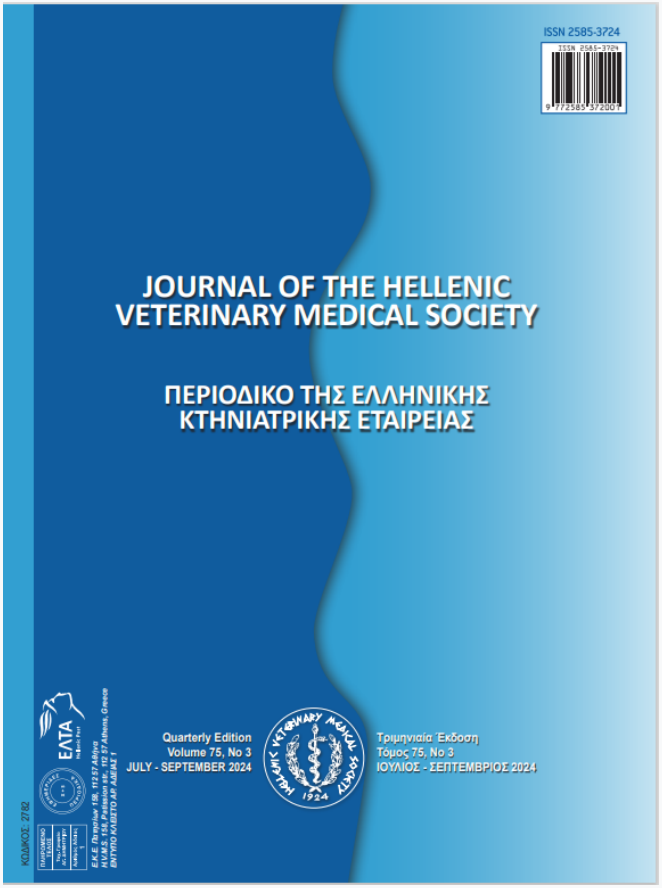
Journal of the Hellenic Veterinary Medical Society

Vol 75, No 3 (2024)



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doi: 10.12681/jhvms.36209

To cite this article:

Psomas, E., Sakaridis, I., Boukouvala, E., Papatsaroucha, E., Apostolidi, E., Skarlatoudi, T., Ekateriniadou, L., & Samouris, G. (2024). Studying enterococci isolated from artisanal feta produced with mountain and low land milk. *Journal of the Hellenic Veterinary Medical Society*, *75*(3), 7967–7976. https://doi.org/10.12681/jhvms.36209

J HELLENIC VET MED SOC 2024, 75 (3): 7967-7976
ΠΕΚΕ 2024, 75 (3): 7967-7976

Research article
Ερευνητικό άρθρο

Studying enterococci isolated from artisanal feta produced with mountain and low land milk

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ABSTRACT: Enterococci are commonly part of natural milk cultures that are used to produce cheese and have a strong influence on ripening, and aroma development in feta cheese. Despite their unique benefits in cheese production, Enterococci have also been identified as opportunistic pathogens that can cause various human diseases, and their use remains controversial due to the possibility of antimicrobial resistance transfer to human strains. The objective of the present study was to detect, identify, and analyze characteristics of Enterococcus spp. from different samples of the artisanal feta cheese produced with mountain or low land milk and finally to evaluate its potential use for enhancing the sensory characteristics of fermented milks and cheeses without compromising the safety of the products. Twenty-five strains of enterococci from mountain areas and twenty-five from low land areas were selected from the cheese making trials. Based on the desired technological characteristics, twelve strains were selected for analytical identification, study of antibiotic resistance and further study by molecular techniques. Eleven of those were found to be sensitive to vancomycin (lower to 10μg/ml), one was resistant up to 20μg/ml and therefore are even more promising, in terms of their safety, for their potential use as adjunct cultures and worth further analysis.

Keywords: enterococci; feta cheese; starter cultures; mountain milk; low land milk

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Date of initial submission: 15-12-2023
Date of acceptance: 03-01-2024

INTRODUCTION

Feta is a Protected Designation of Origin (PDO) cheese made either from sheep milk or from a mixture of sheep and goat milk up to 30% (Anifantakis, 1991). The most important factors affecting the microflora composition of milk and cheese are the milking conditions, the condition of the milk storage, the animal health status/hygiene, the personal hygiene, and the cleaning practices (Settanni and Moschetti, 2010; Litopoulou-Tzanetaki and Tzanetakis, 2014)[8,9]. So far, according to the studies conducted on feta cheese produced from pasteurized milk with the addition of starter cultures, enterococci were found at a level varying from 1 to 7 log cfu/g, based on classical microbiological methods and/or molecular techniques (Sarantinopoulos et al., 2002a; Manolopoulou et al., 2003; Rantsiou et al., 2008; Alexandraki et al., 2016).

