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Characterization and Application of Autochthonous Starter Cultures for Fresh Cheese Production

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Summary

The use of commercial starter cultures in fresh cheese production from pasteurized milk results in the loss of typical characteristics of artisan fresh cheese due to the replacement of complex native microbiota with a defined starter culture. Hence, the aim of this research is to isolate and characterize dominant lactic acid bacteria (LAB) in artisan fresh cheese and to evaluate their capacity as autochthonous starter cultures for fresh cheese production. Fifteen most prevalent Gram-positive, catalase-negative and asporogenous bacterial strains were selected for a more detailed characterization. Eleven lactic acid bacterial strains were determined to be homofermentative cocci and four heterofermentative lactobacilli. Further phenotypic and genotypic analyses revealed that those were two different LAB strains with high acidifying and proteolytic activity, identified as *Lactobacillus fermentum* A8 and *Enterococcus faecium* A7. These two autochthonous strains, alone or in combination with commercial starter, were used to produce different types of fresh cheese, which were evaluated by a panel. Conventional culturing, isolation, identification and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) procedures, applied to the total fresh cheese DNA extracts, were employed to define and monitor the viability of the introduced LAB strains and their effect on the final product characteristics. Production of fresh cheese using a combination of commercial starter culture and selected autochthonous strains resulted in improved sensorial properties, which were more similar to the ones of spontaneously fermented fresh cheese than to those of cheese produced with only starter culture or selected strains. After 10 days of storage, that cheese retained the best sensorial properties in comparison with all other types of cheese. The presence of inoculated autochthonous and starter cultures and their identification was demonstrated by DGGE analysis. The obtained results indicate that autochthonous strains have a strong potential to enrich the flavour of industrially produced fresh type cheese under controlled conditions.

Key words: autochthonous starter strains, fresh cheese, *Lactobacillus fermentum*, *Enterococcus faecium*, PCR-DGGE

Introduction

Fresh cheese is an autochthonous Croatian product, industrially produced by coagulation of pasteurized milk using commercial starter cultures of mesophilic lactic acid bacteria (LAB) and chymosin. It has unique medium acid and refreshing flavour, white colour and soft fragmented consistency (*1*). Traditionally, this cheese is produced

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from unpasteurized milk by spontaneous fermentation with autochthonous LAB during 2–3 days, in the households using simple equipment. The activity of bacteria naturally present in the milk is rather uncontrolled and unpredictable, which results in less uniform sensory characteristics and composition of the product (*2–4*). Industrial production of this kind of cheese requires pasteurization of the milk and the addition of starter cultures (*5,6*). The usage of commercial starter cultures in fresh cheese production from pasteurized milk results in the loss of typical characteristics of artisan cheese due to the replacement of the complex native microbiota with a defined starter culture (*7,8*). Hence, the application of autochthonous starter cultures consisting of LAB isolated from traditionally produced fresh soft cheese and with proven technological capabilities might guarantee improvement of product quality and, at the same time, preservation of characteristics that define the identity of traditional cheese (*6*). The interest of consumers in autochthonous cheese is growing, and the interest of small and medium enterprises (SMEs) in the development of their recognizable trade marks on Croatian and European market in the future is constantly increasing as well (*1*).

Traditionally produced fresh cheese is a valuable source of lactic acid bacteria (LAB) that represent local, geographically specific microbiota, which contributes to differences in flavour, texture and taste among types of cheese of different geographical origin. Isolation of LAB from autochthonous cheese manufactured according to the local tradition, without the addition of commercial starter culture, is the basis for selection of new strains with desirable properties for the development of autochthonous starter cultures (*3,9*).

Progress in molecular biology techniques has led to a pool of information on the natural microbial population present in artisan cheese. This can help to prevent the loss of microbial biodiversity in typical foods and consequently, the loss of a wide range of cheese types produced by different methods, whose typical features depend on local and regional traditions and indigenous microbial population present in raw milk and cheese-making environment (*10,11*). Moreover, these data may be a helpful guideline for the selection of promising autochthonous strains of LAB in cheese production as starters for specific flavour formation or as protective cultures with bacteriocinogenic activity (*4,9,12–14*).

The majority of commercial starter strains for fresh cheese fermentation are homofermentative and were mostly selected and developed from *Lactococcus* species that initiate fermentation by producing lactic acid, which results in curd formation (*15,16*). Therefore, as the *Lactococcus* species are already well characterized as starter cultures for cheese production, the aim of this study is to isolate and characterize other autochthonous lactic acid bacteria species that could be responsible for specific sensory characteristics of traditional fresh cheese, such as pleasant, slightly acidic taste, with characteristic odour and soft fragmented consistency. De Man-Rogosa-Sharpe (MRS) growth medium was used as a selective medium for isolation of autochthonous *Lactobacillus* strains, but it is also suitable medium for other LAB that can be present in the cheese. The aim of this study is identification, characterization and finally, implementation of autochthonous starter cultures in the production of traditional fresh cheese from pasteurized milk, in order to obtain authentic aroma and foliated structure, which cannot be achieved using standard commercial starter cultures in industrial fresh cheese production.

Materials and Methods

Enumeration and isolation of the autochthonous lactic acid bacteria

Lactic acid bacteria were isolated from 3 types of autochthonous fresh cheese, produced traditionally in the household located in Prigorje region in Croatia. A mass of 10 g of each cheese sample was homogenized in 90 mL of sterile saline solution in the BagMixer® 400 blender (Interscience, St Nom, France) for 3 min. For microbial enumeration, cheese homogenates were serially diluted and appropriate aliquots were plated onto plate count agar (PCA) for the total bacterial count (Biolife, Milan, Italy), on M17 agar (Biolife) at 30 °C for *Lactococcus* count, and on MRS agar (Biolife) at 37 °C for *Lactobacillus* count. Plates were incubated at the appropriate temperatures for 48 h. After incubation, the plates with the colony forming units (CFU) ranging from 10 to 300 were selected for enumeration, while 50 colonies grown on MRS agar after inoculation of the highest dilution of samples were chosen for further investigations, in order to focus on dominant *Lactobacillus* bacteria responsible for milk fermentation. The chosen isolates of autochthonous LAB were maintained in the MRS broth (Biolife) containing 15 % glycerol (Alkaloid, Skopje, FYROM) at –80 °C.

