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TOLERANCE OF AUTOCHTHONOUS LACTIC ACID BACTERIA TO DIFFERENT PROCESSING CONDITIONS *IN VITRO*

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Abstract: In this paper, the effect of different temperatures, pH, and NaCl concentration on the growth of autochthonous lactic acid bacteria isolated from traditionally made Serbian cheese (Sokobanja area) was investigated by using the spectrophotometric method. Growth of tested *Lactobacillus* (*Lb. fermentum*, *Lb. plantarum*, and *Lb. brevis*) and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* five isolates were better in acidic pH, while the growth of *Enterococcus* isolates (*E. durans*, *E. faecium*, and *E. faecalis*) was better in basic pH, at 37 °C. At 4 °C after 24 h, none of the tested bacteria showed growth. Since the autochthonous isolates were tolerant to a tested range of dairy processing conditions, further studies need to include the characterization of enzymatic activity of selected isolates, as well as the ability to use these isolates like starter cultures or food supplements in dairy or non-dairy products.

Key words: *lactic acid bacteria, cheese microbiology, processing conditions, growth ability*

INTRODUCTION

Southeastern Serbia is a specific geographical area, which had a long-time tradition of specific manufacturing artisanal dairy products. In this region, inhabitants produce dairy products by spontaneous milk fermentation, without the addition of bacterial starter cultures. The products made in this way represent a source of autochthonous bacterial strains that could have probiotic potential and serve as natural starter cultures. One of the traditionally made fermented products from this area is Sokobanja cheese, made in Southeastern Serbia (Sokobanja area) (Uroić et al., 2014; Muruzović, Mladenović, Žugić Petrović & Čomić, 2018a).

In the process of cheese making, bacterial starter culture needs to be tolerant to a variety of processing conditions, including differences in temperature, salinity, and acid environment (Rao, Pintado, Stevens & Guyot, 2004; Gutiérrez-Méndez et al., 2010). Also, the tolerance to the gastrointestinal conditions is one of the major requirements for auto-chthonous bacteria to be considered as probiotics (Hernandez-Hernandez et al., 2012). Ibourahema, Dauphin, Jacqueline & Thonart (2008) indicated that the capability of lactic acid bacteria (LAB) to grow at high temperatures is a desirable characteristic because a high fermentation temperature decreases contamination by other microorganisms. Several studies showed that temperature and pH have a great influence on the growth of LAB and that optimal pH for the growth of LAB was in the range from 6.3 to 6.9 (Adamberg, Kask, Laht & Paalme, 2003; Muruzović, Mladenović & Čomić, 2018b). According to Fontana, Bermudez-Brito, Plaza-

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Diaz, Muñoz-Quezada & Gil (2013), isolates of *Lactobacillus* spp. showed tolerance to low pH. Menconi et al. (2014) indicated that LABs were tolerant to pH 3.0 and in the presence of 6.5% of NaCl. According to Mohd Adnan & Tan (2007), high osmosis tolerance is a desirable characteristic of LABs to be used as commercial strains in the dairy industry.

The isolates tested in this study present a part of the unexplored microflora of cheese from Serbia. Since the cheese is produced from raw cow's milk in local households, this study aimed to optimize some processing conditions (different temperatures, pH, and NaCl concentrations) for the growth of isolated LAB. Also, the aim was to examine in which combination of factors their growth is higher.

MATERIALS AND METHODS

LAB isolates

The effects of different temperature, pH, and salt concentration (NaCl) were tested against 7 species of autochthonous lactic acid bacteria (13 isolates) isolated from Sokobanja cheese (Southeastern Serbia): *Enterococcus durans* KGPMF10, *E. faecium* KGPMF14, *E. faecalis* KGPMF47, *E. faecalis* KGPMF48, *E. faecalis* KGPMF49, *Lactobacillus fermentum* KGPMF28, *Lb. brevis* KGPMF35, *Lb. plantarum* KGPMF62, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* KGPMF50, *L. lactis* subsp. *lactis* biovar. *diacetylactis* KGPMF54, *L. lactis* subsp. *lactis* biovar. *Diacetylactis* KGPMF55, *L. lactis* subsp. *lactis* biovar. *diacetylactis* KGPMF57, *L. lactis* subsp. *lactis* biovar. *diacetylactis* KGPMF59. These isolates were identified by using API 50CH and Microgen Strep ID tapes for preliminary identification. The final identification was done by using a MALDI-TOF mass spectrophotometry (Muruzović, Mladenović, Žugić Petrović & Čomić, 2018a; Muruzović, Mladenović, Đilas, Stefanović & Čomić, 2018c; Grujović, Mladenović, Žugić Petrović & Čomić, 2019). *E. faecalis* ATCC 29211 (standard strain) and *Lb. plantarum* LP 299v (commercial probiotic strain) were used for comparison of results. All isolates from Sokobanja cheese, as well as standard and probiotic strains were provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The bacterial strains were kept in glycerol stock at -80 °C until their use.