Enterococci are Gram-positive, catalase- and oxidase-negative, facultative anaerobic cocci that do not form spores, belonging to the *Enterococceae* family and are included in the lactic acid bacteria (LAB). Their proteolytic and esterolytic activity, as well as the production of di-ethyl through the metabolism of citrates, significantly affect the ripening and the development of the aroma in feta cheese (Tsakalidou et al., 1993; Centeno et al., 1999). The presence of enterococci in milk comes either directly from animal excrement or from contaminated water, bulk tank or finally milking equipment (Ogier and Serror, 2008). Based on their role in ripening and flavor, as well as their bacteriocin production, enterococci have the potential to be part of the starter cultures in feta cheese (Sarantinopoulos et al., 2002b). *Enterococcus faecium*, *Enterococcus faecalis* and *Enterococcus durans*are considered to be the most prevalent species of enterococci in raw milk cheeses. Enterococci and especially *Enterococcus faecalis* and *Enterococcus faecium*, despite their unique technological properties in cheese production, have also been characterized as opportunistic pathogens that can potentially cause various human diseases, such as bacteremia, urinary tract infections and endocarditis (Terzic-Vidojevic et al., 2021).

Enterococci are also used as probiotics for humans or farm animals, but this use still remains controversial due to the possibility of antimicrobial resistance transfer to human strains and for this reason Canada, for example, banned the use of enterococci as a probiotic (Ogier and Serror, 2008). As for the antimicrobial resistance, resistance to erythromycin, linezolid, chloramphenicol, tetracycline, vancomycin, ciprofloxacin, tetracycline, and erythromycin has been observed in isolates from animal facilities and in foods of animal origin (Rocha et al., 2022). Their antibiotic-resistance genes are located on chromosomes, plasmids, or transposons. Lactic acid bacteria such as *Lactobacillus* and *Lactococcus*, with harbored antibiotic resistance genes could act as reservoirs for the antibiotic resistance transfer to enterococci (Bonham et al. 2017). It is quite significant that although antibiotic-resistant enterococci have been isolated from food, resistance to ampicillin, penicillin, vancomycin and gentamicin is not so widespread; despite this, *Enterococcus* is the only LAB genus characterized as opportunistic pathogen and not considered as "Generally Recognized As Safe" (GRAS) (EFSA BIOHAZ Panel, 2021); in regard to their safety status, enterococci are classified to risk group 2.

The fact that antibiotic-resistant enterococci could be a part of the normal flora of foods such as cheeses may favor the possible spread of antibiotic resistance to other microbial strains of the digestive tract during food consumption. As a result, a wide discussion has opened about the cheese ecosystem as a possible reservoir of antibiotic resistance genes that are transferred to human strains in the gastrointestinal tract. The study of antibiotic resistance in enterococci seems to be more important in the case of their use as starters in cheese production, so a case-by-case analysis of antibiotic resistance may be necessary. According to Výrostková et al. (2021), although there have been numerous studies on antimicrobial resistance, there is currently not enough information from studies on local cheeses and much less on artisanal cheeses. Finally, it is important that there are not enough studies on the prevalence of antimicrobial resistance in enterococci in sheep and goat milk as well as in their products.

The aim of our study was to detect, identify, and analyze the most important characteristics of *Enterococcus* spp. from different samples of the artisanal feta cheese produced with mountain or low land milk. Vancomycin resistance and other possible pathogenic variables were also taken into consideration. Furthermore, we evaluated its perspective use to enhance the sensory characteristics of fermented milks and cheeses without compromising the safety of the products.

MATERIALS AND METHODS

Cheesemaking

Milk from mountain areas of Western Macedonia, Greece was heated up to 50^{o}C for bactofugation

purposes (no starter culture was added), cooled to 36^{O}C and coagulated by adding commercial animal/calf rennet (CHR-Hansen, Denmark) (coagulation took place in 40-45 minutes). The same process was repeated with milk from low land areas of Western Macedonia, Greece.

