Characterization of May bryndza cheese from various regions in Slovakia based on microbiological, molecular and principal volatile odorants examination

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Summary

May bryndza cheese, a type of Slovakian bryndza cheese with a status of Protected Geographical Indication (PGI), produced from raw ewes' milk, was characterized in one season in 7 specialized factories regarding the diversity of bacteria, yeasts and fungi, and regarding aroma-active volatile compounds. The culture-based microbiological analysis showed that all samples contained high numbers of lactococci, lactobacilli and *Galactomyces*/*Geotrichum*. Polymerase chain reaction-based identification classified most of lactobacilli as *Lactobacillus paracasei* and *Lb. plantarum*, and most of lactococci as *Lactococcus lactis.* Culture-independent analysis revealed that the most abundant bacteria were *Lactococcus* spp. followed by *Streptococcus* spp. and *Leuconostoc* spp. The eukaryotic microflora was composed mainly of *Galactomyces*/*Geotrichum*, and yeasts *Yarrowia lipolytica*, *Kluyveromyces lactis* and *Debaryomyces hansenii*. Regarding aroma-active volatiles, 28 compounds were detected by gas chromatography-olfactometry with acetic acid, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2,3-heptanedione, pentanoic acid and 2,4-dimethyl-1-heptene being present in all samples. Other widely detected compounds were decanoic acid, 3-methylbutanal, butanoic acid, heptanoic acid, tentatively identified 4-octanone, tentatively identified 2,4-dimethyl undecane and tentatively identified 1-dodecanol. The results showed that microbial diversity and principal aroma-active compounds did not markedly differ among individual producers from different geographic regions, which was positive in terms of PGI definition of this cheese.

Keywords

ewes' cheese; bryndza; microbial diversity; aroma-active volatiles

Slovakian bryndza cheese (Slovenská bryndza) is a natural, white, spreadable cheese, manufactured according to the traditional method. It is recognized in the European Union by Protected Geographic Indication status (PGI) as cheese produced in specified mountainous regions of Slovakia [1]. However, the parts of Slovakia, where this cheese is produced, differ in altitude, climate, geological and vegetation profile. Until now, scientific evidence about variability of bryndza is lacking in connection to common characteristics of this cheese in the territory of Slovakia.

May bryndza is a type of Slovakian bryndza cheese that is produced from unpasteurized ewes' milk in the beginning of season, i.e. in May. It is a high-valued product, attractive to the consumers due to its pleasant, mild flavour. From raw ewes' milk, lump cheese is produced immediately after

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milking by renneting at 29–32 °C for 30 min. The curd is drained at 18–22 °C for 24 h, left to ripen for 3 days at 18–22 °C, and then the production process continues by further ripening at 12–15 °C during 7–10 days. Mature ewes' lump cheeses are processed by removing the crust, pressed to remove whey and milled with salt solution, in order to obtain the specific creamy texture of bryndza [2, 3].

The distinctive flavour of May bryndza cheese is apparently composed from compounds contained in ewes' milk and from the products of fermentation of the substrate by microflora. Principal volatile aroma-active compounds of May bryndza cheese have been characterized by SÁDECKÁ et al. [4]. Because composition and activity of microflora is believed to have a great impact on the flavour of bryndza cheese, several culture-based as well as culture-independent microbiological studies were carried out in this regard. Data from older culture-based studies, which identified *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Kluyveromyces marxianus* and *Galactomyces geotrichum* as main components of the microflora of bryndza cheese [2, 3, 5], were updated by a study of BERTA et al. [6], in which a range of *Lactobacillus* spp. isolates were identified by 16S rDNA sequencing. Enterococci [7], staphylococci [8] and fungal species [9] were cultured and identified in bryndza cheese. Culture-independent studies [10, 11] provided information on the diversity of bacteria and fungi and its dynamics during the production of bryndza cheese. In the production of bryndza cheese, also interactions between lactic acid bacteria and *Galactomyces*/*Geotrichum* group [12] and competition between lactic acid bacteria and coagulase-positive staphylococci [13] were studied.

Although basic information on May bryndza cheese is available regarding microbiological composition as well as aroma-active compounds, most of the previous experiments were done on a limited geographical basis, sometimes with products of just one factory. In order to obtain a more reliable and representative view, this study aimed to gain data for the products from the entire territory of Slovakia that is relevant to bryndza production, i.e. specified mountainous regions of Slovakia [1]. In Slovakia, the presence of Carpathian Mountains creates different climatic conditions that can influence various characteristics of the produced bryndza cheese. These can relate, in particular, to the sheep diet in terms of different plant species composition in the pasture and, therefore, to the quality of milk used for the production of bryndza [14] and to different temperatures at which the lump cheese is produced, which influence the microbial consortia in the beginning of the ripening process [3].

The aim of this study was to characterize geographical variability of May bryndza cheese in the territory of Slovakia. For this purpose, samples of this cheese produced in seven specialized factories located in different places (valleys), in one production season, were investigated by culture-based methods supplemented with molecular assays, by culture-independent molecular methods and by gas chromatography-olfactometry. Such combined multidisciplinary approach is well established to provide good characterization of various cheeses [15, 16].

MATERIALS AND METHODS

Bryndza cheese samples

Seven samples of May bryndza cheese were provided by 7 different producers (P1–P7). P1, P2, P5 and P7 were small production facilities processing milk originating from several own mountain cottages, chalets, spread in close surroundings; the production process was mostly manual. P4 and P6 were small production facilities processing milk from their own farm; the production process was mostly manual. P3 was a medium-sized specialized factory processing milk or lump cheeses collected from a wider region; the production process was mechanized, using traditional machines. All cheeses were produced exclusively from fresh unpasteurized ewes' milk without the use of starter cultures. Samples were collected from the end of April 2013 and during May 2013. Fresh samples were analysed by culture and gas chromatography-olfactometry (GC-O) within one day after the delivery, and samples frozen at –20 °C for a maximum of 3 weeks were analysed by culture-independent DNA-based methods.

