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Eur Food Res Technol (2011) 233:1029–1040
DOI 10.1007/s00217-011-1600-z

ORIGINAL PAPER

Characterization of enterococci isolated from homemade Bulgarian cheeses and katuk

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Received: 18 July 2011 / Revised: 14 September 2011 / Accepted: 17 September 2011 / Published online: 15 October 2011

Abstract A collection of 107 lactic acid bacteria (LAB) isolates was obtained from traditional Bulgarian dairy products—homemade cheeses and katuk samples, produced from heat-treated cow, goat, ewe and buffalo milk without the addition of any bacterial starter culture. The samples were collected from mountain region of Rhodope (south part of Bulgaria), Tracian valley and mountain region of Stara Planina (west part of Bulgaria). These LAB produced *bacteriocin-like* inhibitory substances (BLIS) and proteinases. Preliminary strain determination was performed according to their fermentation ability using API 50CHL and API 20 Strep. Most of the characterized strains (58) belong to genus *Enterococcus*; 21, 20 and 11 strains were identified as *Lactobacillus* sp., *Streptococcus* sp. and *Lactococcus* sp., respectively. Isolated enterococcal strains were characterized using phenotypic features as well as by DNA typing. The strains were identified as *Enterococcus faecium* (34), *Enterococcus durans* (22) and *Enterococcus faecalis* (2). The proteolytic activity varied from 0.094 to 0.455 mM/L Gly into the group of *E. faecium* and from 0.109 to 0.487 mM/L Gly into the group of *E. durans* strains, while both *E. faecalis* strains showed relatively high proteolytic activity. The samples obtained after 3 h of hydrolysis of β-casein by *E. faecalis* B1 strain were further used for mass spectrometry analysis, and 31 peptides were identified.

Keywords Enterococci · Cheese · RAPD-PCR · Proteolysis · Enzymatic activity

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Introduction

White pickled (brined) cheeses are produced in many varieties in hot-climate countries, among them Beyaz Peynir (Turkey), Feta (Greece), Bjalo salamureno sirene (Bulgaria), Teleme (Turkey, Romania), Domiati (Egypt) and Queso blanco (South America). Originally, they were manufactured from sheep, goat or buffalo milk. A common feature of white-brined cheese technology is that ripening occurs in brine and lasts from a few weeks up to 3 months [1]. Katuk is a dairy product with a salty/sour taste, prepared in Bulgaria. The name actually is used for several products with similar taste, but manufactured by different ways. Traditionally, katuk is made with sheep's milk used for cheese. The resulting product is durable and can be used for several months. Katuk looks like soft or semihard cheese, depending on whether it is just prepared from a

fresh or a mature product. Before inoculation, sheep's milk is boiled on slow heat for several hours to reach more dense texture.

One of main purposes for homemade cheese making is achievement of better taste of the product as compared with commercial one, which is more or less standardized due to technical processing. Using artisanal, wild rennet improves flavor and taste of homemade cheeses, which would cause also contamination with spoilage microflora. Nevertheless, homemade cheeses and katuk combine unique taste and quality. Nowadays, many of citizens of Bulgaria are looking for homemade milk products as natural and tasty.

The genus *Enterococcus* is the most controversial group of lactic acid bacteria (LAB). Enterococci play an important role in the ripening of many cheeses and are also present in other fermented foods, such as sausages and olives [2]. Unlike most of LAB, the *Enterococcus* genus is not considered "generally recognized as safe" (GRAS). Thus, these bacteria have quite contradictory reputation of being useful in dairy fermentations, but also potentially hazardous [3]. Enterococci play beneficial roles in foods and have relatively low virulence. However, they are also considered as a major cause of nosocomial infections like endocarditis, bacteremias, urinary tract infections and other infections [4]. One of the essential factors raising their pathogenicity is that some of their strains are resistant to many antibiotics, especially vancomycin. Nevertheless, the antibiotic resistance cannot by itself explain their virulence. In fact, several virulence factors have been reported for enterococci, linked to *Enterococcus faecalis*, like adherence and tissue invasion factors, cytotoxin production and aggregation substances [5, 6]. Raw milk is colonized by enterococci, originating from animal feces or from contaminated habitats. Their use and persistence in a variety of cheeses, also produced from pasteurized milk, is justified by their ability to survive under adverse conditions, such as temperatures and salinity [2]. Enterococci occur as non-starter (NS) LAB in a variety of cheeses, especially artisan cheeses produced in the Southern Europe from goat, ewe, water buffalo or bovine milk [7] and in cheeses produced from non-pasteurized raw milk in France. In cheeses, in particular in traditionally fermented cheeses made with raw milk, they form the dominant microbiota [8, 9]. They play essential roles in the development of sensory properties during ripening of many cheeses and have also been used as components of cheese starter cultures [10, 11]. It is difficult to clearly classify different isolates into one of the *Enterococcus* species by the physiological tests due to their heterogenic phenotype, regardless of the origin of the isolate [12, 13]. Furthermore, many cheese-related strains display the ability to produce bacteriocins against pathogens or food spoilage bacteria, thus improving food safety [11, 14]. The presence of *E. faecalis* and *Enterococcus faecium* as the dominant enterococcal species in different dairy products was reported by many authors for different cheese varieties [7, 11, 15–18].

The aim of the present work was to make a broad survey of LAB microflora from traditional Bulgarian dairy products with particular interest in Enterococci.

Materials and methods

Preparation of samples

Samples were collected since the beginning of spring to the end of September. The chosen period of sample collection is correlated with increased milk production, which leads to processing of fresh milk into durable product. Cheese, cream, katuk and "yellow cheese" (kashkaval) are the most popular soft, semihard or hard milk products as well as yogurt, prepared by peasants in remote areas. Cheese making follows the next steps: heating of the fresh milk up to 32 °C or pasteurization; addition of rennet, which in most cases may be either commercial rennet or a combination of rennet and some old cheeses, or old cheeses and yogurt as only source of microflora for cheese manufacturing. After milk coagulation, whey is allowed to drain and then the obtained curd is transferred into big dishes or in some cases into clothes made from animal skin. Final ripened cheese product depends on taste of sole manufacturer. The method most often used to preserve spoiling is to put cheese in brine up to 10–12% NaCl. If the milk is of poor quality for cheese making, the coagulum will be soft. This results in heavy losses of fines (casein) and fat as well as poor syneresis during cheese making. An amount (5–20 g) of calcium chloride per 100 kg of milk is normally enough to achieve a constant coagulation time and results in sufficient firmness of the coagulum. During all these processing, contamination of the product may appear, due either to personal hygiene at home or to the use of some animal cloth, dishes, etc.

