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Molecular identification and diversity of enterococci isolated from Slovak Bryndza cheese

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Three hundred and eight presumed enterococcal isolates were recovered from Bryndza, a soft sheep milk cheese. The cheese samples were obtained from five different commercial distributors in Slovakia and were taken at three different seasonal intervals. All isolates were identified to the species level using genotypic tools. Species-specific PCR using *ddl* genes highlighted the predominance of *Enterococcus faecium* (176 isolates) and assigned 50 isolates to the species *Enterococcus faecalis*. The remaining 82 isolates were classified using repetitive element sequence-based polymerase chain reaction (PCR) with primer $(GTG)_5$–$(GTG)_5$-PCR, in combination with phenylalanyl-tRNA synthase gene (*pheS*) sequence analysis and by whole-cell protein analysis (SDS-PAGE). These strains were identified as *Enterococcus durans* (59 strains), *Enterococcus italicus* (8 strains), *Enterococcus casseliflavus* (3 strains), *Enterococcus gallinarum* (3 strains), *Enterococcus hirae* (1 strain), and 8 strains were members of the species *Lactococcus lactis*. Of the seven enterococcal species isolated, three of them, *E. durans*, *E. faecalis* and *E. faecium* were present in all samples studied, with *E. faecium* as the predominant one. The precise identification of enterococci in Bryndza cheese is an essential step in the process of evaluation of their functional properties which will be further studied and assessed.

Key Words——Bryndza cheese; *Enterococcus*; $(GTG)_5$-PCR; *pheS* gene sequence analysis; SDS-PAGE

Introduction

Enterococci are found in high levels in a variety of cheeses, mainly those produced in European Mediterranean countries and which are made from raw or pasteurized cow, ewe, goat or water buffalo milk (Cogan et al., 1997). Enterococci are very relevant to improving and developing the flavor and quality of cheese (Centeno et al., 1999; Sarantinopoulous et al., 2002). Their proteolytic and lipolytic activities contribute to the ripening and the aroma of the final cheese product (Arizcun et al., 1997; Centeno et al., 1999; Giraffa et al., 1997). In contrast to these positive effects, some strains of *Enterococcus faecium* and *Enterococcus faecalis* are now also being recognized as human pathogens, in particular in hospital-associated infections (Morrison et al., 1997; Robredo et al., 2000).

In a few studies rep-PCR has been used for identification of enterococci (Bedendo and Pignatari, 2000; Malathum et al., 1998). Švec et al. (2005) used $(GTG)_5$-PCR as a reliable identification tool for enterococci. There are several other PCR-based methods successfully applied for enterococcal differentiation, e.g. RAPD-PCR (Gelsomino et al., 2001; Yousif et al.,

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2005), ITS-PCR (Tyrrell et al., 1997), tDNA-PCR (Devriese et al., 2002), AFLP (Vancanneyt et al., 2002), and PCR-RFLP (Lu et al., 2000). Multilocus sequence analysis (MLSA) is a recently developed identification approach for enterococci using partial sequences of RNA polymerase alpha subunit (*rpo*A), phenylalanyl-tRNA synthase (*phe*S) and the alpha subunit of ATP synthase (*atp*A) (Naser et al., 2005a, b).

In our previous work (Jurkovič et al., 2006) we analyzed three hundred and eight Bryndza isolates presumably belonging to the genus *Enterococcus* using phenotypical methods and commercial biochemical sets. We identified five enterococcal species, namely *E. faecium*, *E. durans*, *E. faecalis*, *E. mundtii* and *E. casseliflavus*. *E. faecium*, *E. durans* and *E. faecalis* were also confirmed by PCR using species-specific primers for *ddl* genes. Seven isolates remained unidentified.

The aim of this work was to complete precisely the entire enterococcal profile of Bryndza cheese which was accomplished using several molecular methods.

Materials and Methods

Bacterial strains. Bryndza is a soft creamy dairy product from Slovakia. It has been qualified as a "protected designation of origin" (PDO) cheese with characteristic aroma and taste. Bryndza is made from fresh cheese prepared from raw sheep milk or a mixture (1 : 1) of fermented fresh sheep cheese and fermented cow cheese. For the mixture to qualify as Bryndza, the sheep cheese has to represent a minimum of 50% in dry substance. The sheep cheese ripens for 14 days and then is squeezed, ground and mixed with salt and water, with a final salt concentration of up to 3% (w/w). The endproduct is a spreadable cheese known as Bryndza.