Culture-based analysis

Bacteria were grown and quantified on the following media: lactobacilli on de Man-Rogosa-Sharpe agar (MRS; Merck, Darmstadt, Germany) at 37 °C under anaerobic conditions during 72 h; lactococci on M17 agar (Merck) at 30 °C during 72 h under aerobic conditions; total mesophilic aerobes on glucose-tryptone-yeast extract agar (Merck) at 30 °C during 48 h; coliforms and *E. coli* on Chromocult C medium (Merck) at 37 °C during 24 h; *Staphylococcus* spp. on Baird-Parker agar (Merck) at 37 °C during 48 h. Coagulase activity of staphylococci was determined by rabbit plasma tube coagulase test (Bio-Rad, Marnes-la-

Coquette, France) at 37 °C during 24 h. Yeasts (including *Geotrichum* spp.) and moulds were grown and quantified on yeast extract-glucose-chloramphenicol agar (Merck) at 25 °C during 5 days with colony morphology evaluated by microscopy.

Characterization of *Lactococcus* spp. and *Lactobacillus* spp. isolates

Several bacterial strains grown on MRS agar (30 colonies for each sample) and M17 agar (20 colonies for each sample) were selected on the basis of their morphological characteristics and were examined by the specific polymerase chain reaction (PCR). DNA from the isolates was extracted using InstaGene Matrix (Bio-Rad, Hercules, California, USA) following the instructions of the manufacturer. Amplification of DNA was carried out by PCR using *Lactobacillus* genus-specific primers LBLMA 1-rev and R16-1 [17]. For species identification, a two-step multiplex PCR and species-specific PCR were used [18–21]. Lactobacilli were first separated by the multiplex PCR into four groups based on the sizes of the 16S-23S rRNA intergenic spacer region and adjacent 23S rRNA gene, and then identified to species level by two multiplex PCR assays with

Tab. 1. Different PCR approaches and sets of primers used for identification of lactobacilli and lactococci.

PCR assay	Species	Primers (5'–3')	Annealing temperature	References
Genus-specific	*Lactobacillus* spp.	R16-1: CTTGTACACACCGCCCGTCA LBLMA1-rev: CTCAAAACTAAACAAAGTTTC	55 °C	[17]
Two-step multiplex PCR for lactobacilli				
First multiplex PCR	Group I	Ldel-7: ACAGATGGATGGAGAGCAGA	55 °C	[19]
	Group II	LU-1: ATTGTAGAGCGACCGAGAAG		
	Group III	LU-3: AAACCGAGAACACCGCGTT		
	Group IV	LU-5: CTAGCGGGTGCGACTTTGTT		
	Groups I–IV	Lac-2: CCTCTTCGCTCGCCGCTACT		
Second multiplex PCR for Group A	*Lb. paracasei*	Lpar-4: GGCCAGCTATGTATTCACTGA LU-5: CTAGCGGGTGCGACTTTGTT	64 °C	
	Lb. rhamnosus	RhaII: GCGATGCGAATTTCTATTATT LU-5: CTAGCGGGTGCGACTTTGTT		
Second multiplex PCR for Group B	*Lb. fermentum*	Lfer-3: ACTAACTTGACTGATCTACGA Lfer-4: TTCACTGCTCAAGTAATCATC	63 °C	
	Lb. plantarum	Lpla-2: CCTGAACTGAGAGAATTTGA Lpla-3: ATTCATAGTCTAGTTGGAGGT		
Species-specific PCR assays for lactobacilli				
	Lb. casei	Pr I: CAGACTGAAAGTCTGACGG Cas II: GCGATGCGAATTTCTTTTTC	55 °C	[34]
	Lb. fermentum	Lfpr: GCCGCCTAAGGTGGGACAGAT Ferm II: CTGATCGTAGATCAGTCAAG	55 °C	[34]
	Lb. plantarum	Lfpr: GCCGCCTAAGGTGGGACAGAT Plan II: TTACCTAACGGTAAATGCGA	55 °C	[34]
	Lb. delbrueckii	Lac-2: CCTCTTCGCTCGCCGCTACT Ldel-7: ACAGATGGATGGAGAGCAGA	55 °C	[19]
	Lb. rhamnosus	Pr I: CAGACTGAAAGTCTGACGG RhaII GCGATGCGAATTTCTATTATT	58 °C	[34]
	Lb. brevis	BrevI: CTTGCACTGATTTTAACA BrevII: GGGCGGTGTGTACAAGGC	58 °C	[20]
	Lb. paracasei	Para: CACCGAGATTCAACATGG Y2: CCCACTGCTGCCTCCCGTAGGAGT	45 °C	[18]
Lactococcus lactis specific multiplex PCR				
	Lc. lactis	LcLspp-F: GTTGTATTAGCTAGTTGGTGAGGTAAA Lc-R: GTTGAGCCACTGCCTTTTAC	66 °C	[22]
	Lc. lactis subsp. *cremoris*	LcCr-F: TGCTTGCACCAATTTGAAGAG		

Group I – Lactobacilli: *Lb. delbrueckii*; Group II – *Lb. helveticus*, *Lb. acidophilus, Lb. amylovorus*, *Lb. crispatus*, *Lb. gasseri*, *Lb. jensenii;* Group III – *Lb. paracasei*, *Lb. casei*, *Lb. rhamnosus;* Group IV – *Lb. salivarius*, *Lb. reuteri*, *Lb. fermentum*, *Lb. plantarum*.

species-specific primer mixtures and, if necessary, by species-specific PCR assay (using the primers listed in Tab. 1). These PCR assays could identify up to seven species of lactobacilli. Lactococci were identified using the PCR method proposed by Odamaki et al. [22]. The method could differentiate *Lactococcus lactis* (production of one DNA fragment) from *Lc. lactis* subsp. *cremoris* (production of two DNA fragments). The amplified DNA fragments of different PCR assays were analysed by automated flow-through electrophoresis (Qiaxcel Advanced; Qiagen, Hilden, Germany) calibrated with a DNA ladder in the range of 15–3 000 bp.

