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Evaluation of autochthonous lactic acid bacteria as starter cultures for production of white pickled and fresh soft cheeses

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ABSTRACT

In order to preserve the traditional manufacturing of white pickled (WPC) and fresh soft cheeses (FSC), well-characterized autochthonous lactic acid bacteria (LAB) with advantageous characteristics were applied for the production of the cheeses at small industrial scale under the controlled conditions. Selected LAB for design of defined mixed starter cultures belonged to *Lactococcus lactis* ZGBP5-9, *Enterococcus faecium* ZGPR1-54 and *Lactobacillus plantarum* ZGPR2-25 for FSC production and to *Lc. lactis* BGAL1-4, *Lactobacillus brevis* BGGO7-28 and *Lb. plantarum* BGGO7-29 for WPC production. A sensory evaluation indicated that the cheeses obtained by inoculation with selected autochthonous LAB are similar to the traditional cheese and received the best scores. Viable cell counts of LAB used for the production of both type chesses was high, over 10^6 cfu g^{-1}. High viability of the surveyed strains was supported with PCR-DGGE, which confirm the retention of selected LAB strains as starter cultures in cheese production. Next, PFGE analysis showed that each single strains, selected in particular cheese mixed culture, revealed unique *Sma*I PFGE pattern that could enable efficient discrimination and monitoring of the strains in industrial process. As some of the selected LAB strains are attributed as potential probiotics, produced cheeses could be considered as functional food.

1. Introduction

Consumers' requirements for traditional fermented dairy food products are generally increased due to their proved gastronomic quality and positive effects on human health. However, the tightened legislation on food safety results in lower production flexibility, homogeneity in the food production and in the loss of food diversity and traditional specificity. Hence, the preparation of well defined functional autochthonous starter cultures for production of traditional cheeses under controlled conditions, using the standardized traditional technology, is crucial.

Balkan Countries (WBC), a distinct geographical region in Europe, is well known for a variety of traditional, spontaneously fermented dairy products manufactured mainly in households on a small scale. This study is focused on manufacture of traditional cheeses: fresh soft cheese (FSC) and white pickled cheese (WPC). FSC and WPC are artisanal cheeses made from cow's milk by farmers on a small scale in the farmhouse, located in specific continental regions in Croatia (Prigorje, the Bilogorsko-Podravska region and Zagorje) and Serbia (the South Morava, Golija Mountain, mountainous region of Eastern Serbia), respectively, following traditional manufacturing procedures. Nowadays, among Croatian and Serbian population, there is a growing interest in such artisanal cheeses, due to the uniqueness of such foods, in which autochthonous microbiota derived from raw milk shape the specific characteristics and sensory quality of cheeses. Additionally, traditionally produced cheeses exhibit a greater overall intensity of flavour and broader flavour profiles than

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industrially produced, and the typical sensorial properties of these cheeses are a result of the diversity of species and strains of local and specific indigenous milk microbiota (Garabal, Rodríguez-Alonso, & Centeno, 2008). FSC is very popular in the diet of Croatian population. This type of the cheese is usually unsalted. It can be used fresh or for preparation of different salty or sweet dishes (pies, cheese cakes etc). The attractiveness of FSC for modern consumers is also related to its low fat content. On the other hand, Serbian WPC is very salty, older, full fat cheese, but despite that the Serbs like this type of cheese and use it with almost every meal.

Previous studies revealed that numerous lactic acid bacteria (LAB) isolated from the traditional cheeses in WBC region showed desired technological and functional potential such as role in milk protein coagulation, production of proteinases and exopolysaccharides, aggregation phenotype, the presence of S-layer proteins (Leboš Pavunc et al., 2012; Terzic-Vidojevic et al., 2009; Uroić et al., 2014; Veljovic et al., 2007). The isolation and molecular characterisation of autochthonous LAB resulted in formation of the defined collections of natural LAB isolates that could be used for formulation of specific starter cultures for fermented dairy products with geographical origin (Leboš Pavunc et al., 2012; Nikolic et al., 2008).

White pickled cheese (WPC), traditionally manufactured in households in the mountain regions of Serbia, belongs to a group of cheeses with specific cheese texture and ripening process. WPC is characterized by mild salty and sour taste; curd has soft and gentle consistency and its cut section has porcelain shine and compact structure with no gas holes, only mechanical openings in the curd that are size of lens and filled with whey. Autochthonous strains of LAB naturally present in milk, participate in ripening of this cheese, and influence the ripening process and creation of specific taste, characteristic cheese aroma and texture (Irlinger & Mounier, 2009). Similarly, homemade fresh soft cheese (FSC) is the most widely used traditional product in continental lowland regions of Croatia. Production of FSC is based on spontaneous acidification and consequent clothing of almost exclusively raw milk, so this cheese belongs to the group of acidic cheeses (Leboš Pavunc et al., 2012). FSC has a characteristic medium sour and fresh taste; it has white colour and consistency that tends to separate in layers. It contains high percentage of water (55%–80%), and it can be produced from whole milk, partially skimmed or skimmed milk (Leboš Pavunc et al., 2013; Radošević, Tonković, Gregurek, Kos, & Šušković, 2007).

Recently, Golić et al. (2013) characterized autochthonous LAB isolates from nine WPC and nine FSC. The authors reported high LAB species diversity as well as specific technological and probiotic properties, the futures important for formulation and selection of autochthonous starter cultures for the production of white pickled and fresh soft cheeses. Additionally, Uroić et al. (2014) demonstrated that particular dairy LAB isolates exhibit the strain-specific probiotic properties.

