ISSN 1330-9862 *original scientific paper*
doi: 10.17113/ftb.55.03.17.5118

Characterization of Enterococcal Community Isolated from an Artisan Istrian Raw Milk Cheese: Biotechnological and Safety Aspects

Mirna Mrkonjic Fuka[1]*, *Ana Zgomba Maksimovic*[1], *Irina Tanuwidjaja*[1], *Natasa Hulak*[1] *and Michael Schloter*[2]

[1]Department of Microbiology, Faculty of Agriculture, University of Zagreb, Svetošimunska 25, HR-10000 Zagreb, Croatia

[2]Research Unit for Environmental Genomics, Helmholtz Zentrum München, Ingolstaedter Landstrasse 1, DE-85758 Neuherberg, Germany

Received: December 19, 2016
Accepted: June 2, 2017

Summary

In this study, prevalence, biotechnological and safety profiles of 588 *Enterococcus* isolates isolated from raw milk and Istrian cheese during different stages of ripening were analyzed. Despite the low and variable presence of enterococci in milk ((3.65±2.93) log CFU/mL), highly comparable enterococcal populations were established after 30 days of cheese ripening ((7.96±0.80) log CFU/g), confirming *Enterococcus* spp. as a major part of the core microbiota of Istrian cheese. The dominant species were *E. faecium* (53.8 %) and *E. faecalis* (42.4 %), while minor groups, consisting of *E. durans* (2.84 %) and *E. casseliflavus* (0.95 %), also occurred. A pronounced intraspecies variability was noticed based on molecular fingerprinting, with 35 strains (genotypes) detected. Most of the genotypes were farm-specific with one third being shared between the farms. This genotype variability reflected particular differences of Istrian cheese production, mainly variable salt concentration, ripening temperature and air humidity as well as microclimatic or vegetation conditions. There was considerable variation between the strains of the same species regarding wide range of biotechnologically important traits as well as their ability to survive in simulated gastrointestinal conditions. A considerable number of strains were resistant to critically important antibiotics such as tetracycline (43.56 %), erythromycin (35.79 %) and vancomycin (23.48 %). Polymerase chain reaction-based detection did not identify any of the common genetic determinants for vancomycin and erythromycin resistance; for tetracycline *tetM* gene was detected. The presence of virulence genes including *agg*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *esp*, *efaAfm*, *cob* and *cpd* was frequently recorded, especially among *E. faecalis* strains.

Key words: cheese, spontaneous fermentation, *Enterococcus* spp., molecular fingerprinting, antibiotic resistance, virulence genes

Introduction

Enterococci are one of the most common groups of bacteria in diverse ecosystems, mainly due to their high adaptability and resistance to adverse environmental conditions. They have been frequently isolated from natural sources such as soil, water, plants and gastrointestinal

*Corresponding author: Phone: +385 (0)1 239 4034; Fax: +385 1 239 3881; E-mail: mfuka@agr.hr

ORCID IDs: 0000-0002-8494-8805 (Mrkonjic Fuka), 0000-0003-2070-8562 (Zgomba Maksimovic), 0000-0003-1364-2953 (Tanuwidjaja), 0000-0001-8440-128X (Hulak), 0000-0003-1671-1125 (Schloter)

(GI) tract of mammals (*1*). Also, they are an integral part of many spontaneously fermented, traditional cheese varieties in which they contribute to the fermentation and ripening process (*2,3*). Thanks to their proteolytic activity, hydrolysis of milk fat by esterase and production of typical flavour components such as acetaldehyde, acetoin and diacetyl (*2,4*), they are responsible for the development of particular sensory characteristics of many cheese varieties. As indigenous members of the GI tract, enterococci can have beneficial effect on human health by balancing gut microbiota; therefore, they may also act as probiotic bacteria (*1*).

Enterococcus spp. are nowadays recognized for their beneficial effects on the development of cheese flavour and are considered as an integral part of the natural microbiota of many traditional types of cheese; however, some strains may present an emerging threat due to the enterococcal virulence determinants that are able to cause infections in humans (*3,5*). Over the last three decades, epidemiological data have noted the emergence of particular ecotypes of enterococci as leading nosocomial pathogens and they have arisen to become a major cause of human diseases, especially in immunocompromised patients (*6*). Their plasmid and transposon-mediated resistance to tetracycline, erythromycin, chloramphenicol and glycopeptides is considered particularly problematic, since it leads to the increased number of infections related to the acquired antimicrobial resistance of enterococci (*6*). Their capacity to exchange genetic material by conjugation is well documented (*7*) and it can take place in GI tract, where genes coding for resistance to antibiotics and virulence factors can be transferred (*5*). As enterococci are usually present at fairly high levels in ready-to-eat cheese products (*8,9*), they may have significant influence on consumers' health and as such, their safety aspect should be carefully evaluated.

Istrian cheese is a hard type of artisan raw milk cheese that is manufactured at home, on small scale, on the whole Istrian peninsula, without the addition of starter cultures (*10*). It is characterized by ripening of 90–120 days. However, because of its popularity in the region and due to the commercial pressure, the ripening time has been shortened and it has become market practice to sell the cheese as soon as possible. During the ripening of Istrian cheese, the number of enterococci reached up to 10^8 per g when assessed by real-time PCR (*11*). They are also found to be one of the dominant bacterial populations during ripening when denaturing gradient gel electrophoresis (DGGE) or next-generation sequencing approach were applied (*11,12*). Although total microbial diversity of Istrian cheese is analysed in details by culture-independent methods, such as molecular, sequencing-based assays did not allow distinguishing among *Enterococcus* species (*E. faecalis, E. faecium* and/or *E. durans*) or providing information on their intraspecies variability. Due to the bivalent effect of enterococci on food and human health, there is a need for their detailed characterization, not only at the species but also at the strain level. For example, strains of the species *E. faecalis* and *E. faecium* have been recognized as the most dominant species in fermented cheese (*4*), but also strains of the same species have been most frequently associated with the human and animal infections (*13*).

The purpose of this work is to identify and characterize members of the enterococcal community isolated during the ripening of Istrian cheese and to assess their role in cheese ripening as well as to select novel strains with interesting features that could be used as starter or adjunct cultures for fermented foods. Special focus is set towards the surveillance rate of indigenous cheese enterococci in simulated gastrointestinal conditions as well as detection of their antibiotic resistance and virulence traits.

Materials and Methods

Raw milk and cheese sampling

Six batches of Istrian cheese produced by different manufacturers (F1–F6) across the Istrian peninsula (Croatia) were analysed in the frame of this study. All manufacturers are part of a consortium for the preservation of the certified product Istrian cheese and are located in different areas of Istria with different microclimatic conditions, relief and vegetation. At each farm, two independent cheese samples were produced from the same batch of raw, full-cream ewe's milk in accordance with the traditional cheese making procedure (*10*). In brief, milk is heated up to 35 °C prior to the addition of natural rennet. The milk coagulum is left for maximum 1 h at room temperature until the curd formed, which is cut into pieces and cooked at 42 °C for 5 min before moulding. After 2 h of whey drainage with self-pressing, the surfaces of cheese are covered in coarse salt (various mass fractions) and left for 2 days at room temperature. The cheese is placed in a ripening chamber at controlled but variable temperature and air humidity (up to 19 °C and up to 90 %, respectively) for 90–120 days.

The cheese samples were taken with sterile cork borers (1 cm in diameter) from two individual cheese samples of each manufacturer after 30 (Ch30), 60 (Ch60), 90 (Ch90) and 120 (Ch120) days. In addition, milk (M) and cut curd (Cr) samples were taken at day zero. All samples were stored at 4 °C and analyzed within 24 h after sampling. The pH of the ripened cheese samples was 4.7 to 5.4 and their fat and salt contents ranged between 40 and 52 %, and 3.4 and 4.7 %, respectively.

Isolation of enterococci

The milk and cheese samples were subjected to microbiological analysis for enumeration and isolation of *Enterococcus* species. The cheese samples were homogenized with a Stomacher® (BagMixer 400; Interscience; St Nom, France) for 3 min and diluted tenfold in a sterile physiological solution (0.9 % NaCl). Aliquots of 100 μL of the three highest dilutions were inoculated on two selective kanamycin aesculin azide (KAA) agar plates (Merck, Darmstadt, Germany) and incubated under aerobic conditions at 37 °C for 48 h. Ten to fifteen colonies were randomly selected from the highest dilution of each sample and purified twice on KAA agar. Overall 588 isolates were collected and further analyzed. The pure cultures were characterized using the Gram stain method and catalase test. After microscopic examination, Gram-positive and catalase-negative coccoid isolates were grown overnight in 4 mL of brain-heart infusion (BHI) medium (Merck)

supplemented with 20 μg/mL of kanamycin. One aliquot of overnight cultures was used for DNA extractions and another one was stored at –80 °C in BHI (Merck) broth supplemented with glycerol (80:20 by volume).