Phenotypic characterization of autochthonous LAB

All isolates were subjected to the following microbiological and physiological/biochemical tests: cell morphology, Gram staining, catalase test using 3 % H_2O_2 (Kemika, Zagreb, Croatia), spore formation, gas production from glucose in MRS broth containing inverted Durham tubes, growth at different temperatures (10, 15 and 45 °C) and different NaCl concentrations (40 and 65 g/L) (Kemika).

The pH value of the cell-free culture supernatants of isolated strains after overnight incubation in MRS broth at 37 °C was measured by pH meter (Metrohm, Herisau, Switzerland). The concentration of lactic acid in the cell-free supernatants of isolated strains was evaluated by commercial D- and L-lactic acid determination kit (test-combination D-lactic acid/L-lactic acid UV method; Roche Diagnostics GmbH, Mannheim, Germany).

In order to determine the acidifying activity, overnight cultures were inoculated into 10 % by mass per volume of skimmed milk (Dukat, Zagreb, Croatia) and incubated at 37 °C. Lactic acid production and decrease in pH values were recorded after 4 and 24 h of incubation.

Proteolytic activity of the isolated strains was assessed by agar-well diffusion test on MRS agar containing 1.5 % by mass per volume of skimmed milk. Isolates to be tested for proteolytic activity were recovered in MRS broth after overnight incubation at 37 °C. Subsequently, MRS agar containing 1.5 % by mass per volume of

skimmed milk was inoculated with 50 µL of the overnight culture in duplicate, in the wells of 7 mm in diameter, and incubated at room temperature. Clear zones around the inoculated wells appeared after casein hydrolysis as a result of proteolytic activity of the tested isolates and were measured after 4 and 16 h of incubation.

The ability of isolated presumptive LAB to ferment various carbohydrates was determined using API 50 CHL for lactobacilli and API 20 Strep for enterococci (bioMérieux, Marcy l'Etoile, France), performed according to the manufacturer's instructions.

SDS-PAGE of whole cell proteins

The whole cell protein extracts were essentially prepared according to Sánchez *et al.* (*17*). The prepared protein extracts were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % polyacrylamide gel. Protein bands were visualized by staining with Comassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO, USA).

Genotypic characterization of the autochthonous LAB

PCR with genus-specific primers

Genomic DNA was prepared according to Frece *et al.* (*18*). Based on the preliminary phenotypic identification, polymerase chain reactions (PCR) were performed with *Lactobacillus* (*19*) and *Enterococcus* (*20*) genus-specific primers. Reaction mixtures and PCR programs were performed according to the cited authors.

DNA fingerprinting using AFLP™

Identification of the isolated autochthonous LAB was performed using amplified fragment length polymorphism (AFLP™) method provided by Belgian Co-ordinated Collections of Microorganisms/Laboratory for Microbiology (BCCM/LMG, University of Ghent, Ghent, Belgium) identification service.

DNA isolation was peformed according to Gevers *et al.* (*21*). Purified total DNA was digested using restriction enzymes *Taq*I, a 4-base cutter and *Eco*RI, a 6-base cutter. Adaptor 5´-CTCGTAGACTGCGTACC-3´ 3´-CTGACGCATGGTTAA-5´ was used with restriction enzyme *Eco*RI, and adaptor 5´-GACGATGAGTCCTGAC-3´ 3´-TACTCAGGACTGGA-5´ with restriction enzyme *Taq*I. PCR primers EO1 (5´-GACTGCGTACCAATTCA-3´)/T11 (5´-GTTTCTTATGAGTCCTGACCGAA-3´) were specially hybridized to the adaptor ends of the restriction fragments. PCR products were analyzed in ABI PRISM® 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting electrophoretic patterns were normalized and subjected to a band pattern recognition procedure using the GeneMapper v. 4.0 software (Applera, Norwalk, CT, USA). Normalized tables of peaks, containing fragments of 20 to 600 base pairs, were transferred to BioNumerics™ v. 4.61 software (Applied Maths, Ghent, Belgium). For numerical analyses, data interval was delineated between the 40- and 580-bp bands of the internal size standard. The obtained DNA profiles of autochthonous strains were compared with the reference profiles of the lactic acid bacterium taxa from BCCM collection. Clustering of the patterns was done using the Dice coefficient and the UPGMA algorithm.

PCR with primers for proteolytic activity

Genomic DNA was prepared according to Frece *et al.* (*18*). Amplification of two cell envelope-associated proteinase genes, *prtH2* and *prtH*, was performed according to Genay *et al.* (*22*) using primers listed in Table 1. Each PCR was carried out in a final volume of 20 µL containing 1.25 U of *Taq* DNA polymerase, 20 ng of genomic DNA, each deoxynucleoside triphosphate at concentration of 200 µmol/L and each primer at concentration of 25 µmol/L. After the first denaturation step at 95 °C for 4 min, the mixture was subjected to 30 cycles of three steps of 30 s (95, 58 and 72 °C). Final extension was carried out at 72 °C for 10 min. PCR products were checked on a 1.5 % (mass per volume) agarose gel by DNA electrophoresis at 100 V for 45 min.

Table 1. Sequences of primers for the detection of *prtH* and *prtH2* genes by PCR according to Genay *et al.* (*22*)

Primer	Sequence (5′-3′)
PrtH-for-1	GGTACTTCAATGGCTTCTCC
PrtH-rev-1	GATGCGCCATCAATCTTCTT
PrtH-for-3	CTCAGCACCAGGTGGACATA
PrtH-for-2	CGATGATAATCCTAGCGAGC
PrtH-rev-2	TGGCAGAACCTGTGCCTA
PrtH2-for-1	CCAGCTAATAATCAAGACCA
PrtH2-rev-1	AACAGCATATTGAACTGCTC
PrtH2-for-2	GAAGACAAGGTGCTGGTCAA
PrtH2-rev-2	TAGCATTTTGGTCAAAGACA
PrtH2-for-3	GTTGGTGCCGCAACTAAATC

PCR-DGGE analysis of the total cheese DNA extracts

Isolation of genomic DNA from cheese samples after 10 days of storage at 4 °C and denaturing gradient gel electrophoresis (DGGE) in 30–60 % denaturating gradient were performed according to Cocolin *et al.* (*23*).

