Microorganisms and volatile aroma-active compounds in bryndza cheese produced and marketed in Slovakia

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Summary

Ten industrially and traditionally produced versions of bryndza, a typical Slovakian ewes' cheese, covering the dominating offer in the market in Slovakia, were studied regarding the contents of microorganisms and key volatile aroma-active compounds. Culture-based microbiological analysis was complemented by culture-independent analysis by high throughput sequencing on Illumina MiSeq platform using 16S rDNA and internal transcribed spacer amplicons. Aroma-active compounds extracted by headspace solid-phase microextraction were analysed by gas chromatography-olfactometry supported by gas chromatography-mass spectrometry. Bacterial microflora was found to be dominated by lactic acid bacteria, mostly lactococci, followed by streptococci, lactobacilli and leuconostocs. A portion of cheeses contained Enterobacteriaceae, pseudomonads or *Chryseobacterium* spp. and, exceptionally, coagulase-positive staphylococci at a legally acceptable level. Eukaryotic microflora was dominated by Dipodascaceae in most samples. Certain samples contained contaminants such as *Mucor* spp. Key aroma-active compounds comprised butanoic acid, δ-decalactone, acetic acid, diacetyl and guaiacol. Further significant aroma-active compounds were 3-methylbutanol, 3-methylbutanoic acid, 2-phenylethanol, octanoic acid and *p*-cresol. The results demonstrated that geographical location, involvement of pasteurization or admixture of the cows' milk-based component do not entirely determine the aroma profile of bryndza cheese, but it appears to be the result of a complex interplay of the production technology and microorganisms.

Keywords

cheese; lactic acid bacteria; high throughput sequencing; gas chromatography-olfactometry

Bryndza is a typical Slovakian cheese recognized as a Protected Geographical Indication (PGI) product [1]. It 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. The basic intermediate for bryndza production is ewes' lump cheese, produced by a well defined two-stage ripening process lasting for 8–14 days [2].

The traditional bryndza cheese, in its original version [3], is currently produced only by small producers in limited volumes, and is distributed only locally or regionally. On the other hand, different versions of this cheese are produced on industrial basis and are distributed in large volumes by supermarket chains in Slovakia and in neighbouring countries. Problems with safety of cheeses produced from raw ewes' milk by traditional technologies lead industrial producers

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to use altered technologies usually involving pasteurization of milk and application of starter cultures. Another practice is that a part of the ewes' component is replaced by cows' lump cheese, up to the maximum allowed proportion of 1 : 1. These alterations may lead to products of organoleptic quality that is considerably different from the traditional one [4, 5].

In a previous study [5], May bryndza cheese from 7 small producers in Slovakia was characterized, with the main aim to cover microbial diversity and unique aroma variations of the seasonal cheese. In the current study, a representative range of bryndza cheese variants having a main share of the market in Slovakia in 2017–2018 was characterized. Products from 10 producers were characterized, including the product of a major industrial producer dominating the market and products of some medium-volume producers. The samples involved bryndza cheese produced from pasteurized or unpasteurized ewes' milk, 100% ewes' or mixed with up to 49% cows' component, ewes' component being lump cheese or barrelled cheese, or their mixtures. Microflora of the cheeses was characterized by both culture-based and culture-independent methods, and profiles of key volatile aroma-active compounds were determined by gas chromatography-olfactometry.

MATERIALS AND METHODS

Cheese samples

The following bryndza cheese samples were analysed (information was obtained from the label):

1. Full-fat summer bryndza, containing ewes' lump cheese, drinking water and edible salt. Minimum share of the ewes' component made from pasteurized 50 % in dry matter. Dry matter 440 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, package weight 125 g.
2. Full-fat summer bryndza, containing ewes' lump cheese from unpasteurized milk (minimum share 50 %), cows' lump cheese from pasteurized milk, drinking water and edible salt. Dry matter 440 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 25 g·kg^{-1} maximum, package weight 250 g.
3. Ewes' bryndza, containing ewes' lump cheese from unpasteurized milk, drinking water and edible salt. Dry matter 480 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 25 g·kg^{-1} maximum, package weight 382 g.
4. Mixed bryndza, containing ewes' lump cheese produced from pasteurized milk (minimum share 51 %), cows' lump cheese produced from pasteurized milk, drinking water and edible salt. Dry matter 440 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, package weight 125 g.
5. Ewes' bryndza, containing ewes' lump cheese produced from pasteurized milk, drinking water and edible salt. Dry matter 480 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 25 g·kg^{-1} maximum, package weight 250 g.
6. Slovakian bryndza PGI – mixed bryndza produced from barrelled ewes' cheese, containing barrelled ewes' cheese from unpasteurized milk, cows' lump cheese from pasteurized milk, drinking water and edible salt. Dry matter 440 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 25 g·kg^{-1} maximum, package weight 125 g.
7. Ewes' bryndza, containing ewes' lump cheese produced from unpasteurized milk, drinking water and edible salt. Dry matter 480 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 25 g·kg^{-1} maximum, package weight 250 g.
8. Full-fat bryndza, containing barrelled ewes' lump cheese (minimum share 50 %), cows' lump cheese from pasteurized milk, drinking water and edible salt. Dry matter 440 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 30 g·kg^{-1} maximum, package weight 125 g.
9. Full-fat bryndza, containing barrelled ewes' lump cheese from pasteurized milk and barrelled ewes' lump cheese from unpasteurized milk (cumulative share 50 %), cows' lump cheese from pasteurized milk, drinking water and edible salt. Dry matter 440 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 25 g·kg^{-1} maximum, package weight 125 g.
10. Full-fat summer bryndza, containing ewes' lump cheese from unpasteurized milk, cows' lump cheese from pasteurized milk, drinking water and edible salt. Dry matter 440 g·kg^{-1} minimum, fat in dry matter 480 g·kg^{-1} minimum, NaCl 25 g·kg^{-1} maximum, package weight 250 g.