DNA extraction and identification at the genus and species level

Genomic DNA from isolates was extracted from overnight BHI cultures using NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The quantity of DNA extracts was checked with a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

To determine the affiliation of isolates to genus level, PCR assay was carried out with *Enterococcus* genus-specific primers as described by Deasy *et al.* (*14*). A subsequent multiplex PCR reaction with *E. faecalis* and *E. faecium* species-specific primers was carried out in accordance with the PCR protocol of Dutka–Malen *et al.* (*15*). The primers are listed in Table 1 (*5,14–23*). Isolates that were not assigned to *Enterococcus* genus were not characterized further. Enterococci that were not affiliated to *E. faecalis* or *E. faecium* species were additionally amplified and sequenced using the universal bacterial primer sets 27f and 1401R designed by Lane (*16*) and Nübel *at al.* (*17*). The PCR mixture and amplification program were as previously described by Schreiner *et al.* (*24*). The PCR products were separated on 1.5 % agarose gels (100 V, 1 h) and purified by NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). PCR products were sequenced on an ABI PRISM® 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems). Reaction conditions and thermal cycle program were as previously described by Mrkonjić Fuka *et al.* (*25*). Unincorporated dye terminators were removed by ethanol precipitation.

Fingerprinting of Enterococcus spp.

For a strain-specific characterization, PCR assay based on randomly amplified polymorphic DNA (RAPD) was used. Amplification reactions were performed in a 25-μL reaction volume containing 50 ng of template DNA, 1× PCR buffer, 0.1 mM dNTP, 1.5 mM $MgCl_2$, 2 μM primer and 1.25 U of Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The used primers (M13, D8635 and P2) are listed in Table 1. Thermal cycler was programmed to: initial denaturation at 94 °C for 30 s, 94 °C for 5 min for complete denaturation, 37 °C for 5 min for low stringency annealing and 72 °C for 5 min for extension for 2 cycles. This was followed by 40 cycles of 1 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C. Final extension was programmed at 72 °C for 10 min. Amplified PCR products were separated by electrophoresis on 2.5 % (by mass per volume) agarose gels. Clustering was carried out in Bionumerics v. 7.6 software platform (*26*) using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm (*27*) and band-based Dice correlation coefficient for creating the similarity matrix. Based on their RAPD-PCR pattern, representative strains were selected and tested for further phenotypic, technological and safety properties. Monophyletic groups were omitted from further analysis.

Acidifying activity and tolerance to different environmental conditions

Overall 35 representative strains were tested for their acidifying activity. The differences in the pH were measured after 2, 6 and 24 h of incubation at 37 °C using a pH-meter. Combined pH electrodes (InPro® 3030; Metter Toledo, Greifensee, Switzerland) were standardized using two buffers (pH=4.0 and 7.0) and disinfected after each run using a 3 % HCl solution. Strains were inoculated in duplicates at a level of 2 % in reconstituted sterile nonfat dry milk (10 % by mass per volume; Fluka, Sigma-Aldrich, St. Louis, MO, USA). The acidification rate (as ΔpH) was calculated according to Jamalay *et al.* (*28*):

$$\Delta pH = pH_{initial} - pH_{final} \qquad /1/$$

The ability of representative strains to grow at 10 and 45 °C, in 4 and 6.5 % NaCl and at pH=4 and 9.6 was tested in duplicates in BHI broth and recorded after 72 h of growth at 37 °C.

Lipolysis, haemolysis, caseinolytic and proteolytic activity

For screening of lipolytic and caseinolytic activity, the representative strains were cultured overnight at 37 °C in BHI broth. Overnight cultures were subcultured in fresh BHI broth and grown until an absorbance (*A*) of 0.3 was achieved. A volume of 10 μL of cultures was transferred on sterile cellulose discs (Biorad, Berkeley, CA, USA) previously placed on Tributyrin Agar (Oxoid LTD, Basingstoke, UK) to screen for lipolytic activity, or on BHI agar containing 1.5 % of skimmed milk for caseinolytic activity. Plates were incubated for 3 days at 30 °C for lipolytic or at 37 °C for caseinolytic activity and observed daily for halo formation around the discs. The radius of the halo formation (in mm) at the end of incubation was used as measure of the lipolytic or caseinolytic activity. The sizes of clear zones (including the diameter of disk) on the agar surface around the discs were measured in mm and expressed as – (no halo observed or less than 10 mm) or + (≥10 mm). The haemolysis was tested by streaking overnight cultures on BHI agar plates containing 5 % cow's blood. The plates were incubated for 72 h at 37 °C. Zones of clearing around colonies indicated haemolysin production. Proteolytic activity was evaluated by the addition of chromogenic peptide N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (S-Ala, Sigma-Aldrich). Release of *p*-nitroanilide (*p*NA) by the action of enterococcal proteinase is measured at 410 nm as described by Savoy de Giori and Hébert (*29*). Results were expressed in μM of *p*NA.

Survival in simulated gut conditions and autoaggregation assay

To estimate the transit tolerance through a simulated *in vitro* digestion, all representative enterococcal strains were tested as described by Doleyres *et al.* (*30*) and Nueno-Palop and Narbad (*31*) with slight modifications. To simulate the possible hydrolysis of bacteria in the oral cavity, enterococcal strains were grown overnight in skimmed milk (10 %, by mass per volume) and diluted 1:10 in phosphate-buffered saline (PBS), after which lys-

Table 1. List of primers used in this study

Primer	Composition (5′–3′)	PCR product/bp	Reference	Target gene/group
Ddl1	ATCAAGTACAGTTAGTCT	941		*ddl/E. faecalis*
Ddl2	ACGATTCAAAGCTAACTG			
Ddl3	TAGAGACATTGAATATGCC	550	(*15*)	*ddl/E. faecium*
Ddl4	TCGAATGTGCTACAATC			
E1	TCAACCGGGGAGGGT	733	(*14*)	16S rRNA/*Enterococcus*
E2	ATTACTAGCGATTCCGG			
27F	AGAGTTTGATCMTGGCTCAG	1374	(*16*)	16S rRNA/bacteria
1401R	CGGTGTGTACAAGACCC		(*17*)	
M13	GAGGGTGGCGGTTCT	varying	(*18*)	willing
P2	GATCGGACGG	varying	(*19*)	willing
D8635	GAGCGGCCAAAGGGAGCAGAC	varying	(*20*)	willing
tetK	TCGATAGGAACAGCAGTA	169	(*21*)	*tetK*
	CAGCAGATCCTACTCCTT			
tetL	TCGTTAGCGTGCTGTCATTC	267	(*22*)	*tetL*
	GTATCCCACCAATGTAGCCG			
tetM	GTGGACAAAGGTACAACGAG	406	(*21*)	*tetM*
	CGGTAAAGTTCGTCACACAC			
tetO	AACTTAGGCATTCTGGCTCAC	515	(*22*)	*tetO*
	TCCCACTGTTCCATATCGTCA			
tetS	CATAGACAAGCCGTTGACC	667		*tetS*
	ATGTTTTTGGAACGCCAGAG			
ermA	TCTAAAAAGCATGTAAAAGAA	645	(*23*)	*erm*
	CTTCGATAGTTTATTAATATTAGT			
TE 3	AAGAAAAAGTAGACCAAC	1553	(*5*)	*agg*
TE 4	AACGGCAAGACAAGTAAATA			
TE 5	GACAGACCCTCACGAATA	705		*efaAfs*
TE 6	AGTTCATCATGCTGCTGTAGTA			
TE 9	ACCCCGTATCATTGGTTT	419		*gelE*
TE 10	ACGCATTGCTTTTCCATC			
TE 13	CTGATGGAAAGAAGATAGTAT	742		*cylM*
TE 14	TGAGTTGGTCTGATTACATTT			
TE 15	ATTCCTACCTATGTTCTGTTA	843		*cylB*
TE 16	AATAAACTCTTCTTTTCCAAC			
TE 17	TGGATGATAGTGATAGGAAGT	517		*cylA*
TE 18	TCTACAGTAAATCTTTCGTCA			
TE 34	TTGCTAATGCTAGTCCACGACC	933		*esp*
TE 36	GCGTCAACACTTGCATTGCCGAA			
TE 37	AACAGATCCGCATGAATA	735		*efaAfm*
TE 38	CATTTCATCATCTGATAGTA			
TE 49	AACATTCAGCAAACAAAGC	1405		*cob*
TE 50	TTGTCATAAAGAGTGGTCAT			
TE 51	TGGTGGGTTATTTTTCAATTC	782		*cpd*
TE 52	TACGGCTCTGGCTTACTA			

ozyme (Sigma-Aldrich) was added to a final concentration of 100 mg/L. Samples were then incubated for 5 min at 37 °C. In order to estimate the cell survival rate in simulated gastric and intestinal conditions, overnight enterococcal cultures (1 mL) were harvested by centrifugation (Eppendorf 5415R; Eppendorf, Hamburg, Germany) at 10 000×*g* for 10 min at room temperature. The pellets were washed twice with 0.1 % peptone water, resuspended in 100 μL of 0.1 % peptone and stored on ice until use. To simulate the gastric digestion, 0.5 % NaCl and 0.3 % pepsin solution (Sigma-Aldrich) were used. The samples were adjusted to pH=2.5 (with 1 mol/L of HCl). The solu-

tion containing 270 μL of simulated gastric juices and 30 μL of cell suspension was mixed and incubated at 37 °C for 30 min. For cell survival in simulated intestinal conditions, 270 μL of 0.4 % bile salts and 0.2 % pancreatin solution (Sigma-Aldrich) were mixed with 30 μL of cell suspension and incubated for 30 min at 37 °C. Samples were removed for determination of cell counts before and after oral, gastric and intestinal digestion. Aliquots were serially diluted in PBS buffer (pH=7.2) and viable counts were determined after incubation at 37 °C for 48 h. Each strain was tested twice on BHI agar.