Lyophilisation of isolated lactic acid bacteria for the production of fresh cheese

A volume of 10 mL of overnight cultures in MRS broth was collected by centrifugation (10 000 rpm for 20 min), then the cells were washed and resuspended in 1 mL of 10 % (by mass per volume) skimmed milk as lyoprotector. After that the cells were frozen at –80 °C and then lyophilized in Christ Alpha 1–4 benchtop freeze dryer (B. Braun Biotech International, Christ, Melsungen, Germany).

Production of fresh cheese samples

In total, four different fresh cheese samples were analyzed: cheese produced using commercial starter culture, cheese produced using selected autochthonous strains *Lactobacillus fermentum* A8 and *Enterococcus faecium* A7, and cheese made with the combination of commercial culture and selected autochthonous strains, while spontaneously fermented cheese from artisan household served as a control. Before adding commercial starter or selected autochthonous strains, 20 L (5 L for each cheese type)

of cow's milk were pasteurized at 65 °C during 30 min. After cooling to 24 °C, the milk was inoculated with 2 % commercial starter culture CHN22 (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* ssp. *cremoris*), with 2 % of lyophilized *L. fermentum* A8 and *E. faecium* A7 cells or with the combination of 1 % commercial starter culture CHN22 and 1 % of lyophilized *L. fermentum* A8 and *E. faecium* A7. Coagulation was induced by adding commercially available rennet 0.014 g/L (Maxiren® 150, DSM Food Specialties, Montpellier, France). Milk was allowed to curdle at room temperature for 16 h. The curd was cut at pH=4.4–4.8 and left to rest for 30 min. After resting, the curd was subjected to moulding and percolation at room temperature. Produced cheese samples were stored at 4 °C for 10 days.

Physicochemical and sensory evaluation of cheese samples

Chemical composition of each cheese sample was determined by the following standard methods: dry matter by standard drying method at 105 °C, milk fat content by Van Gulik method (*24*), and total protein content by Kjeldahl method (*25*). pH decrease in cheese samples was measured with pH meter (Methrom) and acidity was expressed according to Soxhlet-Henkel (°SH). Cheese yield was determined by the mass of the produced cheese samples and calculated according to the quantity of used raw material (milk), while syneresis was expressed in percentage (*26*). Sensory evaluation (appearance, consistency, colour, odour and taste) of the produced cheese samples was done by a panel consisting of 5 members using weighted point method (*27*).

Viability of lactic acid bacteria in the fresh cheese samples during storage

Each cheese sample (10 g) was homogenized in 90 mL of sterile saline solution for 3 min in a BagMixer® 400 blender (Interscience). Cheese homogenates were serially diluted and appropriate aliquots were plated on certain media. Cheese produced with *Enterococcus faecium* A7 and *Lactobacillus fermentum* A8 strains and cheese produced with a combination of commercial starter culture and *E. faecium* A7 and *L. fermentum* A8 strains were plated on MRS agar and incubated at 45 °C to enumerate *E. faecium* A7 and *L. fermentum* A8 cells. Diluted samples of the cheese produced with commercial starter culture alone or in combination with *E. faecium* A7 and *L. fermentum* A8 strains were additionally plated on M17 medium at 30 °C to enumerate mesophilic bacteria from the commercial starter culture. Spontaneously produced cheese was also plated on both media. Plates were incubated for 48 h. After the incubation, the plates with a number of CFU ranging from 10 to 300 were selected for enumeration.

Statistical analyses

Statistically significant differences among the treatments were determined by the analysis of variance (ANOVA) using STATISTICA v. 9 (*28*). *Post hoc* Duncan's multiple range test was used as a guide for pair comparisons of the treatment mean values. The differences between treatments that were subsequently described as being significant were determined at $p<0.05$.

Results and Discussion

In this study, traditionally produced fresh cheese samples from Prigorje region of Croatia were examined for the total bacterial count, which averaged $9 \cdot 10^7$ CFU/g, then for *Lactococcus* strain count with the average of $3 \cdot 10^5$ CFU/g on M17 agar plates, and for *Lactobacillus* species count with the average of $2 \cdot 10^7$ CFU/g on MRS agar plates. MRS is commonly used selective medium for *Lactobacillus* strains but it is also appropriate for the growth of some other lactic acid bacteria, like *Enterococcus* and *Leuconostoc*.

Fifteen isolates that reduced the pH value of the MRS broth media after overnight incubation below pH=5 and produced lactic acid in the concentration higher than 9 g/L were chosen among 50 isolates tested for further study (data not shown). Production of lactic acid is desirable property as it influences the fresh flavour of the cheese curd and causes curd shrinkage, enables better water separation and provides appropriate curd texture and conditions for the formation of specific cheese aroma (*29*). Microscopy of the isolates has revealed 11 cocci and 4 rod-shaped bacteria, which is in agreement with the literature reporting higher number of cocci than rods in fresh cheese not subjected to ripening, because the number of lactobacilli increases with ripening time (*30*). All eleven coccus isolates were described as Gram-positive, catalase-negative and non-spore forming, without the ability of CO_2 production from glucose and were thus considered homofermentative. Isolated cocci were initially and preliminary identified as enterococci because of their possibility to grow at 10 and 45 °C and also in the presence of 6.5 % (by mass per volume) NaCl. Four rod-shaped isolates were identified as heterofermentative lactobacilli since they are Gram-positive, catalase-negative and non-spore forming bacteria that can produce CO_2 from glucose.