DNA extraction from cheese and first PCR amplification

DNA was isolated from 1 g of bryndza cheese by shaking at 45 °C during 30 min in 20 ml of 2% sodium citrate solution with glass beads, with subsequent removal of the fat layer and then by chaotropic solid phase extraction using DNeasy Tissue kit (Qiagen) [10]. Bacterial 16S rDNA and eukaryotic internal transcribed spacer (ITS) fragment were amplified by two steps, a portion of the PCR product of the first step being used for the construction of clone libraries, and another portion in the second amplification step, a semi-nested PCR facilitating fingerprint analysis based on denaturing gradient gel electrophoresis (DGGE). The first step involved PCR with primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3' [23]) and LAC2 (5'-ATT YCA CCG CTA CAC ATG-3' [24] oriented to 16S rRNA gene. For amplification of ITS region of yeasts and fungi, primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used [25]. The PCR mixture (25 μl) contained 50 pmol of each primer, 200 μmol·l^{-1} of dNTP (Life Technologies, Gaithersburg, Maryland, USA), 1.5 U SuperHot-*Taq* DNA polymerase (Bioron, Ludwigshafen, Germany) and 1× PCR buffer. Three microlitres of extracted DNA were used as a template in the first amplification. The temperature programme consisted of initial denaturation at 94 °C for 5 min, 30 cycles (94 °C for 30 s, 54 °C for 45 s, 72 °C for 1 min) and a final polymerization step at 72 °C for 10 min. For each DNA target (16S rDNA and ITS), four reactions of 25 μl (100 μl altogether) were produced. The four reactions of each DNA target were mixed together and the specificity of amplification was checked by agarose gel electrophoresis.

DGGE fingerprint analysis

The PCR product of the first step (2 μl) was used as a template in the second amplification, a semi-nested PCR for each DNA target. The 16S rDNA was re-amplified with primers LAC1 (5'-AGC AGT AGG GAA TCT TCC A-3' [24]) and LAC2-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGC CCG GGG GCA CCG GGG GAT TYC ACC GCT ACA CAT G-3' [24]). Primers ITS1f-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GTC CGT AGG TGA ACC TGC GG-3') and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') were used for the semi-nested amplification of ITS fragment [25]. The PCR conditions were the same as above. Four semi-nested PCR products (4 reactions) for each DNA target were pooled, checked by electrophoresis in agarose gel, and precipitated with 96% ethanol, resuspended in 20 μl H_2O and the precipitate (10 μl) was analysed by DGGE in 8% polyacrylamide gel (acrylamide-bisacrylamide 37.5 : 1) with the denaturation gradient of 25–55% for separation of 16S rDNA amplicons and 20–50% for separation of ITS amplicons (100% denaturant contained 7 mol·l^{-1} urea and 40% (v/v) formamide). DGGE was run on DCode System (Bio-Rad) in 0.5 × TAE (20 mmol·l^{-1} Tris, 10 mmol·l^{-1} acetate, 0.5 mmol·l^{-1} Na_2 EDTA; pH 8.0) at 200 V and 60 °C for 3 h for bacteria, or for 5 h for fungi.

Construction of clone libraries and sequencing

The rest of the PCR products from the first amplifications were used for the construction of bacterial 16S rDNA and eukaryotic ITS clone libraries. Briefly, the PCR products were purified by QIAquick PCR purification kit (Qiagen), ligated to pGEM-T Easy vector (Promega, Madison, Wisconsin, USA), transformed to *E. coli* XLI-Blue, and spread to LB plates with ampicillin (100 μg·ml^{-1}), X-Gal (0.1 mmol·l^{-1}) and IPTG (0.2 mmol·l^{-1}). A number of about 60 white colonies from each clone library was checked by vector-specific PCR with primers SP6 (5'-ATT TAG GTG ACA CTA TAG AAT AC-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3'). Positive clones of each library were analysed by DGGE at conditions described above, using bacterial primers LAC1 and LAC2-GC, or ITS primers ITS1f-GC and ITS2. Profiles of individual clones were compared with each other and with the profile of the whole community. Clones with different profiles were sequenced using primers SP6 and T7. The obtained sequences were compared with those present in the GenBank database (nucleotide collection database and, in the case of sequences identified as uncultured bacterium or fungus, the 16S ribosomal RNA sequences database or the exclusion of the uncultured/environmental

sample sequences, respectively) by BLAST search (National Center for Biotechnology Information, Bethesda, Maryland, USA). In addition, the sequences were deposited in the GenBank database under the accession numbers KJ804008-KJ804066 (bacterial sequences) and KJ804067-KJ804102 (fungal sequences).

Solid phase microextraction of volatiles

Each sample of bryndza cheese (5.0 g) was incubated statically in a 40 ml vial in a metallic block thermostat at 50 °C for 30 min, with a solid phase microextraction (SPME) fibre placed in the headspace. A DVB/Carboxen/PDMS SPME fibre, film thickness 50/30 μm (2 cm; Supelco, Bellefonte, Pennsylvania, USA), was used. The fibre was initially conditioned by heating in the gas chromatograph injector at 270 °C for 1 h. SPME extracts were desorbed at 250 °C in the injector.

Gas chromatography-olfactometry

Samples extracted by SPME were analysed by gas chromatography-olfactometry (GC-O) using a previously described method [4]. A 7890A gas chromatograph (Agilent Technologies, Santa Clara, California, USA) with flame ionization detector (FID) and an olfactory detector port (ODP3; Gerstel, Mülheim an der Ruhr, Germany) was used. The capillary column was Ultra 1 (50 m × 0.32 mm × 0.52 μm; Agilent Technologies) operated with a temperature programme 40 °C (1 min), 5 °C·min^{-1}, 250 °C (1 min). Hydrogen was used as a carrier gas at a linear velocity of 45 cm·s^{-1} (measured at 143 °C). Pulse splitless injection was used at an injector temperature of 250 °C. The olfactory detector port (ODP) operated at a temperature of 180 °C, interface temperature was 230 °C and the flow of added nitrogen in the ODP humidifier was 12 ml·min^{-1}. The sniffing time of each judge was 30 min. Results of GC-O analyses were expressed as the average values of odour intensities on a scale from 0 to 3 with increments of 0.5, obtained from five independent measurements, complying with the requirement of four citations within every sensory perception. Standard deviation of olfactometry was approx. 0.5.

Identification of volatile compounds

The volatiles were identified on the basis of their linear retention indices (LRI), mass spectra obtained by gas chromatography-mass spectrometry (GC-MS) as described previously [4], GC analysis of standards, and by comparison of data on occurrence and odour description with literature [4, 16]. C_5–C_{15} alkanes were used as reference standards. LRI data were compared and confirmed with LRI data obtained by measurement of standard compounds. For this purpose, our in-house database of LRI data was used. Identification of compounds by comparison of mass spectra was performed with Wiley and NIST MS libraries (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

RESULTS

Culture-based microbiological analysis

Different groups of microorganisms were isolated from the 7 bryndza cheese samples. No quantitatively striking differences among the bryndza samples regarding individual groups of microorganisms were observed (Tab. 2). Generally, the coliform bacteria reached an average density around

Tab. 2. Groups of microorganisms in bryndza cheese samples from producers P1–P7 as detected by culture-based methods.