Autoaggregation assay was performed according to Fortina *et al.* (*32*) and Del Re *et al.* (*33*) with slight modifications. Aggregation was evaluated using 4 mL of cell suspension corresponding to viable counts of approx. 10^8 CFU/mL. The cell suspension was vortexed for 10 s. The absorbance (A) was measured at 610 nm after 3 and 5 h of incubation at room temperature. The aggregation percentage was expressed as:

$$\text{Aggregation}=1-(A_t/A_0)\cdot 100 \qquad /2/$$

where A_t represents the absorbance measured at 610 nm at time t=3 and 5 h, and A_0 the absorbance at t=0.

Phenotypic characterization of antibiotic susceptibility

Antimicrobial susceptibility was determined by standardized agar diffusion test using BBL™ Sensi-Disc™ antimicrobial susceptibility test discs (Becton, Dickinson and Company, Le Pont de Claix, France). The discs impregnated with specific concentrations of antimicrobial agents were placed onto Mueller-Hinton (MH, Merck) agar plates seeded with representative strains at the concentration of 1 McFarland standard. The plates were incubated for 18 h at 35 °C under aerobic conditions. All representative isolates were screened for their susceptibility to penicillin G (10 U), ampicillin (10 μg), tetracycline (5 and 30 μg), vancomycin (5 μg), streptomycin (10 μg), erythromycin (5 and 15 μg), chloramphenicol (10 μg), rifampicin (5 μg), clindamycin (2 and 10 μg) and ampicillin/sulbactam (20 μg) (Becton, Dickinson and Company). After incubation, each strain was classified as sensitive, intermediate or resistant following the recommendations of the National Committee for Clinical Laboratory Standards (*34*).

Detection of antibiotic resistance genes

Detection of the genes responsible for resistance to tetracycline (*tetK, tetL, tetM, tetO* and *tetS*), vancomycin (*vanA* and *vanB*) and erythromycin (*ermA* and *ermB*) was investigated for all strains exhibiting the respective phenotypic resistance pattern by specific PCR assays. Primers used are listed in Table 1. The DNA amplification was performed according to Ng *et al.* (*22*) and Sutcliffe *et al.* (*23*) in 25 μL of reaction mixture containing 100 ng of DNA template. The amplified PCR products were visualized on 1.5 % agarose gel after staining with ethidium bromide.

PCR detection of genes coding for virulence factors

The indices of genes encoding for following virulence factors were investigated: aggregation substance (*agg*), gelatinase (*gelE*), cytolysin (*cylM, cylB*), cytolysin activator (*cylA*), enterococcal surface protein (*esp*), cell wall adhesin (*efaAfs* from *E. faecalis* and *efaAfm* from *E. faecium*) and sex pheromones (*cpd* and *cob*). PCR amplification was performed with primers and conditions described by Eaton and Gasson (*5*), except for *cob* and *agg* genes, whose 29 cycles of elongation were prolonged to 45 s as described by Čanžek Majhenić *et al.* (*35*). The primers are listed in Table 1.

Statistical analysis

Statistical analysis was performed using the R software programming language and R software environment for statistical computing (*36*). The level of significance was $p<0.05$. Student's t-test was used to determine whether there were significant differences in results when *E. faecalis* and *E. faecium* were compared. *Post hoc* Tukey's honestly significant difference (HSD) test was used to ascertain whether there were significant differences between the results of samples from different stages of cheese manufacturing and ripening.

Results and Discussion

Enumeration and identification of enterococci

Despite the available data in other countries, studies on prevalence and safety issue of enterococci in spontaneously fermented cheese in Croatia are limited. In order to improve the knowledge on the role of enterococci in the cheese ripening process and their possible influence on public health as well as to select for potential adjunct/starter cultures, total viable count and isolation of enterococci from 6 different farms during the ripening of Istrian cheese were carried out. The lowest count of enterococci was noticed, as expected, in milk ((3.65±2.93) log CFU/mL) and after 30 days of ripening it reached the maximum value ((7.96±0.80) log CFU/g). Afterwards, the number was 10-fold lower and remained stable until 90 days of ripening. At the end of ripening (120 days) the average number of enterococci was (6.21±0.98) log CFU/g (Fig. 1).

As nowadays Istrian cheese is rarely ripened for 120 days and it is mostly sold and consumed during the first two months, the average load of enterococci within that ripening period is almost 10^8 CFU/g, exceeding the limit of maximum 10^6 CFU/g recommended by International Commission on Microbiological Specifications for Foods

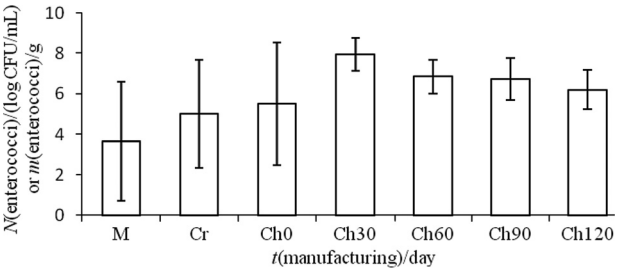

Fig. 1. Number of enterococci during manufacturing and ripening of Croatian artisan raw milk cheese. Results are expressed as mean value±standard deviation. Milk (M) and curd (Cr) samples were taken at t=0, and cheese samples were taken at t=30 (Ch 30), 60 (Ch 60), 90 (Ch 90) and 120 (Ch 120) days

(*37*). The number of enterococci higher than 10^6 CFU/g and even up to 10^8 CFU/g has often been reported in different raw milk products (*9*,*38*) and in some cases irrespective of raw milk or pasteurized/thermally treated milk cheese (*8*).

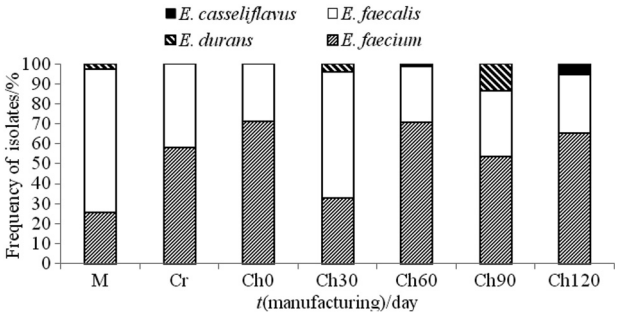

Fig. 2. Frequency of *Enterococcus* species isolated during manufacturing and ripening of Croatian raw milk cheese obtained from six different farms (F1–F6) based on PCR identification at the species level (*E. faecalis* and *E. faecium*) or 16S rRNA gene sequencing (*E. casseliflavus* and *E. durans*). Milk (M) and curd (Cr) samples were taken at *t*=0, and cheese samples were taken at *t*=30 (Ch30), 60 (Ch60), 90 (Ch90) and 120 (Ch120) days

As particular species in the same microbial group, or even the particular strain of a given species specifically affect the organoleptic profile of the final product and may have unique technological or safety traits, a total of 588 presumptive enterococci isolated from KAA agar in this study were identified by PCR-based approach to the genus, species and strain level. In PCR reaction with genus-specific primers, 92.19 % of isolates were confirmed as *Enterococcus* members (data not shown). PCR identification at the species level identified 53.8 % of *Enterococcus* isolates as *E. faecium* and 42.4 % as *E. faecalis* (Fig. 2). They were found at the high level in all samples and their presence remained stable during the fermentation and ripening. The predominance of *E. faecium* and *E. faecalis* over other enterococci is in agreement with other studies on artisan cheese, where high abundance of these two species has been reported (*1*). *E. durans* and *E. casseliflavus* were identified based on the sequencing of 16S rRNA gene and were detected in much lower percentage (2.84 and 0.95 % respectively).