Sample 1 was produced by a major industrial producer, other samples were produced by medium-volume producers. Regional origin could not be attributed to individual samples, as, to our knowledge, producers often purchased lump cheese from other regions. Cheese samples were obtained from the producers or purchased in shops in Slovakia. The samples were analysed dur-

ing the "best before" period of the products as declared on the label. One package of each sample was analysed.

Determination of chemical parameters of cheeses

Dry matter was determined according to ISO 5534:2004 [6]. Fat was determined according to STN 57 0107:1965 [7]. Proteins were determined according to ISO 8968-1:2014 [8]. Sodium chloride (NaCl) content was determined according to STN 57 0107-12:1980 [9].

Culture-based microbiological analysis

Total aerobic counts were determined according to ISO 4833-1:2013 [10]. Coliforms were determined according to ISO 4832:2006 [11]. Coagulase-positive staphylococci were determined according to ISO 6888-2:1999 [12]. Yeasts and moulds were determined according to ISO 6611:2004 [13]. Presumptive lactobacilli were determined by enumeration of colonies after anaerobic culturing on de Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany) for 72 h at 37 °C. Presumptive lactococci were determined by enumeration of colonies after aerobic culturing on M17 agar (Merck) for 72 h at 30 °C. Enterococci were not selectively determined as they do not significantly contribute to the aroma of bryndza cheese [5].

Culture-independent microbiological analysis

DNA was isolated from cheese samples by chaotropic solid-phase extraction using DNeasy Mericon Food Kit (Qiagen, Hilden, Germany) according to the standard protocol for 200 mg of food sample. Bacterial 16S rDNA fragments were amplified by polymerase chain reaction (PCR) using primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1062R (5'-ACA GCC ATG CAG CAC CT-3') oriented to V1-V6 hypervariable regions [14]. Fragments of the eukaryotic internal transcribed spacer (ITS) were amplified by PCR using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC- 3') oriented to ITS1 and ITS2 [15]. The PCR mixture of 25 μl contained 1.25 U thermostable DNA polymerase (Cheetah Hot Start Taq Polymerase; Biotium, Hayward, California, USA), 1× buffer supplied with the polymerase, 1.5 mmol·l^{-1} $MgCl_2$, 340 μmol·l^{-1} dNTP (Applied Biosystems, Foster City, California, USA) and 300 nmol·l^{-1} of each primer. PCR was carried out in a Veriti thermal cycler (Applied Biosystems) using a programme, for 16S rDNA fragment amplification, consisting of initial denaturation at 94 °C for 2 min, 35 cycles (denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and polymerization at 72 °C for 2 min) and final polymerization at 72 °C for 10 min. For amplification of ITS region, a programme consisting of initial denaturation at 94 °C for 2 min, 35 cycles (denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and polymerization at 72 °C for 1 min) and final polymerization at 72 °C for 10 min was used. Amplified products were analysed by agarose gel electrophoresis to check the size and amount of the amplified product. Products of PCR were purified by QIAquick PCR Purification Kit (Qiagen), diluted to equimolar ratio and used as template for library preparation using Nextera XT library preparation kit (Illumina, San Diego, California, USA) according to the standard protocol. Samples were analysed using paired-end (2 × 300 bp) sequencing on MiSeq platform (Illumina). Sequencing data were imported into CLC Genomics Workbench Version 7.5 (Qiagen). Each sequence of sample was treated by merging and trimming. Limit of trimming using quality score was set to 0.001 and reads shorter than 150 nucleotides were discarded. Reads were identified based on their homology to reference *16S rRNA* genes in DNA sequence database of National Center for Biotechnology Information (Bethesda, Maryland, USA) using Basic Local Alignment Search Tool (BLAST). BLAST results were processed by MEtaGenome ANalyzer (MEGAN V5; University of Tübingen, Tübingen, Germany) [16].