Assay for determination of the effect of different temperatures, pHs, and salt concentrations on the growth of LAB

Determination of the effect of different environmental conditions on the growth of bacteria was performed according to Thayer, Muller, Buchanan & Phillips, (1987), with some modifications described in detail in Muruzović, Mladenović & Čomić (2018b). The growth was measured at a spectrophotometer at 600 nm.

The effect of different temperatures on the growth of LAB was examined as follows: in 1 ml of MRS broth (Torlak, Belgrade, Serbia), it was added 10 µl of initial bacterial suspension (contained 10^8 colony-forming units CFU/ml). The turbidity of the initial suspension was adjusted using a 0.5 McFarland densitometer (Biosan, Latvia). Three samples were prepared, each for one tested temperature (4 °C, 20 °C, and 37 °C). These temperatures were selected because the temperature at 4 °C is a temperature of cheese storage in the refrigerator, the temperature of Sokobanja cheese making and preservation in the household is at 20 °C and the temperature for optimal bacterial growth in most cases is at 37 °C. Samples were incubated for 24 h. Each experiment was performed in triplicate. Sterility control was uninoculated MRS broth.

The effect of different pH in different temperatures was examined in modified MRS broths. By adding a concentrated HCl (Zorka Pharma, Šabac, Serbia), it was obtained acidic media (pH 5.5) and with adding a 30% NaOH (Zorka Pharma, Šabac, Serbia), it was obtained neutral and basic media of MRS (pH 7, 7.5, and 8.5). Growth control was at pH 6.5 (pH of pure MRS broth). In 1 ml of each type of modified media, it was added 10 µl of the initial bacterial suspension. Samples were incubated at 4 °C, 20 °C, and 37 °C for 24 h. Each experiment was performed in triplicate. Sterility controls were uninoculated modified MRS broths, prepared in every tested pH value. To examine the effect of different NaCl concentrations on the growth of tested LAB, MRS broths were prepared, modified with the addition of different concentrations of NaCl (Zorka Pharma, Šabac, Serbia) (w/v) (4%, 6.5%, 8%). In 1 ml of modified media, it was added 10 µl of initial bacterial suspension (contained 10^8 colony-forming units CFU/ml). Samples were incubated at 4 °C, 20 °C, and 37 °C for 24 h. Each experiment was

performed in triplicate. Sterility controls were uninoculated modified MRS broths, prepared in each tested salt concentration.

Statistical analysis

All data were presented as means ± standard deviations using Microsoft Excel (Redmond, Washington, DC, USA). Paired – Samples T-test was used to compare the influence of temperatures on the growth of each particular isolate. Data were analyzed using SPSS version 20 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

In this paper, it was investigated the effect of some dairy processing conditions (different temperatures, pH, and NaCl concentrations) on the growth of LABs, previously isolated from traditionally made Sokobanja cheese (South-eastern Serbia). LABs were incubated in standard and modified MRS media, at three different temperatures (4 °C, 20 °C, 37 °C), for 24 h. After incubation, it was noticed that there was no growth at 4 °C. The rest of the results are presented below, in Tables 1 - 4.

Tested LABs showed better growth in acidic media, at 37 °C, compared with growth at 20 °C ($P < 0.05$). *Lactobacillus* and *Lactococcus* isolates showed the growth ability at both tested temperatures. Besides, growth at 37 °C was better. Further, it was noticed that, at 20 °C, growth was better at pH 7 than in growth control (pH 6.5) (Table 1).