Bacterial strains, media and culture conditions

For microbiological analysis, a total of 4 katuk samples collected from mountain region of Rhodope (south part of Bulgaria) and 14 cheese samples collected from mountain region of Rhodope (4 cheeses), Tracian valley (6 cheeses) and mountain region of Stara Planina (west part of Bulgaria) (4 cheeses) were used. Katuk was prepared from ewe's milk. Six cheese samples were from cow milk, 3 from buffalo milk, 3 from goat milk and 2 from ewe's milk. Ten grams of each cheese/katuk sample was homogenized and transferred to 90 mL sterile 2% (w/v) sodium citrate solution (Merck GmbH, Darmstadt, Germany). Decimal dilutions to 10^{-9} of the homogenates were prepared with

sterile 0.9% (w/v) sodium chloride and were plated on suitable for isolation of LAB media: MRS agar pH 5.7 (Merck GmbH, Darmstadt, Germany) in anaerobic conditions with Anaerocult A (Merck) for lactobacilli; LM17 agar pH 7.2 (Merck) for lactococci; and M17 agar (Merck) for enterococci and streptococci. Incubation of inoculated media was performed at 30 °C for lactococci, 37 °C for enterococci and 42 °C for lactobacilli and streptococci for 48 h. Strains from enterococci M17 medium were picked from the plates and subcultured in kanamycin esculin azide agar (KAA) medium (Merck) or D-Coccosel agar (bioMérieux, Marcy l'Etoile, France). After 24 h of incubation at 37 °C, typical colonies of presumptive enterococci (which appear surrounded by a black halo) were observed. Randomly from each sample plate, 2–3 colonies were picked and cultured in M17 broth for 24 h. In order to obtain pure cultures, this procedure was repeated twice (from agar medium to broth), and after second subculture, single colonies were transferred to 5 mL M17 broth and thus were considered as pure strains. Each pure culture after 24 h of cultivation was centrifuged, rinsed twice with 0.9% NaCl sterile solution and stored at −20 °C in 10% (v/v) sterile skim milk (Merck) supplemented with 5% (v/v) yeast extract and 30% glycerol (Sigma Aldrich, Germany) for further analysis.

Phenotypic identification of the strains

Identification analysis was performed to the genus or species level by phenotypic identification based on physiological and biochemical methods as follows: Cell morphology and motility of all isolates of enterococci have been determined by microscopy (Olympus U-RFL-T, BX51, GmbH, Hamburg, Germany); gram staining was performed using Gram stain kit (Merck); catalase test was performed with dropping 3% H_2O_2 at the colonies and observation of the formation of gas. Oxidase tests, production of carbon dioxide from glucose, growth at 10, 37 and 42 °C in M17 broth, growth with 6.5% NaCl in M17 broth, growth at pH 9.6 in M17 and esculin hydrolysis in KAA for cocci test of forming black zone on bile esculin agar were also performed. Hemolytic activity was determined on Columbia Blood Agar supplemented by 5% defibrinised horse's blood after 48 h of incubation at 37 °C.

API galleries assay

Identification at species level was performed using API galleries (bioMérieux) according to the manufacturer's instructions. The fermentation profiles were read after incubation at 37 °C in anaerobic conditions for *Lactobacillus* using API 50 CHL medium and in *microanaerophilic* conditions for *Streptococcus*, *Lactococcus* and *Enterococcus* strains using API 20 STREP medium for 24–48 h. The results were analyzed using APIweb™ stand alone v 1.2.1 software (bioMérieux).

Molecular identification of the strains

DNA isolation

Total DNA from strains was isolated using GeneElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer. All primers used in this study (Table 1) were obtained from "Integrated DNA Technologies" (Belgium) at a concentration of 25 or 100 nmol.

Table 1 Primers used in this study

Primer	Sequence	Product (bp)	Reference
GS-PCR			
Ent1	5′-TACTGACAAACCATTCATGATG-3′	112 bp	[19]
Ent2	5′-AACTTCGTCACCAACGCGAAC-3′		
16S rRNA-PCR			
27F	5′-AGAGTTTGATCMTGGCTCAG-3′	~1.5 kb	[39]
1492R	5′GGTTACCTTGTTACGACTT-3′	~1.5 kb	
RAPD-PCR			
E1	TCACGCTGCA		[33]
E2	AGCCGCCCT		[34]
ERIC–PCR			
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C		
ERIC2	AAG TAA GTG ACT GGG GTG AGC G		
Rep-PCR			
REP1R-Dt	III NCG NCG NCA TCN GCC		
REP2-Dt	NCG NCT ATC NGG CCT AC		

Genus specific (GS)-PCR amplification

GS-PCR amplification was performed with Ent1/Ent2 (Table 1) primers as described by Ke et al. [19] under the following conditions: 50 μL PCR mixes in KCl buffer system at final concentrations 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.1 μg of total bacterial DNA and 1 U of Taq polymerase (Fermentas, Thermo Fisher Scientific, Sofia, Bulgaria). Initial denaturation at 94 °C for 5 min was followed by 35 cycles containing a denaturation step at 94 °C for 15 s, an annealing step at 55 °C for 15 s and an extension step at 72 °C for 45 s. Final extension was performed at 72 °C for 5 min. An aliquot (5 μL) of the PCR reaction was applied on 1.2% agarose gel in 0.5x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) buffer in order to check the presence of amplified fragments. 100 bp ladder was used as a marker.

Sequencing of genes for 16S rRNA

DNA extracted from the strains was used as a template for amplification of the genes for 16S rRNA fragment sequencing. PCR was performed in DNA thermal cycler model (DOPPIO, VWR, Vienna, Austria). Reaction mixtures contained 50 μL PCR mixes in KCl buffer system at final concentrations 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer (Table 1), 0.1 μg of total bacterial DNA and 1 U of Taq polymerase (Fermentas). Amplification was carried out using the following programs: initial denaturation at 94 °C for 10 min, followed by 35 cycles containing a denaturation at 94 °C for 1 s, an annealing step at 48–52 °C for 1 min and an extension step at 72 °C for 3 min followed by a final extension step at 72 °C for 5 min. Amplicons were analyzed on 0.7% agarose. DNA sequencing was carried out by "Macrogen" (Seoul, Korea).

Genotyping by randomly amplified polymorphic DNA (RAPD) analysis, enterobacterial repetitive intergenic consensus–PCR (ERIC–PCR) and repeated sequence PCR (REP-PCR) analysis

All strains were genomically typed by RAPD-, ERIC– and REP-PCR. PCR reaction was carried out in 25 μL PCR mixes in KCl buffer at final concentrations 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of each primer (Table 1), 40 ng of DNA template and 0.5 U "JumpStart™" Taq-polymerase (Sigma-Aldrich). Amplification was carried out using the following programs: initial step of a denaturation at 94 °C for 5 min, 4 cycles of denaturation at 94 °C for 45 s, an annealing step at 30 °C for 2 min and an extension step at 72 °C for 30 s, followed by 30 cycles of 5 s at 94 °C, 30 s at 36 °C and 40 s at 72 °C, and a final extension step for 10 min at 72 °C. An aliquot (6 μL) of each amplicon was applied on 1.2% agarose gel with TAE-buffer using Cleaver Mini Rapid horizontal electrophoresis system (Cleaver Co. Warwickshire, UK).