Analysis of volatile aroma-active compounds

A fraction of volatile compounds was extracted from individual cheese samples of 5.0 g by static incubation 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 above the sample. The SPME fibre DVB/Carboxen/PDMS (2 cm), "For odours", film thickness 50/30 μm (Supelco, Bellefonte, Pennsylvania, USA) was used. The fibre was initially conditioned by heating in the injector block of the gas chromatograph at 250 °C for 1 h. SPME samples were then directly thermally desorbed at 250 °C in the injector block of the gas chromatograph.

The volatile compounds extracted by SPME were analysed by gas chromatography-olfactometry (GC/FID-O) in five replicates, using the concept of detection frequency of posterior assessment as described in our previous study [17]. A sniffing procedure panel was formed of 5 judges (2 men, 3 women, aged 29, 47, 50, 57 and 61) who were chosen from 11 assessors trained in sensory evaluation. Results of GC/FID-O analyses were expressed as average values of odour intensity

in a scale from 0 to 3 with increments of 0.5, obtained from 5 independent measurements, complying with the requirement of 4 citations within every sensory perception. The gas chromatograph Agilent 7890A (Agilent Technologies, Palo Alto, California, USA) was coupled to a flame ionization detector and to an olfactory detector port ODP3 (Gerstel, Mülheim an der Ruhr, Germany). The capillary column was DB-WAX (30 m × 0.32 mm × 0.25 μm; Agilent Technologies) operated with a temperature programme 50°C (1 min), 5 °C·min^{-1}, 240 °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 ODP humidifier was 12 ml·min^{-1}. The sniffing time of each judge did not exceed 30 min.

In order to identify the separated compounds, samples were analysed in parallel by gas chromatography-mass spectrometry (GC-MS) using the gas chromatograph Agilent 6890N (Agilent Technologies) coupled to the mass spectrometric detector 5973 inert (Agilent Technologies) equipped with a column DB-WAXetr (30 m × 0.25 mm × 0.50 μm; Agilent Technologies) operating with a temperature programme 50 °C (1 min), 5 °C·min^{-1}, 240 °C (1 min). The linear velocity of carrier gas helium was 45 cm·s^{-1} (measured at 143 °C). Pulse splitless injection was used at an injector temperature of 250 °C. Ionization voltage (EI) was 70 eV.

Individual volatile aroma-active compounds were identified based on comparison of their linear retention indices, mass spectra, analysis of standards, and by comparison of data on occurrence and odour description with literature, as described in our previous studies [18, 19]. Linear retention indices (*LRI*) for individual compounds were calculated, confirmed and compared with *LRI* data obtained by measurement of C_8–C_{23} alkanes as reference standards. For this purpose, our in-house database of *LRI* data was used. Identification of compounds by comparison of mass spectra was done using Registry of Mass Spectral Data (Wiley, New York, New York, USA) and Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

RESULTS AND DISCUSSION

Chemical parameters of various variants of bryndza cheese from 10 producers are summarized in Tab. 1. The results demonstrate conformity with the specification of Slovakian bryndza PGI within the set of parameters [1].

Culture-based microbiological parameters of the studied cheeses are summarized in Tab. 2. The bacterial microflora was dominated by presumptive lactococci, which were present in all samples at high levels of $\geq 10^7$ CFU·g^{-1}, and by presumptive lactobacilli, which were present at levels of 10^5–10^7 CFU·g^{-1} in bryndza samples from pasteurized milk and at levels of 10^5–10^8 CFU·g^{-1} in bryndza samples from unpasteurized milk. Coliforms as indicators of fecal contamination or unhygienic processing conditions were detected at levels of $< 10^1$–10^2 CFU·g^{-1} in bryndza samples from pasteurized milk and at levels of 10^3–10^8 CFU·g^{-1} in bryndza samples from unpasteurized milk, with the exception of Sample 2 that contained less than 10^1 CFU·g^{-1} coliforms. The determined levels of coliforms as well as differences between cheeses produced from pasteurized and unpasteurized milk were in agreement with the established knowledge in the field [20].

Coagulase-positive staphylococci, which are sporadic toxinogenic contaminants of this type of cheese, were absent from all bryndza samples from pasteurized milk and from 4 out of 6 bryndza samples from unpasteurized milk. Coagulase-positive staphylococci were detected only in Sample 3 and Sample 4, and that was at a level of 10^4 CFU·g^{-1},

Tab. 1. Chemical parameters of the studied bryndza cheese samples.