The genetic diversity of the identified species was analyzed by RAPD-PCR-based fingerprinting (Fig. 3). The RAPD-PCR allowed detailed characterization of isolates at the strain (ecotype) level. Thus, we used this approach

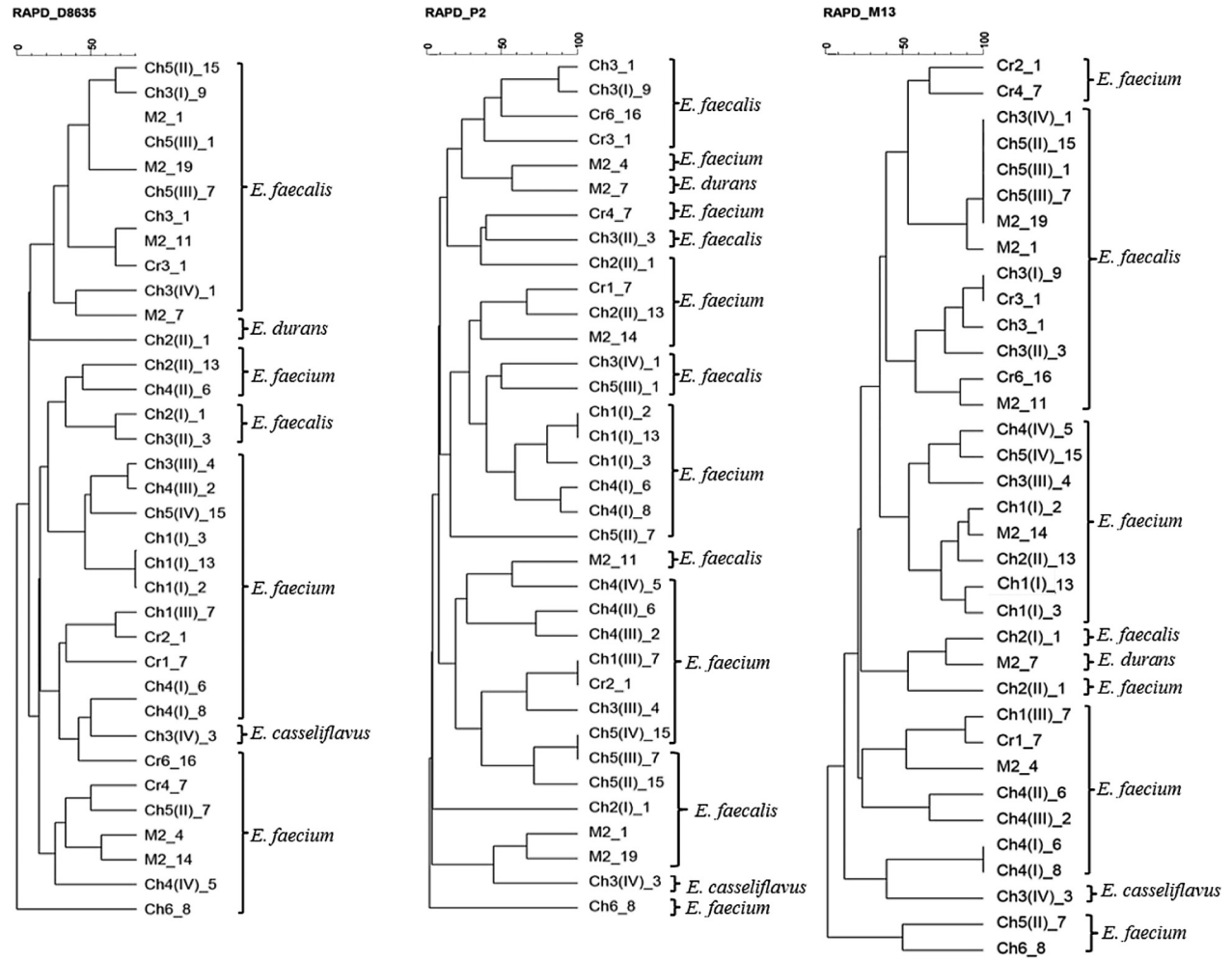

Fig. 3. Dendrograms based on a statistical analysis of the RAPD-PCR fingerprints, obtained using three different primers (D8635, P2 and M13)

to select representative strains for further technological and safety analysis. RAPD-PCR fingerprinting based on M13, D8635 and P2 primers discriminated 35 clusters that comprised two or more isolates. From each of these clusters, one representative strain was selected and further analyzed (a total of 35 strains). The enterococcal isolates (2.4 %) that displayed unique banding patterns were omitted from further analysis. Although the same distribution profile of *Enterococcus* species was noticed among six farms in this study, most of the detected genotypes were farm specific. Of 35 detected genotypes, five dominant ones (approx. 30 % of total isolates) were shared among the farms. Such a genotype distribution reflected the particular differences in traditional Istrian cheese production. Mainly, variable salt concentration, ripening temperature and air humidity as well as microclimatic or vegetation conditions may be the reason for specific strain distribution.

Phenotypic characterization and biotechnological properties of selected strains

During cheese manufacturing, lactic acid bacteria are exposed to different temperatures, pH and salt concentration. The recovery and persistence of enterococci in some types of cheese during ripening can be attributed to their wide range of growth temperatures and their tolerance to pH and salt (*3*). All 35 representative strains tested in this study were able to grow at 10 and 45 °C in the presence of 4 and 6.5 % NaCl as well as at pH=5 and 9.6, which is in line with the high adaptability of enterococci to harsh environmental conditions.

A rapid decrease of milk pH is essential for coagulation and is therefore a crucial step in the cheese manufacturing process. In general, the investigated strains in this study showed low acidifying activity. None of the isolates were able to reduce the milk pH by more than 1.51 units even after 24 h of incubation (Table 2). Only three strains belonging to *E. faecalis*, one *E. faecium*, one *E. casseliflavus* and one *E. durans* strain (19.4, 0.38, 0.95 and 2.84 % of total identified enterococci) were able to lower the pH of 10 % skimmed milk below pH=5 (initial pH=6.33). No significant differences were found between *E. faecalis* and *E. faecium* strains after 2 and 6 h of incubation, whereas after 24 h *E. faecalis* strains exhibited better acidifying potential by lowering the pH of the milk by 1.09 units on average, while *E. faecium* lowered the pH of the milk by 0.87 units ($p<0.01$) on average. The poor acid production by enterococci has also been noticed in other studies and most strains reduce the pH of the milk by only 0.4–0.8 units. Likewise, in our study, overall better acidification properties of *E. faecalis* strains than other enterococcal species were found (*39*,*40*).

Although isolates that show low acidification capacity are not ideal candidates for use as part of starter cultures, such strains can take part in the development of flavour and texture, by proteolytic and lipolytic activity or as bioprotective cultures by producing antimicrobial substances.

The degradation of proteins and lipids plays an important role in the development of texture and taste of the cheese. Almost 40 % of tested enterococci in our study showed caseinolytic activity and lipolytic activity of nearly one third of strains (28.60 %) was measured by agar disc diffusion method (Table 2). Sarantinopoulos *et al.* (*39*) demonstrated the ability of majority of 129 enterococal species isolated from diverse environments to hydrolyse tributyrin and as such to be presumably involved in lipid conversion during the cheese maturation.

Overall, *E. faecium* strains had higher caseinolytic activity than *E. faecalis* and for lipolytic activity opposite trend was noticed. With regard to peptidase activity, as a result of measuring the amount of *p*NA released from the chromogenic peptide (S-Ala), *E. faecalis* had significantly higher ($p<0.01$) activity than *E. faecium* (6641.91 and 6028.90 μM, respectively). It seems that *E. faecium* isolates from Istrian cheese are mainly involved in casein degradation, whereas lipolysis and peptidase activity are mainly due to the activity of *E. faecalis* strains, at least under the applied laboratory conditions.

Surveillance in simulated gut conditions

The ability of entereococcal strains to survive in the GI tract and their autoaggregation ability were tested in order to evaluate their colonization potential and passage through the GI tract, which is a prerequisite for the estimation of their possible influence on human health and their use as probiotics.

The 35 representative enterococci tested in this study were lysed in varying percentage in the conditions of human oral cavity, in the acidic environment of the stomach and in the intestinal conditions (Table 2). The survival rate in the first barrier (human oral cavity) was between 38.52 and 98.30 % (on average 58.68 %). Although low pH and antimicrobial activity of pepsin are known to be very effective in decreasing viability of bacteria, survival rates of the tested strains in simulated environment of the stomach (pH=2 and presence of pepsin) were higher than in the conditions of human oral cavity, and was on average 75.45 % (3.19–99.32 %). While the pH of the small intestine is more favourable for bacterial survival, the presence of pancreatin and bile salts may have detrimental effect. In simulated intestinal conditions (bile salts and pancreatin), survival rate among the tested strains was between 2.75–95.95 % (on average 55.58 %).

