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Indigenous strains of *Lactobacillus* isolated from the Istrian cheese as potential starter cultures

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Abstract

Istrian ewe's milk cheese is an autochthonous product that is manufactured for generations on small family farms in the Croatian peninsula Istria. Traditional Istrian cheese is made from unpasteurized ewe's milk, without the addition of starter cultures. Consequently, the specific flavour and texture of the Istrian cheese is owed to metabolic processes of indigenous microflora of which *Lactobacillus* species play pivotal role. Characterisation and selection of indigenous lactobacilli may result in the potential use of selected strains as starter, bioprotective or even probiotic cultures. This study focuses on potential use of *Lactobacillus plantarum* and *Lactobacillus casei* isolated from traditional Istrian cheese as starter cultures, by using methods that determine their proteolytic, lipolytic, antimicrobial and haemolytic potential, as well as their ability of acidification, autoaggregation and survival in simulated gastrointestinal conditions. Our results indicated that from 12 representative strains most revealed a low or moderate proteolytic activity as well as absence of lipolytic and haemolytic activities. From 12 strains, 5 of them showed a medium to strong acidification ability and lowered the pH of milk below 5.00 after 24 hours of incubation. Furthermore, almost all isolates exhibited antimicrobial activity against *Serratia marcescens*, and lowest number of isolates showed antimicrobial activity against *Staphylococcus aureus* and *Listeria innocua*. The studied *Lactobacillus* strains revealed high survival rate in a simulated oral cavity and duodenum conditions, while the survival ability in a simulated gastric conditions was much lower. Ability to aggregate was low for all tested strains, after 3 hours and after 5 hours of incubation.

Key words: Istrian cheese, *Lactobacillus* spp., starter cultures, antimicrobial activity

Introduction

Traditional Istrian cheese is produced from raw ewe's milk, which is obtained from autochthonous sheep called Pramenka that is breed in Istria (Mulce et al., 2011; Mioč et al., 2012). This cheese is characterized by its unique flavour and texture that derives from the usage of raw sheep milk without the addition of starter cultures. Therefore, its specific characteristics rely on metabolic processes of indigenous microbiota, mostly lactic acid bacteria (LAB) of which lactobacilli play the pivotal role. Lactobacilli are non-sporulating, gram positive, catalase negative rod-shaped bacteria and coccobacilli tolerant to low pH (Claesson et al., 2007; Sieladie et al., 2011). They may be naturally present in milk as falling within the natural microflora of milk (Wouters et al., 2002) and are dominant during the ripening of many artisan cheeses. The technological role of LAB in fermented milk is their ability of acidification (Mäyrä-Mäkinen and Bigret, 2004) and the development of flavour and texture due to processes

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such as metabolism of lactose, lactate, citrate, proteins and lipids. A large number of LAB species, produce lactic acid by taking advantage of various carbohydrates, consequently forming a part of the microorganism present in the human and animal digestive tract. Among LAB, certain strains are used as probiotics in food and pharmaceutical industries. Classified as beneficial for their hosts, probiotics represent an increasing potential in production of varied type of foods. The most commonly used probiotics belong to the species of *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. Apart from the impact on the flavour and texture of cheese, LAB produce bacteriocins that can inhibit not only closely related species but a broad-spectrum of bacteria (Fox et al., 2004). This is well characterized for *Lactobacillus* species as *e.g. Lb. casei*, *Lb. paracasei*, *Lb. plantarum* and *Lb. curvatus* (El Soda et al., 2000; Vasiee et al., 2014).

Since technological and probiotic potential of lactobacilli isolated from the Istrian cheese has not been investigated, it was necessary to thoroughly evaluate their desirable properties, their biotechnological and probiotic potential and assessed their potential application as microbial cultures for the purposes of traditional or industrial cheese production.

Thus, the objectives of this work were to determine qualitative variability of the technological and probiotic potential of 12 cheese strains previously molecularly confirmed as *Lactobacillus casei* or *Lactobacillus plantarum*. The isolates were verified for their proteolytic, lipolytic, haemolytic and acidification activity. Their probiotic potential was assessed in GIT simulated conditions (oral cavity, stomach and intestine). All isolates were tested for their autoaggregation potential as well as for antimicrobial activity against seven types of potential spoilage or pathogenic species commonly found in food.