	Content in a sample [g·kg^{-1}]									
	1	2	3	4	5	6	7	8	9	10
Fat	222.5	236.3	240.0	270.0	285.0	245.0	240.0	270.0	235.0	235.0
Dry matter	444.0	490.0	532.5	481.1	507.6	468.9	482.3	519.6	469.2	449.2
Fat in dry matter	501.1	482.2	450.7	561.2	561.5	522.5	497.6	519.6	500.9	523.2
Proteins	191.1	195.9	221.7	171.9	185.4	181.6	188.8	205.4	181.2	178.7
NaCl	21.2	20.5	16.6	23.4	18.6	22.4	20.3	20.1	24.4	25.0

Tab. 2. Culture-based microbiological parameters of the studied bryndza cheese samples.

	Content of microorganisms in a sample [CFU·g^{-1}]									
	1	2	3	4	5	6	7	8	9	10
Total mesophilic aerobic counts	> 3.0×10^7	> 3.0×10^7	> 3.0×10^7	1.7×10^8	> 3.0×10^8	> 3.0×10^8	> 3.0×10^8	> 3.0×10^8	> 3.0×10^8	> 3.0×10^8
Presumptive lactococci	> 3.0×10^6	> 3.0×10^6	> 3.0×10^6	> 3.0×10^7	> 3.0×10^7	> 3.0×10^7	2.4×10^8	2.9×10^8	2.5×10^8	> 3.0×10^8
Presumptive lactobacilli	1.3×10^6	4.9×10^5	> 3.0×10^6	2.0×10^5	> 3.0×10^6	> 3.0×10^6	1.7×10^8	5.7×10^7	5.0×10^6	1.9×10^8
Coliforms	40	< 10	1.0×10^4	< 10	4.2×10^2	> 1.5×10^5	3.4×10^4	2.1×10^2	2.9×10^3	1.1×10^6
Coagulase-positive styphylococci	< 50	< 50	1.1×10^4	< 50	< 50	< 50	< 50	< 50	< 50	8.3×10^4
Yeasts	1.8×10^5	1.7×10^2	1.3×10^6	2.5×10^3	1.2×10^4	3.9×10^3	> 1.5×10^6	8.0×10^3	6.0×10^4	> 1.5×10^6
Moulds	2.2×10^4	20	1.4×10^5	4.1×10^2	7.4×10^2	6.0×10^3	3.2×10^5	1.5×10^2	1.1×10^3	2.2×10^5

i. e. below the legally stated limit [21]. These results indicated good efficiency of pasteurization and good microbiological quality of milk regarding coagulase-positive staphylococci.

Yeasts and moulds were detected at levels of 10^3–10^6 CFU·g^{-1} and 10^2–10^5 CFU·g^{-1}, respectively, with no systematic differences between cheeses produced from pasteurized and unpasteurized milk. An exception was Sample 2 containing very low amounts of yeasts and moulds, which can be taken as strange in the case of a cheese produced from unpasteurized milk. The determined contents of yeasts and moulds were otherwise in line with previously published data on bryndza cheese [4, 5].

Culture-based data were supplemented by culture-independent analysis using high throughput sequencing. Results on the prokaryotic microflora are summarized in Tab. 3. In all samples, technologically relevant lactic acid bacteria (i. e. *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Leuconostoc* spp.) dominated, forming a share of 57.8–99.6 % of all prokaryotic taxons. Among them, *Lactococcus* spp. dominated in 9 out of 10 samples, involving both bryndza from pasteurized milk produced with starter cultures and also bryndza from unpasteurized ewes' milk. The only exception was Sample 5, in which *Streptococcus* spp. were dominant. Because the latter cheese was produced from pasteurized milk, the high level of *Streptococcus* spp. could have come from a starter culture. *Streptococcus* spp. were detected in all samples at shares of 0.4–44.4 %. The share of *Lactobacillus* spp. varied between the samples in a range of 0.0–16.1 %, being zero in Sample 1, which was bryndza from pasteurized milk containing 500 g·kg^{-1} cows' component. In other cheeses from pasteurized milk (Sample 4, Sample 9), lactobacilli represented a share of 1.2 %, which were probably pasteurization survivors. The high level of *Lactobacillus* spp. of 13.1 % in Sample 5 (produced from pasteurized milk) could have come from a starter culture. *Leuconostoc* spp. were detected at comparatively low shares of 0.2–4.8 % in 6 out of 10 samples.

The culture-independent analysis also revealed certain levels of various contaminants, such as Enterobacteriaceae in 6 out of 10 samples, *Pseudomonas* spp. in 3 out of 10 samples or *Chryseobacterium* spp. in 2 out of 10 samples. Although these results could not be directly matched with those of culture-based analysis, certain interesting relations were evident. For example, the very high share of Enterobacteriaceae in Sample 3 detected by culture-independent analysis coincided with the high level of coliforms of 10^4 CFU·g^{-1}

Tab. 3. Percentage of prokaryotic taxons in bryndza cheese samples, as determined by the culture-independent approach.