Gels were analyzed by DigiGenius (Syngene, Cambridge, UK) photo documentation system followed by data analyzing with GeneTools v. 4.0 software (Syngene). Calculation of similarities of band profiles was based on Pearson product moment correlation coefficient. Dendrograms were obtained by means of the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm.

Antimicrobial assay

Ability of the strains to produce antimicrobial substances was performed on agar-well diffusion assay [20]. Soft M17 or MRS agar (0.7%, w/v) containing approximately 10^6 cells/mL of indicator strains was overlaid onto plates (depending on the indicator strain). Wells were made, and 80 μL of fresh 12–16 h supernatant was poured into the wells. Assay was performed either without adjusting the pH or with neutralized samples. Plates were incubated for 24 h at appropriate temperatures for growth of indicator strains, and then, inhibition zones were observed.

To confirm the proteinaceous nature of antibacterial substances, 80 μL of each sample was incubated for 2 h with 30 μL of 20 mg/mL proteinase K (Merck) or boiled for 5 min. Indicator strains used in this assay were as follows: *Escherichia coli* ATCC 11775, *Lactobacillus helveticus* 1011 ATCC 15009, *E. faecalis* 3360 ATCC 19433, *Enterobacter aerogenes* ATCC 13048 (NBIMCC, Sofia, Bulgaria) and *Listeria innocua* F (ONIRIS, Nantes, France).

Enzymatic activity

All 58 enterococcal strains were assayed using API ZYM tests (bioMérieux) performed according to the manufacturer's instructions. The results were graded from 0 to 5 by comparison of the observed color with the color reaction chart. A value of 0 corresponded to negative reaction and 5 to a reaction of maximal intensity. The approximate number of free nmol hydrolyzed substrate is obtained from the color strength: 0, no activity; 1, liberation of 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, 40 nmol.

Proteolytic activity analysis

Proteolytic activity assay on milk agar

Ability of enterococci to hydrolyze caseins on skim milk agar plates was tested as follows: Overnight cultures of

enterococci were washed twice with 0.9% sterile NaCl, and drops of cell suspensions were applied on the plates with 1.5% milk agar with 5% final milk concentration. After overnight incubation at 37 °C, plates were colored with Coomassie blue R 250 solution for 1 h and proteolytic strains appeared as a clear white spot surrounded by a blue halo.

Milk coagulation test was performed in 10% (w/v) reconstituted skim milk (RSM, Merck). Briefly, overnight cultures growing on M17 broth were washed twice with sterile 0.9% NaCl, and 10 mL sterile milk was inoculated with 0.2% (v/v) cell suspension with optical density of 5. Acidifying activity was evaluated after 24 h at 37 °C. pH was measured using HANNA 200 pH Metter (Hanna Instruments, Kehl am Rhein, Germany).

Proteolytic activity assay with OPA reagent

Overnight cultures of the strains were washed twice with sterile 0.9% NaCl and inoculated in sterile reconstituted (10%, w/v) skim milk. Proteolytic activity was evaluated after 24 h at 37 °C.

The amino acids accumulated in the milk after 24 h of hydrolysis by the tested strains were determined using the *o*-phthaldialdehyde (OPA) method [21]. This test is based on the reaction of the free α-amino groups released by hydrolysis of casein with *o*-phthaldialdehyde, in the presence of β-mercaptoethanol, to form a complex that strongly absorbs at 340 nm. The absorbance of the OPA reagent with aliquot of the control (10% skim milk) was subtracted from each reading. The results were expressed in L-Gly equivalent (mM/L).

Hydrolysis of β-casein

Proteolytic activity of selected enterococci strains was detected according to Kojic et al. [22]. After an overnight pre-culture of the strain in MRS medium, bacteria were inoculated on milk-citrate agar (MCA) plates containing 4.4% skim milk (Merck), 0.8% Na-citrate, 0.1% yeast extract, 0.5% glucose and 1.5% agar (w/v). After incubation of the plates for 48 h at 37 °C, cells were collected and resuspended in 100 mM Na-phosphate buffer pH 7 (10 mg/mL; approximate density of 10^{10} cells/mL). The cell suspension was mixed either with fresh pasteurized milk or with β-casein (5 mg/mL) dissolved in the same buffer at a 1:1 volume ratio. The resulting mixture was incubated at 37 °C for 1 and 3 h. After incubation, the samples were centrifuged to discard cells and then stored at 4 °C before analysis.

SDS electrophoresis

Hydrolysis of milk proteins and pure β-casein was determined by Tricine SDS–PAGE as follows: Samples (8 µL) were suspended in 5 µl of Laemmli buffer [23] and heated at 100 °C for 5 min. The corresponding blanks milk control or β-casein were loaded separately. Tricine SDS–PAGE was carried out on 16.5% (w/v) polyacrylamide gels on vertical slab electrophoresis cells (BioRad Mini PROTEAN 3 System, Hercules, CA, USA) for 4 h at 60 V. Molecular weight standard M3913 (Sigma Aldrich) was used. Either Coomassie brilliant blue R250 or silver staining was used to visualize the proteins.

Reversed phase HPLC analysis

Peptide analysis of β-casein hydrolysate obtained after 1 or 3 h of incubation with *E. faecalis* B1 strain was performed using reversed phase high-performance liquid chromatography (RP-HPLC) with a Waters HPLC apparatus (Alliance system, Waters, Milford, MA). Column (Symmetry C_{18}, 2.1 mm × 150 mm, 300 Å, 5 µ, Waters) was equilibrated with solvent A (H_2O, 0.055% trifluoroacetic acid (TFA), v/v) at a flow rate of 0.2 mL/min. Elution was performed by applying a linear gradient from 0 to 45% solvent B (80% acetonitrile, 20% H_2O, 0.09% TFA, v/v/v) in 15 min, followed by 1 min step at 45% solvent B, then by another linear gradient from 45 to 55% B in 22 min. The column was then rinsed with 100% solvent B for 2 min. Detection of peptides was performed between 220 and 330 nm using a diode array spectrophotometer (model 996, Waters).