Material and methods

Lactobacillus isolates

Twelve indigenous cheese isolates belonging to species *Lb. casei* and *Lb. plantarum* were used in this study. Genotypic characterization (RAPD-PCR analysis) was used to identify these strains and the data was published by Skelin et al. (2012). Isolated lactobacilli proceeded from samples of fresh milk, fresh cheese and different stages of cheese ripening obtained from 3 independent family farms from Istria. Used strains were stored in glycerol at -80 °C and activated in Brain Heart Infusion (Biolife, Milano, Italy) liquid medium before analysis.

Survival in a simulated oral cavity

In order to determine the ability of *Lactobacillus* strains to survive in simulated conditions in the oral cavity, a modified method described by Morandi et al., (2013) was used.

All strains were grown in 2 mL of MRS (Biolife, Milano, Italy) liquid medium with addition of 1.5 % skim milk for 24 h at 30 °C. Part of the overnight culture was diluted ten times in 1X PBS buffer. A series of dilutions was made ranging from 10^{-1} to 10^{-5}, and from 10^{-5} dilution, 100 μL was applied to the MRS solid medium. Each strain was tested twice. The plates were incubated at 30 °C for 48 h in anaerobic conditions.

Overnight cultures of the same portion were also diluted ten times in 1X PBS buffer, which was treated with 200 mL of lysozyme (100 ppm). The suspension was incubated 5 min at 30 °C, after which the dilution 10^{-5} was plated on solid MRS medium. The tests were performed twice. The plates were incubated at 30 °C for 48 h in anaerobic conditions. The result is expressed as a proportion (%) of surviving cells after exposure to simulated oral cavity, and is calculated using the formula:

% = (number of surviving colonies) / (number of colonies) x 100

Survival in simulated gastric and intestinal conditions

To determine the survival of *Lactobacillus* strains in simulated gastric and intestinal environment a modified methodology described by Dolereys et al. (2004) were used.

Gastric test

To test the ability of lactobacilli isolates to survive in the stomach, the gastrointestinal conditions were simulated by mixing 0.5 % NaCl and 0.3 % pepsin (Sigma-Aldrich Co, USA) with the pH 2.5, adjusted with 1 M HCl. The solution was sterilized by filtration through a filter of 0.22 μm pore size.

One millilitre of overnight culture was centrifuged for 10 min at 10000 g, after which the pellet was washed twice with 0.1 % peptone water (Biolife, Milano, Italy) and resuspended in 100 mL of 0.1 % peptone. Thirty millilitres of the lactobacilli suspension was transferred to 270 mL of the simulated gastric condition solution and incubated for 40 min at 30 °C. One millilitre of the suspension (corresponding to dilution of -1) served to prepare a series of dilutions (ranging to 10^{-5}), and 100 μL of 10^{-5} dilution was applied to the solid MRS (Biolife, Milano, Italy) medium. Each strain was tested twice. The plates were incubated at 30 °C for 48 h in anaerobic conditions. The result is expressed as a percentage (%) of surviving cells after exposure to simulated gastric conditions, and is calculated according to the formula mentioned above.

Duodenal test

To determine the ability of isolate's survival in duodenal environment, the solution that simulated those conditions were prepared by mixing 0.4 % bile salts (Biolife, Milano, Italy) and 0.2 % pancreatin (Sigma-Aldrich Co, USA). The methodology used in continuation was the same as previously described when gastric test was carried out. The results were obtained by analysing the number of surviving cells after exposure to simulated duodenal conditions, and are calculated according to the above formula.

Autoaggregation assay

The autoaggregation assay was performed according to the method previously described by Del Re et al., (2000) with certain modifications. The cells were harvested from overnight cultures grown in 6 mL of MRS (Biolife, Milano, Italy) medium at 37 °C. The cultures were centrifuged at 10000 *g* at 4 °C for 15 min. The pellets were washed 2 times and resuspended in phosphate buffered saline (PBS 1x). Absorbance was measured at 610 nm and the aimed cell concentration was of $\pm 10^8$ CFU/mL. The cultures were vortexed and incubated at room temperature. The absorbance was measured at 0, 3 and 5 h after the incubation. Autoaggregation was calculated according to the formula:

$$\text{Autoaggregation (\%)} = (1 - (A_t/A_0)) \times 100$$

Where, A_t represents the absorbance at time 3 and 5 h and A_0 the absorbance at time 0.

