Identification of lactic acid bacteria in Slovakian bryndza cheese

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Summary
Lactic acid bacterial microflora of Slovakian bryndza cheese produced in 10 factories in Slovakia was characterized. Bacterial strains were isolated on de Man-Rogosa-Sharpe agar and identified on the basis of microbiological and biochemical testing, genotyping by randomly-amplified microsatellite polymorhism and, eventually, by 16S rDNA sequencing. Individual Slovakian bryndza cheese samples were found to contain 3–7 strains of *Lactobacillus brevis*, *Lb. parabuchneri*, *Lb. fermentum*, *Lb. helveticus*, *Lb. paracasei*, *Lb. pentosus*, *Lb. plantarum* and *Pediococcus pentosaceus*. Several bryndza cheese samples contained 2 or 3 genotypes of the same species. No clear correlation between the composition of the lactic acid microflora and the involvement of pasteurization of ewes' milk in the technology was observed. Clear correlation was neither observed between the composition of the lactic acid microflora and the organoleptic quality of the studied Slovakian bryndza cheese.

Keywords
cheese; bryndza; *Lactobacillus*; *Pediococcus*; DNA; PCR

Slovakian bryndza cheese (Slovenská bryndza) is a natural, white, mature, spreadable cheese in granular form. It has a delicate odour and taste and has a pleasantly sour ewes' cheese taste that is slightly spicy and salty. It is manufactured according to the traditional method, by milling a lump of matured ewes' cheese or by milling a mixture of lump ewes' cheese and cows' cheese. The percentage of lump ewes' cheese should be greater than 50%. Alternatively, raw ewes' milk may be pasteurized before curdling and then it is inoculated with a starter culture. In this case, the microflora contains microorganisms from the starter culture and non-starter lactic acid bacteria (NSLAB) from milk. The characteristic organolpetic properties of the Slovakian bryndza cheese are believed to originate from the microflora present in the lump ewes' cheese, comprising *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Kluyveromyces marxianus* and *Geotrichum candidum* [1, 2].

The microflora of Slovakian bryndza cheese has been so far studied only to a limited extent, using mostly classical microbiological methods. Modern methods have been applied only to characterization of enterococci from this cheese, as potential probiotic cultures or producers of bacteriocins, or to pathogenic coliforms responsible for safety problems [3–6]. No data obtained by up-to-date methods, including methods of molecular biology, are available on lactic acid bacteria, which are responsible for sensory properties of many types of cheese [7, 8].

In this study, strains of non-*Enterococcus* lactic acid bacteria were isolated from Slovakian bryndza cheese produced in one year in 10 factories producing it on a larger or medium scale from cheese made from non-pasteurized or pasteurized milk. Individual isolates were identified to the species level by microbiological, biochemical and molecular methods.

MATERIALS AND METHODS

Cheese samples

Slovakian bryndza cheese was produced by the following factories: Agrosúča (Horná Súča); Agro-

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farma (Červený Kameň); Bryndziareň (Turčianske Teplice); Peter Makovický – Bryndziareň, vnuk Peter Lajda (Ružomberok); Liptovská mliekareň (Liptovský Mikuláš); Bryndziareň a syráreň (Zvolenská Slatina); Ľuboš Manica – BRYSYRT (Tisovec); PD Goral (Veľká Franková); IGET (Lipany) and Kluknavská mliekáreň (Jaklovce). Samples of Slovakian bryndza cheese were obtained directly from the producers with the exception of Agrofarma, the product of which was obtained from a supermarket in Bratislava. The samples comprised Slovakian bryndza cheese made from varying portions of ewes' lump cheese (made from unpasteurized or pasteurized ewes' milk) and cows' lump cheese made from pasteurized cows' milk; some of the cheese samples were of summer type (made from fresh ewes' lump cheese) and some of winter type (made from ewes' lump cheese stored for several months). All information on the use of pasteurization in the production process, as well as on the contents of ewes' cheese, was obtained from the producers and was not independently verified. The samples underwent preliminary sensory evaluation so that only cheeses of standard organoleptic quality were included in the study. The preliminary sensory evaluation was carried out by a panel of evaluators taking into account aroma (33%), taste (33%), colour (17%) and texture (17%). Cheeses rated at 70–100% of maximum were taken as of high organoleptic quality, cheeses rated at 50–70% of maximum were taken as of medium organoleptic quality. Cheeses from producers 4 and 10 were obtained lately and could not be included in organoleptic evaluation.