Microorganisms	Content [CFU·g^{-1}]						
	P1	P2	P3	P4	P5	P6	P7
Total mesophilic aerobes	1.0×10^8	3.6×10^9	3.4×10^8	4.6×10^8	7.9×10^8	7.6×10^8	2.3×10^8
Coliforms	2.1×10^5	9.0×10^5	3.6×10^4	5.1×10^6	1.3×10^5	1.6×10^5	2.0×10^5
E. coli	9.5×10^4	8.6×10^5	3.6×10^4	1.3×10^6	6.8×10^5	5.7×10^5	2.7×10^6
Staphylococci	6.3×10^6	6.8×10^5	4.8×10^5	5.9×10^6	6.8×10^5	5.7×10^5	2.7×10^6
Coagulase positive staphylococci	9.1×10^3	3.6×10^4	1.8×10^4	$< 5.0 \times 10^3$	4.6×10^3	$< 5.0 \times 10^3$	$< 5.0 \times 10^3$
Presumptive lactobacilli	3.0×10^8	3.6×10^8	5.5×10^7	5.5×10^8	4.6×10^8	3.4×10^8	3.2×10^8
Presumptive lactococci	1.0×10^8	8.2×10^8	3.4×10^8	3.2×10^8	1.3×10^8	2.3×10^8	7.7×10^8
Group *Galactomyces*/*Geotrichum*	1.4×10^5	2.3×10^5	5.0×10^4	1.2×10^5	1.4×10^6	7.2×10^4	1.9×10^5
Other yeasts	7.9×10^4	3.1×10^5	1.0×10^5	3.3×10^5	4.6×10^6	1.9×10^5	8.6×10^5
Other moulds	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	3.6×10^4	1.0×10^4	$< 1.0 \times 10^1$	1.8×10^4

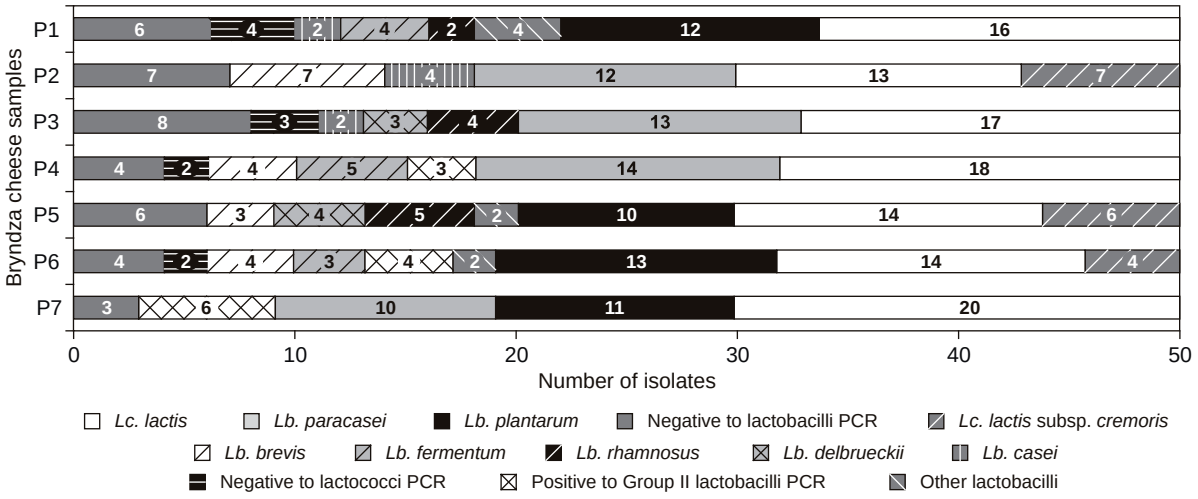

Fig. 1. Distribution of *Lactococcus* and *Lactobacillus* strains in bryndza cheese samples from producers P1–P7 as identified by different PCR assays.

Group II – Lactobacilli: *Lb. helveticus*, *Lb. acidophilus, Lb. amylovorus*, *Lb. crispatus*, *Lb. gasseri*, *Lb. jensenii*.

10^5 CFU·g^{-1} with a peak of 10^6 CFU·g^{-1} recorded for bryndza from producer P4. The density of staphylococci was around 10^5–10^6 CFU·g^{-1} and many of them were coagulase positive, mainly in the bryndza cheeses from P2 and P3 (10^4 CFU·g^{-1}). The eukaryotic microorganisms were represented largely by members of the group *Galactomyces*/*Geotrichum*, which were present at a level of 10^4–10^6 CFU·g^{-1}, and by other yeasts, which were present in all samples at similar levels. The other moulds were present generally at low levels, with the exception of samples P3, P5 and P7, where they were present at a level of 10^4 CFU·g^{-1}.

All samples contained high numbers (from 10^7 CFU·g^{-1} to 10^9 CFU·g^{-1}) of lactic acid bacteria belonging to genera *Lactobacillus* and *Lactococcus*. In order to obtain a better view of the lactic acid bacteria isolates, the *Lactobacillus* and *Lactococcus* strains were identified by several PCR assays. Members of the species *Lactococcus lactis* were isolated and identified in all bryndza cheese samples, while the species *Lc. lactis* subsp. *cremoris* was identified only in the samples P2, P5 and P6, where it constituted 35%, 30% and 22% of the identified *Lactococcus lactis*, respectively (Fig. 1).

Different *Lactobacillus* species, such as *Lb. brevis*, *Lb. casei*, *Lb. delbrueckii*, *Lb. fermentum* and *Lb. rhamnosus*, were identified in the 7 bryndza cheese samples (Fig. 1). The most spread species were *Lb. paracasei* and *Lb. plantarum*, but their co-occurrence was detected only in the bryndza cheese from P7; all other samples contained alternatively either *Lb. paracasei* or *Lb. plantarum*.