Three hundred and eight strains were isolated during microbial analysis of Bryndza cheese from five different commercial distributors from middle and southwest Slovakia, namely Liptovský Mikuláš (LM), Ružomberok (R), Červený Kameň (CK), Tisovec (T), and Zvolenská Slatina (ZS), taken at three different times—in January (I), June (II) and October (III) 2003 (Jurkovič et al., 2006).

Eighty-three representative *E. faecium*, 17 selected *E. faecalis*, and 82 non-identified enterococci isolates were deposited in BCCM/LMG Bacteria Collection, Ghent University, Belgium.

DNA isolation and species-specific PCR. Total DNA from Bryndza cheese isolates was extracted according to Gevers et al. (2001). Genotypic identification of *E. faecium* and *E. faecalis* and detection of vancomycin resistance genes were done using multiplex PCR with species-specific primers ddlF1, ddlF2 and ddlE1, as ddlE2, as well as vanA1, vanA2 and vanB1, and vanB2, as published previously (Dutka-Malen et al., 1995). Primers were synthesized by Sigma-Genosys (UK). For PCR, *Taq* DNA polymerase (TaKaRa, Japan) was used. Strains with negative specific PCR results were analyzed using rep-PCR.

Rep-PCR. $(GTG)_5$-PCR of non-identified enterococcal strains was performed as described by Versalovic et al. (1994) using Red Goldstar DNA Polymerase (Eurogentec, Belgium). PCRs were performed with a DNA Thermal Cycler (Perkin Elmer 9600). PCR products were analyzed on 1.5% agarose gels, stained with ethidium bromide solution (1.5 $\mu g\ ml^{-1}$), visualized under UV light and photographed with TCX-20.M equipment (Vilber Lourmat, France). The resulting fingerprints were analyzed by the BioNumerics v4.0 software package (Applied Maths, Belgium). Digitized and normalized fingerprints of studied isolates were compared to those from the database of BCCM/LMG (Bacteria Collection, Ghent University, Belgium) comprising numerous type and reference strains of LAB. The similarity among digitized profiles was calculated using Pearson correlation and an average linkage (UPGMA) dendrogram was derived.

Sequence analysis of pheS gene. Twenty-four representatives were subjected to species confirmation by using *pheS* gene sequence analysis. Amplification and sequencing of the *pheS* gene were done according to the method described by Naser et al. (2005a). The product of amplification (*pheS*) was purified using the Nucleofast® 96 PCR clean up membrane system (Macherey-Nagel, Germany). For sequencing PCR, the ABI Prism™ BigDye Terminator™ Cycle Sequencing Ready Reaction Mix version 3.1 (Applied Biosystems, USA) was used. Sequencing products were purified using a Montage™ SEQ_{96} sequencing reaction cleanup kit (Millipore, USA). Sequencing of purified DNA was done using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). To determine consensus sequences, sequence data were transferred to Factura™ 1.2or6 and AutoAssembler™ software 1.4.0 (Applied Biosystems). Consensus sequences were imported into Bionumerics v4.0 software (Applied Maths,

Belgium) for determination of phylogenetic relatedness of studied isolates.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole-cell proteins (SDS-PAGE). The same set of 24 representatives subjected to *pheS* gene sequence analysis was also investigated using SDS-PAGE of whole-cell proteins. Isolates were grown for 24 h on Lactobacilli MRS agar (Difco, USA). Whole cell protein extracts were prepared according to the procedure of Pot et al. (1994). The similarity among whole-cell protein profiles was determined by GelCompare v2.0 (Applied Maths) using Pearson correlation and an average linkage dendrogram (UPGMA) was generated.

Results

Using multiplex PCR (Dutka-Malen et al., 1995), 175 isolates were identified as *E. faecium* and 41 isolates as *E. faecalis*. None of them carried the *vanA* or *vanB* genes. The absence of vancomycin resistance was also confirmed using agar dilution assay.