Tested *Enterococcus* isolates showed the growth ability in all tested pH, at both tested temperatures (Table 2), but their growth was better in basic pH. Based on the results, it could be concluded that the temperature had a greater impact, compared to the pH ($P < 0.05$).

Different concentrations of NaCl showed an inhibitory effect on the growth of tested isolates of LABs (Tables 3 and 4). The growth of all isolates was reduced in the presence of 4% of NaCl, at both tested temperatures. Different NaCl concentrations showed a significant influence on bacterial growth at 20 °C when compared to the same concentrations at 37 °C ($P < 0.05$). Tested *Lactobacillus* and *Lactococcus* isolates showed a tolerance up to 6.5% of NaCl (Table 3), while tested *Enterococcus* isolates showed tolerance to the presence of NaCl up to 8%, especially at 37 °C (Table 4).

The process of cheese ripening is very complex, in which the typical cheese characteristics (flavor and texture) are formed by the action of numerous enzymes derived from the milk, the rennet, the starter culture bacteria, and the non-starter bacteria (Fox, Guinee, Cogan & Mcsweeney, 2017). Most of the methods accelerated to cheese ripening is focused on enhancing proteolysis and lipolysis by for instance ripening at elevated temperatures, the addition of enzymes (e.g. lipases, proteinases, and peptidases), using a well-known starter culture, associated culture and genetically modified cultures (Van Mastrigt, Gallegos Tejeda, Kristensen, Abee & Smid, 2018). Van Mastrigt, Gallegos Tejeda, Kristen-sen, Abee & Smid (2018) also indicated that it is interesting to study aroma formation outside the cheese matrix, from a scientific and a technological perspective because such studies could help to optimize or steer to aroma formation by LAB applied as food supplements in dairy or dairy-like products. It is important that, in the process of Sokobanja cheese making, it was not added any bacterial starter culture. A liquid rennet of microbiological origin based on chymosin obtained from the fungi *Rhizomucor miehei* and *Mucor miehei* was used for milk coagulation (Muruzović, Mladenović, Žugić Petrović & Čomić, 2018a). Therefore, it could be assumed that tested isolates of LAB present a part of the autochthonous community of cheese, originated from raw milk and the endogenous culture. According to the results described in Muruzović, Mladenović, Žugić Petrović & Čomić, (2018a) and Muruzović, Đilas, Stefanović & Čomić (2018c) the chese samples were three days old and it belongs to the group of full-fat, acid-curd soft cheese groups. In the process of cheese production, the cheese was salted (6-8%), based on the total weight of the cheese (Muruzović, Mladenović, Žugić Petrović & Čomić, 2018a; Muruzović, Mladenović, Đilas, Stefanović & Čomić, 2018c). These results were the first reason why we tested the influence of the chosen pH and NaCl concentrations on the growth of selected bac-teria. Also, it is well-known that the tolerance to pH is one of the major criteria for LAB to be considered as probiotics and the tolerance to different processing conditions is important for the possible use of LABs as starter cultures in the dairy industry (Zago et al., 2011; Kavitha & Devasena, 2013).

Table 1.
The effect of different pH and temperatures on the growth of isolated *Lactobacillus* spp. and *Lactococcus* spp.