Acidification activity

One hundred μL of overnight isolate strains culture was passed to a sterile 20 mL of 10 % skim milk solution. The samples were incubated at 30 °C, and the pH was monitoring at 2, 6 and 24 h. The ability of the investigated strains to acidify milk was expressed as the change in pH over time, according to Jamaly et al. (2010): ΔpH = pH (final value) - pH (initial value).

Proteolytic activity

Proteolytic activity of examined *Lactobacillus* strains was measured using two different methods.

The *Lactobacillus* strains were grown in 5 mL of BHI (Biolife, Milano, Italy) liquid medium with 1.5 % skim milk (Biolife, Milano, Italy) and were incubated for 24 h at 30 °C. One aliquot of 2 μL of the overnight culture was plated directly into BHI agar with 1.5 % skim milk and simultaneously, 10 μL of respective culture was added on the sterile cellulose disc previously placed on the agar plates. The plates were incubated at 30 °C for 48 h in anaerobic conditions. Degradation of casein was determined by measuring the halos of decomposition around the disc as well as around directly applied cultures.

Proteolytic activity was also measured using a modified chromogenic method described by Savoy de Giori and Hébert, (2000). The measured concentration of released *p*-nitroanilide (*p*NA) from the peptide (S-Ala) was contributed to the proteinase activity of *Lactobacillus* strains. Investigated strains were grown in 50 mL of MRS (Biolife, Milano, Italy) liquid medium with 1.47 grams of $CaCl_2$, and incubated at 37 °C until an optical density of 1.5 at 600 nm was reached. Then the cultures were centrifuged for 10 min at 10000 g at 4 °C, after which the pellet was washed twice with a saline buffer composed of 10 mM $CaCl_2$ and 0.8 % sodium chloride. The cells were resuspended in 2.5 mL of 50 mM Tris buffer with a pH of 7.8. Buffer containing 143.75 μL of 0.2 M phosphate buffer, 112.5 μL of 5 M NaCl and 18.75 μL of 20 mM S-Ala (Sigma) was added to 100 mL of resuspended cells. The suspension was vortexed and incubated at 37 °C for 30 minutes. The reaction was stopped by adding 87.5 mL of 80 % acetic acid. The suspension was centrifuged for 5 min at 10000 *g*. The amount of released *p*NA was measured at 410 nm.

Quantity of released *p*NA is calculated according to the formula:

μM *p*NA = ε x ΔA_{410} F x10^3 where;

ε = molar absorption coefficient, 8.800 /M/cm

ΔA_{410}= Measured absorbance at 410 nm

F = dilution factor, calculated according to the formula: F = final volume/volume

Lipolytic activity

Lipolytic activity was evaluated on tributyrin agar (Acros Organics, Geel, Belgium) that was homogenized with 0.01 % of tributyrin by ultrasound for 2 min at 20 kHz. One aliquot of 2 μL of the overnight grown *Lactobacillus* cultures was plated directly into agar plates and simultaneously, 10 μL of cultures' aliquot was added on the sterile cellulose disc previously placed on the tributyrin agar. The plates were incubated at 30 °C for 72 h in anaerobic conditions. Degradation of lipids was determined by measuring the halos of decomposition around the disc, as well as around the directly applied cultures.

Antimicrobial activity

The antimicrobial activity was performed using a modified method by Domig et al. (2014). The cheese strains, belonging to *Lb. plantarum* and *Lb. casei* were compared to their ability to inhibit the growth of spoilage and food borne pathogens as followed: B1 (*Bacillus cereus* DSMZ 6791), B2 (*Bacillus thuringiensis* DSM2 2046T), B3 (*Pseudomonas fluorescens* WCS 417r), B4 (*Serratia marcescens* DSMZ 30121T), B5 (*Escherichia coli* JM 105), B6 (*Staphylococcus aureus* DSMZ 20231) and B7 (*Listeria innocua* ATCC33090).

