SHORT COMMUNICATION

Antimicrobially active lactobacilli from goats' milk that do not produce biogenic amines

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Summary

Strains of lactobacilli isolated from goats' milk, namely, *Lactobacillus casei* 21L10, *Lactobacillus johnsonii* KB2-1 and *Lactobacillus plantarum* 25/1L, were tested for antimicrobial activity and production of biogenic amines. *Lb. johnsonii* KB2-1 accumulated hydrogen peroxide (6.29 $\mu g \cdot ml^{-1}$). No strain produced acetic acid. Strains *Lb. casei* 21L10 and *Lb. johnsonii* KB2-1 converted glucose into lactic acid at highest levels (19.84 $g \cdot l^{-1}$ and 19.89 $g \cdot l^{-1}$, respectively) after 48 h of cultivation, but specific production rates were the highest for *Lb. johnsonii* KB2-1 (1.42 $g \cdot l^{-1} \cdot h^{-1}$) and *Lb. plantarum* 25/1L (1.50 $g \cdot l^{-1} \cdot h^{-1}$). *Lb. johnsonii* KB2-1 and *Lb. plantarum* 25/1L were more efficient producers of phenyllactic acid (116.63 $mg \cdot ml^{-1}$ and 108.40 $mg \cdot ml^{-1}$, respectively) than *Lb. casei* 21L10 (66.43 $mg \cdot ml^{-1}$) after 72 h of cultivation. All strains considerably inhibited undesirable moulds, and *Lb. casei* 21L10 and *Lb. plantarum* 25/1L even more significantly inhibited undesirable and pathogenic bacteria. None of the strains produced putrescine, tyramine or histamine, while cadaverine was produced only by *Lb. johnsonii* KB2-1 on a low level (8.47 $mg \cdot l^{-1}$). Antimicrobial effects of these lactobacilli, which produce low amounts of biogenic amines, may prevent formation of biogenic amines by contaminating microorganisms in milk processing.

Keywords

lactobacilli; goats' milk; antimicrobial activity; lactic acid; phenyllactic acid; hydrogen peroxide; biogenic amines

In 2013, the total number of goats in Slovakia was estimated at 34 823 heads [1]. Goats' milk is not recommended to be processed without heat treatment because Slovakia is a country with a high risk of tick-borne encephalitis that can be transmitted via consumption of raw goats' milk [2]. Goats' milk is therefore marketed as fresh milk (pasteurized or UHT-treated), or as various processed products like yoghurts or cheeses. In production of these fermented milk products, only commercial starters are used, which is the same as in processing of cows' and ewes' milk. This leads to a lack of diversity of autochthonous lactic acid bacteria that are potentially beneficial to consumers by unique sensory, probiotic or protective properties [3, 4].

In our previous study [5], isolation, identification and processing characterization of lactobacilli from raw goats' milk were described. The strains were used in Slovakia for the first time in the manufacture of new types of original acidified milk and cheese products with unique organoleptic characteristics. The aim of this work was to investigate the protective effect of these strains against several pathogens, eventually, undesirable microorganisms, which may occur in the dairy processes as a primary or secondary contamination, and some of them may form biogenic amines. Metabolites that may be responsible for the antimicrobial effect, namely, hydrogen peroxide, lactic acid, acetic acid and phenyllactic acid, were determined. Likewise, the production of biogenic

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amines by the strains was studied. Cultures that do not produce biogenic amines, while suppressing the growth of microbial contaminants, are promising to improve food safety in processing of cheeses from goats' milk. This was the crucial criterion of evaluation of lactobacilli in this study.

MATERIAL AND METHODS

The strains used in the study were *Lactobacillus casei* 21L10, *Lactobacillus johnsonii* KB2-1 and *Lactobacillus plantarum* 25/1L, which were previously identified by 16S rRNA-oriented polymerase chain reaction [5].

Hydrogen peroxide formation was detected qualitatively on de Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany) with horseradish peroxidase Type VI-A and 3,3',5,5' tetramethylbenzidine (all from Sigma-Aldrich, St. Louis, Missouri, USA) on the basis of formation of blue coloured colonies [6], and quantitatively with horseradish peroxidase Type VI-A and dianizidine (Sigma-Aldrich) by spectrophotometric detection of yellow-orange colour [7].

The growth of lactobacilli in MRS broth (Merck) was examined by measuring the optical density of the culture at $\lambda = 600$ nm. Acetic, lactic and phenyllactic acids, glucose, putrescine, tyramine, histamine and cadaverine were detected after cultivation of the strains (37 °C) in MRS broth by high pressure liquid chromatography methods [8, 9]. In the case of phenyllactic acid, the culture broth was enriched with 0.1% of 2-phenylalanine (Merck) and, in the case of biogenic amines, with 0.1% histidine and tyrosine (all from Merck) .