	pH	**5.5**		**6.5***		**7.0**		**7.5**		**8.5**	
	Temperature	**20 °C**	**37 °C**	**20 °C**	**37 °C**	**20 °C**	**37 °C**	**20°C**	**37 °C**	**20 °C**	**37 °C**
Species	**Isolate**										
Lb. fermentum	KGPMF28	0.22 ± 0.00^{a} (63.67)	1.70 ± 0.04^{b} (97.7)	0.33 ± 0.00^{c}	1.74 ± 0.08^{b}	0.41 ± 0.01^{d} (124.24)	1.93 ± 0.02^{e} (110.92)	0.36 ± 0.02^{f} (109.09)	0.80 ± 0.01^{g} (45.98)	0.04 ± 0.00^{h} (12.12)	0.65 ± 0.05^{i} (37.36)
Lb. brevis	KGPMF35	0.23 ± 0.01^{a} (53.49)	1.98 ± 0.02^{b} (95.65)	0.43 ± 0.02^{c}	2.07 ± 0.03^{d}	0.49 ± 0.02^{e} (113.95)	2.05 ± 0.02^{d} (99.03)	0.38 ± 0.00^{e} (88.37)	1.90 ± 0.00^{f} (91.79)	0.17 ± 0.00^{g} (39.53)	1.66 ± 0.02^{h} (80.19)
Lb. plantarum	KGPMF62	0.13 ± 0.03^{a} (29.55)	1.98 ± 0.02^{b} (97.06)	0.44 ± 0.03^{c}	2.04 ± 0.00^{d}	0.40 ± 0.02^{c} (90.91)	2.00 ± 0.03^{d} (98.04)	0.28 ± 0.00^{e} (63.64)	1.83 ± 0.02^{f} (89.71)	$0.11 \pm 0.00^{g,a}$ (25)	1.69 ± 0.03^{h} (82.84)
	LP 299v	0.25 ± 0.00^{a} (86.21)	0.62 ± 0.02^{b} (126.53)	0.29 ± 0.02^{c}	0.49 ± 0.02^{d}	0.30 ± 0.00^{c} (103.45)	0.70 ± 0.01^{e} (142.86)	0.30 ± 0.00^{c} (103.45)	0.67 ± 0.01^{e} (136.74)	0.01 ± 0.00^{f} (3.15)	0.09 ± 0.02^{g} (18.37)
L. lactis subsp. *lactis* biovar. *diacetylactis*	KGPMF50	0.09 ± 0.00^{a} (39.13)	1.39 ± 0.28^{b} (118.80)	0.23 ± 0.02^{c}	1.17 ± 0.01^{d}	0.24 ± 0.00^{c} (104.35)	1.96 ± 0.14^{e} (167.52)	0.19 ± 0.02^{f} (82.61)	1.68 ± 0.02^{g} (143.59)	0.14 ± 0.01^{h} (60.87)	0.64 ± 0.02^{g} (54.70)
	KGPMF54	0.12 ± 0.01^{a} (70.59)	2.03 ± 0.02^{b} (100.50)	0.17 ± 0.02^{c}	2.02 ± 0.03^{b}	0.19 ± 0.00^{c} (111.76)	1.97 ± 0.00^{d} (97.52)	$0.14 \pm 0.00^{e,a}$ (82.35)	1.86 ± 0.00^{f} (92.08)	0.09 ± 0.00^{g} (52.94)	1.69 ± 0.03^{h} (83.67)
	KGPMF55	0.06 ± 0.02^{a} (23.08)	1.23 ± 0.00^{b} (63.73)	0.26 ± 0.02^{c}	1.93 ± 0.01^{d}	0.26 ± 0.03^{c} (100)	1.79 ± 0.35^{e} (92.75)	0.23 ± 0.02^{f} (88.46)	$1.76 \pm 0.01^{g,e}$ (91.19)	0.18 ± 0.01^{h} (69.23)	1.30 ± 0.08^{i} (67.36)
	KGPMF57	0.21 ± 0.02^{a} (95.45)	2.03 ± 0.02^{b} (99.51)	0.22 ± 0.01^{a}	2.04 ± 0.00^{b}	0.19 ± 0.00^{a} (86.36)	1.98 ± 0.03^{c} (97.06)	0.14 ± 0.01^{d} (63.64)	$1.87 \pm 0.02^{e,c}$ (91.67)	0.11 ± 0.00^{f} (50)	1.72 ± 0.00^{g} (84.31)
	KGPMF59	0.23 ± 0.01^{a} (88.46)	1.98 ± 0.02^{b} (97.06)	0.26 ± 0.01^{a}	2.04 ± 0.00^{c}	0.25 ± 0.02^{a} (96.15)	$1.98 \pm 0.03^{d,b}$ (97.06)	0.24 ± 0.00a (92.31)	1.86 ± 0.00^{e} (91.18)	0.12 ± 0.00^{f} (46.15)	1.71 ± 0.03^{g} (83.82)

Values are expressed as means ± standard deviation measured on 600 nm; the percent of growth was given in brackets;
Means in the two temperature columns or each particular isolate, with superscript with different letters, are significantly different at P<0.05;
**Growth control (100% of growth)*

Table 2.
The effect of different pH and temperatures on the growth of isolated *Enterococcus* spp.