The identity of all non *E. faecium*/*faecalis* strains was genotypically investigated using $(GTG)_5$-PCR and further numerical analysis of the obtained fingerprints and comparison with an in-house reference framework comprising type and representative strains (BCCM/LMG). Of all 92 isolates tested, nine clusters were obtained (Fig. 1). Each cluster comprised reference strain(s) belonging to one enterococcal species. The main cluster (No. 4, see Fig. 1) was assigned to the species *E. durans* (59 isolates), in which several subclusters were delineated according to their cheese origin. The next cluster (No. 2) was assigned to the species *E. faecalis* (9 isolates), followed by *E. saccharominimus* (8 isolates, cluster No. 6), *E. casseliflavus* (3 isolates, cluster No. 7), *E. gallinarum* (3 isolates, cluster No. 8), *E. faecium* (1 isolate, cluster No. 3) and *E. hirae* (1 isolate, cluster No. 1). *E. saccharominimus* was recently assigned to species *E. italicus* (Naser et al., 2006). The distribution of enterococcal species in Bryndza cheese obtained from five commercial distributors is shown in Table 2. By means of $(GTG)_5$-PCR eight strains were identified as *L. lactis*, which were not distinguishable from *Enterococcus* sp. by conventional phenotypic methods. Isolates assigned to the species *Lactococcus lactis* were grouped in two clusters—*L. lactis* subsp. *lactis* (7 isolates, cluster No. 5) and *L. lactis* subsp. *hordinae* (1 isolate, cluster No. 9).

Twenty-four strains (8 *E. durans*, 7 *L. lactis*, 4 *E. italicus*, 1 *E. casseliflavus*, 1 *E. faecalis*, 1 *E. faecium*, 1 *E. gallinarum*, 1 *E. hirae*) representative of the nine clusters were chosen for further SDS-PAGE and *pheS* gene sequence analysis. The selection of these strains was done according to the $(GTG)_5$-relatedness to the type and reference strains. The whole-cell protein profiles and *pheS* sequencing profiles of 24 selected isolates were compared with profiles obtained from 44 type and reference strains (data not shown). Both methods confirmed the $(GTG)_5$-PCR identification of all 24 isolates on the species level.

All *E. faecium*/*faecalis* strains were also tested for susceptibility to vancomycin using agar dilution assays. Intermediate susceptibility to vancomycin (MIC 16 mg/L) was detected in two isolates of *E. gallinarum* (CKII 7 and CKII 23). The remaining strains were susceptible to vancomycin.

When using the $(GTG)_5$-PCR, we detected one isolate of *E. faecium* and nine isolates of *E. faecalis* more than by multiplex species-specific PCR (with primers for *ddl* and *van* genes).

Results of biochemical and molecular identification of enterococcal species were in 90.3% (278 of 308) agreement. Eleven isolates identified as *E. mundtii* using commercial biochemical tests (EN-COCCUStest, STREPTOtest 16 (Pliva-Lachema, Czech Republic), and BBL Crystal Gram-Positive ID kit (Becton Dickinson, MD, USA)) were shown to be *E. faecalis* (9 strains), *E. faecium* (1 strain) and *E. hirae* (1 strain) when using molecular approaches. Similarly, three isolates identified biochemically as *E. casseliflavus* were genotypically determined as *E. gallinarum*. Due to the lack of biochemical sets we did not succeed in the identification of 16 strains. Using molecular approaches, these isolates were proved to be *E. italicus* (8 strains) and *L. lactis* (8 strains).

When evaluating the species diversity over time per distributor, we can conclude that the highest variability of species, including *L. lactis*, was observed in Bryndza from June (II) from distributors Červený Kameň, Ružomberok and Tisovec and from October (III) from distributor Zvolenská Slatina. Less species diversity was found in January (I) Bryndza cheese and this was true for all distributors.