Grown isolates of *Lb. plantarum* and *Lb. casei* were applied in two parallel lines, spaced 17 mm to one-another, in the centre of the Petri dish. Plates were incubated for 48 hours at 30 °C in anaerobic conditions. Target strains were grown in 5 mL of BHI liquid media and 5 μL of culture was applied between the stretched lines of *Lactobacillus*, previously incubated. Target strains were also applied in the same amount on the edges of the Petri dish that served as a control. Plates were additionally incubated for 24 h aerobically at 30 °C or 37 °C, depending on the type of referent strain used. The antimicrobial activity was determined by comparison of the size and density of grown target strains colonies in between lactobacilli lines, and those applied on the edges of the Petri dish, where target strains were not in contact with lactobacilli.

Haemolytic activity

Liquid *Lactobacillus* strains were plated on the solid BHI (Biolife, Milano, Italy) medium supplemented with 5 % bovine blood (Acila AG, Mörfelden-Walldorf, Germany). The plates were incubated for 48 h on 30 °C. Haemolytic activity was determined by visualisation and changes in colour of the medium.

Results

Survival in simulated GIT conditions

Ability of *Lactobacillus* strains to survive in a simulated oral and GIT environment was tested in laboratory conditions. Survival rate was species or even strain dependent, or depending on precise test applied (Table 1). The best survival properties were noticed for strains MLK 2-2, CH3/3/3 and CH1/3/6 for all three environments tested whereas strain CH2/3/20 were not able to survive in any of simulated conditions.

Autoaggregation ability

The investigated strains showed a weak ability to aggregate after 3 h, and after 5 h of incubation. Percentage of autoaggregation of *Lactobacillus* strains are shown in Table 2.

Proteolytic and lipolytic potential

Based on the casein degradation, by using the disc diffusion method, *Lactobacillus* strains showed different proteolytic activity. Results of proteolytic (caseinolytic) activity were categorized into four groups based on the halo formation around the disc and around cultures applied directly into the agar medium: (-) no halo observed, (+) those with weak activity and halo size <8 mm, (++) those with moderate activity and halo size between 8-10 mm and (+++) those with pronounced activity which halo size was >10 mm. The majority of strains exhibited pronounced caseinolytic activity; CH2/3/7, CH2/2/3, CH1/3/9, CH1/3/4, CH1/3/6, MLK 2-1

Table 1. Survival ability of *Lactobacillus* strains in GIT conditions. Results are presented as percentage (%) of survived cells after exposure to simulated oral cavity, intestine and gastric conditions

Strain label	*Lactobacillus* species	Survival (%) in simulated		
		Oral conditions	Duodenal conditions	Gastric conditions
CH3/0/1	*Lb. plantarum*	85.33	100.00	0.00
CH2/3/20	*Lb. plantarum*	0.00	0.00	0.00
CH2/3/7	*Lb. plantarum*	40.00	24.00	33.00
CH2/2/3	*Lb. plantarum*	35.67	0.00	100.00
CH1/3/1	*Lb. casei*	86.84	46.27	53.73
CH1/3/12	*Lb. casei*	100.00	18.64	44.77
CH1/3/9	*Lb. plantarum*	52.78	47.83	52.17
CH3/3/3	*Lb. plantarum*	100.00	100.00	72.00
CH1/3/4	*Lb. casei*	50.00	46.15	25.64
CH1/3/6	*Lb. casei*	92.52	100.00	54.37
MLK 2-1	*Lb. plantarum*	88.29	100.00	2.90
MLK 2-2	*Lb. casei*	91.67	92.86	85.71

Table 2. Autoaggregation ability of *Lactobacillus* strains. The average initial number of cells was $\pm 10^8$ CFU/mL

Strain label	*Lactobacillus* species	Autoagreggation (%)	
		3 h	5 h
CH3/0/1	*Lb. plantarum*	10.73	14.69
CH2/3/20	*Lb. plantarum*	15.56	16.73
CH2/3/7	*Lb. plantarum*	11.38	12.60
CH2/2/3	*Lb. plantarum*	11.89	20.00
CH1/3/1	*Lb. casei*	15.13	23.03
CH1/3/12	*Lb. casei*	9.55	15.17
CH1/3/9	*Lb. plantarum*	11.91	16.60
CH3/3/3	*Lb. plantarum*	13.64	18.94
CH1/3/4	*Lb. casei*	5.68	21.59
CH1/3/6	*Lb. casei*	11.89	14.10
MLK 2-1	*Lb. plantarum*	16.05	17.90
MLK 2-2	*Lb. casei*	16.14	16.67