DGGE and identification of clones

The seven bryndza cheese samples were examined by DGGE fingerprinting combined with the construction of respective bacterial and fungal clone libraries. The most abundant bacterial strains detected belonged to the genus *Lactococcus*, being present in all examined samples, reaching an average percentage of 59%. The other frequently detected lactic acid bacteria were *Streptococcus* spp. and *Leuconostoc* spp. Streptococci were detected in all samples with the exception of P2, while *Leuconostoc* spp. were detected in all samples with the exception of P7. Other members of the Lactobacillales, including species of the genera *Enterococcus* and *Lactobacillus*, were not detected in all samples and did not attain so high percentage as the other abovementioned Lactobacillales (Fig. 2A). Members of the class γ-proteobacteria were represented by different Enterobacteriaceae (*Enterobacter ludwigii*, *Hafnia alvei* and *Serratia* spp.) and by *Pseudomonas* spp. These were identified in the samples from each of the three farms located in central Slovakia. For example, the γ-proteobacteria detected in the sample P1 reached a percentage of 19% (Fig. 2A). The richest bacterial diversity was detected in the samples P1 and P3 (Tab. 3). Strains belonging to the group *Galactomyces*/*Geotrichum* were identified in all analysed bryndza cheese samples, being the dominant fungi in all samples with percentages ranging from 44% to 73% (Fig. 2B). The other most prevalent species were *Yarrowia lipolytica*

(detected in four samples – P3, P4, P6, P7), *Kluyveromyces lactis* (detected in P3, P4, P5) and *Debaryomyces hansenii* (detected in P4, P5, P6). The most diverse fungal community was found in the bryndza sample from P4 in which, in addition to the fungal species mentioned above, also strains of the species *Candida zeylanoides*, *Gymnoascus reesii* and *Pichia fermentans* were detected (Fig. 2B, Tab. 4).

Aroma-active volatile compounds

Twenty-eight aroma-active volatile compounds were detected in bryndza cheese samples by GC-O (Tab. 5, Fig. 3). Findings showed that principal odorants detected in all samples were acetic acid, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2,3-heptanedione, pentanoic acid and 2,4-dimethyl-1-heptene. Another compound detected in all samples, but at varying intensities, was decanoic acid. Further widely detected compounds (detected in six or five samples out of seven samples) at an odour intensity equal or greater than 1 were 3-methylbutanal, butanoic acid, heptanoic acid, tentatively identified 4-octanone, tentatively identified 2,4-dimethyl undecane, tentatively identified 1-dodecanol and an unknown compound No. 17, detected only by olfactometry with odour characteristics of moist cardboard-like, smoky.

DISCUSSION

May bryndza cheese is a typical Slovak product made from raw ewes' milk. In this study, new data regarding microbial diversity and profiles of principal volatile aroma-active compounds were obtained for the entire territory relevant to the production of bryndza cheese. Microbiological culture-based methods supplemented with molecular

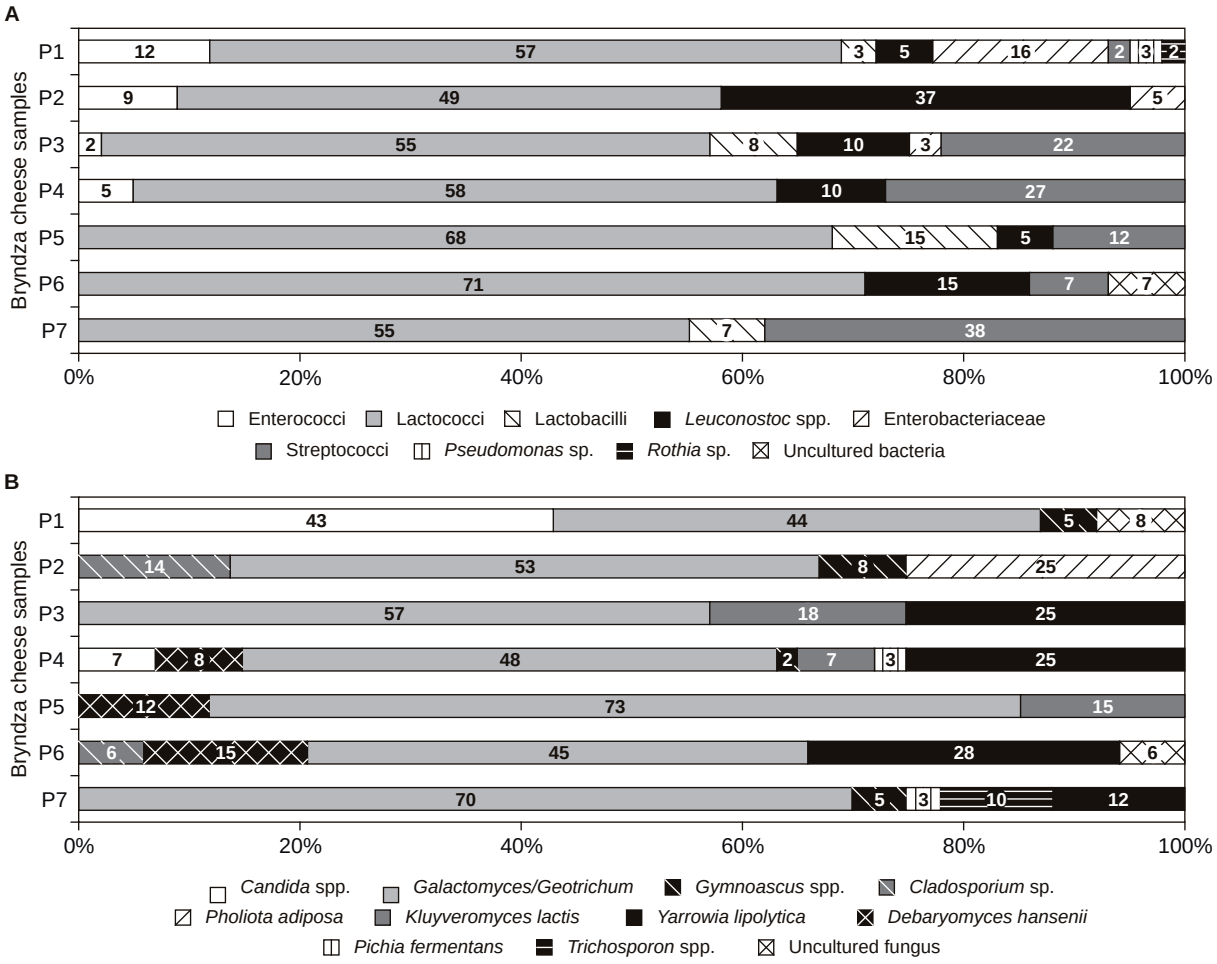

Fig. 2. Bacterial and fungal diversity detected in bryndza cheese samples from producers P1–P7 by clone library construction and DGGE fingerprinting.