Specific growth rates of lactobacilli (μ_G), as well as specific production rates of lactic acid formation (μ_{LA}), were calculated by Origin 8.1 software (OriginLab, Northampton, Massachusetts, USA).

The antimicrobial activity of lactobacilli was tested against bacterial (Tab. 1) and mould (Tab. 2) strains by modified bi-layer agar method [10] on the basis of measuring cleared inhibition zones in comparison with the whole area of the Petri dish, by expression of the percentage of inhibition.

For evaluation of interactions between lactobacilli and growth of undesirable microorganisms, the variability $x \pm$ standard deviation was defined as the parameter for efficacy of inhibition of lactobacilli against the relevant set of microorganisms. The differences between means for the mentioned parameters and pairwise combinations of lactobacilli were tested by *t*-test criterion. Each measurement mentioned above was performed in duplicate, the means were calculated and reported in the results.

RESULTS AND DISCUSSION

Lactobacilli may form hydrogen peroxide. Both qualitative [6] and quantitative [7] methods confirmed that, from the tested strains, *Lb. johnsonii* KB2-1 was the best producer, producing 6.29 $\mu g \cdot ml^{-1}$ of hydrogen peroxide during a 24-h cultivation at 37 °C, and subsequent incubation at 5 °C. This hydrogen peroxide concentration was higher than that reported by Smetanková et al. for *Lb. plantarum* K816 2.30 $\mu g \cdot ml^{-1}$ [11]. Lavilla-Lerma et al. [4] reported formation of hydrogen peroxide in 38.9% *Lb. plantarum* strains and in

Tab. 1. Antimicrobial activity of lactobacilli against bacteria.

Strain	*Lb. casei* 21L10	*Lb. johnsonii* KB2-1	*Lb. plantarum* 25/1L
	Inhibition [%]		
Escherichia coli CCM 3988	24	7	18
Bacillus cereus OPT	21	6	18
Bacillus subtilis CCM 2216	21	7	15
Staphylococcus aureus CCM 3953	18	6	15
Listeria monocytogenes NCTC 4886	16	4	13
Pseudomonas aeruginosa CCM 3955	23	6	15
$x \pm SD$ ($n = 6$)	20.50 ± 2.75	6.00 ± 1.00	15.67 ± 1.80

($x \pm SD$) – the mean of inhibition data ± standard deviation. CCM – Czech Collection of Microorganisms (Brno, Czech Republic), OPT – Collection of Department of Food Science and Technology, Slovak Technical University (Bratislava, Slovakia), NCTC – National Collection of Type Cultures (Salisbury, United Kingdom).
Differences: *Lb. casei* 21L10 – *Lb. johnsonii* KB2-1, $t = 11.07$, $p < 0.001$; *Lb. casei* 21L10 – *Lb. plantarum* 25/1L, $t = 3.29$, $p < 0.01$; *Lb. johnsonii* KB2-1 – *Lb. plantarum* 25/1L, $t = 10.52$, $p < 0.001$).

Tab. 2. Antimicrobial activity of lactobacilli against moulds.

Strain	*Lb. casei* 21L10	*Lb. johnsonii* KB2-1	*Lb. plantarum* 25/1L
	Inhibition [%]		
Alternaria alternata CCM F-128	19	30	31
Aspergillus flavus CCM F-171	9	22	22
Cladosporium herbarum CCM F-159	41	24	30
Mucor rouxii CCM F-220	13	13	10
Penicillium purpurogenum CCM F-257	19	22	13
Rhizopus oryzae CCM 8284	22	22	22
$x \pm SD$ ($n = 6$)	20.50 ± 10.13	22.17 ± 4.98	21.33 ± 7.82

($x \pm SD$) – the mean of inhibition data ± standard deviation. CCM – Czech Collection of Microorganisms (Brno, Czech Republic). Differences: *Lb. casei* 21L10 – *Lb. johnsonii* KB2-1, $t = 0.33$, $p > 0.05$; *Lb. casei* 21L10 – *Lb. plantarum* 25/1L, $t = 0.15$, $p > 0.05$; *Lb. johnsonii* KB2-1 – *Lb. plantarum* 25/1L, $t = 0.2$, $p > 0.05$.

9.1% *Lb. paracasei* strains isolated from the Spanish farmers' goats' cheese. Accumulation of hydrogen peroxide at refrigeration temperatures is important, because it strengthens the inhibitory effect on the growth of the contaminating psychotrophic microflora, which may produce biogenic amines [12, 13].