Significant differences in the ability to survive in simulated GI tract conditions were noted among *Enterococcus* species and among the strains of the same species (Table 2). *E. faecalis* showed higher survival rate in the conditions of human oral cavity than *E. faecium* ($p<0.01$), while *E. faecium* showed higher survival rate in gastric conditions ($p<0.01$). These findings are in agreement with those reported by other authors for *E. faecium* probiotic strains that show higher resistance to low pH than other enterococci (*41*). No significant differences were found between the survival rates of *E. faecalis* and *E. faecium* in intestinal conditions.

All tested strains exhibited poor autoaggregation ability, with values after 5 h ranging from 4.64 to 46.66 % (on average 13.22 %), therefore exhibiting low capacity of adhesiveness.

Table 2. Technological characteristics and probiotic potential of representative enterococcal strains

Species	Representative strain	(*N*(strain)/*N*(total enterococci))/%	Caseinolytic activity	Chromogenic method	Lypolitic activity	Acidifying activity			Bacterial survival/%			Autoaggregation/%	
												t/h	
						$\Delta pH_{2\,h}$	$\Delta pH_{6\,h}$	$\Delta pH_{24\,h}$	Oral test	Duodenum test	Gastric test	3	5
E. faecalis	Cr6_16	17.05	+	5942.2	+	0.07±0.00	0.47±0.00	1.43±0.01	43.86	53.41	84.91	1.70	5.96
	Cr3_1	4.16	+	7855.1	+	0.23±0.00	0.35±0.05	0.69±0.06	59.36	75.79	77.04	5.21	6.37
	Ch3(I)_9	1.32	++	6715.5	–	0.07±0.00	0.42±0.00	1.12±0.01	95.41	44.71	84.55	13.33	14.54
	M2_11	1.32	+	7488.8	–	0.23±0.01	0.32±0.09	0.92±0.00	50.45	28.12	85.00	3.08	10.36
	Ch2(I)_1	1.32	++	8180.7	+	0.07±0.00	0.45±0.00	1.35±0.00	80.95	70.39	75.63	0.54	4.64
	Ch3(II)_3	2.08	++	5087.5	–	0.25±0.00	0.30±0.01	0.97±0.00	93.50	67.22	73.13	9.93	10.25
	Ch5(II)_15	5.87	+	8384.2	–	0.26±0.00	0.59±0.01	0.82±0.00	81.63	5.24	28.22	6.36	14.01
	Ch5(III)_1	1.32	++	9198.2	–	0.25±0.00	0.50±0.00	0.94±0.09	40.68	6.36	43.40	4.95	12.21
	Ch5(III)_7	3.41	+	8465.6	–	0.26±0.00	0.31±0.01	0.68±0.00	77.77	83.43	95.09	5.19	7.35
	M2_1	1.7	++	5942.2	+	0.24±0.00	0.41±0.00	1.03±0.09	80.83	32.94	61.17	4.33	9.28
	M2_19	0.76	+	7610.9	–	0.24±0.00	0.30±0.00	0.87±0.01	38.52	31.05	81.98	2.12	8.86
	Ch3(IV)_1	0.57	++	8628.4	–	0.08±0.02	0.42±0.01	1.51±0.00	98.30	21.38	76.21	6.94	17.01
	Ch3_1	1.52	+	5128.2	+	0.06±0.07	0.38±0.04	1.08±0.04	72.17	58.88	55.00	19.19	23.21
E. casseliflavus	Ch3(IV)_3	0.95	++	5494.5	–	0.08±0.00	0.38±0.00	1.38±0.00	78.78	41.52	79.66	12.26	21.93
E. faecium	Ch6_8	0.76	+	5046.8	–	0.17±0.05	0.45±0.04	0.62±0.05	71.87	40.00	95.13	6.68	10.14
	M2_14	0.38	+	7407.4	+	0.13±0.04	0.38±0.06	0.56±0.02	77.27	40.78	88.18	5.03	10.69
	Ch2(II)_13	7.2	++	6593.4	–	0.18±0.02	0.30±0.03	0.43±0.03	68.95	51.85	98.47	8.91	16.56
	Ch1(I)_13	0.95	++	7081.8	+	0.06±0.02	0.15±0.02	0.26±0.01	48. 38	74.07	93.44	1.56	4.81
	Ch3(III)_4	0.38	+	4761.9	–	0.09±0.00	0.47±0.00	1.37±0.00	70.58	50.98	84.31	5.99	9.35
	Ch4(IV)_5	4.17	+	5128.2	–	0.29±0.00	0.47±0.00	1.03±0.01	67.70	11.70	77.92	3.66	15.13
	Ch5(IV)_15	2.65	++	5413.1	–	0.27±0.00	0.50±0.05	0.59±0.09	43.90	48.67	81.45	3.63	7.87
	Ch4(I)_6	3.03	++	6593.4	–	0.21±0.05	0.27±0.00	0.94±0.00	59.21	63.08	98.99	3.31	13.90
	Ch1(I)_2	0.57	++	7488.8	–	0.28±0.01	0.52±0.09	1.03±0.04	50.78	95.95	99.32	6.50	17.84
	Ch1(I)_3	2.84	++	7041.1	–	0.06±0.00	0.44±0.00	1.24±0.01	39.85	89.45	98.29	1.90	3.81
	Cr1_7	5.11	+	6023.6	–	0.06±0.01	0.36±0.09	0.54±0.03	62.89	25.83	64.20	7.14	15.81
	Ch5(II)_7	1.52	++	5779.4	–	0.28±0.00	0.49±0.05	0.70±0.00	44.02	24.59	66.39	3.28	7.89
	Cr4_7	2.65	+	4599.1	–	0.23±0.01	0.56±0.09	1.25±0.06	88.52	72.72	72.72	16.13	18.06
	Ch4(I)_8	6.06	+	5168.9	–	0.06±0.02	0.57±0.10	1.23±0.04	48.64	75.00	45.00	19.27	22.39
	Ch4(III)_2	2.27	+	6959.7	–	0.05±0.02	0.51±0.07	1.34±0.07	82.78	93.12	3.19	46.66	46.66
	M2_4	0.95	+	4802.6	–	0.03±0.03	0.45±0.13	1.06±0.00	43.13	79.45	73.97	13.04	19.13
	Ch2(II)_1	2.65	+	5291.0	–	0.08±0.04	0.52±0.05	0.94±0.06	41.69	15.36	60.00	5.16	9.14
	Ch4(II)_6	0.76	+	5087.5	–	0.03±0.01	0.47±0.00	1.08±0.04	40.88	42.52	71.55	3.54	7.21
	Ch1(III)_7	1.14	++	5372.4	–	0.06±0.04	0.47±0.05	0.70±0.09	88.71	2.75	47.41	8.22	17.82
	Cr2_1	7.77	++	7000.4	–	0.24±0.06	0.75±0.11	0.90±0.02	45.45	88.13	94.91	16.34	16.82
E. durans	M2_7	2.84	++	8424.9	+	0.10±0.00	0.39±0.06	1.35±0.06	42.98	81.95	64.43	2.68	5.78

Safety issues

Enterococci in food of animal origin have been shown to harbour different virulence factors, suggesting that this type of food could act as an important environmental reservoir for human infections. The presence of 10 genes coding for virulence factors and potential pathogenicity of enterococcal strains was evaluated by PCR (Table 3). Many of virulence genes, *e.g.* aggregation substances, cytolysin and gelatinase genes are known to have a silent state, making their genotypic study more reliable than phenotyping one because they might remain undetected under the *in vitro* conditions but be expressed *in vivo*, so the pathogenic potential of such strains might be underestimated (*42*).

PCR amplification of the aggregation substance (*agg*) and surface protein (*esp*) genes revealed 27.65 and 9.85 % of positive strains in the current study. The *esp* gene is known to be associated with the biofilm production, endocarditis and nosocomial infections and is usually detected at low frequency (*43,44*), quite opposite to a study of Semedo *et al.* (*45*) who detected 62 % of *esp*-positive strains, 57 % of which being of food origin *versus* 22 % from clinical samples (*N*=164).