Discussion

The distribution of *Enterococcus* species in Bryndza

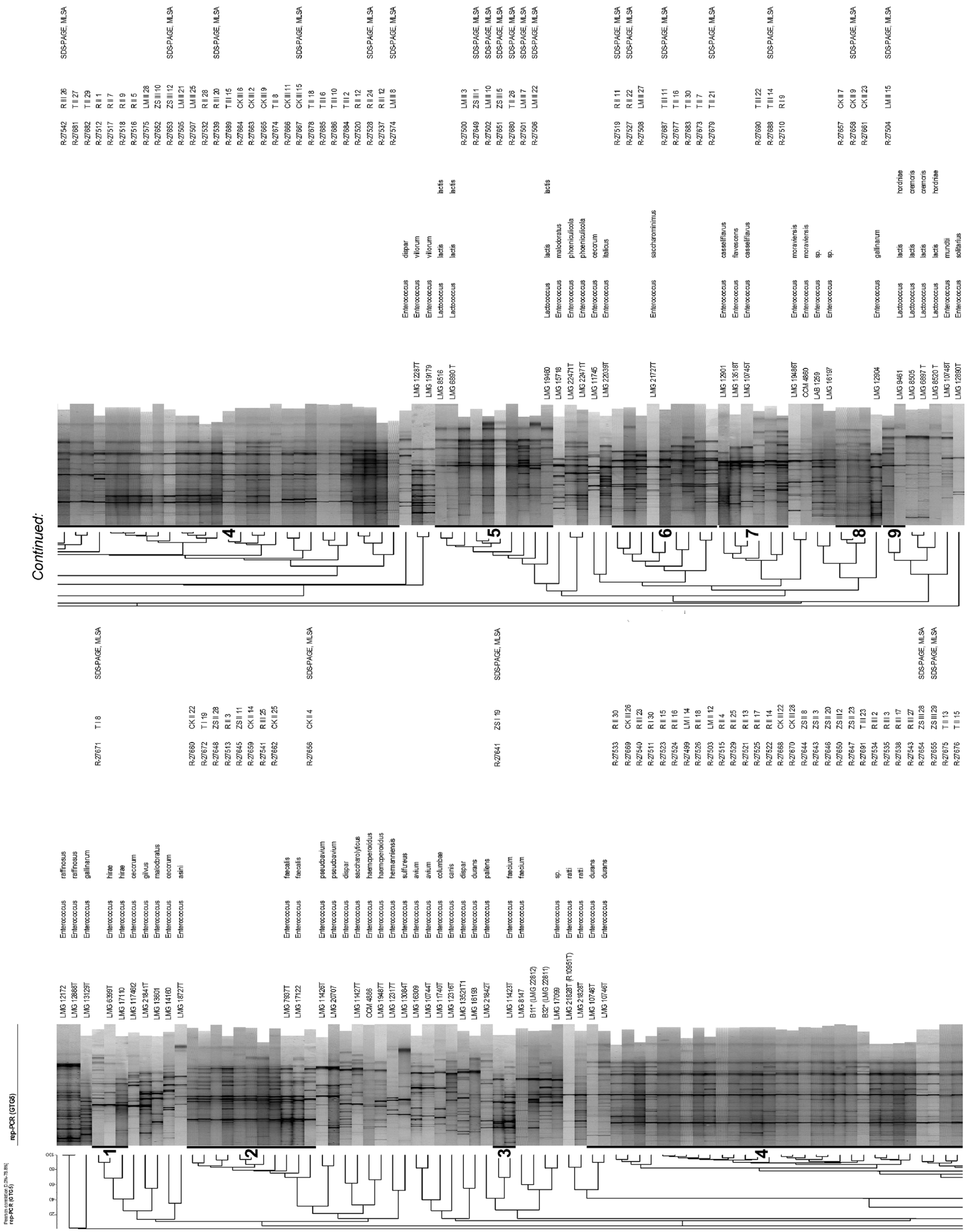

Fig. 1

cheese of all five investigated commercial distributors (Table 2), is as follows. The predominant enterococcal species was *E. faecium* (176 isolates), followed by *E. durans* (59), *E. faecalis* (50), *E. italicus* (8), *E. casseliflavus* (3), *E. gallinarum* (3) and *E. hirae* (1). *E. faecium* predominated in Bryndza samples obtained from all five distributors. *E. durans* was the second most frequently determined species, in all except in Zvolenská Slatina samples. Other enterococcal species were found with lower frequencies. The highest species diversity was detected in Bryndza cheese from Tisovec (6 species), while in Bryndza cheese from Zvolenská Slatina only three enterococcal species were found. This is the first report of the presence of *E. italicus* in the Slovakian Bryndza cheese and was confirmed in the samples from the aforementioned three distributors: Tisovec, Ružomberok, and Liptovský Mikuláš. *E. faecium*, *E. durans* and *E. faecalis* were found in all three seasons (January, June and October), and *E. italicus* and *L. lactis* in two seasons (June and October). The distribution of other Bryndza species (*E. casseliflavus*, *E. gallinarum* and *E. hirae*) was not equal through the seasons or regions (Table 1).