Mass spectrometry analysis

Peptide analysis was performed using a Waters HPLC system (Waters 616 pump controlled by a Waters 600 controller) coupled to a Finnigan LCQ ion trap spectrophotometer (Finnigan MAT, San Jose, CA). Peptides were separated using the column, and the elution conditions were described earlier. Spectra were acquired in automated MS/MS mode. The scan rate of MS mode was set between the masses of 400 to 2,000 Da. Peptide identification was performed using the Mascot software (version 2.5, Matrix Science) on the MS/MS ion search mode with the following parameters: enzyme: none; peptide mass tolerance: 1 Da; fixed modification: phosphorylation. Identification was performed by comparing peptides with β-casein sequence. Mass spectrometry analyses were conducted in the platform "Biopolymers-Structural Biology" located at the INRA Center of Nantes (INRA Research Unit 1268) (http://www.angers-nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs).

Results and discussion

Isolation and characterization of the strains

A total of 107 LAB were isolated from traditional Bulgarian cheeses and katuk. The samples were collected from mountain region of Rhodope (south part of Bulgaria), Tracian valley and mountain region of Stara Planina (west part of Bulgaria). Lactobacilli, lactococci and streptococci represent together half of total isolated strains. Enterococci are dominant among LAB isolates. Both phenotypic and genetic methods were applied for the identification of enterococci strains to genus and species levels.

Preliminary analyses of patterns from API galleries showed that three main enterococcal species were presented. API 20 STREP galleries showed sufficient coefficient of similarity with *E. faecium* (99.5% within the assayed group of isolates), *Enterococcus durans* (99.3% within the group) and 99.6% similarity for both *E. faecalis* strains. However, for LAB species in general, accurate species identification and typing is important for selecting bacteria for the use either in food or as probiotics [24]. Within this research, all strains were identified by genus specific-PCR and sequencing of genes for 16S rRNA. Among a total of 58 enterococci isolates, 34 isolates were identified as *E. faecium*, 22 belong to *E. durans* and 2 belong to *E. faecalis* according to the results of sequencing of the 16S rRNA genes (data not shown). The repartition of the 58 enterococci isolates in the 4 katuk and 14 cheese samples studied is shown in Table 2. Their geographical origin is shown in Table 3. Two *E. faecalis* strains isolated during this study were obtained from cheeses made from buffalo milk, made in the Rhodope region. One of two cheeses made with ewe's milk did not contain any *enterococcus* isolate. Enterococci are the main NSLAB generally occurring in cheeses [11]. Both *E. faecium* and *E. faecalis* have been reported to be largely prevalent species in fully ripened cheeses and to lower degree *E. durans* [25–27]. *Enterococcus faecalis* has been shown to be dominant species in Roncal and Idiazabal cheeses [28]. Both *E. faecium* and *E. durans* constitute the dominant species in Batzos cheese [29]. Slightly different ratios in enterococcal microflora in cheese, where *Enterococcus devriesei*, *E. faecalis* and *Enterococcus malodoratus* dominate, have been reported [30]. Unlike other reported results on prevalence of *E. faecalis*, our results demonstrate that enterococcal microflora is dominant in typical Bulgarian cheeses with both *E. faecium* and *E. durans* as main species. Moreover, in Feta type cheese, which has more or less the same technology of manufacturing of typical Bulgarian cheeses, *E. durans* was reported to be the main enterococcal species [31, 32]. Phenotypic characterization of the strains was based on physiological and biochemical characteristics of the strains, cell morphology, gram staining and hemolysis. All strains were able to hydrolyze esculin, to grow in 6.5% NaCl and were cocci.

Table 2 Repartition of the 58 enterococci isolates in the 4 katuk and 14 cheese samples studied

	E. faecium	*E. durans*	*E. faecalis*
Katuk 1	2	2	0
Katuk 2	2	1	0
Katuk 3	2	2	0
Katuk 4	3	1	0
Cow milk			
Cheese 1	2	1	0
Cheese 2	2	1	0
Cheese 3	2	1	0
Cheese 4	0	3	0
Cheese 5	3	1	0
Cheese 6	3	0	0
Buffalo milk			
Cheese 7	0	1	1
Cheese 8	2	2	1
Cheese 9	3	2	0
Goat milk			
Cheese 10	2	1	0
Cheese 11	2	2	0
Cheese 12	1	1	0
Ewe milk			
Cheese 13	3	1	0
Cheese 14	0	0	0
Total	34	22	2

Table 3 Geographical localization of the 58 enterococci isolates

	Rhodope	Tracian valley	Stara Planina
E. faecium	16	11	7
E. durans	8	5	9
E. faecalis	2	0	0

RAPD-, ERIC– and REP-PCR diversity among *Enterococcus* strains

Many fingerprinting methods have been applied to the study of microbial biodiversity. In this context, RAPD-, ERIC– and REP-PCR have proved to be reliable tools for the identification and typing of LAB from foodstuffs [33–35]. The calculated distances in the dendrograms constructed on the basis of the similarity matrices obtained from three types of analyses are sufficiently