A – bacterial diversity, B – fungal diversity.

Tab. 3. Bacterial representatives in bryndza cheese samples from producers P1–P7 as detected by the culture-independent approach.

Identification on the basis of similarity score	Accession number and similarity score	Number of clones						
		P1	P2	P3	P4	P5	P6	P7
Enterobacter ludwigii	KC355279 100%/99%	1	–	–	–	–	–	–
Enterococcus durans	KF060267 100%/99%	–	–	1	–	–	–	–
Enterococcus faecium	HM218625 100%/100% KF358453 100%/100% CP006620 100%/99%	6	5	–	3	–	–	–
Enterococcus hirae	KC707584 100%/99%	1	–	–	–	–	–	–
Hafnia alvei	JX860524 100%/100%	–	3	–	–	–	–	–
Lactobacillus helveticus	HM218719 100%/99%	–	–	–	–	6	–	4
Lactobacillus paracasei	AY735405 100%/100%	–	–	2	–	–	–	–
Lactobacillus plantarum	FJ749733 100%/100% KF030756 100%/99% KF583521 99%/96%	2	–	3	–	3	–	–
Lactococcus lactis	DQ340069 100%/99%	13	–	–	–	–	–	–
Lactococcus lactis subsp. *cremoris*	KF234767 100%/99% AB819486 100%/99%	6	–	2	8	–	–	–
Lactococcus lactis subsp. *lactis*	AB854187 100%/100% AB819489 100%/100% FJ749328 100%/99%	14	28	31	25	41	43	33
Lactococcus raffinolactis	AB681294 100%/99%	1	–	–	–	–	–	–
Leuconostoc mesenteroides	KC959399 100%/99%	2	–	4	–	–	–	–
Leuconostoc sp.	AB194668 100%/100% AB858504 100%/100% AB749361 99%/96%	1	9	2	–	–	9	–
Leuconostoc pseudomesenteroides	AB854208 100%/99% AB854217 100%/99% AB854206 100%/97%	–	12	–	6	3	–	–
Pseudomonas sp.	FJ937924 100%/99%	2	–	–	–	–	–	–
Rothia sp.	AJ969174 100%/99%	1	–	–	–	–	–	–
Serratia sp.	HM063908 100%/99%	1	–	2	–	–	–	–
Serratia plymuthica	JF756168 100%/99%	1	–	–	–	–	–	–
Serratia proteamaculans	NR_074820 100%/100%	7	–	–	–	–	–	–
Streptococcus sp.	KF639825 100%/100%	–	–	–	–	–	–	6
Streptococcus lutetiensis	CP003025 100%/99%	–	–	–	–	–	4	–
Streptococcus macedonicus	HE613569 100%/99%	–	–	5	–	1	–	–
Streptococcus parauberis	JN630844 100%/99% NR_102798 100%/99%	–	–	1	15	–	–	–
Streptococcus uberis	KC510224 100%/100%	1	–	–	–	–	–	–
Streptococcus thermophilus	AB797142 100%/99% NR_074827 100%/99%	–	–	7	–	5	–	15
Uncultured bacterium (*Streptococcus thermophilus*) [a]	GQ267985 100%/100% (NR_074827 100%/97%) [a]	–	–	–	–	1	–	2
Uncultured bacterium (*Leuconostoc mesenteroides*) [a]	JN620473 100%/94% (NR_074957 99%/91%) [a]	–	–	–	–	–	1	–
Uncultured bacterium (*Streptococcus lutetiensis*) [a]	EU774882 100%/99% (NR_037096 99%/95%) [a]	–	–	–	–	–	3	–

Similarity score is expressed as query coverage/maximum identity.
a – Data in brackets represent results of the search in bacterial and archaeal 16S rRNA database. (–) – not detected.

Tab. 4. Fungal representatives in bryndza cheese samples from producers P1–P7 as detected by the culture-independent approach.

Identification on the basis of similarity score	Accession number and similarity score	Number of clones						
		P1	P2	P3	P4	P5	P6	P7
Candida sp.	HQ631020 100%/99%	25	–	–	–	–	–	–
Candida zeylanoides	EF687774 100%/99%	–	–	–	4	–	–	–
Cladosporium sp.	HQ832966 100%/100% KF472149 100%/100%	–	8	–	–	–	3	–
Debaryomyces hansenii	JQ912667 100%/100% KF726846 100%/100%	–	–	–	5	7	8	–
Galactomyces sp.	HQ657282 100%/99%	–	–	–	–	–	–	5
Galactomyces geotrichum	JQ668729 100%/100% KF225047 100%/99%	26	32	34	29	35	25	37
Galactomyces candidum	JN974290 100%/100%							
Galactomyces reessii	HQ436459 100%/100%	–	–	–	–	3	–	–
Gymnoascus sp.	JX270460 100%/99%	–	5	–	–	–	–	–
Gymnoascus reesii	JQ387570 100%/100%	3	–	–	1	–	–	3
Kluyveromyces lactis	KF646161 100%/100% KF646180 100%/100%	–	–	11	4	9	–	–
Pichia fermentans	KF646205 100%/99%	–	–	–	2	–	–	–
Pholiota adiposa	FJ464595 100%/99%	–	15	–	–	–	–	–
Trichosporon coremiiforme	NR_073249 100%/100%	–	–	–	–	–	–	5
Trichosporon insectorum	HM802133 100%/100%	–	–	–	–	–	–	1
Yarrowia lipolytica	HF545663 100%/100% JX420122 100%/100% KC254114 100%/99%	–	–	15	15	–	16	7
Uncultured fungus (*Epacris microphylla*) [a]	JN847480 100%/99% (AY268192 100%/98%) [a]	5	–	–	–	–	3	–
Uncultured fungus (*Geotrichum* sp.) [a]	AM711445 100%/100% (DQ325460 100%/100%) [a]	–	–	–	–	5	–	–
Uncultured fungus (*Pichia fermentans*) [a]	FM173054 100%/100% (GQ458040 100%/99%) [a]							2

Similarity score is expressed as query coverage/maximum identity.
a – Data in brackets represent results of the BLAST search performed excluding the uncultured/environmental sample sequences. (–) – not detected.

assays, culture-independent molecular methods and gas chromatography-olfactometry were used as complementary approaches that are well established to provide good characterization of various cheeses [15, 16].