All three tested lactobacilli converted glucose into lactic acid very efficiently. The formation of acetic acid was not observed. After 48 h of cultivation, residual glucose (0.29 g·l^{-1}) was demonstrated in the medium only in the case of *Lb. plantarum* 25/1L, which produced the least lactic acid (16.49 g·l^{-1}) from the strains. In cultures of *Lb. casei* 21L10 and *Lb. johnsonii* KB2-1, the final concentrations of lactic acid were higher (19.84 g·l^{-1} and 19.89 g·l^{-1}). The value of μ_{LA} nearly corresponded with the μ_G, as for *Lb. johnsonii* KB2-1 (1.42 g·l^{-1}·h^{-1} and 0.29·h^{-1}) and *Lb. plantarum* 25/1L (1.50 g·l^{-1}·h^{-1} and 0.28·h^{-1}) these rates were greater in comparison to *Lb. casei* 21L10 (0.94 g·l^{-1}·h^{-1} and 0.24 h^{-1}). Although the acidic pH is optimal for bacterial decarboxylases, which are the crucial enzymes in the formation of biogenic amines, a rapid decrease in pH can reduce the growth of decarboxylating microorganisms [12, 14]. The protective effect of lactic acid is based on the decline in pH when the non-dissociated form is inhibitory to several technologically undesirable or pathogenic bacteria [15].

Lb. johnsonii KB2-1 and *Lb. plantarum* 25/1L were significantly more effective in phenyllactic acid production [16] (producing it at rates of 116.63 mg·l^{-1} and 108.40 mg·l^{-1}, respectively) in comparison with *Lb. casei* 21L10 (producing it at a rate of 66.43 mg·l^{-1}) after 72 h of anaerobic cultivation at 37 °C. Phenylalanine, as the precursor, is commonly found in goats' milk [17], so the amount of phenyllactic acid formed during fermentation by lactobacilli significantly depends on the strain. Valerio et al. [18] tested 29 strains belonging to 12 species of lactic acid bacteria for the production of phenyllactic acid. In the case of the genus *Lactobacillus*, the highest recorded concentration was 76.44 mg·l^{-1}, which was reached by *Lb. brevis* ATCC 14869. Smetanková et al. [11] evaluated 34 *Lb. paracasei* and *Lb. plantarum* strains for the production of phenyllactic acid and found the highest concentration of 101.62 mg·l^{-1} produced by *Lb. plantarum* 816K.

Tab. 1 and Tab. 2 show results of the antagonistic action of lactobacilli against selected undesirable or pathogenic microorganisms, which are hazardous as contaminants [19–21] or biogenic amines producers [22, 23]. *Lb. casei* 21L10 and *Lb. plantarum* 25/1L inhibited the bacteria stronger (Tab. 1; $p < 0.001$) than *Lb. johnsonii* KB2-1. However, in comparison with the two, *Lb. casei* 21L10 was more effective ($p < 0.01$). The differences in the inhibitory effect of the set of lactobacilli on moulds were not significant (Tab. 2; $p > 0.05$). The strongest inhibition was found for *Lb. casei* 21/L10 against *Cladosporium herbarum* CCM F-159 (41%). Antimicrobial activity of lactobacilli is mainly important for long-ripening cheeses [24].

The tested lactobacilli had low proteolytic activity [8], which is an advantage for avoiding the production of biogenic amines [25]. Moreover, even with the availability of precursors and ideal growth conditions (MRS medium supplemented with histidine and tyrosine, cultivation at 37 °C), *Lb. casei* 21L10 and *Lb. plantarum* 25/1L did not produce putrescine, tyramine, cadaverine or histamin during 48 h. Only *Lb. johnsonii* KB2-1 produced small amounts of cadaverine, its concentration reaching 8.47 mg·l^{-1}. Buňková et al. reported higher contents of tyramine, putrescine and cadaverine in cheeses made from ewes' milk or in longripened cheeses, as in 10% of the tested cheeses the sum of biogenic amines and polyamines was higher than 200 mg·kg^{-1} [26].

CONCLUSION

Lactobacillus casei 21L10 and *Lb. plantarum* 25/1L seem promising strains for application in the production of cheeses from goats' milk. They effectively suppress growth of possible microbial contaminants, bacteria and moulds, and do not produce biogenic amines, which is an important factor for preventing their accumulation in dairy products. *Lb. johnsonii* KB2-1, as the best producer of hydrogen peroxide and phenyllactic acid, exhibited a strong antifungal activity.

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