pH		**5.5**		**6.5***		**7.0**		**7.5**		**8.5**	
Temperature		**20 °C**	**37 °C**	**20 °C**	**37 °C**	**20 °C**	**37 °C**	**20 °C**	**37 °C**	**20 °C**	**37 °C**
Species	**Isolate**										
E. durans	KGPMF10	0.09 ± 0.01^{a} (14.06)	1.16 ± 0.01^{b} (68.64)	0.64 ± 0.02^{c}	1.69 ± 0.01^{d}	0.63 ± 0.00^{c} (98.44)	1.72 ± 0.01^{d} (101.77)	0.52 ± 0.02^{e} (81.25)	1.81 ± 0.01^{f} (107.10)	0.44 ± 0.02^{g} (68.75)	1.65 ± 0.02^{h} (97.63)
E. faecium	KGPMF14	0.01 ± 0.00^{a} (11.11)	0.19 ± 0.00^{b} (38.78)	0.09 ± 0.01^{c}	0.49 ± 0.00^{d}	0.16 ± 0.01^{e} (177.78)	0.55 ± 0.01^{f} (112.24)	0.12 ± 0.00^{g} (133.33)	0.69 ± 0.00^{h} (140.81)	0.11 ± 0.00^{g} (122.22)	0.76 ± 0.00^{i} (161.23)
E. faecalis	KGPMF47	0.07 ± 0.00^{a} (87.5)	0.46 ± 0.02^{b} (95.83)	0.08 ± 0.01^{a}	0.48 ± 0.01^{b}	0.11 ± 0.00^{a} (137.5)	0.67 ± 0.01^{c} (139.58)	0.12 ± 0.01^{a} (150)	0.79 ± 0.00^{d} (164.58)	0.09 ± 0.01^{a} (112.5)	0.81 ± 0.01^{d} (168.75)
	KGPMF48	0.06 ± 0.00^{a} (50)	0.28 ± 0.03^{b} (35.44)	0.12 ± 0.02^{c}	0.79 ± 0.01^{d}	0.14 ± 0.01^{c} (116.67)	0.89 ± 0.01^{e} (112.66)	0.16 ± 0.01^{c} (133.33)	1.11 ± 0.01^{f} (140.51)	0.12 ± 0.00^{c} (100)	1.22 ± 0.01^{g} (154.43)
	KGPMF49	0.03 ± 0.00^{a} (16.67)	0.16 ± 0.02^{b} (31.37)	0.18 ± 0.00^{c}	0.51 ± 0.00^{d}	0.16 ± 0.01^{c} (88.89)	0.56 ± 0.00^{d} (109.80)	0.15 ± 0.00^{c} (83.33)	0.68 ± 0.01^{e} (133.33)	0.16 ± 0.00^{c} (88.89)	0.79 ± 0.00^{f} (154.90)
	ATCC 29211	0.01 ± 0.00^{a} (7.69)	0.06 ± 0.01^{b} (12.5)	0.13 ± 0.01^{c}	0.48 ± 0.03^{d}	0.16 ± 0.02^{c} (123.08)	0.63 ± 0.01^{e} (131.25)	0.14 ± 0.00^{c} (107.69)	0.59 ± 0.01^{f} (122.92)	0.14 ± 0.00^{c} (107.69)	0.62 ± 0.02^{f} (129.17)

Values are expressed as means ± standard deviation measured on 600 nm; the percent of growth was given in brackets.
Means in the two temperature columns or each particular isolate, with superscript with different letters, are significantly different at $P<0.05$.
**Growth control (100% of growth).*

Table 3.
The effect of different NaCl concentrations and temperatures on the growth of isolated *Lactobacillus* spp. and *Lactococcus* spp.