The general goal of the present study was the evaluation of autochthonous LAB starter cultures for production of WPC and FSC at semi-industrial scale under controlled conditions, in order to preserve typical sensorial properties of the cheeses. First objective was the selection of the autochthonous LAB strains of technological and functional interest for construction of novel mixed starter cultures. Next, selected strains were used in the production of FSC and WPC, which were analysed for their quality parameters and yield and evaluated for sensory properties.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Lactobacillus strains were cultivated on MRS medium (pH 5.7) (Merck GmbH, Darmstadt, Germany), whereas *Lactococcus* and *Enterococcus* strains were grown on M17 medium (pH 7.2) (Merck) supplemented with 0.5% (w/v) glucose (GM17). The cultures were incubated overnight at 37 °C for *Lactobacillus* and *Enterococcus* strains, and at 30 °C for *Lactococcus* strains.

2.2. Acidifying activity of autochthonous LAB strains

Acidifying activity was determined by recording the pH variations of milk cultures. Actively growing cultures were inoculated (2%) in 100 ml of sterile 11% reconstituted skimmed milk (RSM) (w/v), (RSM, Subotica, Serbia) and the pH measured (pH metre, glass electrode, EUTECH Instruments, CyberScan pH 510, Bukit Raja, Malaysia) at time 0 and after 6 and 24 h of incubation at 30 °C. The same procedure was repeated three times for each strain.

2.3. Physiological characterization of the LAB isolates

The phenotypic characterization of LAB isolates was performed according to Terzic-Vidojevic, Vukasinovic, Veljovic, Ostojic, and Topisirovic (2007). Utilization of citrate was tested as previously described by Kempler and McKay (1981). The Voges-Proskauer test was used for determination of acetoine production from organic acids that result from glucose metabolism (Ijutov, 1963). Diacetyl production was assessed after the inoculation of the LAB strains in RSM for 16 h. The addition of 0.1 g of creatinine and 0.1 ml of 30% NaOH (w/v) to 1 ml of coagulated milk followed. Diacetyl production by LAB strains was considered positive if the red ring formation was observed at the top of the tubes after 2 h incubation.

2.4. Antimicrobial and proteolytic activity of the LAB isolates

Antimicrobial activity of the LAB isolates was detected by an agar-well diffusion assay (Tagg & Mc Given, 1971) using *Lactococcus lactis* subsp. *cremoris* NS1, *Lactococcus lactis* subsp. *lactis* BGMN1-596, *Lactobacillus paracasei* subsp. *paracasei* K4, *L. paracasei* BGHN14, *Listeria innocua* ATCC33090, *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923 as indicator strains. A clear zone of inhibition around the well, but not near the pronase E crystal, was taken as a positive signal for bacteriocin production.

Proteolytic activity of LAB isolates was assayed as described by Kojic, Fira, Banina, and Topisirovic (1991) following the degradation of β-casein. For this purpose, the strains were grown on milk citrate agar (MCA) plates for 48 h at 30 °C prior to cell collection.

2.5. Composition and acidity of milk used for cheese production

Physicochemical analysis were performed according to the following analytical methods: milk fat by Soxhlet (Carpenter, 2010), proteins by IS0:1871:2009, lactose by gravimetric method (ISO 1211:2010a), total dry matter by drying at 102 °C (ISO 6732:2010b) and non fat dry matter by calculation. Active acidity was measured by Knick digital pH meter type 911 and titratable acidity by titration with 0.1 M NaOH (ISO/TS 22113:2012).

2.6. Selection of LAB isolates and production of cheeses

Autochthonous LAB strains, selected among twenty well characterized LAB isolates according to their physiological properties, acidification, antimicrobial and proteolytic activity and were analysed as potential starter culture for production of WPC (*Lactococcus lactis* subsp. *lactis* BGAL1-4, *Lactobacillus plantarum* BGGO7-29, *Lactobacillus brevis* BGGO7-28) and FSC (*Lactococcus lactis* ZGBP5-9, *L. plantarum* ZGPR2-25, *Enterococcus faecium* ZGPR1-54) (Golić et al., 2013; Uroić et al., 2014). The BGGO7-28 and ZGPR1-

54 strains were chosen due to their good probiotic properties (Uroić et al., 2014).

2.6.1. White pickled cheese (WPC) production

For production of WPC the technique for traditional cheese manufacturing was modified in order to adjust the technological demands of modern industrial production. For production of control cheese A commercial starter culture DX-31E (*Lactococcus lactis* subsp. *lactis*/*cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* spp.) (DSM Food Specialties, The Netherlands) was used. Selected autochthonous LAB strains for production of cheese B were cultivated overnight in pasteurized RSM. Total amount of selected LAB strains was 3% related to the total amount of milk in previously determined ratios: BGAL1-4 (25%), BGGO7-29 (45%) and BGGO7-28 (30%). After 30 min of resting 0.15 g l^{-1} of rennet Maxiren 1800 (DSM Food Specialties) and $CaCl_2$ (0.02% of milk mass) were added to milk. Coagulation took 60 min after which cheese curd was processed and cut to 0.5 × 0.5 cm^3 pieces, stirred and left to rest for 10 min. Curd was transferred in moulds covered with cheese cloths for whey draining. For additional whey draining, weight was put on top of the cheese (2 kg of weight per 1 kg of cheese). After 2 h of pressing, curd was cut to nine rectangular pieces. Cheese pieces were salted and arranged in a cheese vat into layers and covered with "presolac" (pasteurized and cooled whey with addition of 20% NaCl) creating anaerobic ripening conditions. "Presolac" is traditionally used in production of autochthonous WPC in Serbia, and is rich in lactose, whey proteins, minerals and especially B vitamins. After ten days "presolac" was replaced by brine (20 g NaCl l^{-1}). WPC was ripened in brine at 15–18 °C for 30 days, and later was stored in brine at 4–8 °C. The difference from industrial production was using the "presolac" only for first 10 days of cheese ripening.