Table 3. Proteolytic activity of *Lactobacillus* strains

Strain label	*Lactobacillus* species	Proteolytic activity
CH3/0/1	*Lb. plantarum*	+
CH2/3/20	*Lb. plantarum*	++
CH2/3/7	*Lb. plantarum*	+++
CH2/2/3	*Lb. plantarum*	+++
CH1/3/1	*Lb. casei*	-
CH1/3/12	Lb. casei	-
CH1/3/9	*Lb. plantarum*	+++
CH3/3/3	*Lb. plantarum*	+
CH1/3/4	*Lb. casei*	+++
CH1/3/6	*Lb. casei*	+++
MLK 2-1	*Lb. plantarum*	+++
MLK 2-2	*Lb. casei*	+++

(-) no halo observed, (+) halo size < 8 mm, (++) halo size between 8 - 10 mm, and (+++) halo size > 10 mm

and MLK 2-2. Only one strain had moderate caseinolytic activity; CH2/3/20 and weak caseinolytic activity was observed for two strains, CH3/0/1 and CH3/3/3. Strains CH1/3/1 and CH1/3/12 did not display caseinolytic activity (Table 3).

Proteolytic activity was also determined using a chromogenic method. The calculated amount of released chromogenic peptide *p*NA, due to the enzymatic action of lactobacilli, is shown in Table 4. The minimum of proteolytic activity was observed for isolate MLK 2-2 and it was 4477 μM *p*NA, and the highest value was in isolate CH1/3/4 which was 7448.1 μM *p*NA. Lipolytic potential was not notice for any of the *Lactobacillus* strains tested (data not shown).

Acidification potential

The ability of *Lactobacillus* strains to acidify milk is shown in Table 5. The initial pH value of milk was of 6.34. The measurement was carried out in two replications, and the results are shown as mean values. From a total of 12 *Lactobacillus* isolates, 5 strains; CH1/3/9, CH3/3/3, CH1/3/6 , MLK 2-1 and MLK 2-2, were able to lower the pH of milk to 5.00, after 24 hours of incubation and showed moderate to strong acidification potential. According to Beresford et al. (2001), strains with ΔpH in 24 h between 1 and 2 belong to the group of bacteria with moderate acidification ability while strains with ΔpH in 24 h ≥2 are considered to be strong acidifiers.

Antimicrobial potential and haemolysis

The investigated lactobacilli were classified into 4 categories, based on their ability to limit the proliferation of food pathogens in terms of the reduction of the size and/or the density of target strain between the lines of *Lactobacillus* isolates, in comparison to those grown at the edge of the Petri dish as demonstrated in Domig et al. (2014). Those marked as (-) did not show any antimicrobial activity, (+) is referred to weak antimicrobial activity/ weak reduction of the size and/or the density of target strain, (++) was marked as medium antimicrobial activity, (+++) as pronounced antimicrobial activity and (++++) complete inhibition of target referent strains. Results of antimicrobial activity of the *Lactobacillus* isolates against target strains are shown in Table 6.

Table 4. Proteolytic activity of *Lactobacillus* strains determined using a chromogenic method. Results are presented as μM of *p* - nitroamilide (*p*NA)

Strain label	*Lactobacillus* species	μM *p*NA
CH3/0/1	*Lb. plantarum*	4761.9
CH2/3/20	*Lb. plantarum*	5982.9
CH2/3/7	*Lb. plantarum*	5046.8
CH2/2/3	*Lb. plantarum*	5250.3
CH1/3/1	*Lb. casei*	5372.4
CH1/3/12	*Lb. casei*	4517.7
CH1/3/9	*Lb. plantarum*	4843.3
CH3/3/3	*Lb. plantarum*	5006.1
CH1/3/4	*Lb. casei*	7448.1
CH1/3/6	*Lb. casei*	4884.0
MLK 2-1	*Lb. plantarum*	4477.0
MLK 2-2	*Lb. casei*	7000.4