When using multiplex PCR with primers for *ddl* and *van* genes, the identification of 10 isolates (1 *E. faecium*, 9 *E. faecalis*) was puzzling as multiple PCR products of various size were amplified. Because of given discrepancies and for possible risk of multiplex PCR usage, the identity of these strains was confirmed by means of individual species-specific *ddl* gene-based PCR tests.

When analyzing the intraspecies genotypic variability in *E. durans* (59 isolates), we discovered that few subclusters contain isolates originating from the same producer, which supports our hypothesis about the uniqueness of samples origin. Moreover, each cheese brand was dominated by strains from different subclusters, confirming the high genetic diversity of enterococcal strains. This observation is interesting when monitoring the strain dissemination of *E. durans* during all three seasons in 2003. The evaluation of strain diversity of the remaining enterococcal species using a dendrogram based on $(GTG)_5$-PCR gave ambiguous results due to the low number of identified isolates (9 *E. faecalis*, 8 *E. italicus*, 3 *E. casseliflavus*, 3 *E. gallinarum*, 1 *E. faecium*, and 1 *E. hirae*).

Enterococci are a heterogeneous group of Gram-positive cocci sharing many characteristics with other LAB, e.g. genus *Lactococcus* (Gram staining, aesculin hydrolysis, growth at 10°C, 45°C, and in 6.5% NaCl, and in presence of 40% of bile salts) (Devriese et al., 1993; Facklam and Elliot, 1995). As a result, their identification is challenging; therefore *Lactococcus* sp. was not identified using conventional phenotypic tests and neither did we succeed in biochemical identification of any *Lactococcus* sp. isolate on the species level. It is difficult to unequivocally categorize isolates into one of the *Enterococcus* species by only physiological tests because heterogeneity in phenotypic features is very high, regardless of the origin of the isolate (Giraffa, 2002). Identification of enterococci using traditional phenotypic differentiation can be a tedious process requiring numerous tests. Although more than 20 species can be identified using these methods, tests often require long incubation periods before results can be interpreted. Time constraints and number of samples to be processed can be overcome by using commercial biochemical kits. The accuracy of these, however, has been assessed using only type strains and strains from clinical sources, and they usually identify a maximum of 10 enterococcal species (Jackson et al., 2004). Similarly, atypical biochemical profiles of Bryndza isolates may be due to different origin of strains (clinical vs. environmental). Our results derived from biochemical tests did not reveal the accurate or comprehensive enterococcal composition of Bryndza cheese, namely 4.1% (12 of 292) of enterococci were not identified to species level and 7.2% (21 of 292) did not agree with genotypic findings. Andrighetto et al. (2001) isolated 124 enterococcal

Fig. 1. Dendrogram based on cluster analysis of digitized $(GTG)_5$-PCR fingerprints of type, reference and Bryndza cheese strains.

E. saccharominimus was recently reclassified to species *E. italicus* (Naser et al., 2006). The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using correlation levels expressed as percentage values of the Pearson correlation coefficient. Twenty-four selected strains marked as SDS-PAGE, MLSA were next analyzed by SDS-PAGE and *pheS* sequencing to confirm species identification. Fingerprints marked as R-represent those from Bryndza isolates, and LMG those from BCCM/LMG Bacteria Collection, Ghent University, Belgium (http://www.belspo.be/bccm). Numbered clusters (1–9) correspond to identified species of *Enterococcus* sp. and *Lactococcus* sp.

Table 1. Bryndza isolates obtained from five different distributors in 2003 and used in this study.