SDS-PAGE of whole cell proteins derived from 15 chosen isolates was performed within the phenotypic characterization (data not shown). Comparison of the protein profiles of those isolates indicated that there are probably two different bacterial strains, showing identical whole-cell protein profile for all identified enterococci (11 isolates) on the one hand, and also identical profile for rod-shaped isolates (4 isolates) on the other hand. As they were isolated from the highest decimal dilution of cheese samples, it was presumed that these two strains were predominant in the microbiota of chosen fresh cheese samples. One representative of enterocooci, marked A7, and one of lactobacilli, marked A8, were chosen for further study. Phenotypic characterization of the isolated strains was done by API tests (API 50 CHL for lactobacilli and API 20 Strep for enterococci). Strain A7 was identified as *Enterococcus faecium* and strain A8 as *Lactobacillus fermentum*, with 84.6 and 92.7 % similarity with the species from the API system database, respectively.

To avoid possible misclassification, phenotypic methods were supported with genotypic methods for the reliable identification. According to the results of microbio-

logical and physiological/biochemical tests, identification on the genus level was verified and confirmed by PCR using primers specific for *Lactobacillus* and *Enterococcus* genera (data not shown). Bulut *et al.* (*15*) also isolated high percentage of strains of enterococci from traditional type of Turkish cheese from Central Anatolia. In Mediterranean type of cheese, enterococci are present at concentration of 10^7 CFU/g and it is shown that they have beneficial effects on the flavour development in many types of cheese (*16*,*31*).

DNA fingerprinting using AFLP™ was applied for identification at species level and dendrograms of cluster analyses are presented in Fig. 1. Identification of cheese isolates was quite straightforward since there was a good correlation between the results of API test and AFLP analysis. However, the carbohydrate fermentation pattern obtained by API test can be affected by experimental conditions such as incubation time and temperature, and hence is limited in terms of its discriminating ability and accuracy. In contrast, molecular methods such as AFLP offer the advantage of having a high level of taxonomic resolution at species or subspecies level (*32*).

In order to incorporate the identified lactic acid bacteria *E. faecium* A7 and *L. fermentum* A8 as autochthonous starter cultures for fresh cheese production, it was necessary to examine metabolic activity of these strains in skimmed milk by measuring the pH and lactic acid concentration produced during 16 h of incubation (Table 2). Both strains displayed coagulation properties, lowering the pH value of skimmed milk below 5.0 after 4 h of incubation, whereby strain *E. faecium* A7 decreased the pH value for 0.23 units more in comparison with *L. fermentum* A8. However, after 16 h of incubation, pH was equal for both strains showing the value reduced for approx. 2.1 units. Faster acidifying ability of *E. faecium* A7 at the very start of incubation is in agreement with the results of Bulut *et al.* (*15*), who compared the acidifying profiles of lactococci, enterococci and lactobacilli.

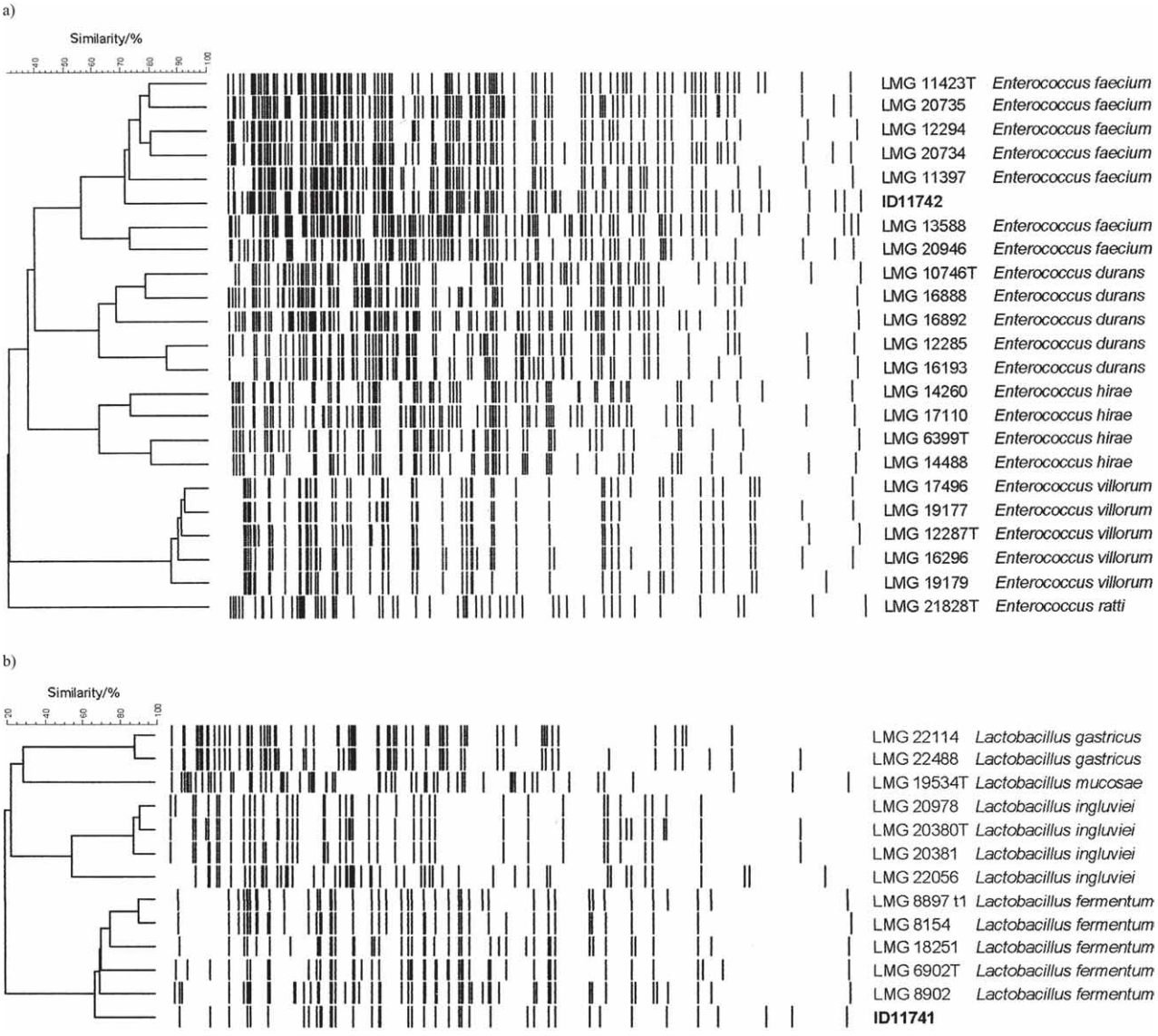

Fig. 1. Computer-generated profiles of: a) *Enterococcus faecium* A7 (ID 11742), reference strains of *Enterococcus faecium* from BCCM and phylogenetically related species; b) *Lactobacillus fermentum* A8 (ID11741), reference strains of *Lactobacillus fermentum* from BCCM and phylogenetically related species, and a dendrogram of the cluster analyses of these profiles