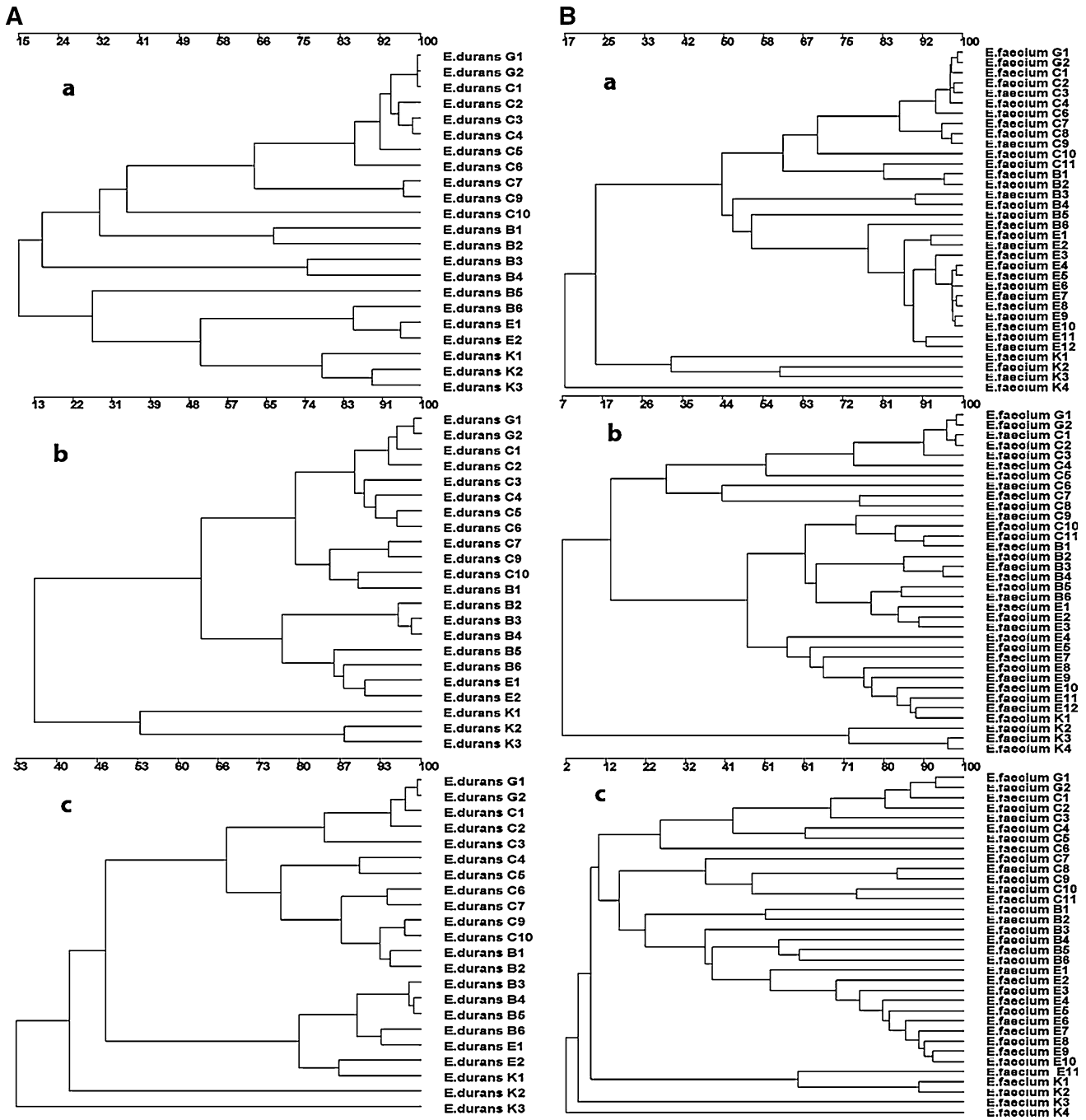

Fig. 1 **A** Dendrograms generated from ERIC– (*a*), RAPD- (*b*) and REP- (*c*) PCR profiles obtained from the *Enterococcus durans* isolates studied. Profiles were grouped using UPGMA. *G* goat milk strains, *C* cow milk strains, *B* buffalo milk strains, *E* ewe milk strains, *K* katuk. **B** Dendrograms generated from ERIC– (*a*), RAPD- (*b*) and REP- (*c*) PCR profiles obtained from the *Enterococcus faecium* isolates studied. Profiles were grouped using UPGMA. *G* goat milk strains, *C* cow milk strains, *B* buffalo milk strains, *E* ewe milk strains, *K* katuk

articulated among the most closely related isolates, which eliminates the prospect of multiple isolation of one strain.

Several trends appear from the results obtained by the phylogenetic diversity studies. First of all, similar dendrogram clustering in three types of analyses of isolates was obtained in both *E. faecium* and *E. durans*, corresponding and reflecting to some extent their origins. This coincidence suggests that the results obtained are enough reliable. This finding probably also corresponds to the fact that *E. faecium* and *E. durans* are very closely related species among the phylogeny of the genus.

Table 4 Extracellular enzymatic activity of the tested strains using API-ZYM galleries

Enzyme	*E. faecium* (34)		*E. durans* (22)		*E. faecalis* (2)	
	Mean[a]	Strain producing	Mean[a]	Strain producing	Mean[a]	Strain producing
Alkaline phosphatase	1.62	16	1.70	10	0	0
Esterase	3.59	34	3.63	22	4.0	2
Esterase and lipase	3.40	34	3.30	22	3.0	2
Lipase	1.00	8	1.00	5	0	0
Leucine arylamidase	4.21	34	4.15	22	4.0	2
Valine arylamidase	4.43	34	4.24	22	4.5	2
Cystine arylamidase	4.21	34	4.09	22	4.5	2
Acid phosphatase	4.67	34	4.60	22	5.0	2
Naphtol phosphohydrolase	2.79	24	2.95	21	2.0	2
α-Galactosidase	1.70	17	1.87	16	0	0
β-Galactosidase	4.51	34	2.66	22	5.0	2
β-Glucuronidase	1.1	10	1.00	11	0	0
α-Glucosidase	2.92	25	1.88	17	4.0	2
β-Glucosidase	2.04	22	1.41	17	1.5	2
N-acetyl-β-glucosaminidase	2.23	13	1.70	10	0.5	1
α-Chymotrypsin	1.20	4	1.42	3	4.0	2

[a] The results were graded from 0 to 5 by comparison of the observed color with the color reaction chart. A value of 0 corresponded to negative reaction and 5 to a reaction of maximal intensity. The approximate number of free nmol hydrolyzed substrate is obtained from the color strength: 0, no activity; 1, liberation of 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, 40 nmol

Interesting results were obtained by the evaluation of the relationship between isolates from different origins. For both species, isolates from cow and goat milk products were grouped in one cluster whatever the type of analyze used; another cluster contained mainly isolates of buffalo milk products. Similar clustering was observed also for the *E. faecium* ewe milk product isolates, in which also some buffalo milk isolates were present. However, the same statement cannot be formulated for *E. durans* ewe milk isolates because of their limited number. All "katuk" isolates express tendency to group in one small cluster, which diverges from other big clusters. From all milk product isolates, the most heterogeneous groups are those obtained from buffalo milk, again rich in *E. faecium* as well as in *E. durans*, representatives of what can be observed in all major clusters in the dendrograms generated by RAPD-, ERIC– and REP-PCR (Fig. 1A, B).

Antimicrobial activity

Antimicrobial activities of the culture supernatants of all strains were tested against the indicator strains, after treatment with proteinase K or heat treatment. A total of 33 strains showed antibacterial activity. Obtained results ranged from weak halo to some strong antibacterial activities mainly against *L. helveticus* and *E. faecalis* strains. Parts of the tested strains do not possess antibacterial activity. Many of the strains have lost their antibacterial activities after heat or proteinase K treatment, demonstrating the proteinaceous nature of the antimicrobial substance. However, two strains kept their antibacterial activity against *L. helveticus* and *E. faecalis* after heating. That could mean either that antibacterial substance is of proteinaceous nature, which is stable even after heat treatment, or that antibacterial substance with no proteinaceous nature is produced. These strains belong to *E. faecium* (data not shown).