Taxon	Share [%]									
	1	2	3	4	5	6	7	8	9	10
Sequences total	14355	13654	14246	23596	34157	28082	60802	60022	57990	59095
Lactococcus spp.	96.0	49.9	54.9	76.9	35.7	93.8	66.9	79.3	90.1	49.8
Streptococcus spp.	0.5	22.6	1.8	18.1	44.4	0.4	4.5	14.1	5.8	30.6
Lactobacillus spp.	0.0	1.9	1.1	1.2	13.1	0.6	16.1	3.0	1.2	10.0
Leuconostoc spp.	1.1	0.2	0.0	0.0	0.2	4.8	3.4	2.0	0.0	0.0
Enterococcus spp.	1.9	2.4	1.6	0.2	2.2	0.0	1.8	0.0	0.0	1.1
Acetobacter spp.	0.2	1.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Enterobacteriaceae	0.0	6.4	26.3	2.5	0.9	0,0	4.6	0.0	0.0	3.3
Staphylococcus spp.	0.0	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
Pseudomonas spp.	0.0	2.2	5.4	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Acinetobacter spp.	0.0	0.6	4.5	0.2	1.9	0.0	0.0	0.0	2.0	1.8
Chryseobacterium spp.	0.0	2.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Vagococcus spp.	0.0	0.0	0.0	0.3	0.4	0.0	0.0	0.0	0.0	0.0
Other	0.2	9.9	3.1	0.3	0.8	0.2	2.3	1.5	0.9	3.0
Unassigned	0.1	0.4	1.2	0.2	0.1	0.2	0.4	0.1	0.0	0.4

determined by the culture-based method. On the other hand, presence of coliforms at a similar or even higher level in samples 6, 7 and 10 was not adequately registered by culture-independent analysis in any taxon including Enterobacteriaceae. These discrepancies may reflect different composition of the enterobacterial microflora in individual cheese samples, which might have been quantified with different efficiency by the culture-based method. *Staphylococcus* spp. were detected only in two samples and the culture-independent analysis failed to detect these bacteria in Sample 3 and Sample 10, in which 10^4 CFU·g^{-1} of coagulase-positive staphylococci were detected by the culture-based method. These results demonstrate a problematic reliability of the culture-independent analysis at detection of contaminating and potentially pathogenic bacteria in bryndza cheese.

Results of the culture-independent analysis of the eukaryotic microflora are summarized in Tab. 4. In 8 out of 10 samples. Dipodascaceae were dominant, representing a share of 78.0–97.0 %. This fungal family contains members of the group *Galactomyces*/*Geotrichum*, which had been previously identified as very important for the production of bryndza cheese [18, 22, 23],

Tab. 4. Percentage of eukaryotic taxons in bryndza cheese samples as determined by the culture-independent approach.

Taxon	Share [%]									
	1	2	3	4	5	6	7	8	9	10
Sequences total	20865	15484	20034	33010	26230	17529	74890	64806	75976	64782
Dipodascaceae	96.8	97.0	93.0	6.4	5.3	93.3	93.9	96.0	78.0	89.0
Debaryomyces spp.	0.0	1.3	0.5	0.0	81.4	4.6	0.1	0.3	2.0	0.9
Kluyveromyces spp.	2.4	0.3	0.5	0.0	0.1	0.0	3.6	2.6	15.1	0.3
Pichia spp.	0.0	0.7	1.3	93.6	10.8	0.5	1.1	0.3	3.0	2.1
Rhodotorula spp.	0.0	0.3	2.4	0.0	0.0	0.1	0.0	0.1	0.3	0.4
Candida spp.	0.0	0.0	0.7	0.0	2.3	0.0	1.3	0.2	1.0	0.1
Mucor spp.	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.1	0.4
Other or unassigned	0.8	0.4	1.0	0.0	0.1	1.5	0.0	0.5	0.5	6.8

Tab. 5. Volatile aroma-active compounds in bryndza cheese samples.