At microbiological characterization, both approaches were able to identify, in the 7 bryndza cheese samples, members of the species *Lc. lactis*, *Lb. plantarum* and *Lb. paracasei*. Diverse *Lactobacillus* spp. were identified through cultivation and subsequent PCR assays strategy. *Lb. helveticus* was unambiguously detected by the clone libraries in the samples from P5 and P7, while by cultivation, perhaps, this species remained hidden among the PCR-positive results for Group II lactobacilli (Fig. 1). The clone libraries permitted to detect enterococci, streptococci and *Leuconostoc* strains, which by culture-based strategy probably grew on M17 and MRS, but were negative in PCR-assays specific for *Lactococcus* and *Lactobacillus*. These three groups of bacteria constituted a comparatively high percentage of the microflora identified by the culture-independent approach.

Divergences regarded mainly the presence of coliform bacteria and staphylococci, which were detected in all bryndza cheeses by cultivation but, by culture-independent analysis, Enterobacteriaceae were detected in the samples P1, P2 and P3. The high levels of Enterobacteriaceae and pseudomonads in sample P1, confirmed also by DNA-based approach, might have been caused by problematic storage conditions of milk. These characteristics could contribute to a stronger growth of psychrotrophic bacteria belonging to the

Tab. 5. Aroma-active volatile compounds in bryndza cheese samples from producers P1–P7 as detected by gas chromatography-olfactometry.

No.	*LRI*	Compound	Odour description	Basis for identification	Odour intensity						
					P1	P2	P3	P4	P5	P6	P7
1	511.1	Acetic acid	Strong, pungent sour odour, vinegar, fresh cottage cheese	*LRI*, MS, ST, OD, LIT	3	3	2	2	3	3	3
2	600.0	Ethyl acetate	Fruity, sweet-solvent	*LRI*, MS, ST, OD, LIT							
3	610.7	2-Methyl-1-propanol (Isobutanol)	Penetrating, cottage cheese-like, wine-like, fusel-alcohol	RI, MS, ST, OD	2						
4	627.2	3-Methylbutanal (Isovaleraldehyde)	Malty, unpleasant fermented, penetrating rancid yeasty odour	*LRI*, MS, ST, OD, LIT	1	–	–	1	1.5	1	1.5
5	673.5	3-Hydroxy-2-butanone	Pleasant buttery, creamy, weak earthy	*LRI*, MS, ST, OD, LIT	–	–	–				
6 7	717.0 + 720.2	3-Methyl-1-butanol + 2-Methyl-1-butanol	Straw-like, earthy, yeast odour gets on with rancid, unpleasant fermented	*LRI*, MS, ST, OD, LIT	3	2.5	2	3	3	3	3
8	770.7	Butanoic acid	Cheesy, unpleasant fermented, yeasty, vinegary-like odour	*LRI*, MS, ST, OD, LIT	3	–	2	1	2	3	1.5
9	nc	(*E*)-3-octene[T]	Pleasant fruity, slight flowery odour	MS, OD, LIT	–	1	–	–	–	–	–
10	859.4	2,3-Heptanedione	Pungent buttery, cheesy, sweaty, rancid	*LRI*, MS, ST, OD	3	3	3	3	3	3	3
11	868.6	Pentanoic acid	Rancid yeast, unpleasant fermented	*LRI*, MS, ST, OD, LIT							
12	nc	2,4-Dimethyl-1-heptene	Fermented, earthy	MS, OD, LIT							
13	nc	Unknown[O]	Pleasant, flowery, weakly sweetish	–	1	–	–	–	–	–	0.5
14	nc	Dihydroxy acetophenone[T] (unknown isomer)	Roasted, caramel-like, nutty, vegetable	MS, OD	–	–	0.5	–	–	–	1
15	1039.5	3-Methylbutyl butanoate	Sweet, fruity	*LRI*, MS, ST, OD	0.5	–	–	–	–	–	–
16	1065.0	Heptanoic acid	Unpleasant, rancid, moist cardboard-like, plastic-like	*LRI*, MS, ST, OD	–	1	–	2	1	1.5	1
17	nc	Unknown[O]	Moist cardboard-like, smoky	–	1	2	–	1	2	2	–
18	1079.1	2-Phenyl-1-ethanol	Sweet-flowery, rose	*LRI*, MS, ST, OD	–	–	1.5	3	1	–	1
19	nc	4-Octanone[T]	Caramel-like, baked	MS, OD	1	–	1	2	–	1	1
20	nc	4-Methyl undecane[T]	Bitter, herbaceous, medicinal, varnish-like	MS, OD	1	2	1	–	1	–	–
21	1157.4	Octanoic acid	Unpleasant, fatty, rancid, waxy, goat odour	*LRI*, MS, ST, OD, LIT	1.5	–	–	0.5	1	–	0.5
22	nc	Unknown[O]	Smoky odour, weak herbaceous	–	–	1	0.5	–	0.5	–	1
23 24	1180.3 + 1183.4	Ethyl octanoate + Decanal	Pleasant, fruity, floral, fresh, clove-like, sweet, soap	*LRI*, MS, ST, OD, LIT	–	1.5	0.5	1	0.5	–	0.5
25	nc	2,4-Dimethyl undecane[T]	Pleasant, cooling, fresh, menthol-like	MS	1.5	1	1	–	1	1	1
26	1349.3	Decanoic acid	Unpleasant rancid odor, putrescent plants-like, mild animal odour typical for bryndza cheese	*LRI*, MS, ST, OD, LIT	1	2	2	3	2	1	1
27	1456.8	1-Dodecanol[T]	Coconut-like, sweet odour	*LRI*, OD	1	–	1.5	1.5	1	1	–
28	nc	Unknown[O]	Pleasant flower, linden-like, gentle softener-like	–	1	1.5	–	–	–	1	–

LRI – linear retention index calculated from the data measured on Ultra 1 GC column, MS – mass spectrum, ST – comparison with standard compound, OD – odour quality, LIT – literature reference; T – tentative identification, O – detected only by olfactometry, (–) – not detected, nc – not calculated.