2.6.2. Fresh soft cheese (FSC) production

FSC were produced following traditional procedures established in households in Croatia. In control trial (sample cheese C) commercial starter culture LL-50Y (*Lactococcus lactis* subsp. *lactis*/*cremoris*) (DSM Food Specialties, The Netherlands) was added, while in a trial with autochthonous LAB strains (sample cheese D) three LAB isolates in previously determined ratios were applied: ZGPB5-9 (40%), ZGPR2-25 (30%) and ZGPR1-54 (30%). The cultures were added in pasteurized (65 °C for 30 min) and cooled (at 23 °C) milk. Total amount of LAB strains was 3% related to total amount of milk. Afterwards, 0.2% rennet and 0.02% $CaCl_2$ were introduced in both cheeses (C and D). Calcium chloride ($CaCl_2$) is commonly added to pasteurized milk used for production of FSC, as calcium improves coagulation properties and strength of the curd. After reaching pH value of 4.60 (approximately 16 h), curd was cut in cubes 2 × 2 cm^3 followed by resting (for 1 h), shaping and draining in warm ambient (for 2 h). Afterwards, FSC were stored at 4 °C during 10 days.

2.7. Determination of the quality parameters after production and during storage of cheeses

2.7.1. Determination of pH, acidity, syneresis, dry matter and yield in produced cheeses

Active acidity of cheeses during production, ripening and storage was measured by pH meter Knick type 911, syneresis by measurement of whey volume during draining of cheese, cheese mass by weighing, total dry matter by drying method at 102 °C and cheese yield by calculation. Fat content was determined by the milk fat by Soxhlet method (Carpenter, 2010), and expressed as fat percentage in dry matter. Moisture in non-fat cheese (Mffb) was calculated based on the content of moisture and fat in the cheese (Codex General Standard for Cheese, 2006). Mffb equals percentage moisture on fat-free basis:

$$\frac{\text{Weight of moisture in the cheese}}{\text{Total weight of cheese} - \text{Weight of fat in the cheese}} \times 100$$

2.7.2. Viability of LAB in the cheese samples during maturation and storage

Ten grams of WPC and FSC samples were homogenized with 90 ml of a sterile saline solution for 3 min in a blender BagMixer® 400 (Interscience, St Nom, France). The cheese homogenates were serially diluted, plated in triplicate onto MRS and GM17 agar plates and incubated for 48 h at 37 °C and 30 °C. The plates with a number of colony forming units (cfu) ranging from 10 to 300 were selected for enumeration (Leboš Pavunc et al., 2012). The results were expressed as average number of cfu g^{-1} of cheese. Three repetitions of each analysis were performed. The arithmetic means and standard deviations were calculated.

2.7.3. Analyses of sensory properties of produced cheeses

Sensory analysis (appearance, consistency, colour, odour and taste) of produced cheese was performed by five sensory reviewers (university stuff) trained to recognize cheese tastes and f1avors. For sensory evaluation, ISO/DIS 22935-2 IDF 99-2 Draft International standard Milk and milk products – sensory analysis was used.

Control cheeses produced without starter cultures and cheeses produced with selected autochthonous LAB were tasted and the most frequently cited descriptors: appearance, taste, odor, consistency, and in addition for WPC cuts and colour were selected. FSC were evaluated on 1st, 5th and 10th day throughout the storage, while WPC were tested on 10th, 20th and 30th day of ripening. The panelists were asked to rank the cheese samples according to their intensity, in the range of 1–5, for each of the descriptors. The averages of sensory evaluation data with standard deviations were determined.

2.8. PCR-DGGE analysis of the total cheese DNA extracts

Isolation of genomic DNA from cheese samples after 10 days of storage at 4 °C, PCR and denaturing gradient gel electrophoresis (DGGE) in 30–60% denaturing gradient were performed according to Cocolin, Manzano, Cantoni, and Comi (2001).

2.9. Pulse-field gel electrophoresis (PFGE)

PFGE was performed using a contour-clamped homogeneous electric field system (2015 Pulsafor unit; LKB Pharmacia, Sweden) as described previously (Kojic, Strahinic, & Topisirovic, 2005), using *Sma*I restriction enzyme. Phage Ladder PFG Marker (New England BioLabs Inc., Whitby, Canada), was used as a molecular size marker.

3. Results and discussion

In this study, among previously characterized LAB natural isolates, strains with prominent specific technological (salt tolerance, CO_2 and diacetyl production, L-arginine and esculin hydrolysis, citrate utilisation, curd formation etc.) and probiotic properties, were selected for construction of mixed starter cultures for the production of WPC and FSC, respectively (Golić et al., 2013; Uroić et al., 2014). Additionally, surveyed LAB strains were subjected for further analysis of technological characteristics important for cheese manufacturing, as described below.