Enzymatic activity

Table 4 describes extracellular enzymatic activity of the tested strains using API-ZYM galleries. All strains exhibited C-4 esterase and C-8 esterase lipase activity, while several strains of *E. faecium* and *E. durans* showed C-14 lipase activity. This confirms previous results on esterolytic activity of enterococci [11] and demonstrates their lower proteolytic capacity as compared with more efficient lipolytic system. Those C-4 and C-8 esterase lipase activities play a role in flavor compound formation in cheeses made from ewe and goat milk [36]. However, other important enzymatic activities, acid phosphatase and phosphohydrolase, which are of great importance for the hydrolysis of phosphopeptides during cheese ripening were observed on all tested strains. Most of the strains showed glucosidase activity, which is essential for their aptitude to utilize milk sugars during ripening. Very low level of

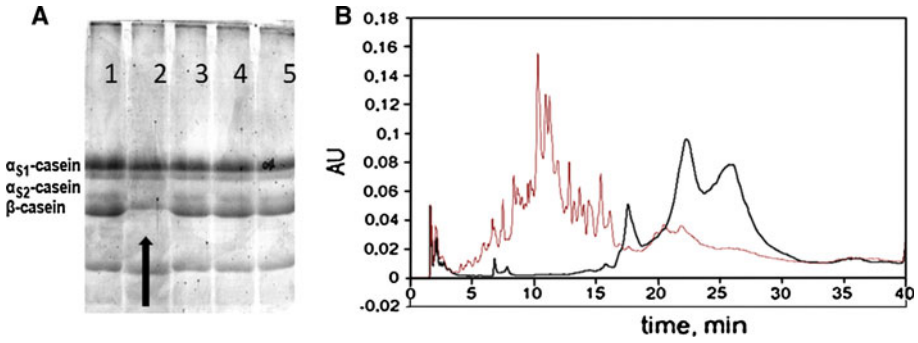

Fig. 2 **a** SDS–PAGE of UHT milk (*1*) incubated for 3 h at 37 °C with *Enterococcus faecalis* B1 (*2*), *Enterococcus durans* C2 (*3*), *Enterococcus durans* C7 (*4*) and *Enterococcus durans* K2 (*5*). **b** RP-HPLC profile was obtained after 3 h of incubation of UHT milk with *Enterococcus faecalis* B1

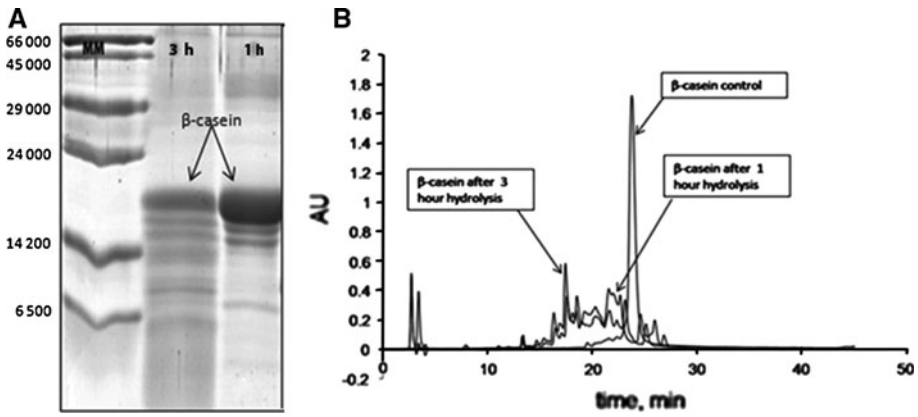

Fig. 3 Hydrolysis of *β*-casein by *Enterococcus faecalis* B1 strain after 1 and 3 h of incubation. **a** SDS–PAGE profile: 1 h means hydrolysis after 1 h of incubation; 3 h means hydrolysis after 3 h of incubation; *MM* molecular mass marker. **b** RP-HPLC of *β*-casein hydrolysate

α-chymotrypsin activity was shown from both *E. faecium* and *E. durans* strains but two *E. faecalis* strains showed higher levels of proteolytic activities.

Proteolytic activity

Milk coagulation and proteolytic activity

Determination of proteolytic activity on skim milk agar was used as a qualitative method for preliminary characterization of the strains 31 isolates assigned to *E. faecium*, 20 isolates assigned to *E. durans* and both *E. faecalis* isolates showed clear zones illustrating their proteolytic activities. On the basis of the area of the halo, the strains were classified as highly proteolytic and non-proteolytic. All tested strains were able to coagulate RSM and decreased pH to 4.9–5.2 after 24 h at 37 °C with no observed intra-species variability. Other authors have reported lower acidifying capacity of cheese originating enterococci [2, 37]. Only *E. faecalis* has been reported like a strong acidifier in skim milk among genus *Enterococcus* [35], whereas El-Ghaish et al. [15] reported efficient hydrolysis of milk proteins by 14 enterococcal strains.

The proteolytic activity varied from 0.094 to 0.455 mM/L Gly in the group of *E. faecium* and from 0.109 to 0.487 mM/L Gly in the group of *E. durans* strains, while both *E. faecalis* strains showed relatively high proteolytic activity (Table 4). The highest activity was detected for strains *E. faecium* B6 and *E. durans* C2. Regarding casein degradation, the tested strains showed low proteolytic activity, what remains in agreement with other results [7, 18].

Relatively high proteolytic activity in all homemade cheese samples correlates with main microflora represented by enterococci. Despite different origin of the fresh milk and areas of sampling, proteolysis was observed in all samples. Concerning other enzymatic activities, slight variations occurred, which could be explained by period frame of sampling, age of the products or milk type.

Hydrolysis of β-casein

As estimated by SDS–PAGE (Fig. 2a) after 3 h of incubation at 37 °C on milk, casein hydrolysis of *E. faecalis* B1 strain could be observed. In case of two strains of *E. durans* (*E. durans* C2 and *E. durans* C7), a weak hydrolysis was observed but only after 6 or 12 h of hydrolysis (data not shown). Lower proteolytic activity was detected for *E. faecium* strains (data not shown). These results confirm data previously reported, indicating that proteolytic activity of *E. faecalis* was higher than that of *E. faecium* [7, 18, 38]. No reduction in quantity of major whey proteins (*β*-lactoglobulin and α-lactalbumin) was observed.

Table 5 Identification by mass spectrometry of the peptides obtained after hydrolysis of β-casein by *Enterococcus faecalis* B1 strain