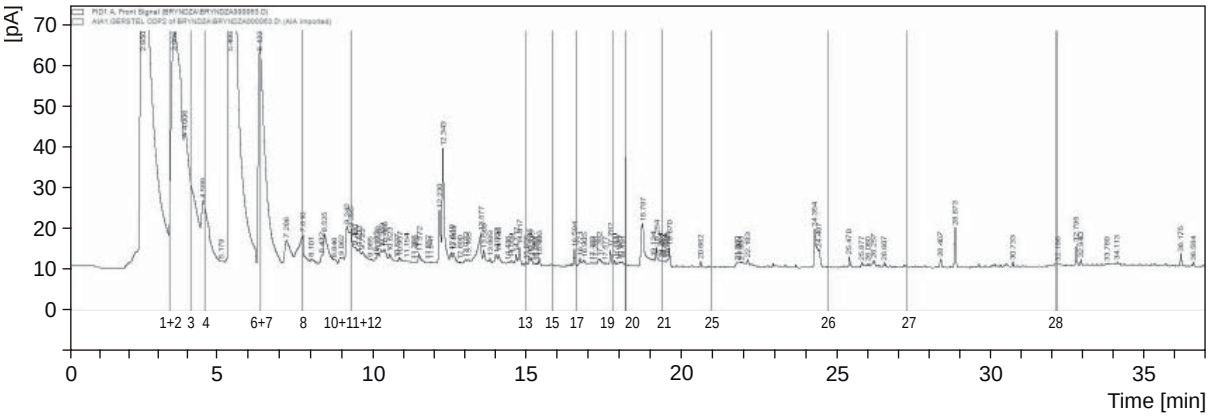

Fig. 3. Chromatogram of volatiles in May bryndza cheese from producer P1 as detected by GC-FID-olfactometry.

Vertical lines indicate the positions where aroma-active compounds (Tab. 5) were registered by olfactometry.

genera *Serratia* and *Pseudomonas*, which was described previously by other authors [26].

On the other hand, the culture-independent approach was successful at a better description of the eukaryotic microflora. This was found to be composed mainly of yeast members belonging to the group *Galactomyces*/*Geotrichum* and to the species *Yarrowia lipolytica*, *Kluyveromyces lactis* and *Debaryomyces hansenii*. Species such as *Pholiota adiposa* and strains of the genus *Trichosporon* were only detected in the bryndza cheeses from P2 and P7, respectively. *Trichosporon* species were previously detected in bryndza [9, 11] but, to our knowledge, this is the first detection, in a cheese sample, of a member of the species *Trichosporon insectorum* [27]. Members of this species can be considered as oleaginous yeasts, which can synthesize and accumulate lipids up to approximately 70% of their dry weight [28]. *Pholiota adiposa* reached the percentage of 25% in P2 bryndza, which is quite surprising as it is an atypical fungus for cheese environment, having been reported in milk-related samples never before.

In our previous study of the microbial dynamics during the production of May bryndza cheese from P4 [11], universal primers oriented to the region V3-V5 of the 16S rRNA gene were used in PCR to investigate the bacterial community. In that study, only *Lactococcus* strains, *Mannheimia glucosida* and *Streptococcus parauberis* were detected. Since using those universal primers, it might have been impossible to reveal all members of the microflora, we decided to change the primers combination. In this investigation, the region V1-V4 known to be more suitable for identification of certain groups of bacteria [29] was amplified, using a universal primer (27f) combined with a lactic acid bacteria-specific primer (LAC2). The comparison of current results with those obtained in the previous study of bryndza cheese from P4 showed that the new primers facilitated confirmation of some bacterial groups and detection of some new ones, such as *Leuconostoc* and *Enterococcus* spp. Generally, the new primers permitted the detection of a more diverse bacterial microflora, compared to the previous combination of primers (Fig. 2A, Tab. 3). Indeed, this time also the presence of lactobacilli was confirmed by the culture-independent approach.

The microflora detected in May bryndza cheese in this study contained mostly genera and species that are common in raw milk-based cheeses. The composition of the microflora was, to a certain extent, similar to other fresh and/or soft ewes' cheeses from Central Europe [30–33].

The high contents of staphylococci in bryndza cheese are well known and widely discussed from the hygienic point of view. Despite the introduction of guidelines based on HACCP principles in the production, it is difficult to avoid staphylococcal contamination of this type of cheese. The efforts aim at such improvement in the technology, which would suppress the growth of staphylococci and, in particular, production of toxins [3, 13].

Concerning the profiles of principal aroma-active compounds, the obtained results indicated large similarities between bryndza cheeses from different factories, but some of the principal aroma-active compounds were typical or unique to regions or individual producers. For example, compound No. 9 (Tab. 5) tentatively identified as (*E*)-3-octene with pleasant, fruity, slight flowery odour, was specifically characteristic for P2 bryndza cheese. Another comparable example

was 3-methylbutyl butanoate (No. 15) with sweet, fruity notes, which was specifically characteristic for bryndza cheese from P1.

The results were analysed by formal statistical methods, factor analysis and principal component analysis, which produced trivial results displaying homogenaity in the studied samples (data not shown). If there was a causal relation between the presence of certain microorganisms and the presence of certain aroma-active volatiles in May bryndza cheese, or was this just co-incidence, requires further research on the basis of transcriptional and enzymological characterization of the microflora.

By the use of microbiological culture-based methods, molecular culture-independent methods and GC-O analysis of aroma-active volatiles, it was possible to characterize May bryndza cheese produced in one season in 7 different factories, representing the whole relevant part of Slovakia. In products from certain cheese producers, certain unique microorganisms and certain unique aroma-active volatiles were detected. The results that microbial diversity and principal aroma-active compounds showed no apparent differences among the individual producers from different regions are positive in terms of PGI labeling of this cheese produced in the entire territory of Slovakia. This study provides new knowledge regarding the microbial and aroma characteristics of this semi-soft ewes' cheese, which can be also important for other short-ripened raw ewes' milk-based cheeses.

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