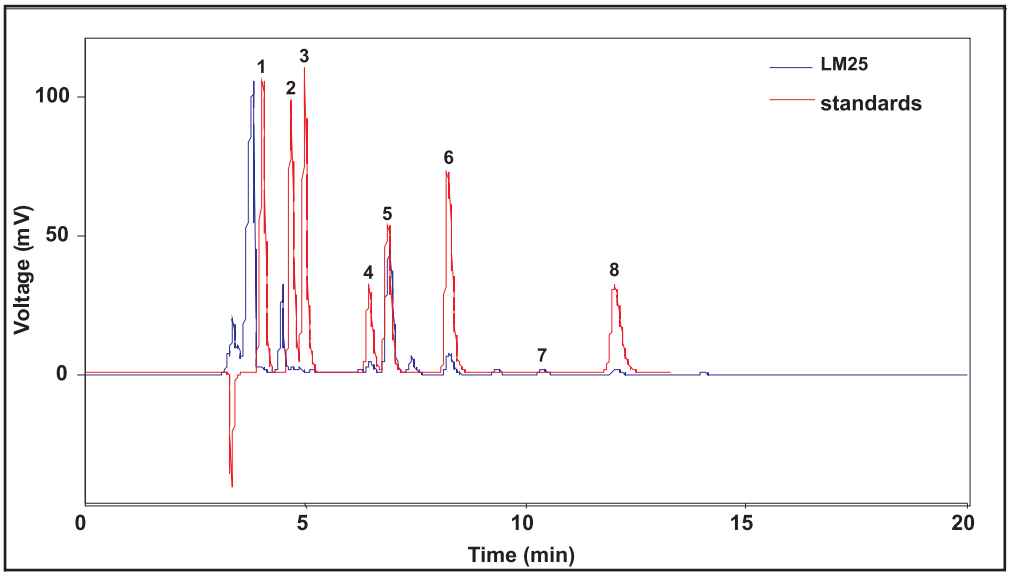


Fig. 2. Chromatogram of standards (1 — lactose, 2 — glucose, 3 — galactose, 4 — succinic acid, 5 — lactic acid, 6 — acetic acid, 7 — propionic acid, 8 — ethanol) and of the sample LM25 cultivated in broth.

the cell to a degree depending on the intracellular pH. H^+ ions released during the dissociation are reported to acidify the cytoplasm to cause collapse of the electrochemical proton gradient, resulting in bacteriostasis and eventual death of the susceptible bacteria (Tharmaraj and Shah, 2009; Piard and Desmazeaud, 1991; Eklund, 1989).

Table 4 shows that all strains produced lactic acid in concentration in broth (2.877—15.282 g/L). They also produced acetic acid (0.696—0.945 g/L), succinic acid (0.187—0.421 g/L) and ethanol (8.462—12.286 g/L) except strain *Pediococcus* sp. G5 (production of ethanol was undetectable). Chromatogram of standards and of the sample LM25 cultivated in broth is illustrated in Figure 2. Lactic acid was the main organic acid in case of tested strains.

Conclusion

Antimicrobial activity of 4 tested cocci (*Lactococcus lactis* ZS25, *Lactococcus lactis* LM25, *Streptococcus thermophilus* M37, *Pediococcus* sp. G5) against used indicator microorganisms was the largest in the sample G5. The growth of *Asperglillus flavus*, *Penicillium funiculosum* and *Rhizopus oryzae* was not inhibited by all of tested cocci. Cocci produced lactic acid, acetic acid and succinic acid during growth in broth. Production of phenyllactic acid from phenylalanine as precursor during the 72 hours was confirmed by *Pediococcus* sp. (49.65 mg/L). The samples ZS25, LM25 and M37 did not produce phenyllactic acid.

In this study, we could show that various LAB strains are able to produce organic acids and to inhibit the growth of some pathogenic bacterial strains.

Acknowledgement

This work was supported by grant APVV no. 07/0158 and by the Slovak State Committee for Scientific Research VEGA, grant 1/0879/12.

References

Eklund T (1989) Elsevier Applied Sciences 196—200.

Izco JM, Tormo M, Jiménez-Flores R (2002) Journal of Agriculatural and Food Chemistry 50: 1765—1773.

Jamuna M, Jeevaratnam K (2004) Applied Microbiology and Biotechnology 65: 433—439.

Jeanson S, Hilgert N, Coquillard MO, Seukpanya C, Faiveley M, Neveu P, Abraham CH, Georgescu V, Fourcassié P, Beuvier E (2009) International Journal of Food Microbiology 131: 75—81.

Kieronczyk A, Skeie S, Langsrud T, Yvon M (2003) Applied and Enviromental Microbiology 69: 734—739.

Lavermicocca P, Valerio F, Visconti A (2003) Applied and Enviromental Microbiology 69: 634—640.

Liu S, Han Y, Zhou Z (2011) Food Research International 44: 643—651.

Magnusson J, Ström K, Roos S, Sjögren J, Schnürer (2003) FEMS Microbiology Letters 219: 129—135.

Mezaini A, Chihib NE, Bouras AD, Nedjar-Arroume N, Hornez JP (2009) Journal of Environmental and Public Health Article ID 678495: 6 p.

Nieto-Lozano JC, Reguera-Useros JI, Peláez-Martínez MC, Sacristán-Pérez-Minayo G, Gutiérrez-Fernández AJ, Hardisson de la Torre A (2010) Food Control 21: 679—685.

Piard JC, Desmazeaud M (1991) Lait 71: 525—541.

Rattanachaikunsopon P, Phumkhachorn P (2010) Annals of Biological Research 1: 218–228.
Silva J, Carvalho AS, Teixeira P, Gibbs PA (2002) Letters in Applied Microbiology 34: 77–81.
Tadesse G, Ephraim E, Ashenafi M (2005) Internet Journal of Food Safety 5: 13–20.
Tharmaraj N, Shah NP (2009) International Food Research Journal 16: 261–276.
Valerio F, Lavermicocca P, Visconti A, Pascale M (2004) FEMS Microbiology Letters 233: 289–295.
Wong HC, Chen YL (1988) Applied and Enviromental Microbiology 54: 2179–2184.
Yvon M, Berthelot S, Gripon JC (1998) International Dairy Journal 8: 889–898.
Yvon M, Thirouin S, Rijnen L, Fromentier D, Gripon JC (1997) Applied and Enviromental Microbiology 63: 414–419.