3.1. Definition of mixed starter cultures and testing at laboratory scale

Physiological and technological properties of six selected LAB strains, as potential starter cultures were studied in order to preserve authentic aroma and texture of the traditional cheeses (Table 1). The results showed that lactococci BGAL1-4 and ZGBP5-9 strains curdled milk faster than other analysed strains, after 5 and 6 h, respectively. Additionally, acidifying activity of *Lc. lactis* strains was much better than *Lb. plantarum* and *Ec. faecium* strains, while the strain BGGO7-28 had the lowest activity in milk (Table 2). Lactococci strains were previously shown to be able for rapid milk acidification and are usually employed in starter cultures, influencing improved curd firmness and the cheese aroma (Leboš Pavunc et al., 2012; Terzic-Vidojevic et al., 2014). The strains BGAL1-4, BGGO7-29, ZGPR2-25 and ZGBP5-9 formed a hard compact curd without of whey separation. Moreover, the taste of the fermented products prepared with mentioned individual cultures was mildly sour and very pleasant (data not shown). Moreover, most of the strains (Table 1) provided good sensory characteristics of the fermented product producing diacetyl, acetoine and utilizing citrate (ZGPR1-54 and ZGPR2-25), which are considered the main aroma compound of fermented milk products (Terzic-Vidojevic et al., 2013). Further, BGAL1-4 and ZGBP5-9 strains showed bacteriocin activity inhibiting the growth of *S. aureus* ATCC25923, while the strain ZGPR1-54 inhibited the growth of two food-related pathogens, *S. aureus* ATCC25923 and *E. coli* ATCC25922. LAB cultures are often used to prevent and control undesirable microorganisms by production of antimicrobial compounds such as lactic acid and bacteriocins (Lozo et al., 2007; Settanni, Franciosi, Cavazza, Cocconcelli, & Poznanski, 2011; Šušković et al., 2010). Hence, the strains BGAL1-4, ZGBP5-9 and ZGPR1-54 were chosen due to their biopreservation potential. Moreover, all strains, except ZGPR2-25, showed good proteolytic activity (Table 1), as it directly influences the cheese flavour and texture development (Beganović et al., 2013; Bergamini, Hynes, & Zalazar, 2006). Finally, the strain *Lb. brevis* BGGO7-28 was chosen due to its probiotic potential (Uroić et al., 2014).

3.2. Determination of the quality parameters of cheeses

Composition of cheese can vary within a broad range due to the differences in the raw milk composition (Meijer & Brandsma, 2005). Quality parameters of milk used for production of the cheeses are presented in Table 3A. Our results confirmed that the composition of milk used in this study was in standard values (Tratnik & Božanić, 2012).

3.2.1. White pickled cheese characterisation

During draining and pressing of WPC, greater amount of whey was separated from cheese B than from cheese A (Table 3B). Accordingly, mass and yield of the cheese B was 13.45% and 13.43%, respectively, less compared to the mass and yield of the cheese A (Table 3B). The both WPC (cheese A and cheese B) are classified as medium fat cheeses (according to fat content in dry matter), the soft cheese type (related to cheese's firmness) and as cheese in brine (according to the principal ripening) (Codex General Standard for Cheese, 2006). Further, dry matter values of both produced cheeses (A and B) are in ranges common for WPC and are in compliance with industrially produced WPC "Kriška" (47.04%) and autochthonous WPC type Sjenica (45.59%) (Popović-Vranješ et al., 2011). Viable cell number of autochthonous LAB strains were reduced during the ripening of cheese B for 1.36 log units and 2.86 log units, respectively (Fig. 1A). Interestingly, viable cell count of commercial starter culture DX-31E was decreased by 3.33 log units at the end of maturation period of cheese. The results showed that the viable cell number of the probiotic strain BGGO7-28, at the end of storage period was in range recommended for number of live probiotic cells (above 10^6 cfu/g) in fermented milk products (Rogelj, Bogovič Matijašić, Majhenič, & Stojković, 2002). In addition, although the number of potential probiotic strains ZGPR1-54 and ZGPR2-25 was decreased by 0.68 and 0.85 log units, respectively, at the end of storage period, it is still in a range of recommended number of live probiotic cells (Sanders & Huis in't Veld, 1999).

The pH values of WPC B, measured during 30 days of ripening, varied more and appeared to be not considerably lower compared to the cheese A (Fig. 2A). Our results suggest that the post-

Table 1
Physiological and technological properties of autochthonous LAB strains selected as for evaluation potential cheese starter cultures.

Test	LAB strains selected as starters for production of					
	White-pickled cheese			Fresh soft cheese		
	BGAL1-4 *Lc. lactis* subsp. *lactis*	BGGO7-28 *Lb. brevis*	BGGO7-29 *Lb. plantarum*	ZGPR1-54 *Ec. faecium*	ZGPR2-25 *Lb. plantarum*	ZGBP5-9 *Lc. lactis*
Growth at 15 °C	+	−	+	−	+	+
Growth at 30 °C	+	+	+	+	+	+
Growth at 37 °C	+	+	+	+	+	+
Growth at 45 °C	−	−	−	±	−	−
Growth in 2% NaCl	+	−	+	+	+	+
Growth in 4% NaCl	+	−	+	+	+	+
Growth in 6.5% NaCl	±	−	±	±	+	+
Hydrolysis of arginine	+	−	−	+	−	+
Hydrolysis of esculin	+	+	+	+	+	+
Citrate utilization	+	−	±	+	+	+
Acetoine production (V.P.test)	+	+	+	+	+	−
Diacetyl production	−	−	−	+	+	−
Production of CO_2 from glucose	−	−	−	−	−	−
Survival at 63.5 °C for 30 min	−	−	±	±	+	+
Milk curdling time	5 h	72 h	10 h	24 h	22 h	6 h
Slime production	−	−	−	−	−	−
Proteolytic activity	+	+	+	+	−	+
Production of bacteriocins	+	−	−	+	−	+

Lc. – *Lactococcus, Lb.* – *Lactobacillus, Ec.* – *Enterococcus*.
+ Positive reaction; − Negative reaction; ± Weak reaction.
Note: LAB strains were identified according to $(GTG)_5$ fingerprint analyses and 16S rDNA sequencing previously (Golić et al., 2013).