Table 5. Ability of *Lactobacillus* strains to acidify milk. The pH values were measured after 2, 6 and 24 hours of incubation

Strain label	*Lactobacillus species*	ΔpH_{2h}	ΔpH_{6h}	ΔpH_{24h}
CH3/0/1	*Lb. plantarum*	0.03	0.05	1.06
CH2/3/20	*Lb. plantarum*	0.03	0.05	1.18
CH2/3/7	*Lb. plantarum*	0.03	0.08	0.32
CH2/2/3	*Lb. plantarum*	0.03	0.06	0.18
CH1/3/1	*Lb. casei*	0.02	0.06	0.21
CH1/3/12	*Lb. casei*	0.00	0.14	1.04
CH1/3/9	*Lb. plantarum*	0.04	0.05	1.46
CH3/3/3	*Lb. plantarum*	0.09	0.11	2.35
CH1/3/4	*Lb. casei*	0.03	0.07	0.35
CH1/3/6	*Lb. casei*	0.04	0.04	1.85
MLK 2-1	*Lb. plantarum*	0.04	0.06	1.80
MLK 2-2	*Lb. casei*	0.04	0.07	1.74

Strains CH3/0/1, CH2/2/3, CH1/3/1, CH1/3/6 and MLK 2-2 showed none to moderate antimicrobial activity against to most target strains. Strains CH2/3/20, CH1/3/12 and CH1/3/4 displayed moderate or pronounced antimicrobial activity against the majority of target bacteria.

Majority of the *Lactobacillus* isolates showed antimicrobial activity against *Serratia marcescens*, and none to poor antimicrobial activity against *Staphylococcus aureus* and *Listeria innocua*.

In the food industry the lack of haemolytic activity is one the main criteria in the selection for potential food starters (Giraffa, 1995; Hawaz, 2014). Lactobacilli as well as most members of the group LAB show no haemolytic activity (Munoz-Atienza et al., 2013). This was confirmed with *Lactobacillus* isolates from Istrian cheese, none of the isolates exhibited haemolytic activity (data not shown).

Discussion

Ripening of cheese is a complex biochemical process. It involves changes in both, physico-chemical and microbiological properties. Microbial interactions during ripening determine certain characteristics of the product, such as sensory properties and hygienic safety (Ahola et al., 2002). Since Istrian cheese is produced from raw ewe's milk it contains more complex microbiota from those produced with pasteurized milk (Fuka et al., 2013). There are four known groups of lactic acid bacteria (LAB) that are dominant during cheese ripening and that have major impact on characteristics of the ripened cheese; mesophilic lactobacilli, pediococci, enterococci and *Leuconostoc*. Since strains of *Lactobacillus casei* and *Lactobacillus plantarum* used in this study proceed from indigenous LAB group it was necessary to explore their technological and probiotic potential such to select for potential bioprotective, starter or even probiotic cultures. This was achieved by measuring their proteolytic, lipolytic and haemolytic activity besides their acidification ability and survival ability in simulated GIT conditions.

The *Lactobacillus* isolates tested in our study displayed moderate acidification capacity. However, 5 isolates lowered the milks pH below 5.00 (ΔpH = 2.35; 1.85; 1.80; 1.74; 1.46), indicating a higher acidification potential, that is similar to the results published by Anas and colleagues (2008). According to research published by Perez et al., (2003), Hidalgo-Morales et al., (2005) and Haddad et al., (2005) certain strains such as

Table 6. Antimicrobial activity of *Lactobacillus* strains based on Domig et al., (2014)