Time	Species	Bacterial isolates
January (I)	*E. casseliflavus*	RI 9†
	E. durans	LMI 14†
		RI 30†
	E. faecalis	TI 19†
		ZSI 10, 12, 17, 21, 24, 25, 27†
	E. faecium	LMI 2, 3, 4, 5, 6, 7, 8, 13, 15†, 17, 18†, 19, 21, 22, 23, 24, 25, 26, 27†, 28, 29†, 30
		RI 2†, 3, 4, 5†, 6†, 8, 10†, 11†, 12, 13†, 14†, 15, 19†, 20†, 22†, 23, 24†, 25†, 26†, 27†, 28†, 29†
		TI 1, 14†
		ZSI 1†, 2†, 3†, 5†, 6, 7, 9†, 11, 13, 14, 15†, 18†, 19†, 26, 28†, 29†, 30†
	E. gallinarum	—
	E. hirae	TI 8†
	E. italicus	—
	Lactococcus lactis	—
June (II)	*E. casseliflavus*	—
	E. durans	LMII 8†, 12†, 21†, 25†, 28†
		RII 1†, 4†, 5†, 7†, 9†, 12†, 13†, 14†, 15†, 16†, 17†, 18†, 24†, 25†, 28†, 30†
		TII 8†, 13†, 15†, 18†, 27†, 29†
		ZSII 3†, 8†, 20†, 23†
	E. faecalis	CKII 4†, 14†, 22†, 25†
		LMII 14, 19, 20, 30†
		RII 2†, 3†, 8†, 26†, 27†
		ZSII 4, 7, 11†, 14, 16, 21, 22, 24, 25, 28†, 29
	E. faecium	CKII 1, 5, 6, 8, 10, 11, 12, 13†, 15, 16, 17, 18, 20, 21, 26†, 29, 30
		LMII 1, 4†, 5†, 9†, 13†, 17, 18, 23†, 24†, 26, 29†
		RII 10, 20, 21†, 23†, 29
		TII 3†, 4, 5, 6, 9, 11, 12, 14, 17, 20†, 22†, 23†, 24†, 25†
		ZSII 1, 2, 5, 6, 9, 10, 12†, 13, 15, 17†, 18, 19, 26†, 27†, 30
	E. gallinarum	CKII 7†, 9†, 23†
	E. hirae	—
	E. italicus	LMII 27†
		RII 11†, 22†
		TII 7†, 16†, 21†, 30†
	Lactococcus lactis	LMII 3†, 7†, 10†, 15†, 22†
		TII 26†
October (III)	*E. casseliflavus*	TIII 14†, 22†
	E. durans	CKIII 2†, 6†, 9†, 11†, 15†, 22†, 26†, 28†
		RIII 2†, 3†, 12†, 17†, 20†, 23†, 26†, 27†
		TIII 2†, 6†, 10†, 15†, 23†
		ZSIII 2†, 10†, 12†, 28†, 29†
	E. faecalis	RIII 8†, 25†, 28†
		ZSIII 3, 4, 6, 7, 8, 9, 11, 13, 14, 15, 16, 18, 23, 25, 27
	E. faecium	CKIII 1†, 3†, 4, 5, 7†, 10†, 12, 13†, 14†, 16†, 17†, 18, 19, 20†, 21†, 23†, 24†, 25†, 27†, 30†
		RIII 1†, 4, 5, 7, 9†, 10, 11, 13, 14, 15†, 16†, 18, 19†, 21†, 22†, 29, 30
		TIII 1, 16†, 17, 18†, 24, 25†, 26†, 27†, 28†, 30
		ZSIII 11†, 24, 26, 30†
	E. gallinarum	—
	E. hirae	—

Table 1. Continued

Time	Species	Bacterial isolates
	E. italicus	TIII 11†
	Lactococcus lactis	ZSIII 1†, 5†

Distributors: Červený Kameň (CK), Liptovský Mikuláš (LM), Ružomberok (R), Tisovec (T), Zvolenská Slatina (ZS).
All isolates deposited in Institute of Cell Biology, Comenius University, Bratislava, Slovakia.
† isolates also deposited in BCCM/LMG Bacteria Collection, Ghent University, Belgium—83 *E. faecium*, 17 *E. faecalis*, 82 *non-faecium* and *non-faecalis* (http://www.belspo.be/bccm).

Table 2. Proportional representation of enterococcal species in Bryndza cheese recovered from five commercial distributors in 2003.