Isolation of strains

An amount of 1 g of Slovakian bryndza cheese was mixed with 9 ml of Buffered peptone water (BPW; Merck, Darmstadt, Germany) and homogenized in Stomacher 400 (Seward, Basingstoke, United Kingdom) for 1 min at medium intensity. The homogenate was decimally diluted in BPW and 1 ml of the dilution of 10^{-5}, 10^{-6} and 10^{-7} was pipetted in a Petri dish in duplicate. Subsequently, warm liquid de Man-Rogosa-Sharpe (MRS) agar (Merck) was poured to the Petri dish and, after it solidified, the plates were incubated microaerobically in an anaerostat using Anaerocult A (Merck) at 37 °C for 48 h. Out of plates with an appropriate density of colonies, a loopful from 20 distinct colonies including those of different morphology was inoculated in 5 ml of MRS broth (Merck) and incubated at 37 °C for 24–48 h. A loopful of the culture was streaked on the surface of MRS agar and incubated microaerobically at 37 °C for 48 h. If the presence of a mixed culture was indicated by the morphology of colonies, the previous step was repeated. If all the colonies had identical morphology, colonies from this plate were used for strain characterization and for storage by freeze-drying.

Microbiological and biochemical characterization

Isolates were presumptively identified by colony morphology, Gram staining, cell morphology, KOH-test, catalase test, assay for gas formation using Durham bells at aerobic and anaerobic conditions, and growth in MRS broth at 15 °C for 5 days and at 45 °C for 3 days [9]. Saccharide fermentation profiles were obtained using API CHL 50 kit (Bio-Mérieux, Marcy l'Etoile, France) with incubation at 37 °C for 48 h.

Randomly-amplified microsatellite polymorphism

Randomly-amplified microsatellite polymorphism was performed by a modification of a previously described method [10]. A loopful of the colony material was transferred to 1 ml of distilled water, mixed and centrifuged at 10 000 *g* for 5 min. InstaGene suspension (Bio-Rad, Hercules, California, USA) was added to the sediment and incubated at 56 °C for 25 min. Then the mixture was vortexed, incubated at 100 °C for 8 min, vortexed, centrifuged at 10 000 *g* for 5 min and the supernatant containing DNA was removed. A volume of 2 μl of the DNA solution was added to the PCR reaction mixture (total volume, 25 μl) containing 500 nmol·l^{-1} primer K7 (5'-caactctctctctct-3'), 500 nmol·l^{-1} primer 1254 (5'-ccgcagccaa-3'; both oligonucleotides synthesized by Qiagen Operon, Cologne, Germany), 600 μmol·l^{-1} each dNTP (Applied Biosystems, Foster City, California, USA), 6 mmol·l^{-1} $MgCl_2$, 2.5 U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) and 2.5 μl of 10× concentrated PCR buffer supplied with the polymerase. PCR was carried out in a Biometra Personal thermal cycler (Whatman Biometra, Göttingen, Germany) using a thermal programme consisting of the initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 95 °C for 60 s, annealing at 40 °C for 90 s, ramping at 0.1 °C·s^{-1}, and polymerization at 72 °C for 120 s, followed by the final polymerization at 72 °C for 10 min. A volume of 12 μl of the PCR product was mixed with 1.5 μl of the loading buffer and analysed by electrophoresis in a 1.5% agarose gel (Seakem LE, FMC Bioproducts, Rockland, Maine, USA) for 5 h at 2.3 V·cm^{-1}. Molecular size standard $n \times 250$ bp (Invitrogen, Carlsbad, California, USA) was electrophoresed in every fourth lane along with samples. The gel was stained with ethidium bromide for 30 min, destained in distilled water for 5 min, visualized under UV light and photographed with

a digital camera. RAMP profiles were compared with those of standard strains from culture collections.

Analysis of 16S rDNA sequences

A single colony of the bacterial isolate was inoculated into 5 ml MRS medium and cultivated for 18–24 h at 37 °C. Bacterial DNA was isolated using DNeasy Tissue kit (Qiagen) according to manufacturer's instructions. In order to amplify the 16S rDNA fragment, the published primers 27F (agagtttgatcctggctcg) and 1492R (cggctaccttgttacgactt) were used [11]. PCR was performed in a volume of 60 μl. Each reaction contained 2 μl of template DNA, 1.25 U DNA polymerase HotStarTaq Plus (Qiagen), 1× reaction buffer for the DNA polymerase, 3.5 mmol·l^{-1} $MgCl_2$ (final concentration), 340 μmol·l^{-1} dNTP mixture (Applied Biosystems), 700 nmol·l^{-1} of the forward primer and 700 nmol·l^{-1} of the reverse primer. Oligonucleotides were synthesized by Qiagen Operon. PCR was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) using a thermal programme consisting of the initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and polymerization at 70 °C for 30 s, followed by the final polymerization at 70 °C for 7 min. PCR products were purified using the QiaQuick PCR purification kit (Qiagen) and sequenced at Department of Molecular Biology, Faculty of Natural Sciences, Comenius University in Bratislava, using the primers 27F and 1492R. The sequences were processed using the LaserGene software (DNAstar, Madison, Wisconsin, USA). Species identification was achieved by comparison to nucleotide database GenBank using the online software Blast (National Center for Biotechnology Information, Bethesda, Maryland, USA).