Virulence traits involved in the production of the cytolysin (*cylA, cylB* and *cylM*) are one of the most studied virulence traits associated with enterococci and are usually detected in low number of isolates (*46*). Of all entero-

Table 3. Detection of virulence determinants

Species	Representative strain	(*N*(strain)/*N* (total enterococci))/%	Virulence determinant									
			agg	*efaAfs*	*cylM*	*cylA*	*esp*	*efaAfm*	*cob*	*gelE*	*cylB*	*cpd*
E.faecalis	Cr6_16	17.05	+	+	+	+	–	+	+	+	–	+
	Cr3_1	4.16	–	+	+	–	–	+	+	+	–	+
	Ch3(I)_9	1.32	–	+	+	–	+	+	+	+	–	+
	M2_11	1.32	+	+	+	+	+	+	+	+	–	+
	Ch2(I)_1	1.32	–	+	+	–	+	+	+	+	–	+
	Ch3(II)_3	2.08	–	+	–	–	+	+	+	–	–	+
	Ch5(II)_15	5.87	+	+	–	–	–	–	+	–	–	+
	Ch5(III)_1	1.32	–	+	–	–	+	–	+	–	–	+
	Ch5(III)_7	3.41	–	+	–	–	–	–	+	–	+	+
	M2_1	1.7	–	+	–	–	+	+	+	+	+	+
	M2_19	0.76	–	+	–	–	+	+	+	+	–	+
	Ch3(IV)_1	0.57	–	+	–	–	–	+	+	–	–	+
	Ch3_1	1.52	–	+	+	+	–	–	–	+	+	+
E. casseliflavus	Ch3(IV)_3	0.95	–	–	–	–	–	+	–	+	–	+
E. faecium	Ch6_8	0.76	+	+	–	–	–	–	–	+	+	+
	M2_14	0.38	–	+	–	–	–	–	–	+	+	+
	Ch2(II)_13	7.2	–	–	+	–	–	+	–	–	–	–
	Ch1(I)_13	0.95	–	+	–	–	–	+	–	–	–	–
	Ch3(III)_4	0.38	–	–	–	–	–	–	–	–	–	–
	Ch4(IV)_5	4.17	–	–	–	–	–	+	–	–	–	–
	Ch5(IV)_15	2.65	–	–	–	–	–	+	–	–	–	–
	Ch4(I)_6	3.03	–	+	+	–	–	+	–	+	–	+
	Ch1(I)_2	0.57	–	+	–	–	–	+	–	+	+	+
	Ch1(I)_3	2.84	–	+	+	–	–	+	–	+	+	+
	Cr1_7	5.11	–	+	+	–	–	+	–	–	–	–
	Ch5(II)_7	1.52	–	+	–	–	–	–	+	–	+	+
	Cr4_7	2.65	+	+	+	–	–	+	–	+	+	+
	Ch4(I)_8	6.06	–	+	–	–	–	+	+	–	–	+
	Ch4(III)_2	2.27	–	–	–	–	–	+	–	–	–	–
	M2_4	0.95	–	+	+	–	–	–	–	–	–	–
	Ch2(II)_1	2.65	–	+	+	–	–	–	–	–	–	–
	Ch4(II)_6	0.76	–	–	–	–	–	+	–	–	–	–
	Ch1(III)_7	1.14	–	–	–	–	–	+	–	–	–	–
	Cr2_1	7.77	–	+	–	–	–	+	–	–	–	–
E. durans	M2_7	2.84	–	–	–	–	–	+	–	–	–	–

cocci from Istrian cheese, cytolysin components (*cylM, cylB* and *cylA*) were present in 51.14, 15.34 and 19.88 % of strains respectively, which is high in comparison with other studies (*44,46*).

The genes *gelE* and *cpd*, associated with toxin production and sex pheromones, were also reported to be commonly found in commensal isolates (*31*). The sex pheromone determinants *cpd* and *cob* occurred with the frequency of 61.17 and 48.48 % respectively and gelatinase (*gelE*) gene was present in 40.34 % of our enterococcal isolates.

The widespread occurrence of adhesion-associated protein in enterococci, irrespective of origin and species allocation, is reported in many food and clinical isolates (*45*) and was confirmed in our study with 77.65 % *efaAfs*- and 81.25 % *efaAfm*-positive enterococci.

Overall, *E. faecalis* strains harboured 56.70 % more virulence genes than the tested *E. faecium* strains. Especially high incidence of sex pheromone (*cpd* and *cob*) and *efaAfs* determinants was noticed among *E. faecalis* isolates (100, 99 and 100 % respectively). Among *E. faecium*, 0.70 % of strains were lacking all virulence determinants and among 34.86 % of strains only one virulence gene was detected (*efaAfm*). The *cylA* and *esp* genes were absent from all *E. faecium* strains. A significantly different pattern was noticed in *E. faecium* isolated in our study compared to strains tested by Eaton and Gasson (*5*), who did not detect *agg, cylM, cylB* or *cylA* and sex pheromone genes among the analyzed *E. faecium* strains. Although several studies have reported *agg* gene only in *E. faecalis* (*5,47*), in this study the presence of *agg* was demonstrated also in *E. faecium*. The aggregation substance (*agg*) promotes the conjugative transfer of sex pheromone plasmids by formation of mating aggregates between donor and recipient cells (*48*) and may support the strain probiotic characteristics (*49*).

For *cob* and *cpd* genes our data are in line with the literature on *E. faecalis* strains; however, the respective genes were also detected among *E. faecium* isolates (14.08 and 33.09 %, respectively). The detection of sex pheromones in species other than *E. faecalis* is confirmed by the work of Heaton and Handwerger (*50*), who suggested that sex pheromone cross talk between *E. faecium* and *E. faecalis* has been established, which is also indicated in our study.

The number of virulence determinants was the highest in milk samples and was decreasing until the 30th day of cheese ripening, when it rapidly increased. After 30 days of ripening, the number of virulence determinants continued to decrease and was significantly lower ($p<0.05$) at the end of ripening. This implies a reduction of the number of strains possessing multiple virulence genes or virulence genes in general, rather than correlation to the total number of enterococci, as in milk samples the lowest CFU count and the highest number of virulence determinants was noticed.

Although enterococci can induce haemolytic reaction, none of the 35 tested strains in this study were found to cause haemolysis.

Food that is not heat treated before consumption, *e.g.* spontaneously fermented cheese may contain viable antibiotic-resistant bacteria that are assumed to contribute to the transmission of antibiotic resistance genes into the GI tract. Species of the genus *Enterococcus* are intrinsically resistant to a wide variety of antimicrobials including cephalosporins, low levels of aminoglycosides, lincomycine and quinupristin/dalfopristin (*7,44*). However, enterococci efficiently acquire and transmit antibiotic-resistant genes *via* mobile genetic elements as conjugative plasmids and transposons (*51*), which is pivotal in the dissemination and persistence of antimicrobial resistance in enterococci.

In the current study, only 0.76 % of strains were non-resistant to any of the tested antibiotics, 7.57 % of strains were resistant to one antibiotic and 7.95 % of the strains were resistant to two antibiotics, while others (83.72 %) were multiresistant (Table 4). None of the strains were resistant to penicillin (10 μg), ampicillin (10 μg) and ampicillin/sulbactam (30 μg). The vast majority of strains were resistant to clindamycin (2 and 10 μg; 92.80 and 63.07 %, respectively), streptomycin (10 μg; 82.00 %) and rifampicin (5 μg; 72.35 %), while total of 28.41 % of strains were resistant to chloramphenicol (10 μg). A considerable number of strains were resistant to critically important antibiotics such as tetracycline (5 and 30 μg; 43.56 and 17.99 %, respectively), erythromycin (5 and 15 μg; 35.79 and 29.35 %, respectively) and vancomycin (5 μg; 23.48 %). A high frequency of resistance to erythromycin and tetracycline in enterococci from various foods was also reported in Europe, Canada, Turkey and Tunisia (*35,52–54*). The frequency of vancomycin-resistant enterococci in food is mostly low and varied between 0–25 % (*6*) and higher resistance to vancomycin in our study (although of low concentration) has to be considered as vancomycin is one of the few alternatives in treating enterococcal infections.

With respect to the genes responsible for resistance to tetracycline (*tetK, tetL, tetM, tetO* and *tetS*), vancomycin (*vanA* and *vanB*) and erythromycin (*ermA* and *ermB*), only *tetM* gene could be detected in the analyzed strains. The *tetM* gene is the most frequently detected in tetracycline-resistant enterococci worldwide and it is known to be mainly based on the transfer of Tn916-type transposons (*55*).