% of NaCl		4.0		6.5		8.0	
Temperature		20 °C	37 °C	20 °C	37 °C	20 °C	37 °C
Species	**Isolate**						
Lb. fermentum	KGPMF28	0.06 ± 0.00^{a} (18.18)	0.46 ± 0.02^{b} (26.44)	0.04 ± 0.01^{a} (12.12)	0.12 ± 0.01^{c} (24.14)	n.g.	0.02 ± 0.00^{d} (1.15)
Lb. brevis	KGPMF35	0.32 ± 0.00^{a} (74.42)	1.62 ± 0.01^{b} (78.26)	0.12 ± 0.01^{c} (27.91)	0.22 ± 0.02^{d} (10.63)	n.g.	0.09 ± 0.01^{e} (4.35)
Lb. plantarum	KGPMF62	0.32 ± 0.02^{a} (72.72)	1.70 ± 0.02^{b} (83.33)	0.09 ± 0.00^{c} (20.45)	0.13 ± 0.01^{c,d} (6.37)	n.g.	0.10 ± 0.00^{d} (4.91)
	LP 299v	0.19 ± 0.01^{a} (65.52)	0.45 ± 0.01^{b} (91.84)	0.08 ± 0.00^{c} (27.59)	0.17 ± 0.01^{d} (34.69)	n.g.	0.03 ± 0.00^{e} (6.12)
L. lactis subsp. *lactis* biovar. *diacetylactis*	KGPMF50	0.10 ± 0.00^{a} (43.49)	0.61 ± 0.01^{b} (52.14)	0.05 ± 0.01^{c} (21.74)	0.22 ± 0.01^{d} (18.80)	0.02 ± 0.0^{e,c} (8.70)	0.05 ± 0.01^{f} (4.27)
	KGPMF54	0.08 ± 0.01^{a} (47.06)	1.42 ± 0.01^{b} (70.30)	0.04 ± 0.01^{c} (23.53)	0.11 ± 0.00^{d} (5.45)	0.03 ± 0.00^{c} (17.65)	0.09 ± 0.00^{d} (4.46)
	KGPMF55	0.05 ± 0.01^{a} (31.25)	0.78 ± 0.01^{b} (45.09)	0.02 ± 0.00^{a} (12.5)	0.20 ± 0.01^{c} (11.56)	0.01 ± 0.00^{a} (6.25)	0.03 ± 0.00^{d} (1.73)
	KGPMF57	0.12 ± 0.02^{a} (54.55)	1.69 ± 0.02^{b} (82.84)	0.05 ± 0.01^{c} (22.73)	0.13 ± 0.00^{d} (6.37)	0.02 ± 0.00^{c} (9.09)	0.10 ± 0.00^{d} (4.90)
	KGPMF59	0.14 ± 0.02^{a} (53.85)	1.62 ± 0.02^{b} (79.41)	0.12 ± 0.00^{a} (46.15)	0.16 ± 0.00^{c} (7.84)	0.03 ± 0.00^{d} (11.54)	0.12 ± 0.00^{c} (5.88)

Values are expressed as means ± standard deviation measured on 600 nm; the percent of growth was given in brackets; Means in the two temperature columns or each particular isolate, with superscript with different letters, are significantly different at P<0.05; n.g.-no growth.

Table 4.
The effect of different NaCl concentrations and temperatures on the growth of isolated *Enterococcus* spp.

% of NaCl		4.0		6.5		8.0	
Temperature		20 °C	37 °C	20 °C	37 °C	20 °C	37 °C
Species	**Isolate**						
E. durans	KGPMF10	0.32 ± 0.01^{a} (50)	0.97 ± 0.03^{b} (75.40)	0.11 ± 0.01^{c} (17.19)	0.22 ± 0.00^{d} (13.02)	0.02 ± 0.00^{e} (3.13)	0.08 ± 0.01^{f} (4.73)
E. faecium	KGPMF14	0.06 ± 0.01^{a,f} (66.67)	0.45 ± 0.01^{b} (91.84)	0.03 ± 0.00^{a} (33.33)	0.10 ± 0.01^{c} (20.41)	n.g.	0.03 ± 0.00^{d} (6.12)
E. faecalis	KGPMF47	0.03 ± 0.00^{a} (37.5)	0.40 ± 0.00^{b} (83.33)	0.01 ± 0.0^{a} (12.5)	0.07 ± 0.00^{c} (14.58)	n.g.	0.03 ± 0.00^{d} (6.25)
	KGPMF48	0.09 ± 0.00^{a} (75)	0.31 ± 0.11^{b} (39.24)	0.06 ± 0.00^{a} (50)	0.10 ± 0.01^{c} (12.66)	0.02 ± 0.00^{a} (16.67)	0.03 ± 0.01^{d} (3.80)
	KGPMF49	0.12 ± 0.00^{a} (66.67)	0.43 ± 0.01^{b} (84.31)	0.03 ± 0.00^{c} (16.67)	0.08 ± 0.00^{d} (15.69)	n.g.	0.03 ± 0.00^{e} (5.88)
	ATCC 29211	0.08 ± 0.01^{a} (61.54)	0.43 ± 0.01^{b} (89.58)	0.03 ± 0.00^{c} (23.08)	0.09 ± 0.01^{d} (18.75)	0.01 ± 0.00^{c} (2.08)	0.03 ± 0.00^{e} (6.25)

Values are expressed as means ± standard deviation measured on 600 nm; the percent of growth was given in brackets; Means in the two temperature columns or each particular isolate, with superscript with different letters, are significantly different at P<0.05; n.g.-no growth.