Table 2. pH changes and lactic acid concentrations in skimmed milk inoculated with autochthonous strains *Enterococcus faecium* A7 and *Lactobacillus fermentum* A8 and their proteolytic activity determined on the MRS agar with the addition of 1.5 % (by mass per volume) of skimmed milk after incubation at room temperature

Activity in skimmed milk	Incubation time/h	*E. faecium* A7	*L. fermentum* A8
ΔpH	4	$(0.8\pm0.05)^a$	$(0.57\pm0.08)^b$
	16	$(2.1\pm0.14)^a$	$(2.11\pm0.12)^a$
γ(lactic acid)/(g/L)	4	$(4.14\pm0.09)^a$	$(3.78\pm0.19)^b$
	16	$(7.94\pm0.29)^b$	$(8.74\pm0.28)^a$
Proteolytic activity*	4	$(10.0\pm0.5)^a$	$(10.0\pm1.0)^a$
	16	$(15.0\pm1.0)^b$	$(19.0\pm1.5)^a$

*clear zone diameter (in mm)
Values are mean±standard deviations of results from three separate experiments
Values concerning certain parameter in the same row having different letters in superscript differ significantly ($p<0.05$)

Their results revealed that pH value determined in milk decreased sharply between the 3rd and 6th hour of incubation for lactococci, after which it slowed down for the enterococci, while lactobacilli displayed the slowest acidifying activity. Furthermore, *E. faecium* A7 produced 0.36 g/L more lactic acid than *L. fermentum* A8 after 4 h of incubation in skimmed milk, but at the end of incubation, *L. fermentum* A8 produced 0.8 g/L more lactic acid (Table 2). These results are supported with literature data which report that lactobacilli produce more lactic acid than enterococci, although more slowly (*15*). Besides lactic acid production, bacteriocin production by enterococci could also be beneficial because of the growth inhibition of some food pathogens, if it does not inhibit the applied starter cultures. Some preliminary results suggest that bacteriocinogenic activity of *Enterococcus faecium* A7 should be further examined. Antibacterial activity together with other desirable technological and metabolic properties is the main reason to suggest enterococci as part of a defined starter culture for fresh cheese production, as well as for production of different European types of cheese (*15*,*33*).

Since milk does not contain appropriate quantity of free amino acids and low molecular peptides that are necessary for growth of lactic acid bacteria, in order to hydrolyze milk proteins to the amino acids and peptides they need to have an active system of proteinases (*22*, *34*). Although the production of fresh cheese does not include ripening and the main function of lactic acid bacteria is the production of lactic acid from lactose, products of milk protein degradation (such as casein) are important flavour compounds or flavour precursors. It has clearly been demonstrated that there is a broad differentiation in the activity and specificity of proteolytic enzymes from different LAB species or even strains, which can contribute to specific sensory properties of cheese (*7*,*9*,*16*). Proteolytic activity of bacterial strains *E. faecium* A7 and *L. fermentum* A8 was examined using the diffusion test in agar wells containing skimmed milk. The appearance of clear zones in skimmed milk agar around the inoculated strains was the indicator of casein hydrolysis (Table 2). Proteolytic activity was observed already after 4 h of incubation and it was equal for both of the strains, reaching zone diameter of about 10 mm in size. Moreover, zone diameters were increased as the incubation proceeded, which could be attributed to the continuous proteolytic activity of each strain. After 16 h of incubation, clear zone around strain *L. fermentum* A8 was increased for additional 4 mm compared to the zone around *E. faecium* A7, probably due to better proteolytic activity of *L. fermentum* A8 under given conditions. Furthermore, the presence of the cell envelope-associated proteinases PrtH and PrtH2 for the degradation of milk casein, originally identified in *Lactobacillus helveticus* strains, was examined in *E. faecium* A7 and *L. fermentum* A8 strains using six pairs of primers. PrtH proteinases are the most extensively studied proteolytic system in *Lactobacillus* species with up to 45 % similarity to the lactococcal PrtP proteinases (*22*). As shown in Fig. 2, strain *E. faecium* A7 exhibited bands corresponding to the *prtH2* gene using all three PrtH2 sets of primers, while *L. fermentum* A8 exhibited no such bands. Although the probability to obtain a PCR product with chosen primers in other genera or species is considered to be very low, positive signals indicate the presence of proteinase genes, similar to the ones present in some *Lactobacillus* strains, in the examined *Enterococcus* strain. As this is the first report about the presence of *prtH* genes that are known as proteolytic system of *Lactobacillus* species in *Enterococcus* strain, further studies are needed to clarify proteolytic profile of the examined strain.

Fig. 2. Products of PCR for *E. faecium* A7 (lanes 1, 5 and 9), *L. fermentum* A8 (lanes 2, 6 and 10), *L. plantarum* H13 as negative control (lanes 3, 7 and 11) and water as negative control (lanes 4, 8 and 12) with PrtH2-for-1/PrtH2-rev-1 (lanes 1–4), PrtH2--for-2/PrtH2-rev-2 (lanes 5–8) and PrtH2-for-3/PrtH2-rev-2 (lanes 9–12) sets of primers. S=standard λ-*Hind*III and Gene ruler 100 bp