No.	Aroma compound	Aroma description	Aroma intensity in individual samples									
			1	2	3	4	5	6	7	8	9	10
1	Ethyleneoxide [a]	Ethereal	–	–	1	–	1	1	1	–	–	–
2	Ethanethiol [a]	Unpleasant, rotten cabbage-like, stinking	–	0.5		0.5	–	1	–	–	1.5	0.5
3	2-Octene (unknown isomer) [a]	Unpleasant, plastics-like, musty undertone	–	–		–	0.5	–	–	0.5	–	–
4	Ethylacetate + 2-butanone [b]	Ethereal, sweetish, acetone-like	–	–	1	–	–	1	2	–	–	1
5	2-Methylpropanal [a]	Unpleasant, yeasty, malty, cheesy, fermented, wet straw-like	0.5	1	1	–	1	–	–	–	–	–
6	3-Methylbutanal [b]	Unpleasant, sweaty, malty	0.5		2	–	1	–	2	–	1	1
7	Ethanol [a]	Solvent-like, fresh	–	–	–	–	–	–	–	–	–	1
8	Diacetyl [b]	Buttery, fresh cheese-like	1.5	2	2	1.5	2	2	2	2	1.5	2
9	Ethylbutanoate [b]	Pleasant, sweetish, fruity	0.5	1	1	1	1	2	1.5	2	–	1.5
10	Toluene [a]	Sweetish, solvent-like	0.5	0.5	0.5	0.5		1	1	0.5	–	0.5
11	2-Methylethylbutanoate [a]	Sweetish, fruity	–	–	0.5	0.5	0.5	–	–	–	–	–
12	3-Methylbutylacetate [b]	Sweetish, solvent-like, ethereal	–	–	1	–	0.5	–	1.5	–	–	1
13	Butanol [a]	Pleasant, fruity, ripened banana-like	–	–	–	0.5	–	1	–	1	–	–
14	β-Myrcene [b]	Terpenic, herbaceous	–	–	–	0.5	–	–	–	0.5	–	–
15	2-Heptanone + D-limonene [b]	Pleasant, fruity, slightly spicy + terpenic, fresh	–	–	–	0.5	0.5	1	1	1	–	0.5
16	3-Methylbutanol [b]	Unpleasant, sourish, stinking, rotten cheese-like	0.5	1.5	2.5	0.5	1	2	2.5	1.5	2.5	2.5
17	Ethylhexanoate [b]	Fruity, winy, green apple-like, slightly sweetish, brandy-like	–	–	–	–	–	2	1	1.5	1	2
18	Pentanol [b]	Sweetish, vitamin B complex-like, balsamic	–	0.5	0.5	0.5	–	0.5	–	0.5	–	–
19	Acetoin [b]	Pleasant, sweetish	0.5	0.5	–	0.5	–	2	–	1	–	–
20	Octanal [b]	Bitterish, vegetable	–	–	0.5	–	–	–	–	–	–	0.5
21	Unknown	Milky, sweetish	0.5	–	–	0.5	0.5	–	–	–	0.5	–
22	Hexanol [b]	Grassy, plant-like	–	0.5	0.5	–	–	–	–	0.5	–	–
23	2-Nonanone [b]	Pleasant, sweetish, fruity, fragrant	–	–	–	–	–	1	1.5	0.5	1	0.5
24	Nonanal [b]	Plant-like, fresh, sharp, slightly citrus peel-like	–	–	–	–	–	1	1	1	–	–
25	Ethyloctanoate [b]	Sweetish, green-fruity, fresh	–	–	0.5	–	0.5	1.5	0.5	1.5	0.5	1
26	Unknown	Sweetish, milky	–	–	–	0.5	–	1	–	–	–	–
27	Acetic acid [a]	Vinegar-like, sour	2	1.5	2	2	2	2	2	2	1.5	2
28	3-(Methylthio)propanal [c]	Cooked potato-like, slightly garlic-like	–	–	–	–	–	1	1.5	–		1.5
29	2-Nonanol [a]	Fatty, waxy, green, plant-like	–	0.5	0.5	–	1	–	–	1	–	–