Previously, isolates of LAB showed a good acidification activity in original and enriched milk (Muruzović, Mladenović, Žugić Petrović & Čomić, 2018a; Muruzović, Mladenović & Čomić, 2018b; Grujović, Mladenović, Žugić Petrović & Čomić, 2019). We selected isolates that showed the ability to decrease pH value to almost 4 within 24 h because this was a promising result which indicated the ability to use the selected isolate as starter cultures for food fermentations. They showed the ability to consume sugars from the food matrix converting them to acid via the fast fermentation process. Such produced acids are also attractive criteria for selected strains to be protective cultures where pathogenic bacteria cannot grow, due to a low pH value of the medium.

Some of the research increasingly suggests that the acid resistance of the LAB might be strain-specific and stress-specific. The reason can be found in the genetic diversity of these acid alleviating systems among different strains (Wu, Tun, Law, Khafipour & Shah, 2017; Lyu et al., 2018). *Lb. fermentum* KGPMF29, isolated from Sokobanja cheese, was able to to-

lerate the acidic pH (Muruzović, Mladenović & Čomić, 2018b). Rao, Pintado, Stevens & Guyot (2004) and Muruzović, Mladenović & Čomić (2018b) indicated that *Lb. fermentum* was not tolerant to higher NaCl concentrations. Soliman, Sharoba, Bahlol, Soliman & Radi (2015) showed that *Lb. plantarum* had the ability of tolerance to acidic medium (pH 2.0 and 3.0) and bile salts (0.1, 0.3, 0.5, and 0.7 %), at 37 °C for 24 h. *Lb. plantarum* ATCC 14917 showed the ability of growth in 6% of NaCl (Wang et al., 2016), while *Lb. plantarum* B282 showed a survival rate in 8% of NaCl (Blana, Grounta, Tassou, Nychas & Panagou, 2014), which was confirmed in our study, too. Xia et al. (2017) indicated that optimal NaCl concentration in the medium is 6% because in this concentration *Lb. brevis* AR123 and a commercial starter could rapidly lower the pH of the pickles. The results from our investigation confirmed that tested *Lactobacillus* isolates were more tolerant to acidic pH, but their tolerance to the presence of NaCl concentration higher than 6.5% was low.

Lactococcus lactis subsp. *lactis* is one of the most important starter bacteria used in dairy technology. Its use in the production of dairy products, such as cheese, butter, cream, and fermented kinds of milk is of great economic importance (Yerlikaya, 2019). It is well-known that a starter culture bacterium, *L. lactis* needs to be tolerant to a variety of processing conditions (high or low temperature, osmotic and acid environment) in the process of cheese making (Gutiérrez-Méndez et al., 2010). Velly, Fonseca, Passot, Delacroix-Buchet & Bouix (2014) investigated the effects of fermentation parameters (different temperatures (22 °C, 30 °C and 38 °C) and pH (5.6, 6.2 and 6.8)) on the cell growth and on the tolerance to each step of the freeze-drying process of natural cheese isolate *L. lactis* subsp. *lactis* TOMSC161. They concluded that, in the whey-based medium, *L. lactis* showed the best growth at 32 °C, pH 6.2. Khemariya, Singh, Nath & Gulati (2017) indicated that the growth of *Lactococcus* spp. at higher NaCl concentrations (>4% sodium chloride) vary depending on the species. Muruzović, Mladenović & Čomić (2018b) showed that *L. lactis* subsp. *lactis* KGPMF23 showed the ability of low growth in 6.5% of NaCl, at 37 °C. The results from this paper indicated that tested *Lactococcus* isolates were tolerant to acidic pH, as well as in the different NaCl concentrations, but in concentrations above 6.5%, the growth was reduced. Van Mastrigt, Gallegos Tejeda, Kristensen, Abee & Smid (2018) indicated that aroma production by *L. lactis* was clearly affected by the type of medium and the cultivation method. Therefore, the optimal conditions for the growth of bacteria might be strain-specific and stress-specific.