RESULTS AND DISCUSSION

Typical organoleptic properties of Slovakian bryndza cheese originate from the ewes' milk and from the compounds produced by microflora present in the lump ewes' cheese during fermentation. However, the "typical microflora" involved in the production of this cheese has not been appropriately characterized yet with regard mainly to species from the genera *Lactobacillus* and *Lactococcus*, which are known to significantly contribute to organoleptic properties of various types of cheese [7, 8]. It should be noted that phenotypic methods are known to be insufficient for identification of lactic acid bacteria and a combination of phenotypic and genotypic analyses is accepted as a better alternative [9, 12].

In this study, Slovakian bryndza cheese produced in 10 large- or medium-scale production facilities in the same year were analysed. All samples were of high or medium organoleptic quality. A fraction of microflora culturable on MRS agar at microaerobic conditions was isolated and identified to the genus and species level. Strains of *Enterococcus* sp., which were eventually also present, were not further processed. Microbiological methods, saccharide fermentation profiling by the API kit and genotyping by randomly-amplified microsatellite polymorphism (RAMP) were used. Out of 244 isolated strains, 54 were identified as unique genotypes. In cases when API test and RAMP produced ambiguous or conflicting results, analysis of 16S rDNA sequence was carried out and identification based on its result was taken as definitive. Results are presented in Tab. 1.

In the identification process, typing by RAMP was mostly used to identify identical strains from the same cheese sample, which was helpful in reducing the number of strains to be handled. Comparison of RAMP profiles of strains of unknown identity with a panel of RAMP profiles of authentic strains from culture collections was poorly productive as no sufficient similarities were usually observed (data not shown). On the other hand, RAMP profiles were able to distinguish unique genotypes in frames of species (Tab. 1, Producers 2, 3, 7, 10).

The method of 16S rDNA sequencing was very informative. A strain isolated from the cheese produced by Producer 8 was classified according to its fermentation profile by API as *Lb. acidophilus* (99%), however, based on 16S rDNA sequencing it was classified as *Lb. helveticus*. Several strains isolated from the cheeses produced by Producers 2, 3, 4 were classified according to their fermentation profiles by API as *Lb. curvatus* (99%), however, based on 16S rDNA sequencing they were classified as *Lb. casei* or *Lb. paracasei*. Unambiguous distinction between *Lb. casei* and *Lb. paracasei* was not attempted in this study, since it is problematic and re-classification of these two species under the single taxon *Lb. casei* was repeatedly suggested in the past [13, 14], albeit unsuccessfully [15]. The identification table in the API test defines the fermentation profiles only for *Lb. paracasei* and not for *Lb. casei*. For these reasons, both species are referred to as *Lb. paracasei* in this work. A strain isolated from the cheese produced by Producer 3 was classified according to its fermentation profile by API as *Lb. paracasei* (91%), however, based on 16S rDNA sequencing it was classified

Tab. 1. Lactic acid bacteria isolated from Slovakian bryndza cheese.