Tab. 5. *continued*

No.	Aroma compound	Aroma description	Aroma intensity in individual samples									
			1	2	3	4	5	6	7	8	9	10
30	2-Ethylhexanol [b]	Mixed aroma of balsamic, fragrant, waxy, slightly sweetish	–	–	–	0.5	–	1	–	–	–	–
31	2,3-Butanediol [b]	Sweetish, creamy	–	–	–	0.5	–	0.5	–	–	–	1
32	Propanoic acid [a]	Stale leaves-like, rotten fruits-like, putrid herbs-like	–	–	–	1	–	0.5	–	–	–	–
33	2-Methylpropanoic acid [b]	Cheesy, slightly rancid butter-like	–	–	0.5	–	–	–	0.5	–	1	–
34	Butanoic acid [a]	Unpleasant, putrid, sweaty, rancid, spoilt cheese-like	1.5	3	3	3	3	3	3	3	3	3
35	Ethyldecanoate [b]	Sweetish, honey-like, brandy-like	–	–	–	–	–	–	–	–	1	1.5
36	3-Methylbutanoic acid [b]	Unpleasant, fermented, rotten, repulsive	0.5	1	2	0.5	1	1.5	2	0.5	3	3
37	3-(Methylthio)propanol [b]	Cooked potato-like, earthy	–	–	–	–	–	–	1.5	0.5	1	1.5
38	D-Carvone [c]	Herbaceous, fresh, peppermint-like	–	–	1	1	1	1	1	0.5	–	–
39	Pentanoic acid [a]	Sharp cheesy, acidic	–	–	–	1	–	0.5	–	–	–	–
40	Methoxyphenyloxime [a]	Earthy, musty	–	0.5	–	1	–	0.5	0.5	–	–	–
41	2-Phenylethylacetate [a]	Sweetish, honey-like	–	–	0.5	–	1	–	1.5	1	–	2
42	Hexanoic acid [a]	Unpleasant, fatty, pungent, goat-like, rancid, burnt, leather-like	–	–	–	–	1.5	1.5	2.5	2	–	1.5
43	Guaiacol [a]	Smoky, burnt, slightly maggi-like	1.5	1	1.5	1	1	1.5		1	1	
44	Unknown	Bitterish-green, plant-like	1	–	–	–	–	–	1	–	0.5	–
45	2-Phenylethanol [b]	Rosy, sweetish, fragrant	–	1	1.5	1	1	1.5	1.5	1	2.5	2
46	Heptanoic acid [a]	Cheesy, rancid fat or oil-like, waxy	–	–	–	–	–	1	1	0.5	–	1.5
47	Unknown	Bitterish, plant-like	–	0.5	–	–	–	–	–	–	–	–
48	Phenol + unknown [a]	Burnt, caramel-like, sweet, slightly tarry	2	0.5	–	1.5	1.5	2	1.5	1.5	1	2
49	Octanoic acid + *p*-cresol [b]	Mixed aroma of rancid, fatty + medicinal, exhaust fumes-like	–	1	1	0.5		2	1.5	1.5	1	2
50	Nonanoic acid [a]	Unpleasant, irritating, waxy, stale citrus peel-like, plastics-like	–	–	–	0.5	0.5	1	1	–	0.5	1
51	δ-decalactone [c]	Sweetish, milky, coconut-like	2	1	1.5	3	3	2	2	2.5	2	1.5
52	Decanoic acid + hedion [b]	Pleasant, herbaceous, fresh, citrus-like	1	0.5	1	1	1.5	1.5	1	1	0.5	1
53	Benzoic acid [a]	Pleasant, herbaceous, fresh, balsamic	0.5	0.5	0.5	1	1	1	0.5	0.5	0.5	0.5
54	Dodecanoic acid [a]	Soapy, slightly bay leaves-like	0.5	0.5	–	–	–	–	0.5	1	0.5	0.5
55	Unknown	Unpleasant, putrid, heavy stale air-like	1	–	–	–	1.5	1.5	1	0.5	–	1

a – Compound identification based on electron impact mass spectrum, aroma description and literature data, b – compound identification based on electron impact mass spectrum, linear retention index, reference compound, aroma description and literature data, c – compound identification based on linear retention index, reference compound, aroma description and literature data.

and also genus *Yarrowia*, members of which were previously detected in ewes' lump cheese and May bryndza [4, 22]. The remaining two cheese samples contained other dominant fungi, namely, Sample 4 contained dominant *Pichia* spp. and Sample 5 contained dominant of *Debaryomyces* spp. accompanied with a significant admixture of *Pichia* spp. Both of these fungi were previously detected in ewes' lump cheese and May bryndza, similar to *Klyuveromyces* spp. [4, 22], which was here detected in 8 out of 10 samples at a share of 0.1–15.1 %. These results suggest that the dominant fungal microflora reflects the composition of the microflora of the environment of individual production facilities, in particular the ripening chambers [24]. Other detected fungi, in particular *Mucor* spp., are established contaminants causing spoilage and their presence may indicate hygienic problems in the cheese production [22, 25].

Comprehensive summary of the chemical analytical-sensory results for bryndza cheese from all ten producers are summarized in Tab. 5. The chemical profiles are dominated by carboxylic acids including free fatty acids, esters, alcohols, ketones, aldehydes, terpenoids, one lactone, one oxime and a small group of yet unidentified compounds. The reason for no success at identification of the latter ones was their presence at very low, trace or ultra-trace levels, which did not allow GC-MS to record relevant mass spectra. However, these compounds were not remarkably aroma-active (intensities 0.5–1.5) in most cases, with the exception of compound No. 48.

Based on the GC/FID-O method used in this study, essential aroma-active compounds that were present in all analysed bryndza cheese samples and presenting highest aroma intensity scores were butanoic acid, δ-decalactone, acetic acid, diacetyl and guaiacol. Further significant aroma-active compounds were 3-methylbutanol, 3-methylbutanoic acid, 2-phenylethanol, octanoic acid and *p*-cresol. 3-Methylbutanol and 3-methylbutanoic acid, together with less stable 3-methylbutanal, are products of microbial metabolism that can be mutually converted, but all have similar aroma characteristics and are known as principal aroma-active compounds of various cheeses including ewes' ones [26, 27].