Table 2
Acidifying activity of autochthonous LAB strains in reconstituted skimmed milk (RSM).

Autochthonous LAB strains (potential starter cultures)	pH of inoculated skim milk after 0, 6 and 24 h of incubation at 30 °C		
	0	6	24
Lc. lactis subsp. *lactis* BGAL1-4	6.24 ± 0.02	4.52 ± 0.16	4.24 ± 0.32
Lb. brevis BGGO7-28	6.26 ± 0.01	6.23 ± 0.21	6.16 ± 0.09
Lb. plantarum BGGO7-29	6.09 ± 0.03	5.46 ± 0.28	4.67 ± 0.41
Ec. faecium ZGPR1-54	6.26 ± 0.02	5.48 ± 0.19	4.74 ± 0.18
Lb. plantarum ZGPR2-25	6.13 ± 0.01	5.46 ± 0.24	4.49 ± 0.22
Lc. lactis ZGBP5-9	6.24 ± 0.01	4.70 ± 0.27	4.31 ± 0.34

Lc. – *Lactococcus, Lb.* – *Lactobacillus, Ec.* – *Enterococcus.*
Note: pH of inoculated skim milk was 6.35.
The results represent mean values and standard deviations of three independent measurements on different samples.

acidification in WPC was more efficient in the cheese B, produced with autochthonous starter culture, which produce high amount of lactic acid.

3.3. Fresh soft cheese characterisation

The characterisation of FSC revealed that both cheeses (C and D) belong to the fresh, low fat cheese group (Regulations on milk and dairy products, 2009). Total dry matter for the cheese D was higher (17.99%) compared to total dry matter in the cheese C (14.60%) due to the higher content of milk fat and proteins (Table 3A). Dry matter of FSC depends on present fat content in milk (Radošević et al., 2007). FSC C has shown less syneresis (4.44%) than FSC D (6.45%). Syneresis was measured at the end of production and during storage of FSC according to mass of cheeses obtained at 1st day of storage (data not shown). The higher cheese syneresis in the cheese D, produced with autochthonous starter culture, may be partly due to slower proteolysis (Fox, Guinee, Cogan, & McSweeney, 2000). Mass of the FSC D was 9.7% less than mass of the cheese C after 10 days of storage, while the yield of the cheese D was for 2.41% less compared to the cheese C (Table 3B).

Table 3
Physicochemical parameters and titratable acidity of milk used for production of the cheeses (A) and physicochemical parameters, mass, yield and acidity of produced white pickled cheese and fresh soft cheese (B).

A. Parameters	Milk used for production of	
	White pickled cheese	Fresh soft cheese
Fat (%)	3.34 ± 0.02	0.19 ± 0.09
Proteins (%)	3.20 ± 0.06	3.45 ± 0.19
Lactose (%)	4.29 ± 0.09	4.59 ± 0.02
Non fat dry matter (%)	8.37 ± 0.03	8.95 ± 0.06
pH	6.50 ± 0.06	6.53 ± 0.03
Titratable acidity (°SH)	7.80 ± 1.55	6.73 ± 0.25

B. Parameters	White pickled cheese		Fresh soft cheese	
	Cheese A[b]	Cheese B[c]	Cheese C[d]	Cheese D[e]
Dry matter (g g^{-100})	47.04 ± 0.02	47.30 ± 0.01	14.60 ± 0.01	17.99 ± 0.01
Proteins (g g^{-100})	20.60 ± 0.88	17.21 ± 0.56	8.52 ± 0.96	11.58 ± 1.14
Fat (g g^{-100})	20.09 ± 0.26	26.64 ± 0.19	0.08 ± 0.01	0.58 ± 0.04
Fat in dry matter (g g^{-100})	42.71 ± 0.18	36.38 ± 0.12	0.55 ± 0.02	3.22 ± 0.07
Mffb[a] (g g^{-100})	66.27 ± 0.64	71.84 ± 0.83	85.47 ± 1.89	82.49 ± 1.54
Mass (g)	920.00 ± 109.51	796.25 ± 100.28	443.67 ± 79.29	401.75 ± 47.25
Yield (%, m V^{-1})	23.00 ± 1.97	19.91 ± 2.5	27.94 ± 4.24	25.53 ± 2.76
pH	4.26 ± 0.04	4.18 ± 0.04	4.5 ± 0.13	4.49 ± 0.04

Note: Mean values and standard deviations of three independent measurements on different cheese samples.
[a] Mffb – moisture on a free-fat basis.
[b] Cheese A: white pickled cheese produced with commercial starter culture DX-31E.
[c] Cheese B: white pickled cheese produced with *Lactococcus lactis* BGAL1-4, *Lactobacillus brevis* BGGO7-28 and *Lactobacillus plantarum* BGGO7-29.
[d] Cheese C: fresh cheese produced with commercial starter culture LL-50Y.
[e] Cheese D: fresh cheese produced with *Lactococcus lactis* ZGBP5-9, *Enterococcus faecium* ZGPR1-54 and *Lactobacillus plantarum* ZGPR2-25.