Producer	Type	Strain - genotype[a]	Basis of identification							Organoleptic quality
			GS	CM	GP	G	API	RAMP	16S	
1	summer type 80% of ewes' lump cheese made from unpasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	–	+	+	high (rated at 71% of maximum)
		Lactobacillus fermentum	+	+	HeF	45 °C	–	+	+	
		Lactobacillus paracasei	+	+	HoF	15 °C	–	+	+	
		Lactobacillus plantarum 1	+	+	HoF	15 °C	+	+	+	
		Lactobacillus plantarum 2	+	+	HoF	–	–	–	+	
		Pediococcus pentosaceus	+	+	–	15, 45 °C	–	+	+	
2	winter type mixture of cows' lump cheese and ewes' lump cheese made from pasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	+	high (rated at 73% of maximum)
		Lactobacillus parabuchneri	+	+	HeF	–	+	+	+	
		Lactobacillus paracasei 1	+	+	HoF	–	+	+	–	
		Lactobacillus paracasei 2	+	+	HoF	–	+	+	–	
		Lactobacillus paracasei 3	+	+	–	–	–	–	+	
		Lactobacillus plantarum	+	+	HoF	–	+	+	–	
3	summer type 100% ewes' lump cheese made from unpasteurized ewes' milk	*Lactobacillus brevis* 1	+	+	HeF	–	+	+	–	high (rated at 70% of maximum)
		Lactobacillus brevis 2	+	+	HeF	–	+	+	–	
		Lactobacillus paracasei	+	+	–	–	+	+	–	
		Lactobacillus plantarum 1	+	+	HoF	15 °C	–	+	+	
		Lactobacillus plantarum 2	+	+	HoF	15 °C	–	+	+	
		Lactobacillus plantarum 3	+	+	HoF	15 °C	+	+	+	
3	summer type 50% ewes' lump cheese made from unpasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	–	medium (rated at 55% of maximum)
		Lactobacillus fermentum	+	+	HeF	45 °C	+	–	–	
		Lactobacillus paracasei 1	+	+	HoF	15 °C	–	+	–	
		Lactobacillus paracasei 2	+	+	HoF	15 °C	+	+	–	
		Lactobacillus pentosus	+	+	–	–	+	–	+	
		Lactobacillus plantarum 1	+	+	–	15 °C	–	+	+	
		Lactobacillus plantarum 2	+	+	–	15 °C	–	–	+	
4	summer type ewes' lump cheese made from unpasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	–	medium (rated at 59% of maximum)
		Lactobacillus paracasei	+	+	–	15 °C	+	+	+	
		Lactobacillus plantarum	+	+	HoF	–	+	+	–	
		Pediococcus pentosaceus	+	+	–	–	–	+	–	

Tab. 1. *continued*

Producer	Type	Strain - genotype[a]	Basis of identification							Organoleptic quality
			GS	CM	GP	G	API	RAMP	16S	
4	mixture of cows' lump cheese and ewes' lump cheese made from pasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	–	not determined
		Lactobacillus fermentum	+	+	HeF	45 °C	–	+	+	
		Lactobacillus helveticus	+	+	HoF	45 °C	–	–	+	
		Lactobacillus plantarum	+	+	HoF	–	+	+	–	
		Pediococcus pentosaceus	+	–	–	–	–	–	+	
5	summer type mixture of cows' lump cheese and ewes' lump cheese made from pasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	–	medium (rated at 57% of maximum)
		Lactobacillus fermentum	+	+	–	–	+	+	–	
		Lactobacillus paracasei	+	+	HoF	–	–	+	–	
6	summer type mixture of cows' lump cheese and ewes' lump cheese made from pasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	+	medium (rated at 55% of maximum)
		Lactobacillus plantarum	+	+	HoF	–	+	+	–	
		Lactobacillus paracasei	+	+	–	–	+	+	–	
7	summer type 100% ewes' lump cheese made from unpasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	–	high (rated at 86% of maximum)
		Lactobacillus fermentum	+	+	HeF	45 °C	–	+	–	
		Lactobacillus plantarum 1	+	+	HoF	–	–	+	–	
		Lactobacillus plantarum 2	+	+	HoF	–	–	+	–	
8	winter type mixture of cows' lump cheese and ewes' lump cheese made from pasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	–	+	+	+	medium (rated at 56% of maximum)
		Lactobacillus helveticus	+	+	HoF	–	+	–	+	
		Lactobacillus fermentum	+	+	–	–	+	–	+	
		Lactobacillus paracasei	+	+	HoF	–	+	+	–	
9	ewes' lump cheese made from unpasteurized ewes' milk	*Lactobacillus brevis*	+	+	HeF	45 °C	+	+	–	high (rated at 71% of maximum)
		Lactobacillus fermentum	+	+	HeF	45 °C	–	+	–	
		Lactobacillus plantarum	+	+	HoF	–	+	–	+	
		Lactobacillus paracasei	+	+	HoF	15 °C	+	+	–	
10	mixture of cows' lump cheese and ewes' lump cheese made from pasteurized ewes' milk	*Lactobacillus fermentum* 1	+	+	HeF	45 °C	+	+	+	not determined
		Lactobacillus fermentum 2	+	+	HeF	45 °C	+	+	+	
		Lactobacillus paracasei 1	+	+	HoF	15 °C	+	+	–	
		Lactobacillus paracasei 2	+	+	HoF	–	–	+	–	

a – If more than one genotype of a species per sample were distinguished by RAMP, then individual genotypes were numbered starting from 1 for each sample.
GS – Gram staining + cell morphology, CM – colony morphology, GP – gas production, HoF – homofermentative metabolism, HeF – heterofermentative metabolism, G – growth at the given temperature, API – biochemical identification using the API kit, RAMP – Randomly-amplified microsatellite polymorphism, 16S – sequencing 16S rDNA, (+) – determined and relevant, (–) – not determined or irrelevant.