Enterococci can grow in a wide range of pH, but the optimum for growth is pH 7.5 (Van Den Berghe, De Winter & De Vuyst, 2006). Fisher & Phillips (2009) also indicated that *E. faecalis* showed the ability of growth in 6.5% of NaCl. Some authors indicated that *E. durans*, *E. hirae*, and *E. faecium* strains isolated from raw milk and various dairy products were resistant to low pH and to the different bile NaCl concentrations (Nami et al.,2014; Guo, Li, Tang, Yang & Huo, 2016; Muruzović, Mladenović, & Čomić 2018b; Yerlikaya & Akbulut, 2020). Ivanov, Boytcheva & Mihailova (1999) indicated that temperature influenced *E. faecalis* membrane permeability for salts. In our study, tested *Enterococcus* isolates showed a high tolerance in the basic pH and presence of NaCl. Although the role of bacteria from genus *Enterococcus* in the formation of cheese flavor is proved (Abeijón, Medina, Katz & Gonzalez, 2006), a very few *Enterococcus* strains have been used as food additives because of the safety concern associated with their pathogenic trait as opportunistic microorganisms (Braiek & Smaoui, 2019).

In most cases, traditionally made dairy products are produced by spontaneous milk fermentation, without the addition of any bacterial starter culture. Autochthonous lactic acid bacteria (aLAB), that are present in milk, are responsible for fermentation and quality of dairy products. Most of the research conducted so far confirmed that aLAB has great potential in natural food preservation and good probiotic properties. There are studies which indicated that the adding of natural isolates, with a desirable probiotic's characteristic, in the production of the dairy product have a positive effect on the sensory properties, especially on flavor (Silva et al., 2017: Baher Abd EL Khalek et al., 2018; Santos et al., 2018; Yerlikaya & Akbulut, 2019).

CONCLUSIONS

The tested processing conditions (different temperatures, pH, and NaCl concentrations) showed a different influence on the growth of the tested LAB. After 24 h of incubation, growth at 4 °C was not noticed. Generally, all tested isolates showed the ability of growth in low pH (acidic media), as well as up to 6.5% of NaCl, which is a desirable characteristic for potential starter culture. This paper indicated that the tests showed certain positive technological properties of the tested LAB isolates. This is an important issue because if we use these bacteria like starter cultures, the safety aspect and hygiene of cheese will be increased together with the conservation of traditional heritage, while the aroma and taste will be preserved. Further studies need to include the characterrization of enzymatic activities and amine formation, the effect on texture and organoleptic properties of products of selected isolates, in order to investigate their role in the formation of cheese flavor and taste. Also, *Enterococcus* strains investigated for the potential use must be well characterized and perfectly assessed regarding safety aspects.

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ТОЛЕРАНЦИЈА АУТОХТОНИХ БАКТЕРИЈА МЛЕЧНЕ КИСЕЛИНЕ НА РАЗЛИЧИТЕ УСЛОВЕ ПРЕРАДЕ *IN VITRO*

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Сажетак: У овом раду је испитиван утицај различитих температура, pH и концентрација соли на раст аутохтоних бактерија млечне киселине изолованих из традиционално произведеног српског сира (подручје Сокобање) помоћу спектрофотометријске методе. Раст испитиваних *Lactobacillus* изолата (*Lb. fermentum*, *Lb. plantarum* и *Lb. brevis*), као и пет изолата *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* је био бољи у киселој pH, док је раст изолата *Enterococcus* (*E. durans*, *E. faecium* и *E. faecalis*) био бољи у базној pH, на 37 °C. На 4 °C након 24 часа, ниједна тестирана бактерија није показала способност раста. Будући да су аутохтони изолати били толерантни на тестирани спектар услова прераде млека, даље студије морају да обухвате карактеризацију ензимске активности одабраних изолата, као и могућност употребе ових изолата као стартер култура или додатака исхрани у млечним или немлечним производима.

Кључне речи: *бактерије млечне киселине, микробиологија сира, услови прераде, способност раста*

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