Post-acidification of the FSC D was similar to one measured in the cheese C (Table 3B). Regarding the post-acidification, it depends on LAB strains' ability to produce lactic acid and in FSC pH values after 10 days of production can vary from 4.20 to 4.60 (Leboš Pavunc et al., 2012). Viable cell count of autochthonous LAB strains and commercial starter culture in produced FSC (C and D) was determined on 1st, 5th and 10th day of storage at 4 °C (Fig. 1B). Results revealed that milk was suitable carrier for the use of LAB strains, since more than 10^7 cfu g^{-1} was reached during the cheese storage. The number of potential probiotic strains ZGPR1-54 and ZGPR2-25 decreased by 0.68 and 0.85 log units, respectively, at the end of storage period.

3.4. Analyses of sensory properties of produced cheeses

Sensory evaluation of produced WPC was made on 10th, 20th and 30th day of cheese ripening (Fig. 2A). The results of sensor evaluation generally showed better sensory parameters for WPC A than for cheese B. This can be explained by fact that functional autochthonous starter culture used in this study did not contain *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* nor *Leuconostoc* sp., which are known as diacetyl and acetoin producers, and are present in commercial starter culture, that could be significant in aroma formation (Tratnik & Božanić, 2012). Similarly, Leboš Pavunc et al. (2012) showed in previous work that in some cases it was necessary to combine autochthonous strains with commercial starter culture for cheese production in order to obtain satisfactory sensory properties.

Sensory evaluation of produced FSC was made on 1st, 5th and 10th day of storage (Fig. 2B). In contrast, sensory evaluation of FSC C and D gave approximately equal scores for all parameters, appearance, colour, taste and odour in both cheeses. However, the consistency of cheese C got better average score, than FSC D made with autochthonous strains, while taste parameter got better score in cheese D than in cheese C. This could be attributed to the lack of specific traditional flavour and aroma in the cheese C. Hence, desirable sensory properties of FSC were achieved only with addition of autochthonous starter cultures.

3.5. Monitoring and identification of the starter cultures in cheeses

DNA-based techniques such as PCR-DGGE and PFGE are of great discriminatory strength, even up to differentiation of individual strains (Cocolin et al., 2001). Thus, by using PCR-DGGE, to detect selected autochthonous strains at the end of the each cheese manufacture, we aim to support microbiological analysis and to show whether selected starter cultures can sustain high viability in produced cheeses. The DGGE fingerprint of the autochthonous LAB starter was performed in order to check their presence in the WPC B and FSC D at the end of ripening and storage time, respectively. Obtained DGGE patterns from cheese samples were compared with DGGE patterns obtained with autochthonous LAB strains used for the cheese production (Fig. 3A). The results showed that DGGE patterns obtained from FSC D corresponds to the DGGE pattern of mixed autochthonous LAB. The DGGE pattern was in agreement with the number of the live cell count of ZGBP5-9, ZGPR2-25 and