To examine the capacity of autochthonous strains *E. faecium* A7 and *L. fermentum* A8 as starter cultures, two different samples of fresh cheese were produced, one with *E. faecium* A7 and *L. fermentum* A8 and another with a combination of these strains and commercial starter cul-

tures. Produced cheese samples were compared with the one produced only with the addition of commercial starter culture and also with the traditional one, produced without the addition of starter cultures. Chemical composition and yield of all produced fresh cheese types are presented in Table 3. Dry matter of all produced cheese types is common for fresh cheese and according to milk fat content they all belong to low fat cheese types (*35*). Nevertheless, cheese produced with bacterial strains *E. faecium* A7 and *L. fermentum* A8 was significantly different from the other produced cheese types in terms of higher content of dry matter (approx. 13.8 %) and milk fat (approx. 2.65 %), and lower content of proteins (approx. 4.3 %) and yield (approx. 6.58 %). Additionally, cheese produced with commercial starter culture slightly differed from the traditional cheese and the one produced with the combination of *E. faecium* A7 and *L. fermentum* A8 strains and commercial starter culture in terms of higher dry matter content (approx. 6.3 %) and lower yield (4.2 %). However, there were no significant differences in physical and chemical parameters between the traditional cheese and the one produced with the combination of *E. faecium* A7 and *L. fermentum* A8 strains and commercial starter culture after production and after the 1st day of storage (Tables 3 and 4). The values of the chemical parameters and yield of investigated cheese samples were similar to the literature data for fresh cheese (*5,36,37*). However, it is important to note that the composition of artisan fresh cheese varies within a broad range due to the differences in the composition of raw milk and processing parameters (*5*).

Changes in pH, acidity, syneresis and mass of produced fresh cheese samples during 10 days of storage are presented in Table 4. The pH values for all cheese samples corresponded to the value relevant for fresh cheese (approx. 4.47), except for the one produced with bacterial

Table 3. Chemical composition and yield of produced fresh cheese samples

Cheese sample	*w*(dry matter) g/100 g	*w*(proteins)* g/100 g	*w*(milk fat)* g/100 g	Yield %, *m/V*
A7+A8	(35.7±2.1)^{c}	(54.62±3.08)^{b}	(18.21±1.96)^{a}	(27.0±2.04)^{a}
CHN22	(28.2±1.1)^{b}	(68.44±4.61)^{a}	(14.89±1.77)^{b}	(26.18±1.57)^{a}
A7+A8+CHN22	(22.5±1.6)^{a}	(70.22±5.13)^{a}	(17.33±1.33)^{a}	(22.39±1.84)^{b}
spontaneous	(21.3±1.3)^{a}	(70.89±3.76)^{a}	(17.84±0.94)^{a}	(20.01±1.02)^{b}

*per mass of dry matter
A7+A8=fresh cheese produced with autochthonous strains *E. faecium* A7 and *L. fermentum* A8 from pasteurised milk; CHN22=fresh cheese produced with commercial starter culture CHN22 from pasteurised milk; A7+A8+CHN22=fresh cheese produced with *E. faecium* A7, *L. fermentum* A8 and commercial starter culture CHN22 from pasteurised milk; spontaneous=spontaneously produced fresh cheese from unpasteurised milk
Values are mean±standard deviations of results from three separate experiments
Values in the same column having different letters in superscript differ significantly ($p<0.05$)

Table 4. Changes in acidity, syneresis and mass of the produced fresh cheese samples during 10 days of storage at 4 °C

Storage/day	Cheese sample	Acidity/°SH	pH	Syneresis/%	Mass/g
1	A7+A8	(50.33±0.58)^{b}	(5.15±0.04)^{b}	(29.40±1.5)^{a}	(400.23±10.20)^{c}
	CHN22	(54.67±0.58)^{a}	(4.46±0.02)^{a}	(24.90±2.1)^{a}	(447.84±18.40)^{b}
	A7+A8+CHN22	(53.67±0.58)^{a}	(4.49±0.04)^{a}	(26.50±2.3)^{a}	(523.69±15.70)^{a}
	spontaneous	(54.00±1.00)^{a}	(4.47±0.05)^{a}	(27.80±3.7)^{a}	(540.00±20.40)^{a}
4	A7+A8	(50.67±0.58)^{b}	(5.13±0.03)^{b}	(11.60±0.5)^{a}	(353.80±3.99)^{c}
	CHN22	(55.00±1.00)^{a}	(4.45±0.03)^{a}	(7.60±0.6)^{b}	(413.80±5.37)^{b}
	A7+A8+CHN22	(54.00±0)^{a}	(4.49±0.03)^{a}	(8.50±0.5)^{b}	(479.18±5.24)^{a}
	spontaneous	(55.30±1.15)^{a}	(4.46±0.02)^{a}	(10.42±1.2)^{a}	(483.73±12.75)^{a}
6	A7+A8	(52.00±1.00)^{c}	(5.11±0.05)^{b}	(9.50±0.8)^{a}	(320.19±6.30)^{c}
	CHN22	(55.33±1.15)^{ab}	(4.45±0.02)^{a}	(3.20±0.5)^{b}	(400.56±4.14)^{b}
	A7+A8+CHN22	(54.67±0.58)^{b}	(4.48±0.05)^{a}	(4.40±0.8)^{b}	(458.09±7.64)^{a}
	spontaneous	(57.33±1.15)^{a}	(4.43±0.03)^{a}	(8.30±1.1)^{a}	(443.58±10.64)^{a}
10	A7+A8	(52.67±0.58)^{c}	(5.10±0.04)^{c}	(8.10±0.4)^{a}	(294.25±2.86)^{d}
	CHN22	(56.33±1.15)^{b}	(4.43±0.03)^{b}	(3.20±0.3)^{b}	(387.74±2.40)^{c}
	A7+A8+CHN22	(55.33±1.15)^{b}	(4.47±0.04)^{b}	(4.00±0.5)^{b}	(439.77±4.56)^{a}
	spontaneous	(63.33±0.58)^{a}	(4.31±0.05)^{a}	(7.70±0.5)^{a}	(409.42±4.43)^{b}