Strain label	*Lactobacillus* species	*Bacillus cereus*	*Bacillus thuringiensis*	*Pseudomonas fluorescens*	*Serratia marcescens*	*E. coli*	*Staphylococcus aureus*	*Listeria innocua*
CH3/0/1	*Lb. plantarum*	-	-	-	-	++	-	++
CH2/3/20	*Lb. plantarum*	++	+	+	+++	++	+	-
CH2/3/7	*Lb. plantarum*	+	+	-	+	+	+	+
CH2/2/3	*Lb. plantarum*	-	-	-	+	-	-	+
CH1/3/1	*Lb. casei*	-	-	-	+	-	-	-
CH1/3/12	*Lb. casei*	+	+++	++	++	++	+	-
CH1/3/9	*Lb. plantarum*	+	-	+	++	+	-	-
CH3/3/3	*Lb. plantarum*	++	+	++	+	-	-	+
CH1/3/4	*Lb. casei*	+++	+	+	++	+	+	-
CH1/3/6	*Lb. casei*	-	-	+	++	-	-	+
MLK 2-1	*Lb. plantarum*	++	-	+	+	-	-	-
MLK 2-2	*Lb. casei*	+	++	+	++	-	+	-

(-) = no activity, (+) = weak reduction of size and/or density, (++) = medium reduction of size and/or density, (+++) = pronounced reduction of size and/or density of target strain and (++++) = no pathogen growth.

Lb. reuteri, *Lb. plantarum* and *Lb. casei*, as well as other members of the genus *Lactobacillus* have a low acidification capacity. Furthermore, a study published by Cheriguene et al. (2006) and Akabanda et al. (2014) confirmed the higher acidification ability of *Lactobacillus* species, but only after 24 h post incubation, when the produced acid reaches the same quantities as those produced by *Lactococcus* species. Since *Lactobacillus* is a genus of LAB which is usually isolated from milk and cheese; hence the presence of different strains possesses different abilities of acidification (Carnerio Ratao, 2010). Although, *Lactobacillus* isolates that show low acidification capacity are not ideal candidates to be used as part of starter cultures, nevertheless they can take part in the development of flavour and texture, by proteolytic and lipolytic activity or by producing bacteriocins.

Our results indicated that all 12 tested isolates showed low or moderate proteolytic activity and no lipolytic activity, like previously stated in bibliography (Ljungh and Wadström, 2003; Jay et al., 2005; Hidalgo-Morales et al., 2005). Lactobacilli are generally known for their limited protease activity. Interestingly, the type of protease may vary within same genus (Haddadi et al., 2005). Hebert et al. (2000) reported that some of the *Lactobacillus* species can display high; while others exhibit weak proteolytic activity. Weak lipolytic activity and absence of proteolytic activity in *Lactobacillus* was published by Munoz-Atienza et al. (2013) as well.

Lactic acid bacteria have the capability of producing antimicrobial agents called bacteriocins. Bacteriocins have inhibitory activity (Daeschel, 1989; Bhattacharya and Das, 2010) which influences the growth of other pathogens and spoilage microorganisms. The genus *Lactobacillus* is known for its production of bacteriocins (Munoz-Atienza et al., 2013) which can be linked to their antagonistic ability to compete and to colonize GIT (Garriga et al., 1993; Šušković et al., 2010; Leboš Pavunc et al., 2013).

Our study of antimicrobial activity against 7 potentially spoilage or pathogenic species revealed that almost all isolates were able to inhibit proliferation of *Serratia marcescens*. Five isolates showed low antimicrobial activity against *Staphylococcus aureus* and *Listeria innocua*, while the other 7 isolates showed no activity against these two pathogens. Herreros et al. (2003) observed that *S. aureus* and *L. innocua* were not affected in their proliferation by *Lactobacillus* isolates. The study from Anas et al. (2008) suggested that *Lactobacillus plantarum* had antimicrobial activity against *S. aureus*. This variation in ability to inhibit the pathogen proliferation is species or strain specific and needs to be further studied.

Numerous studies have been carried out in order to determine the commercial application of *Lactobacillus* as probiotics and their survival during gastric transit (Lilly et al., 1965; Havenaar et al., 1992; Daly et al., 1998; Gregurek 1999; Fernandez et al., 2003; Soccol et al., 2010; Sabir et al., 2010; Jankovic et al., 2012; Beganović et al., 2013; Domig et al., 2014; Terzić-Vidojević et al., 2015). Our isolates showed a high percentage of survival in a simulated oral cavity and 5 isolates showed more than 90 percent survival in simulated duodenum conditions. Simulated gastric conditions disclosed much lower survival, with an exception of 3 isolates that have revealed more than 70 % survival rate.