Experimental mass (Da)	Peptide sequence	Theoretical mass (Da)
558.0	(77–81) P.LTQTP.V	558.3
656.1	(82–87) P.VVVPPF.L	656.4
680.5	(52–57) P.FAQTQS.L	680.3
689.5	(127–132) T.LTDVEN.L	689.3
1,103.4	(155–164) T.VMFPPQSVLS.L	1,103.6
1,172.8	(177–186) K.AVPYPQRDMP.I	1,172.6
1,196.7	(77–87) P.LTQTPVVVPPF.L	1,196.7
1,209.9	(166–176) L.SQSKVLPVPQK.A	1,209.7
1,323.9	(165–176) S.LSQSKVLPVPQK.A	1,322.8
1,360.5	(127–138) T.LTDVENLHLPLP.L	1,359.7
1,363.6	(196–209) P.VLGPVRGPFPIIV	1,362.8
1,468.7	(143–154) S.WMHQPHQPLPPT.V	1,467.7
1,472.9	(127–139) T.LTDVENLHLPLPL.L	1,472.8
1,514.8	(88–101) F.LQPEVMGVSKVKEA.M	1,513.8
1,622.9	(162–176) S.VLSLSQSKVLPVPQK.A	1,622.0
1,687.9	(125–139) S.LTLTDVENLHLPLPL.L	1,687.0
1,725.7	(82–97) P.VVVPPFLQPEVMGVSK.V	1,725.0
1,796.4	(140–154) L.LQSWMHQPHQPLPPT.V	1,795.9
1,905.1	(1–16) RELEELNVPGEIVESL.S + 1 Phospho S	1,904.9
1,928.4	(190–206) A.FLLYQEPVLGPVRGPFP.I	1,928.1
1,951.8	(77–94) P.LTQTPVVVPPFLQPEVMG.V	1,951.0
2,072.0	(1–17) RELEELNVPGEIVESLS.S + 2 Phospho S	2,071.9
2,254.1	(190–209) A.FLLYQEPVLGPVRGPFPIIV	2,253.3
2,265.6	(77–97) P.LTQTPVVVPPFLQPEVMGVSK.V	2,265.2
2,408.8	(155–176) T.VMFPPQSVLSLSQSKVLPVPQK.A	2,408.4
2,434.3	(162–183) S.VLSLSQSKVLPVPQKAVPYPQR.D	2,433.4
2,565.8	(187–209) P.IQAFLLYQEPVLGPVRGPFPIIV	2,565.5
2,579.3	(116–138) P.VEPFTESQSLTLTDVENLHLPLP.L	2,578.3
2,705.1	(102–124) A.MAPKHKEMPFPKYPVEPFTESQS.L	2,704.3
2,937.3	(165–190) S.LSQSKVLPVPQKAVPYPQRDMPIQAF.L	2,936.6
2,994.8	(139–164) P.LLQSWMHQPHQPLPPTVMFPPQSVLS.L	2,994.5

Hydrolysis of pure β-casein was estimated for the strain *E. faecalis* B1 according to Kojic et al. [22]. SDS–PAGE profile (Fig. 3a) indicated hydrolysis of β-casein after 3 h of incubation.

RP-HPLC profile after 1 h of hydrolysis of pure β-casein by *E. faecalis* B1 strain showed that all β-casein disappeared (Fig. 3b). A lot of more hydrophilic peptides were observed on the chromatograms. After 3 h of hydrolysis, the obtained chromatogram was comparable with that obtained after 1 h of hydrolysis.

The samples obtained after 3 h of hydrolysis were further used for mass spectrometry analysis, and 31 peptides were identified (Table 5, Fig. 4). Due to the scan rate used (400–2,000 Da), recovery of sequence was incomplete. On the N-terminal side, except for the peptides 52–57, no peptide corresponding to the sequence between 18 and 76 was found. β-Casein was hydrolyzed preferentially after hydrophobic residues. Moreover, a lot of cleavage occurred after prolyl residues, which should be highlighted since Pro-X bond is generally difficult to cleave.

In conclusion, enterococci form the main part of the microbial populations of homemade white-brined cheeses and katuk in Bulgaria, confirming the importance of the presence of these microorganisms during cheese making and ripening. Differences in the microflora of different products occurred, being either due to the use of different rennets or to contamination risk during cheese processing at home or to milk type. A total of 58 enterococcal strains were isolated from traditional Bulgarian homemade cheeses and well characterized. Three species in the genus were identified: *E. faecium* and *E. durans* in comparable quantities and only two isolates belonging to *E. faecalis*.

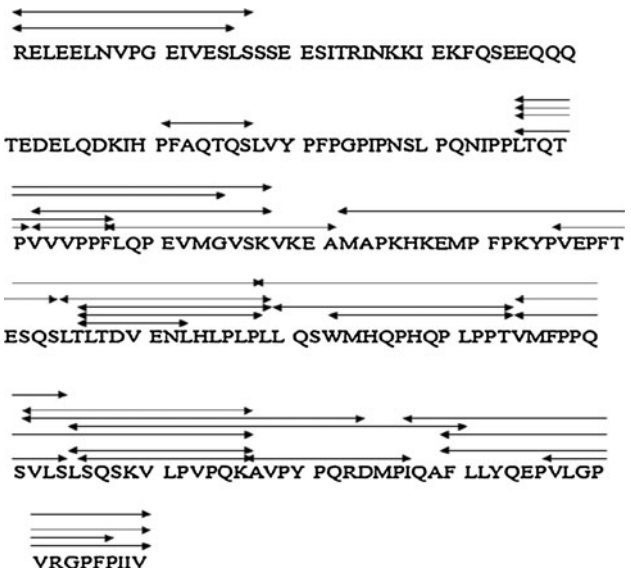

Fig. 4 Location in the primary structure of β-casein of 31 identified peptides (*arrows*) released by *Enterococcus faecalis* B1 strain proteinase

Other authors described the presence of *Enterococcus avium* in cheeses [28] as well as *Enterococcus hirae* and *Enterococcus gallinarium* [35].

Phenotypic and some physiological properties of the strains as well as their genetic diversity were well characterized. None of studied strains was doted of β-hemolytic activities.

Acknowledgments We would like to express our gratitude for the support of this work in the frame of project 982164 "Study of antimicrobial and hypoallergenic products of lactic acid bacteria" to NATO Science for Peace program and RILA project 4/1001.