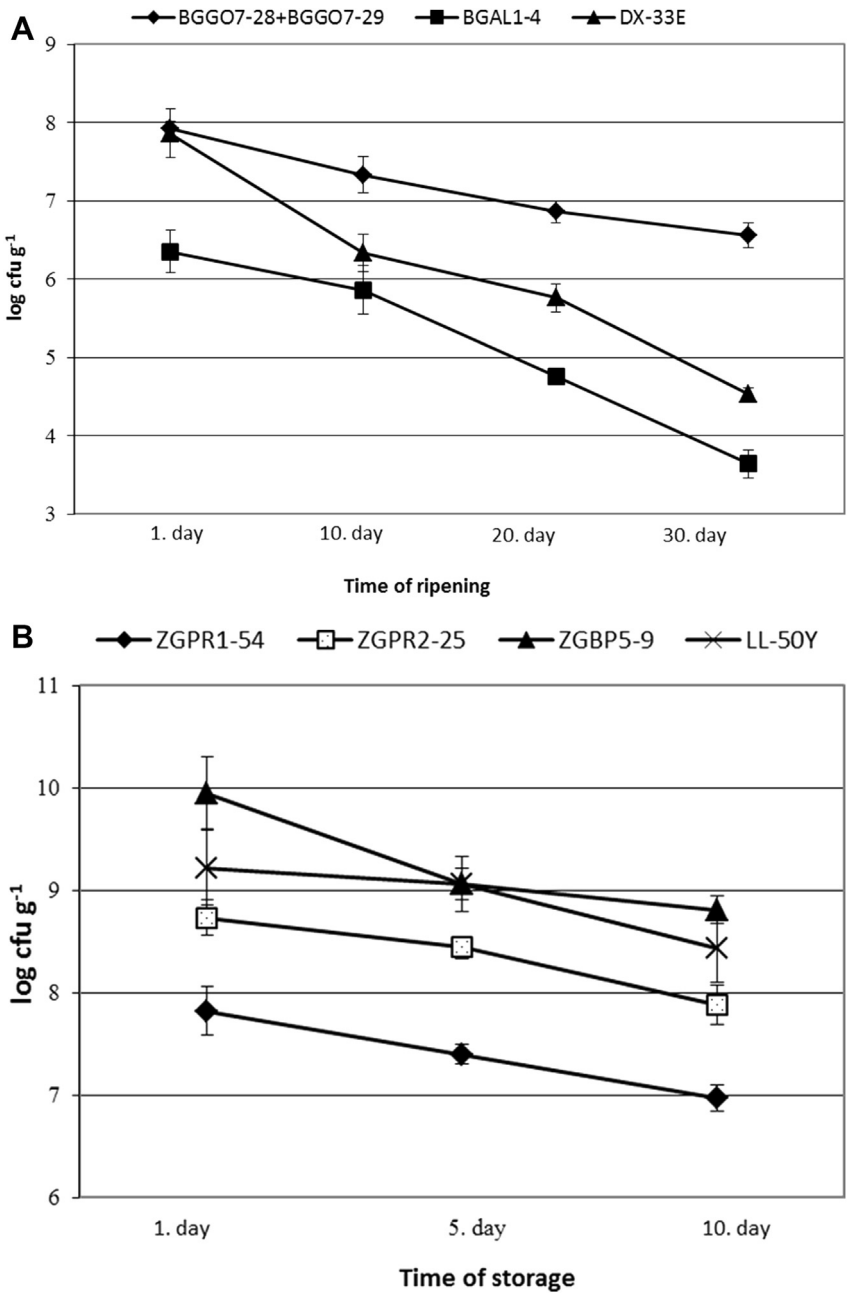

Fig. 1. Average change of: **A**. *Lactococcus* sp. and *Lactobacillus* sp. cell count in white pickled cheese during 30 days of ripening; **B**. *Lactococcus lactis* ZGBP5-9, *Lactobacillus plantarum* ZGPR2-25 and *Enterococcus faecium* ZGPR1-54 cell count during 10 days storage of fresh cheese.

ZGPR1-54 after 10 days storage of FSC (Fig. 1A). However, comparing the DGGE pattern obtained from the mixture of autochthonous LAB cultures used for WPC production with the DGGE pattern obtained from cheese B after 30 days of ripening it can be seen that the band corresponding to the strain BGAL1-4 is missing. The result was in agreement with the number of live cell count of BGAL1-4 and two *Lactobacillus* strains after 30 days of WPC ripening (Fig. 1A).

In addition, in order to discriminate the LAB strains used for mixed starter cultures for production of WPC and FSC, PFGE was performed (Fig. 3B). The results showed that all used strains have unique PFGE profiles that could be efficiently used for discrimination and monitoring of the strains in industrial process.

4. Conclusions

The results of the study suggest promising use of autochthonous LAB strains for production of traditional cheeses under controlled conditions, with preserved traditional taste and aroma and with the same quality. The autochthonous starter culture composed of strains *Lc. lactis* ZGBP5-9, *Lb. plantarum* ZGPR2-25 and *Ec. faecium* ZGPR1-54 could be successfully used for the fresh cheese production. In addition, autochthonous starter culture composed of *Lc. lactis* subsp. *lactis* BGAL1-4, *Lb. plantarum* BGGO7-29 and *Lb. brevis* BGGO7-28 are suitable for white pickled cheese production, although it could be combined with commercial starter culture containing faster producers of diacetyl such as *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* sp. in order to improve sensory