Conclusion

Our study confirmed the presence of enterococci as a major part of the indigenous core microbiota of Istrian cheese during fermentation and ripening, with *Enterococcus faecalis* and *E. faecium* being the predominant species. Considerable variation between strains of the same species was recorded in their ability to exhibit a wide range of biotechnologically important traits as well to survive in gastrointestinal (GI) tract. Even though probably none of enterococcal strains were able to colonize the GI tract, they can survive in harsh GI environment to different extents. The high rate of multiresistant enterococci and high load of virulence determinants detected in our study should be taken into consideration due to the high frequencies of transmission of genes encoding for antibiotic resistance and virulence factors in GI tract. The role of enterococci in cheese ripening remains questionable, because several isolates possess virulence or antibiotic resistance genes and thus have potential of endangering

Table 4. Susceptibility of 35 representative enterococcal strains to antibiotics using the disc diffusion method

Species	Representative strain	(*N*(strain)/*N*(total enterococci))/%	Disc potency/μg												
			TET (30)	TET (5)	RIF (5)	ERY (15)	ERY (5)	VAN (5)	PEN (10)	AMP (10)	STR (10)	CLI (10)	CLI (2)	CHL (10)	SAM (30)
E. faecalis	Cr6_16	17.05	R	R	R	S	S	S	S	S	R	I	R	I	S
	Cr3_1	4.16	I	I	R	I	I	S	S	S	R	R	R	R	S
	C3(I)_9	1.32	I	I	I	I	I	S	S	S	R	R	R	R	S
	M2_11	1.32	I	I	I	S	S	S	S	S	R	R	R	I	S
	Ch2(I)_1	1.32	S	S	R	S	S	S	S	S	R	R	R	S	S
	Ch3(II)_3	2.08	I	R	R	I	I	R	S	S	R	R	R	R	S
	Ch5(II)_15	5.87	I	I	R	I	I	R	S	S	R	R	R	I	S
	Ch5(III)_1	1.32	I	R	R	I	I	R	S	S	R	R	R	R	S
	Ch5(III)_7	3.41	I	R	I	I	R	R	S	S	R	R	R	R	S
	M2_1	1.7	I	I	R	S	S	R	S	S	R	R	R	I	S
	M2_19	0.76	I	R	R	S	S	R	S	S	R	R	R	R	S
	Ch3(IV)_1	0.57	S	S	R	S	S	S	S	S	I	S	S	R	S
	Ch3_1	1.52	I	I	R	I	I	R	S	S	R	R	R	R	S
E. casseliflavus	Ch3(IV)_3	0.95	S	S	R	R	R	S	S	S	R	R	R	R	S
E. faecium	Ch6_8	0.76	I	I	S	S	S	S	S	S	S	S	I	I	S
	M2_14	0.38	S	S	I	I	I	S	S	S	I	I	R	I	S
	Ch2(II)_13	7.2	I	I	S	I	I	S	S	S	I	I	R	I	S
	Ch1(I)_13	0.95	S	S	R	I	I	S	S	S	I	R	R	R	S
	Ch3(III)_4	0.38	I	I	R	I	R	S	S	S	I	S	I	I	S
	Ch4(IV)_5	4.17	S	S	S	S	S	S	S	S	R	R	R	I	S
	Ch5(IV)_15	2.65	I	I	R	S	S	S	S	S	R	R	R	R	S
	Ch4(I)_6	3.03	I	I	R	R	R	S	S	S	R	R	R	I	S
	Ch1(I)_2	0.57	I	R	R	R	R	S	S	S	R	R	R	I	S
	Ch1(I)_3	2.84	I	I	R	R	R	S	S	S	R	R	R	I	S
	Cr1_7	5.11	I	I	R	R	R	S	S	S	R	R	R	R	S
	Ch5(II)_7	1.52	I	I	S	I	R	S	S	S	I	I	R	S	S
	Cr4_7	2.65	S	S	S	S	S	S	S	S	R	I	R	S	S
	Ch4(I)_8	6.06	I	I	R	R	R	R	S	S	R	R	R	I	S
	Ch4(III)_2	2.27	I	R	R	R	R	S	S	S	I	R	R	I	S
	M2_4	0.95	R	R	S	S	S	S	S	S	R	I	R	I	S
	Ch2(II)_1	2.65	I	R	R	I	S	S	S	S	R	I	I	S	S
	Ch4(II)_6	0.76	I	R	R	R	R	R	S	S	R	R	R	R	S
	Ch1(III)_7	1.14	I	R	I	I	R	S	S	S	I	R	R	I	S
	Cr2_1	7.77	I	R	R	R	R	S	S	S	R	R	R	I	S
E. durans	M2_7	2.84	I	R	S	S	S	S	S	S	I	S	I	R	S

TET=tetracycline (30 and 5 μg), RIF=rifampicin (5 μg), ERY=erythromycin (5 and 15 μg), AMP=ampicillin (10 μg), VAN=vancomycin (5 μg), PEN=penicillin (10 μg), STR=streptomycin (10 μg), CLI=clindamycin (2 and 10 μg), CHL=chloramphenicol (10 μg), SAM=ampicillin/sulbactam (30 μg)

human health. In agreement with the literature, the majority of enterococci isolated from ripened Istrian cheese cannot fulfil the strict safety rules needed for starter or adjunct cultures. Based on their good technological properties, absence of most of the virulence determinants and antibiotic resistance genes, only one strain, *E. durans* M2_7, is a good candidate for the application as starter or adjunct culture for fermented food production.

Funding

This study was partially funded by the German Academic Exchange Service (DAAD) and the Ministry of Science and Education of the Republic of Croatia (MZO).

References

1. Franz CMAP, Holzapfel WH, Stiles ME. Enterococci at the crossroads of food safety? Int J Food Microbiol. 1999;47:1–24. https://doi.org/10.1016/S0168-1605(99)00007-0
2. Moreno MRF, Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of enterococci in food and health. Int J Food Microbiol. 2006;106:1–24. https://doi.org/10.1016/j.ijfoodmicro.2005.06.026
3. Giraffa G. Enterococci from foods. FEMS Microbiol Rev. 2002;26:163–71. http://doi.org/10.1111/j.1574-6976.2002.tb00608.x
4. Giraffa G. Functionality of enterococci in dairy products. Int J Food Microbiol. 2003;88:215–22. https://doi.org/10.1016/S0168-1605(03)00183-1
5. Eaton TJ, Gasson MJ. Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol. 2001;67:1628–35. https://doi.org/10.1128/AEM.67.4.1628-1635.2001
6. Wang JS, Muzevich K, Edmond MB, Bearman G, Stevens MP. Central nervous system infections due to vancomycin-resistant enterococci: case series and review of the literature. Int J Infect Dis. 2014;25:26–31. https://doi.org/10.1016/j.ijid.2014.01.009
7. Murray BE. The life and times of the Enterococcus. Clin Microbiol Rev. 1990;3:46–65. http://doi.org/10.1128/CMR.3.1.46
8. Jamet E, Akary E, Poisson MA, Chamba JF, Bertrand X, Serror P. Prevalence and characterization of antibiotic resistant Enterococcus faecalis in French cheeses. Food Microbiol. 2012;31:191–8. https://doi.org/10.1016/j.fm.2012.03.009
9. Rehaiem A, Fhoula I, Faouzi Slim A, Ben Boubaker IB, Boudabous Chihi A, Ouzari HI. Prevalence, acquired antibiotic resistance and bacteriocin production of Enterococcus spp. isolated from Tunisian fermented food products. Food Control. 2016;63:259–66. https://doi.org/10.1016/j.foodcont.2015.11.034
10. Magdić V, Kalit S, Mrkonjić Fuka M, Skelin A, Samaržija D, Redžepović S, Havranek J. A survey on hygienic and physicochemical properties of Istrian cheese. Mljekarstvo. 2013;63: 55–63.
11. Mrkonjić Fuka M, Wallisch S, Engel M, Welzl G, Havranek J, Schloter M. Dynamics of bacterial communities during the ripening process of different Croatian cheese types derived from raw ewe's milk cheeses. PloS One. 2013;8:e80734. https://doi.org/10.1371/journal.pone.0080734
12. Mrkonjić Fuka M, Engel M, Skelin A, Redžepović S, Schloter M. Bacterial communities associated with the production of artisanal Istrian cheese. Int J Food Microbiol. 2010;142:19–24. https://doi.org/10.1016/j.ijfoodmicro.2010.05.008
13. Mundy LM, Sahm DF, Gilmore M. Relationships between enterococcal virulence and antimicrobial resistance. Clin Microbiol Rev. 2000;13:513–22. https://doi.org/10.1128/CMR.13.4.513-522.2000
14. Deasy BM, Rea MC, Fitzgerald GF, Cogan TM, Beresford TP. A rapid PCR based method to distinguish between Lactococcus and Enterococcus. Syst Appl Microbiol. 2000;23:510–22. https://doi.org/10.1016/S0723-2020(00)80025-9
15. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995;33:24–7.
16. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. Chichester, UK: John Wiley & Sons; 1991. pp. 115–75.
17. Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, et al. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J Bacteriol. 1996;178:5636–43. https://doi.org/10.1128/jb.178.19.5636-5643.1996
18. Huey B, Hall J. Hypervariable DNA fingerprinting in Escherichia coli: minisatellite probe from bacteriophage M13. J Bacteriol. 1989;171:2528–32. https://doi.org/10.1128/jb.171.5.2528-2532.1989
19. Křížova J, Španová A, Rittich B. RAPD and rep-PCR fingerprinting for characterization of Bifidobacterium species. Folia Microbiol. 2008;53:99–104. https://doi.org/10.1007/s12223-008-0014-1
20. Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. DNA diversity among clinical isolates of Helicobacter pylori detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. 1992;20:5137–42. https://doi.org/10.1093/nar/20.19.5137
21. Warsa UC, Nonoyama M, Ida T, Okamoto R, Okubo T, Shimauchi C, et al. Detection of tet(K) and tet(M) in Staphylococcus aureus of Asian countries by the polymerase chain reaction. J Antibiot. 1996;49:1127–32. https://doi.org/10.7164/antibiotics.49.1127
22. Ng LK, Martin I, Alfa M, Mulvey M. Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probe. 2001;4:209–15. https://doi.org/10.1006/mcpr.2001.0363
23. Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemoter. 1996;40:2562–6.
24. Schreiner K, Hagn A, Kyselková M, Moënne-Loccoz Y, Welzl G, Munch JC, Schloter M. Comparison of barley succession and take-all disease as environmental factors shaping the rhizobacterial community during take-all decline. Appl Environ Microbiol. 2010;76:4703–12. https://doi.org/10.1128/AEM.00481-10
25. Mrkonjić Fuka M, Engel M, Haesler EF, Welzl G, Munch JC, Schloter M. Diversity of proteolytic community encoding for subtilisin in an arable field: spatial and temporal variability. Biol Fertil Soils. 2008;45:185–91. https://doi.org/10.1007/s00374-008-0319-x
26. BioNumerics v. 7.6. Gent, Belgium; Applied Maths NV; 2016. Available from: http://www.applied-maths.com.
27. Vauterin L, Vauterin P. Computer-aided objective comparison of electrophoretic patterns for grouping and identification of microorganisms. Eur Microbiol. 1992;1:37–41.
28. Jamaly N, Benjouad A, Comunian R, Daga E, Bouksaim M. Characterization of Enterococci isolated from Moroccan dairy products. Afr J Microbiol Res. 2010;4:1768–74.