In addition, all isolates showed a low ability to aggregate after 5 hours of incubation; this diminishes their probiotic properties since the aggregation ability is associated with adherence properties of the cell (Li et al., 2015) which also challenges their ability to colonize the GIT.

One of the emphasized criterions in the selection of starter or probiotic cultures is the lack of haemolytic activity which implies non-virulence of the isolate and their safe use in food production (Anas et al., 2008). Haemolysis is not characteristic for genus *Lactobacillus* and our selected cheese isolates did not show any haemolytic activity (Maragkoudakisa et al., 2006; Hawaz, 2014; Munoz Atienza et al., 2013).

Conclusions

Obtained results from our study showed that *Lactobacillus* isolates from Istrian cheese displayed high survival rate in a simulated oral cavity and the duodenum while the survival rate of most examined *Lactobacillus* isolates in a simulated gastric conditions was much lower. Generally, all strains demonstrated antimicrobial activity against *Serratia marcescens*, with an exception of strain CH3/0/1. Certain

strains *e.g.*, CH2/3/20, CH1/3/12, SIR1/3/4 exhibited antimicrobial activity against majority of food spoilage and pathogens tested. Although autoaggregation rate for all strains was low, the tendency of this isolates to autoaggregate showed exponential growth in between 2 hours. Moderate to strong milk acidification ability within 24 hours of incubation was measured for several strains which contributed to their potential application as starter cultures. Additionally, all investigated isolates showed low or moderate proteolytic activity and absence of lipolytic activity. Haemolytic activity was not observed in any of the tested isolates, which states their safety application.

Because of their moderate or strong acidification ability, pronounced proteolytic activity as well as medium or pronounced antimicrobial activity against tested spoilage or pathogenic bacteria, four *Lactobacillus* strains, CH3/3/3, CH1/3/6, MLK2-1 and MLK2-2 should be further investigated. As no isolate was superior for all tested properties, combination of two or more strains may be the best promising strategy for the application as starter cultures.

Autohtoni sojevi roda Lactobacillus *izolirani iz Istarskog sira kao potencijalne starter kulture*

Sažetak

Tradicionalni Istarski sir proizvodi se od nepasteriziranog ovčjeg mlijeka, bez dodatka starter kultura. Prema tome, specifična aroma i tekstura Istarskog sira pripisuje se, uz ostale faktore, metaboličkim procesima autohtone mikroflore od kojih vrste roda *Lactobacillus* imaju ključnu ulogu. Karakterizacija i izbor autohtonih sojeva laktobacila može dovesti do potencijalnog korištenje odabranih sojeva kao starter, zaštitnih ili probiotičkih kultura. U ovom istraživanju željeli smo istražiti tehnološki i probiotički potencijal sojeva *Lactobacillus plantarum* i *Lactobacillus casei* izoliranih iz tradicionalnog Istarskog sira pomoću metoda koje određuju njihov proteolitički, kazeinolitički, lipolitički, antimikrobni i hemolitički potencijal, kao i njihovu sposobnost zakiseljavanja, autoagregacije i preživljavanja u simuliranim gastrointestinalnim uvjetima. Svih 12 reprezentativnih sojeva pokazuju nisku do umjerenu proteolitičku aktivnost te odsutnost lipolitičke i hemolitičke aktivnosti. Od 12 sojeva, 5 je pokazalo jaku sposobnost zakiseljavanja, spuštajući pH mlijeka ispod 5,00 nakon 24 sati inkubacije. Nadalje, gotovo svi izolati pokazuju antimikrobno djelovanje u odnosu na vrstu *Serratia marcescens* te slabo antimikrobno djelovanje u odnosu na *Staphylococcus aureus* i *Listeria innocua*. Istraživani sojevi pokazali su visoku prosječnu stopu preživljavanja u simuliranim uvjetima usne šupljine i dvanaesnika, dok je prosječna stopa preživljavanja u simuliranim želučanim uvjetima bila znatno niža. Nakon 5 sati inkubacije, autoagregacijska sposobnost svih istraživanih sojeva bila je niska.

Ključne riječi: Istarski sir, *Lactobacillus* spp., starter kulture, antimikrobno djelovanje

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