For description of cheese samples see Table 3
Values are mean±standard deviations of results from three separate experiments
Values in the same column having different letters in superscript differ significantly ($p<0.05$)

strains *E. faecium* A7 and *L. fermentum* A8, which was significantly higher (5.15). Since this cheese was produced without the addition of commercial starter culture, it could be concluded that the addition of *E. faecium* A7 and *L. fermentum* A8 was not sufficient for the fresh cheese production and that the addition of *Lactococcus* strain is necessary for faster development of acidity. During 6 days of storage, there was no significant difference in the pH values among the other three cheese samples, but at the end of storage period, the lowest pH value was determined in the traditional cheese (4.31), which points out the higher post-acidification in the cheese sample that was spontaneously produced with naturally present microbiota and in the absence of controlled process parameters. The increase in acidity was in correlation with the decrease of pH values during storage. There were no significant differences in the percentage of separated whey among the cheese samples after the production, but during storage the lowest syneresis was obtained in the cheese produced with commercial starter culture and in the one produced with a combination of *E. faecium* A7 and *L. fermentum* A8 strains and commercial starter culture (Table 4). The obtained mass values of produced cheese samples were proportional to the yield values (Table 4). During storage, mass values were decreasing as a consequence of whey separation, so at the end of storage time the highest decrease in mass was obtained in the traditional cheese (130 g) and in the one produced with *E. faecium* A7 and *L. fermentum* A8 strains (109 g). This result also points out the influence of commercial starter cultures and controlled cheese production conditions in terms of lower syneresis, which is important because syneresis can cause distortion of cheese appearance and consistency (*5*,*36*). The main characteristic of the examined artisan fresh cheese samples obtained by Kirin (*1*) is great variability, especially in fat content and acidity, which results in varying organoleptic quality. Higher acidity obtained in some of those cheese types negatively affected odour and taste. Mean values of pH (3.81–4.45) and acidity (53.6–91.6 °SH) in these cheese samples were higher than in those examined in this work.

As the aim of this work was to produce fresh cheese from the pasteurized milk with the addition of a well-defined culture under controlled conditions but with aroma and texture characteristics typical for traditional cheese, sensory characteristics of the produced cheese samples were compared with that produced spontaneously during 10 days of storage at 4 °C (Table 5). All cheese samples were described as commercial grade with compact appearance, white colour, pleasant, sour milk odour, soft foliated consistency and mildly sour taste, which are the main characteristics of fresh cheese (*5*,*36*, *37*). Only the cheese sample produced with *E. faecium* A7 and *L. fermentum* A8 strains received significantly lower score for the taste due to the lack of acidity, which was in accordance with the higher pH value (5.15) and also lower score for the consistency, which was evaluated as a compact and firm due to the higher dry matter content. Also, the cheese sample produced with the commercial starter culture significantly differed in terms of odour due to the lack of traditional flavour. Interestingly, cheese sample produced with combination of commercial starter culture and *E. faecium* A7 and *L. fermentum* A8 strains was evaluated as the most similar to the spontaneously produced one and received higher score than the cheese produced only with commercial starter culture because of the absence of traditional aroma.

Table 5. Sensory evaluation of produced fresh cheese samples during 10 days of storage at 4 °C

Storage/day	Cheese sample	Sensory properties (max. points)					
		Appearance (1)	Colour (2)	Odour (3)	Consistency (4)	Taste (10)	Total (20)
1	A7+A8	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(3.73±0.27)^{a}	(8.0±0)^{b}	(17.73±0.54)^{b}
	CHN22	(1.0±0)^{a}	(2.0±0)^{a}	(2.4±0)^{b}	(4.0±0)^{a}	(9.33±0.67)^{a}	(18.73±0.67)^{b}
	A7+A8+CHN22	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(4.0±0)^{a}	(10.0±0)^{a}	(20.0±0)^{a}
	spontaneous	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(4.0±0)^{a}	(10.0±0)^{a}	(20.0±0)^{a}
4	A7+A8	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(3.73±0.27)^{a}	(8.0±0)^{b}	(17.73±0.54)^{b}
	CHN22	(1.0±0)^{a}	(2.0±0)^{a}	(2.4±0)^{b}	(4.0±0)^{a}	(9.33±0.67)^{a}	(18.73±0.67)^{b}
	A7+A8+CHN22	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(4.0±0)^{a}	(10.0±0)^{a}	(20.0±0)^{a}
	spontaneous	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(4.0±0)^{a}	(9.33±0.67)^{a}	(19.33±0.67)^{a}
6	A7+A8	(0.93±0.07)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(3.73±0.27)^{a}	(8.0±0)^{b}	(17.66±1.6)^{bc}
	CHN22	(0.93±0.07)^{a}	(2.0±0)^{a}	(2.4±0)^{b}	(4.0±0)^{a}	(9.33±0.67)^{a}	(18.66±1.08)^{b}
	A7+A8+CHN22	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(4.0±0)^{a}	(10.0±0)^{a}	(20.0±0)^{a}
	spontaneous	(0.87±0.12)^{a}	(1.6±0)^{b}	(3.0±0)^{a}	(3.2±0)^{b}	(8.0±0)^{b}	(16.67±0.32)^{c}
10	A7+A8	(0.93±0.07)^{a}	(1.6±0)^{b}	(2.0±0.35)^{b}	(3.2±0)^{b}	(8.0±0)^{b}	(16.73±0.4)^{b}
	CHN22	(0.8±0)^{b}	(1.73±0.23)^{a}	(2.4±0)^{b}	(4.0±0)^{a}	(9.33±0.67)^{a}	(18.26±0.5)^{a}
	A7+A8+CHN22	(1.0±0)^{a}	(2.0±0)^{a}	(3.0±0)^{a}	(4.0±0)^{a}	(9.0±1.0)^{a}	(19.00±1.0)^{a}
	spontaneous	(0.67±0.12)^{c}	(1.6±0)^{b}	(2.4±0)^{b}	(2.4±0)^{c}	(7.0±1.0)^{b}	(14.07±0.8)^{c}

For description of cheese samples see Table 3
Values are mean±standard deviations of results from three separate evaluations
Values in the same column having different letters in superscript differ significantly ($p<0.05$)

During the storage period, the biggest changes in the consistency were observed for the traditional cheese, probably as the consequence of a higher syneresis, which caused lower scores for the appearance at the end of storage period. Other cheese samples showed minimal changes at the end of storage period, which could be attributed to the commercial culture used for the production and to the controlled process of production.