The samples analysed in this study were bryndza cheese produced in various geographical locations, employing various variants of technology including or excluding pasteurization, and containing or not containing lump cheese made from cows' pasteurized milk. Reflecting this, the aroma profiles of the samples varied in a certain range. However, no clear correlation with individual geographical or technological parameters could be seen. For example, samples 3 and 7, both made in a traditional way from 100% unpasteurized ewes' milk, were similar in aroma intensities of butanoic acid, acetic acid, 3-methylbutanal, diacetyl, 3-methylbutanoic acid, δ-decalactone, 2-phenylethanol, ethylbutanoate and 3-methylbutylacetate, but differed in aroma intensities of 2-methylpropanal, 3-(methylthio)propanol, hexanoic acid, heptanoic acid, phenol + unknown compound and nonanoic acid. Considering the effect of pasteurization and aroma profiles of samples 3 and 7 (unpasteurized) versus sample 5 (pasteurized) are compared, very similar aroma intensities were recorded for butanoic acid, 3-methylbutanol, diacetyl, ethyloctanoate, acetic acid, D-carvone and 2-phenylethanol, while aroma intensities of ethylacetate + 2-butanone, 3-methylbutanal, 3-methylbutyl acetate, 3-methylbutanol, 2-methylpropanoic acid and 3-methylbutanoic acid decreased, and aroma intensities of δ-decalactone increased.

The picture is further more complex when samples made from pasteurized ewes' milk with admixture of the component made from pasteurized cows' milk are taken into account (samples 1 and 4). In this case, a markedly lower number of odour zones was recorded in Sample 1, which was produced in an industrial way. The latter sample was also characterized by the poorest aroma profile of all bryndza cheese samples analysed in this study. This sample was also characterized by overall lower intensities of individual aroma-active compounds. When samples 2 and 10 made from unpasteurized ewes' milk with an admixture of the component made from pasteurized cows' milk are taken into account, their aroma profiles were similar in aroma intensities recorded for diacetyl, acetic acid, guaiacol, decanoic acid + hedion, benzoic acid and toluene. Samples 1, 4 resembled samples 2, 10 by intensities of the following aroma-active compounds, albeit recorded at low intensities: ethyleneoxide, ethanethiol, butanol, β-myrcene, 2-heptanone + D-limonene, 2-methylethylbutanoate, 2-nonanol, 2-ethylhexanol, pentanol, acetoin, octanal, nonanal, 2-methylpropanoic acid, hexanol and 2-nonanone. Comparing samples 2 and 10, 34 odour zones were recorded for Sample 10, with an evident dominance of high aroma intensities, intensities of 2–3 being detected in 10 odour zones, and only 23 odour zones were recorded for sample 2, with intensities of 2–3 being detected in only 2 odour zones.

Another interesting comparison is that between samples 6 and 9, which are both composed of barrelled ewes' cheese and of cows' lump cheese. Sample 6 has a qualitatively as well as

quantitatively rich profile of aroma-active compounds, containing 37 odour zones (the highest number of all samples) with high aroma intensities. In contrast, Sample 9 contained only 24 odour zones with overall lower aroma intensities.

Our results demonstrate that geographical location, involvement of pasteurization or admixture of the cows' milk-based component do not entirely determine the aroma profile of bryndza cheese, but the specific way of production in individual production premises is crucial.

Common key or significant aroma-active compounds determined in bryndza cheese in this study were previously encountered in our studies of May bryndza cheese [5, 19] or barrelled ewes' cheese [18]. Compared to May bryndza cheese, butanoic acid was detected at higher intensities, while 3-methylbutanol at lower intensities in samples in this study. δ-Decalactone, which was a key aroma-active compound in this study, was not detected previously in May bryndza cheese but belonged to key aroma-active compounds of barrelled ewes' cheese as well as to essential odourants of ewes' lump cheese produced particularly from pasteurized milk. However, it should be noted that we achieved improved separation of aroma-active compounds in this study due to the use of the gas chromatographic column with a polar stationary phase. Therefore, direct comparability of our current and previous results is limited.

Variability in the sensory profile of bryndza cheese samples did not show a clear connection to microflora as relatively quantified at genus level. For example, Sample 6 that had the richest sensory profile, was not outstanding in its microflora. Composition of eukaryotic microflora appeared to have no significant effect on aroma profile, including aroma intensities of medium-chain fatty acids that are known to be generated from lipids by lipases [27]. However, the specific aroma profile of bryndza cheese appears to be the result of a complex interplay of the production technology and microorganisms.

In this study, we defined the dominating microorganisms and key aroma-active compounds, as well as characterized their range in bryndza cheese produced and marketed in Slovakia. The obtained data can be used for objective specification of this cheese type, its definition and differentiation from other cheese types.

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