References

1. Erdem YK (2005) Effect of ultrafiltration, fat reduction and salting on textural properties of white-brined cheese. J Food Eng 71:366–372
2. Fortina MG, Ricci G, Borgo F, Manachini PL, Arends K, Schiwon K, Abajy MY, Grohmann E (2008) A survey on biotechnological potential and safety of the novel *Enterococcus* species of dairy origin, *E. italicus*. Int J Food Microbiol 123:204–211
3. Ogier J-C, Serror P (2008) Safety assessment of dairy microorganisms: the *Enterococcus* genus. Int J Food Microbiol 126: 291–301
4. Ben Omar N, Castro A, Lucas R, Abriouel H, Yousif NMK, Franz CMAP, Holzapfel WH, Pérez-Pulido R, Martínez-Cañamero M, Gálvez A (2004) Functional and safety aspects of enterococci isolated from different spanish foods. Syst Appl Microbiol 27:118–130
5. Eaton TJ, Gasson MJ (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microb 67: 1628–1635
6. Dogru AK, Gençay YE, Ayaz ND (2010) Comparison of virulence gene profiles of *Enterococcus faecium* and *Enterococcus faecalis* chicken neck skin and faeces isolates. Kafkas Universitesi Veteriner Fakultesi Dergisi 16:129–133
7. Andrighetto C, Knijff E, Lombardi A, Torriani S, Vancanneyt M, Kersters K, Swings J, Dellaglio F (2001) Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. J Dairy Res 68:303–316
8. Lopes MFS, Pereira CI, Rodrigues FMS, Martins MP, Mimoso MC, Barros TC, Figueiredo Marques JJ, Tenreiro RP, Almeida JS, Barreto Crespo MT (1999) Registered designation of origin areas of fermented food products defined by microbial phenotypes and artificial neural networks. Appl Environ Microbiol 65:4484–4489
9. Mannu L, Paba A, Daga E, Comunian R, Zanetti S, Duprè I, Sechi LA (2003) Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. Int J Food Microbiol 88:291–304
10. García MC, Rodríguez MJ, Bernardo A, Tornadijo ME, Carballo J (2002) Study of enterococci and micrococci isolated throughout manufacture and ripening of San Simón cheese. Food Microbiol 19:23–33
11. Giraffa G (2003) Functionality of enterococci in dairy products. Int J Food Microbiol 88:215–222
12. Devriese LA, Pot B, Collins MD (1993) Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J Appl Bacteriol 75:399–408
13. Ulrich A, Müller T (1998) Heterogeneity of plant-associated streptococci as characterized by phenotypic features and restriction analysis of PCR-amplified 16S rDNA. J Appl Microbiol 84:293–303
14. Sarantinopoulos P, Leroy F, Leontopoulou E, Georgalaki M, Kalantzopoulos G, Tsakalidou E, de Vuyst L (2002) Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. Int J Food Microbiol 72:125–136
15. El-Ghaish S, Dalgalarrondo M, Choiset Y, Sitohy M, Ivanova I, Haertlé T, Chobert J-M (2010) Screening of strains of lactococci isolated from Egyptian dairy products for their proteolytic activity. Food Chem 120:758–764
16. El-Ghaish S, Hadji-Sfaxi I, Ahmadova A, Choiset Y, Rabesona H, Sitohy M, Haertlé T, Chobert J-M (2011) Characterization of two safe *Enterococcus* strains producing enterocins isolated from Egyptian dairy products. Benef Microbes 2:15–27
17. López-Díaz TM, Alonso C, Román C, García-López M, Moreno B (2000) Lactic acid bacteria isolated from a hand-made blue cheese. Food Microbiol 17:23–32
18. Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea MC, Lombardi A, Cogan TM, Kalantzopoulos G, Tsakalidou E (2001) Biochemical properties of enterococci relevant to their technological performance. Int Dairy J 11:621–647
19. Ke D, Picard FJ, Martineau F, Menard C, Roy PH, Ouellette M, Bergeron MG (1999) Development of a PCR assay for rapid detection of enterococci. J Clin Microbiol 37:3497–3503
20. Batdorj B, Trinetta V, Dalgalarrondo M, Prévost H, Dousset X, Ivanova I, Haertlé T, Chobert J-M (2007) Isolation, taxonomic identification and hydrogen peroxide production by *Lactobacillus delbrueckii* subsp. *lactis* T31, isolated from Mongolian yoghurt: inhibitory activity on food-borne pathogens. J Appl Microbiol 103:584–593
21. Church FC, Swaisgood HE, Porter DH, Catignani GL (1983) Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci 66:1219–1227
22. Kojic M, Fira D, Banina A, Topisirovic L (1991) Characterization of the cell wall-bound proteinase of *Lactobacillus casei* HN14. Appl Environ Microbiol 57:1753–1757

23. Laemmli UK (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227:680–685
24. Martin B, Garriga M, Hugas M, Aymerich T (2005) Genetic diversity and safety aspects of enterococci from slightly fermented sausages. J Appl Microbiol 98:1177–1190
25. Teuber M, Perreten V, Wirsching F (1996) Antibiotikumresistente bakterien: Eine neue dimension in der lebensmittelmikrobiologie. Lebensm Wiss Technol 29:182–199
26. Franz CMAP, Holzapfel WH, Stiles ME (1999) Enterococci at the crossroads of food safety? Int J Food Microbiol 29:255–270
27. Valenzuela AS, Ben Omar N, Abriouel H, López RL, Veljovic K, Martinez Cañamero M, Kojic M, Topisirovic L, Gálvez A (2009) Virulence factors, antibiotic resistance, and bacteriocins in enterococci from artisan foods of animal origin. Food Control 20: 381–385
28. Arizcun C, Barcina Y, Torre P (1997) Identification and characterization of proteolytic activity of *Enterococcus* spp isolated from milk and Roncal and Idiazábal cheese. Int J Food Microbiol 38:17–24
29. Psoni L, Kotzamanides C, Andrighetto A, Lombardi A, Tzanetakis N, Litopoulou-Tzanetaki E (2006) Genotypic and phenotypic heterogeneity in *Enterococcus* isolates from Batzos, a raw goat milk cheese. Int J Food Microbiol 109:109–120
30. Martín-Platero AM, Valdivia E, Maqueda M, Martínez-Bueno M (2009) Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. Int J Food Microbiol 132:24–32
31. Litopoulou-Tzanetaki E, Tzanetakis N, Vafopoulou-Mastrojiannaki A (1993) Effect of the type of lactic starter on microbiological, chemical and sensory characteristics of Feta cheese. Food Microbiol 10:31–41
32. Tzanetakis N, Vafopoulou-Mastrojiannaki A, Litopoulou-Tzanetaki E (1995) The quality of white-brined cheese from goat's milk made with different starters. Food Microbiol 12:55–63
33. Barbier N, Saulnier P, Chachaty E, Dumontier S, Andremont A (1996) Random amplified polymorphic DNA typing versus pulsed-field gel electrophoresis for epidemiological typing of vancomycin-resistant enterococci. J Clin Microbiol 34:1096–1099
34. Monstein HJ, Quednau M, Samuelsson A, Ahrné S, Isaksson B, Jonasson J (1998) Division of the genus *Enterococcus* into species groups using PCR-based molecular typing methods. Microbiology 144:1171–1179
35. Suzzi G, Caruso M, Gardini F, Lombardi A, Vannini L, Guerzoni ME, Andrighetto C, Lanorte MT (2000) A survey of the enterococci isolated from an artisanal Italian goat's cheese (semicotto caprino). J Appl Microbiol 89:267–274
36. Mc Sweeney PLH, Sousa MJ (2000) Biochemical pathways for the production of flavour compounds in cheeses during ripening: a review. Lait 80:293–324
37. Morea M, Baruzzi F, Cocconcelli PS (1999) Molecular and physiological characterization of dominant bacterial populations in traditional Mozzarella cheese processing. J Appl Microbiol 87: 574–582
38. Morandi S, Brasca M, Andrighetto C, Lombardi A, Lodi R (2006) Technological and molecular characterisation of enterococci isolated from north–west Italian dairy products. Int Dairy J 16: 867–875
39. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York