The lactic acid bacteria count during the storage of cheese samples presented in Fig. 3a demonstrates changes in the viable cell count of *E. faecium* A7 and *L. fermentum* A8 strains in the samples and changes in the count of LAB in traditional cheese that are capable of growing on MRS medium at 45 °C (thermophilic LAB), while Fig. 3b presents changes in viable cell count of mesophilic starter cultures in cheese samples and changes in the count of LAB in traditional cheese that are capable of growing on M17 medium at 30 °C (mesophilic lactococci). In the

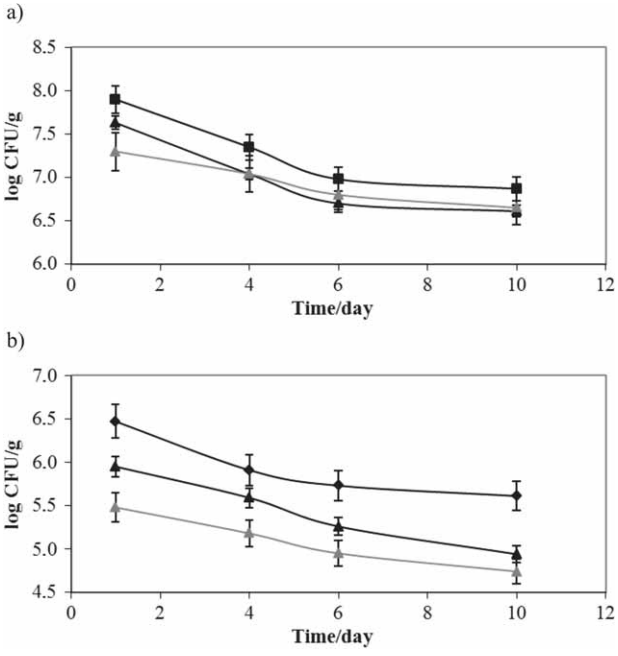

Fig. 3. Viability of bacteria in cheese samples during 10 days of storage tested under two different conditions: a) MRS medium at 45 °C and b) M17 medium at 30 °C. Cheese samples: cheese produced with *Enterococcus faecium* A7 and *Lactobacillus fermentum* A8 (■); cheese produced with commercial starter culture CHN22 (◆); cheese produced with *E. faecium* A7, *L. fermentum* A8 and commercial starter culture CHN22 (▲); spontaneously produced cheese (▲)
Values are mean±standard deviations of results from three separate experiments

cheese sample produced with the combination of *E. faecium* A7 and *L. fermentum* A8 strains and commercial starter culture, *E. faecium* A7 and *L. fermentum* A8 cell count slightly exceeded the number of mesophilic starter culture cells, by 0.68 log units, suggesting that the milk was a suitable carrier for the tested strains, as high levels were reached during manufacture. Besides, the high rate of total bacterial count in each cheese sample was maintained during the whole storage period. The number of *E. faecium* A7 and *L. fermentum* A8 cells, in both cheese samples, decreased by approx. 1.02 log units, while the number of mesophilic starter culture cells decreased by approx. 1.2 log units. In the traditionally produced cheese, the number of thermophilic LAB decreased by 0.65 log units and the number of mesophilic lactococci by 0.74 log units.

In order to monitor the presence of all applied bacterial starter culture strains in the produced cheese samples by controlled fermentation, DGGE analysis was performed (Fig. 4). This method is based on the direct extraction of DNA from food sample and subsequent PCR amplification, which enables rapid and reliable bacteria identification (*38–40*). Analysis of the reference strains showed a single, species-specific DGGE band for all of the analyzed cultures. Other, nondominant bands present in DGGE profiles of strains A7 and A8 (lanes 1 and 2) were not obtained when these two strains were mixed together (lane 3), nor when the DNA extracted from cheese samples was amplified (lane 6), so only dominant bends can be considered as positive signals (Fig. 4). DGGE analysis was performed for pure autochthonous and commercial starter cultures used for the fresh cheese production and the obtained patterns were compared with the patterns obtained for cheese samples.

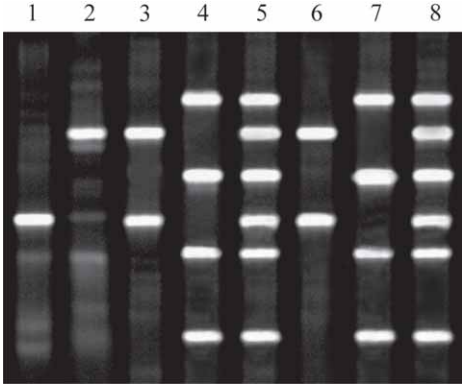

Fig. 4. DGGE profiles of reference strains and fresh cheese samples
Lane 1: *Lactobacillus fermentum* A8; lane 2: *Enterococcus faecium* A7; lane 3: *E. faecium* A7+*L. fermentum* A8; lane 4: commercial starter culture CHN22; lane 5: *E. faecium* A7+*L. fermentum* A8+CHN22; lane 6: cheese produced with *E. faecium* A7 and *L. fermentum* A8; lane 7: cheese produced with commercial starter culture CHN22; lane 8: cheese produced with *E. faecium* A7, *L. fermentum* A8 and commercial starter culture CHN22

Therefore, three samples of fresh cheese (the one produced with commercial starter culture, the one produced with *E. faecium* A7 and *L. fermentum* A8 strains, and the one produced with a combination of commercial starter culture and *E. faecium* A7 and *L. fermentum* A8 strains) were also screened by DGGE after direct DNA extraction and PCR amplification. DGGE profiles generated from the cheese samples did not show any differences in the number of bands or migration distance, meaning that all samples showed bands corresponding to the electrophoretic profiles of pure autochthonous and commercial starter cultures (Fig. 4).

Conclusions

The results of this research indicate that starter culture prepared from selected autochthonous lactic acid strains *Enterococcus faecium* A7 and *Lactobacillus fermentum* A8, in the combination with commercial starter culture, can be successfully used for fresh cheese production in order to achieve specific sensory characteristics, typical for the traditionally made fresh cheese.

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