29. Savoy de Giori G, Hébert EM. Methods to determine proteolytic activity of lactic acid bacteria. In: Spencer JFT, de Ragout Spencer AL (editors). Food microbiology protocols. Methods in biotechnology vol. 14. Totowa, New Jersey, USA: Humana Press Inc; 2001. pp. 197–202. https://doi.org/10.1385/1-59259-029-2:197
30. Doleyres Y, Fliss I, Lacroix C. Increased stress tolerance of Bifidobacterium longum and Lactococcus lactis produced during continuous mixed-strain immobilized-cell fermentation. J Appl Microbiol. 2004;97:527–39. https://doi.org/10.1111/j.1365-2672.2004.02326.x
31. Nueno-Palop C, Narbad A. Probiotic assessment of Enterococcus faecalis CP58 isolated from human gut. Int J Food Microbiol. 2011;145:390–4. https://doi.org/10.1016/j.ijfoodmicro.2010.12.029
32. Fortina MG, Ricci G, Borgo F, Manachini PL, Arends K, Schiwon K, et al. A survey on biotechnological potential and safety of the novel Enterococcus species of dairy origin, E. italicus. Int J Food Microbiol. 2008;123:204–11. https://doi.org/10.1016/j.ijfoodmicro.2008.01.014
33. Del Re B, Sgorbati B, Miglioli M, Palenzona D. Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum. Lett Appl Microbiol. 2000;31:438–42. https://doi.org/10.1046/j.1365-2672.2000.00845.x
34. M100-S15. Fifteenth informational supplement. Performance standards for antimicrobial susceptibility testing. Wayne, PA, USA: Clinical and Laboratory Standards Institute (CLSI); 2005.
35. Čanžek Majhenič A, Rogelj I, Perko B. Enterococci from Tolminc cheese: population structure, antibiotic susceptibility and incidence of virulence determinants. Int J Food Microbiol. 2005;102:239–44. https://doi.org/10.1016/j.ijfoodmicro.2004.12.021
36. The R project for statistical computing, R software v. 3.1.2, The R foundation, Vienna, Austria; 2002. Available from: http://www.r-project.org.
37. International Commission on Microbiological Specifications for Foods (ICMSF). Microorganisms in foods 1: their significance and methods of enumeration represents a major step in establishing a common understanding of, and developing standard methods for, important foodborne microorganisms. Toronto, Canada: University of Toronto Press; 1978.
38. Teuber M, Perreten V, Wirsching F. Antibiotic-resistant bacteria: The new dimension in food microbiology. Lebensm Technol. 1996;29:182–199 (in German). https://doi.org/10.3929/ethz-a-004270972
39. Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea MC, Lombardi A, Cogan TM, et al. Biochemical properties of enterococci relevant to their technological performance. Int Dairy J. 2001;11:621–47. https://doi.org/10.1016/S0958-6946(01)00087-5
40. Özdemir GB, Oryaşın E, Bıyık HH, Özteber M, Bozdoğan B. Phenotypic and genotypic characterization of bacteriocins in enterococcal isolates of different sources. Indian J Microbiol. 2011;51:182–7. https://doi.org/10.1007/s12088-011-0143-0
41. Hosseini SV, Arlindo S, Böhme K, Fernández-No C, Calo-Mata P, Barros-Velázquez J. Molecular and probiotic characterization of bacteriocin producing Enterococcus faecium strains isolated from nonfermented animal foods. J Appl Microbiol. 2009;107:1392–403. https://doi.org/10.1111/j.1365-2672.2009.04327.x
42. Morandi S, Silvetti T, Brasca M. Biotechnological and safety characterization of Enterococcus lactis, a recently described species of dairy origin. Antonie Leeuwenhoek. 2013;103:239–49. https://doi.org/10.1007/s10482-012-9806-z
43. Olsen RH, Schønheyder HC, Christensen H, Bisgaard M. Enterococcus faecalis of human and poultry origin share virulence genes supporting the zoonotic potential of E. faecalis. Zoonoses Public Health. 2012;59:256–63. https://doi.org/10.1111/j.1863-2378.2011.01442.x
44. Yılmaz EŞ, Aslantaş Ö, Önen SP, Türkyılmaz S, Kürekci C. Prevalence, antimicrobial resistance and virulence traits in enterococci from food of animal origin in Turkey. LWT – Food Sci Technol. 2016;66:20–6. https://doi.org/10.1016/j.lwt.2015.10.009
45. Semedo T, Santos MA, Lopes MFS, Figueiredo Marques JJ, Barreto Crespo MT, Tenreiro R. Virulence factors in food, clinical and reference enterococci: a common trait in the genus? Syst Appl Microbiol. 2003;26:13–22. https://doi.org/10.1078/072320203322337263
46. Jahan M, Holley RA. Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25 °C and 37 °C. Int J Food Microbiol. 2014;170: 65–9. https://doi.org/10.1016/j.ijfoodmicro.2013.11.002
47. Barbosa J, Gibbs PA, Teixeira P. Virulence factors among enterococci isolated from traditional fermented meat products produced in the north of Portugal. Food Control. 2010;21: 651–6. https://doi.org/10.1016/j.foodcont.2009.10.002
48. Aberna RA, Prabakaran K. Evaluation for the association of virulence determinants among E. faecalis with its clinical outcome. Int J Biol Med Res. 2011;2: 523–7.
49. Domann E, Hain T, Ghai R, Billion A, Kuenne C, Zimmermann K, Chakraborty T. Comparative genomic analysis for the presence of potential enterococcal virulence factors in the probiotic Enterococcus faecalis strain Symbioflor 1. Int J Med Microbiol. 2007;297:533–9. https://doi.org/10.1016/j.ijmm.2007.02.008
50. Heaton MP, Handwerger S. Conjugative mobilization of a vancomycin resistance plasmid by a putative Enterococcus faecium sex pheromone response plasmid. Microb Drug Resist. 1995;1:177–83. https://doi.org/10.1089/mdr.1995.1.177
51. Huycke MM, Sahm DF, Gilmore MS. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg Infect Dis. 1998;4:239. https://doi.org/10.3201/eid0402.980211
52. Aslam M, Diarra MS, Checkley S, Bohaychuk V, Masson L. Characterization of antimicrobial resistance and virulence genes in Enterococcus spp. isolated from retail meats in Alberta, Canada. Int J Food Microbiol. 2012;156:222–30. https://doi.org/10.1016/j.ijfoodmicro.2012.03.026
53. Klibi N, Said LB, Jouini A, Slama KB, López M, Sallem RB, et al. Species distribution, antibiotic resistance and virulence traits in enterococci from meat in Tunisia. Meat Sci. 2013;93: 675–80. https://doi.org/10.1016/j.meatsci.2012.11.020
54. Özmen Toğay S, Çelebi Keskin A, Açık L, Temiz A. Virulence genes, antibiotic resistance and plasmid profiles of Enterococcus faecalis and Enterococcus faecium from naturally fermented Turkish foods. J Appl Microbiol. 2010;109:1084–92. https://doi.org/10.1111/j.1365-2672.2010.04763.x
55. Agersø Y, Pedersen AG, Aarestrup FM. Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene tet(M) in enterococci from humans, pigs and poultry. J Antimicrob Chemother. 2006;57: 832–9. https://doi.org/10.1093/jac/dkl069