Distributor	Number and percentage representation (%) of isolated enterococci							
	E. casseliflavus	*E. durans*	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. hirae*	*E. italicus*	*E.* sp. (total)
Červený Kameň (CK)	0 (0)	8 (15.4)	4 (7.7)	37 (71.1)	3 (5.8)	0 (0)	0 (0)	52 (100)
Liptovský Mikuláš (LM)	0 (0)	6 (13.6)	4 (9.1)	33 (75.0)	0 (0)	0 (0)	1 (2.3)	44 (100)
Ružomberok (R)	1 (1.3)	25 (31.2)	8 (10.0)	44 (55.0)	0 (0)	0 (0)	2 (2.5)	80 (100)
Tisovec (T)	2 (4.3)	11 (23.9)	1 (2.2)	26 (56.5)	0 (0)	1 (2.2)	5 (10.9)	46 (100)
Zvolenská Slatina (ZS)	0 (0)	9 (11.5)	33 (42.3)	36 (46.2)	0 (0)	0 (0)	0 (0)	78 (100)

strains from traditional Italian cheeses. Identification using the rapid ID 32 Strep galleries gave different results from SDS-PAGE in 12.1% of the cases. Franzetti et al. (2004) showed that among 64 *Enterococcus* sp. from different origins, biochemical identification (API 20 Strep) correlated with PCR using species-specific primer only in 57.8% of the cases. Harwood et al. (2004) reported that among 139 *Enterococcus* sp. isolated from various sources, 58 (42.2%) *E. faecalis* determined biochemically were also confirmed by PCR. However, of 27 (19.5%) biochemically classified *E. faecium*, only seven (5.0%) showed PCR-positive results.

Finding two *E. gallinarum* Bryndza isolates with intermediate susceptibility to vancomycin (MIC 16 mg/L) was not suprising since *E. galinarum* is known as intrinsically resistant to low-level vancomycin concentration (VanC resistance). Confirmation that no Bryndza cheese isolates possessed *vanA* or *vanB* genes was desirable. This fact is favorable, because resistance against vancomycin dramatically reduces its therapeutic utility in treating enterococcal infections.

The enterococcal composition of Bryndza cheese corresponds with those of other European artisanal cheeses, where *E. faecium* and *E. faecalis* represent the dominant enterococcal microflora (Franz et al., 1999; Giraffa et al., 1997), although there are exceptions. Cosentino et al. (2004) showed that in artisanal Fiore Sardo cheese *E. durans* is more frequently detected (20.3%) than *E. faecalis* (8.5%). Arizcun et al. (1997) identified *E. faecalis* as the most frequent (85%) enterococcal species in Roncal and Idiazábal cheese in which *E. faecium*, *E. durans* and *E. avium* were present in lower counts, while in traditional Italian cheeses, *E. faecalis* was found to be the predominant enterococcal species (Andrighetto et al., 2001) and *E. casseliflavus* predominates in the Irish farmhouse cheese Baylough (Gelsomino et al., 2002).

Enterococci are considered to play an important role in the fermentation of many European cheeses (Franz et al., 1999). As a high amount of enterococci, especially *E. faecium*, was detected in Bryndza cheese, their function and properties should be further determined, namely as to their overall positive or negative

effect on human health (Giraffa et al., 1997).

To summarize, more bacterial species (seven *Enterococcus* sp. and *L. lactis*) were found in Bryndza cheese in comparison to five species determined in a recent study (Jurkovič et al., 2006). The present study highlights the importance of exact identification of natural microflora in Bryndza cheese using genotypic approaches. It was shown that rep-PCR ($(GTG)_5$-PCR) can serve as a reliable DNA-grouping tool for DNA-based identification of Gram-positive cocci. Rep-PCR, in conection with other molecular methods, is a valuable technique for precise determination of bacterial species. Although other additional methods such as SDS-PAGE and gene sequencing are cost- and time-consuming, when combined with DNA-grouping techniques they are an effective confirmation tool in microbial diagnostics. For monitoring of intraspecies variability within enterococcal species (originating from both clinical and environmental sources), all rep-PCR methods should be used and further evaluated. Work on the further identification of the *Lactococcus* species continues.

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