as *Lb. plantarum* or *Lb. pentosus*. This strain was further identified as *Lb. plantarum* based on the failure to produce acid from D-xylose and glycerol, as suggested in [16] and in the API identification table. D-xylose and glycerol fermentation criterium was used also in other cases of doubt between *Lb. plantarum* and *Lb. pentosus*, since the 16S rDNA sequences are identical in these two species [17]. According to the 16S sequence, strain exhibiting fermentation profiles consistent with *Lb. buchneri* was identified as *Lb. parabuchneri*, a closely related species [18], which is not included in the API characterization list. Cocci isolated from the cheese produced by Producers 1 and 4 were classified according to their fermentation profiles by API as *Lactococcus lactis* (63–80%), however, based on 16S rDNA sequencing they were classified as *Pediococcus pentosaceus*. Another strain isolated from the cheese produced by Producer 1 was classified according to its fermentation profile by API as *Lc. lactis*, however, based on 16S rDNA sequencing it was classified as *Enterococcus faecium* and was not further dealt with.

In our study, individual Slovakian bryndza cheese samples were found to contain 3–7 strains of *Lactobacillus* sp. or *Pediococcus pentosaceus*. Typing by RAMP revealed that several bryndza cheese samples contained 2 or 3 genotypes of the same species. *Lb. paracasei* was the most abundant species, being present in all but two bryndza cheeses, followed by *Lb. plantarum*, which was present in all but three bryndza cheeses. Together with other *Lactobacillus* species identified in bryndza cheese in this study, i. e. *Lb. brevis*, *Lb. parabuchneri*, *Lb. fermentum*, *Lb. helveticus* and *Lb. pentosus*, they are well established components of microflora of various cheeses and may positively contribute to flavour development by peptidolytic activities and aminoacid catabolism [7, 8, 19, 20]. *P. pentosaceus* is known to be present in various types of cheese but its contribution to their quality has not been unambiguously established yet [21, 22].

In Slovakian bryndza cheeses produced from both cows' and ewes' lump cheeses made from pasteurized milk, the microflora might have originated in starter cultures used in the technology. In bryndza cheeses produced from ewes' lump cheese made from unpasteurized milk, or from a mixture of ewes' lump cheese made from unpasteurized milk and cows' lump cheese made from pasteurized milk, the microflora might have originated in starter cultures, if used in the technology, and in non-starter lactic acid bacteria (NSLAB) from milk. Unfortunately, reliable data on the use of specific starter cultures in the production of individual Slovakian bryndza cheeses in this study were not available. However, no big differences could be observed in the variety of species between the bryndza cheeses produced from the ewes' lump cheese made from pasteurized and unpasteurized milk.

No clear correlation could be observed between the composition of the lactic acid microflora and the organoleptic quality of the studied bryndza cheese samples. This may be attributed to the relatively short, max. 10-days fermentation of ewes' lump cheese, which is the main starting material for the production of bryndza cheese. This period is probably not sufficient for lactic acid bacteria, which generally have only weak proteolytic activity, to produce the characteristic organoleptically active compounds. Deeper proteolysis of ewes' lump cheese is thought to be carried out by fungi such as *Geotrichum candidum* or yeasts such as *Kluyveromyces* spp. [2, 23, 24].

CONCLUSIONS

Individual Slovakian bryndza cheese samples produced by 10 producers were found to contain 3–7 strains of *Lactobacillus brevis*, *Lb. parabuchneri*, *Lb. fermentum*, *Lb. helveticus*, *Lb. paracasei*, *Lb. pentosus*, *Lb. plantarum* and *Pediococcus pentosaceus*. Several bryndza cheese samples contained 2 or 3 genotypes of the same species. No clear correlation between the composition of the lactic acid microflora and the involvement of pasteurization of ewes' milk in the technology was observed. Clear correlation was neither observed between the composition of the lactic acid microflora and the organoleptic quality of the studied Slovakian bryndza cheese.

Acknowledgements

This work was supported by the Slovak Research and Development Agency under the contract No. LPP-0136-07. Authors thank to Ing. B. Glončáková for methodological advice on sensory evaluation and to Ing. K. Ženišová for technical assistance.

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Received 1 April 2009; revised 21 April 2009; accepted 4 May 2009.