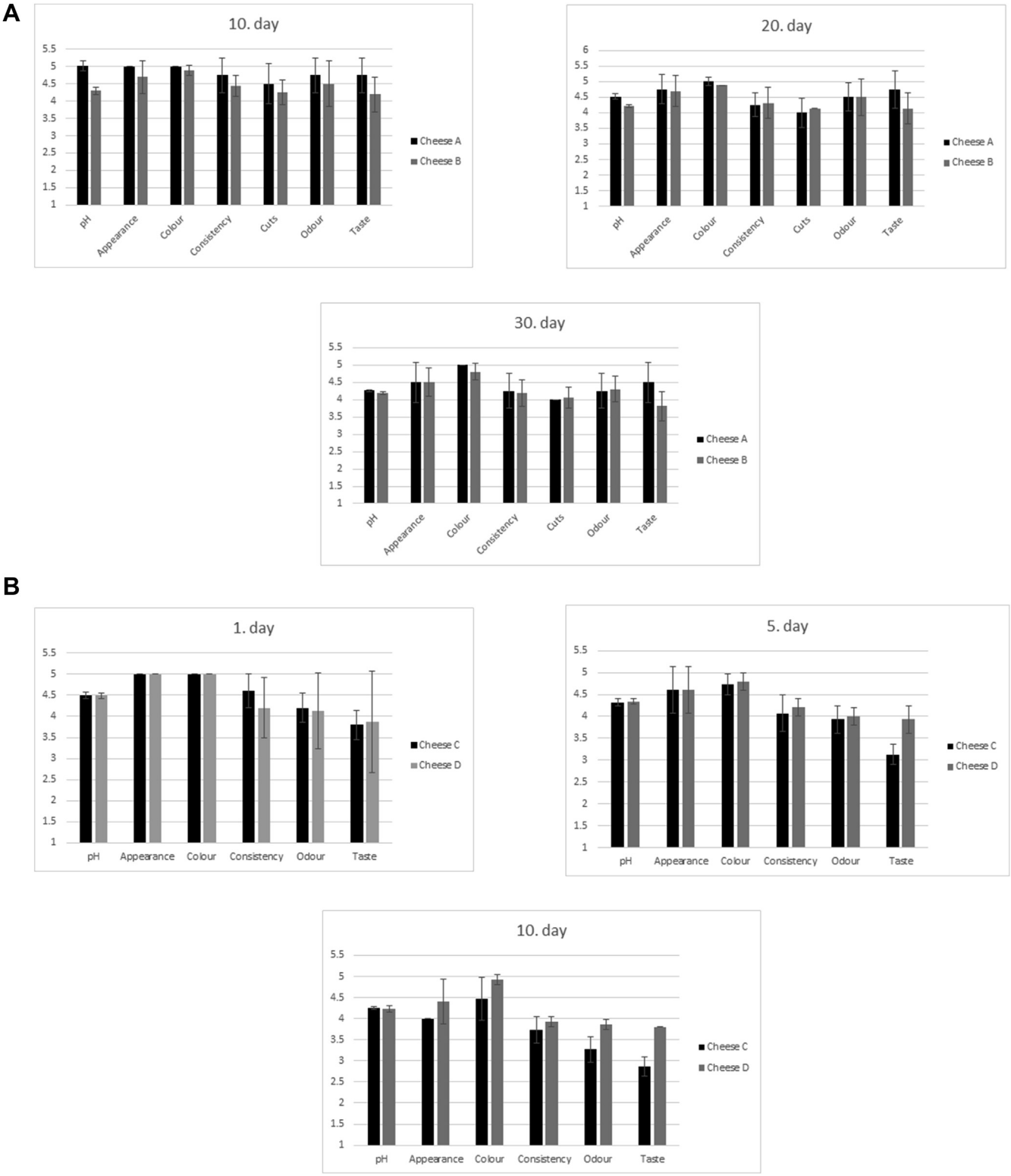

Fig. 2. Changes in sensory properties: **A**. In white pickled cheese samples during the ripening; **B**. In fresh soft cheese samples during the storage.

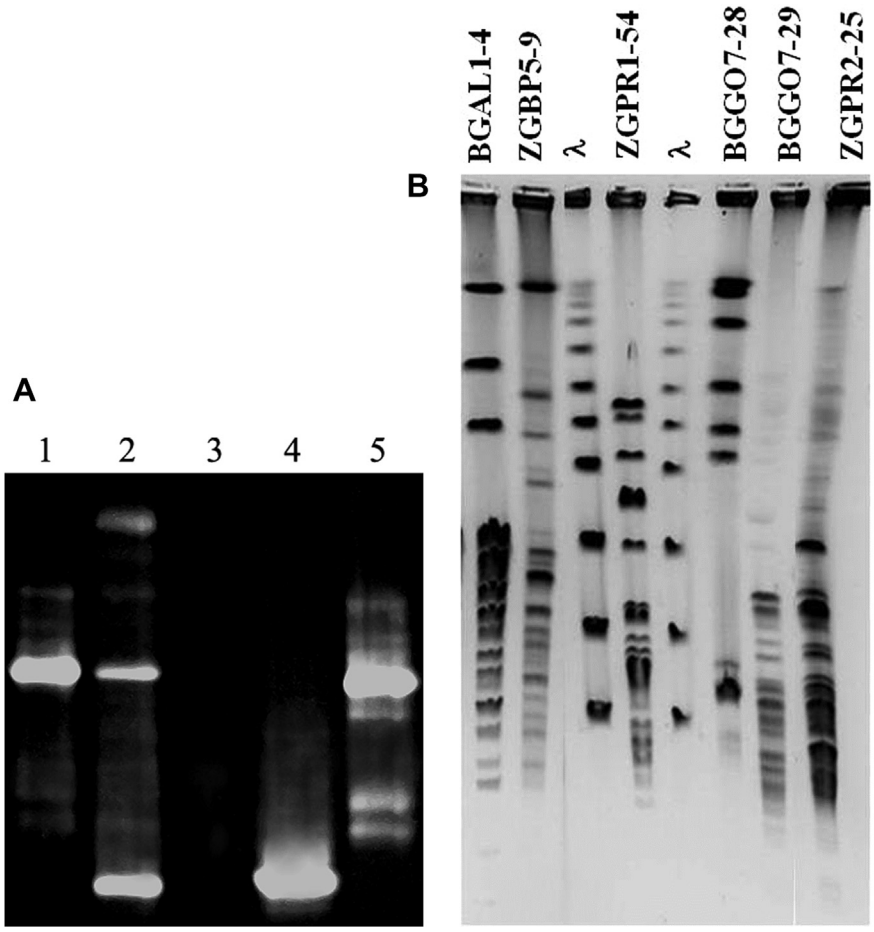

Fig. 3. A. DGGE profiles of autochthonous LAB strains contained in designed starter cultures: line 1 – autochthonous LAB strains used as starter cultures for fresh soft cheese production: *Lactococcus lactic* ZGBP5-9, *Enterococcus faecium* ZGPR1-54 and *Lactobacillus plantarum* ZGPR2-25; line 2- autochthonous LAB strains used as starter cultures for white-pickled cheese production: *Lactococcus lactis* BGAL1-4, *Lactobacillus brevis* BGGO7-28 and *Lactobacillus plantarum* BGGO7-29; line 3 – milk used for cheese production (control); line 4 – white-pickled cheese sample after 30 day of ripening; line 5 – fresh soft cheese sample after 10 days of storage; **B**. PFGE profiles of autochthonous LAB contained in designed starter cultures used for cheese production.

properties. In addition, since three probiotic LAB strains were used in starter culture design, the cheeses based on the suggested starter cultures may have additional functional properties.

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