Samples and isolation of enterococci

Samples of milk, curd and cheese were analyzed. Specifically, double milk samples (10 mL), curd samples and cheese samples (10 g) were homogenized with 90 mL of buffered peptone water (BPW) (Oxoid, Basingstoke, UK). Then they were diluted with the help of a Stomacher Lab- Blender 400 (Seward). The homogenization took place for about 5 minutes at 180 rpm. The homogenates were cultured at 37^{O}C for 24-48 hours, in Kanamycin-Esculin-Azide (KAA) medium from Oxoid, Basingstoke, UK. After macroscopic observation, enterococcal colonies were visually identified and five different colonies were randomly selected from each petri dish. A total of 50 colonies were selected and cultivated in the same selective medium. All colonies were examined microscopically by Gram staining, catalase production and spore staining. Gram-positive, catalase-negative, non-sporulating cocci were stored at -18^{O}C and -80^{O}C in two series, in polypropylene tubes (1.5 mL) containing their corresponding isolation medium with addition of glycerol (70:30).

Technological characterization

The technological characteristics of the 50 isolates were studied according to methods described by Durlu-Ozkaya et al. (2001), Floros et al. (2012), Georgieva et al. (2014) and Fusco et al. (2019).

For the final selection of isolations with special technological characteristics, the following criteria were applied: reduction of pH (acidification intensity at 24h and 48h), best temperature growth (mesophilic 32^{O}C and thermophilic 42^{O}C), curd formation (clot, extraction of whey (serum separation), cohesiveness, texture, intensity, virtual attributes) and specific/of aroma formation(Fusco et al., 2019).From this trial 12 strains were selected, 9 from mountainous and 3 from low land area.

The proteolytic activity of the selective strains was determined using the plate count skim milk Agar, Plate Count Agar (PCA) (Oxoid, Basingstoke, UK) with (10% w/v) sterile reconstituted skimmed milk. Activated cultures in MRS Broth at 30^{O}C for 24h were inoculated at plate and incubated at 37^{O}C for 24h/48h and at 7^{O}C for 10 days. The production of a clear halo around the colony meant positive trial (Floros et al., 2012; Georgieva et al., 2014; Fusco et al., 2019).

The lipolytic activity of the selective strains was determined using the Rosco Tributyrin Diatabs TM diagnostic tablets (Rosco, Taastrup, Denmark) according to the manufacturer's instructions. Single colonies from KAA agar were selected and suspended in 0.5 ml saline to achieve a turbidity corresponding to McFarland No. 4-5. One Tributyrin diagnostic tablet was added and the tube (Eppendorf 1.5ml) was incubated at 35-37^{O}C for 4 hours. It was also possible to read after overnight incubation. As for the enzymatic hydrolysis of tributyrin into butyric acid and glycerol the release of butyric acid lowers pH and results in a color change from red to yellow.

Phenotypic Characterization of Isolates

All strains were phenotypically characterized according to the following criteria:

Gas production from glucose; Gram reaction; Endospore formation; Catalase activity; Growthat 10^{O}C for 7 days and 45^{O}C for 48 h;growth in 6.5% and 18% NaCl at 30^{O}C for 48 h; Growth at pH 4.4 e 4.5 at 30^{O}C for 48 h; Esculin Hydrolysis (ESC); Pyrrolidonyl Aminopeptidase.

The ability to ferment carbohydrates was determined using Carbohydrates Differentiation Discs (Sigma-Aldrich, St. Louis, MO, USA) in sugar free Phenol Red Broth Base that was prepared according to the manufacturer's instructions. The isolates were identified to species level according to the methodology and characteristics given by Schillinger and Lücke (1989), Bergey's Manual of Systematic Bacteriology (2009) and Yerlikaya O. and Akbulut N. (2020).

Haemolytic activity

The haemolytic activity of Enterococcal strains was evaluated on Columbia agar with sheep blood plates (Oxoid, Basingstoke, UK) (Jurkovic et al., 2006, Ispirli et al., 2017)..

Antibiotic resistance

The antibiotic resistance testing was performed according to CLSI document M100-S30 [34] on Petri dishes with Müeller-Hinton agar (Oxoid, Basingstoke, UK) in duplicate using a Disc Diffusion Method

for the following nineteen antibiotics: Amoxicillin (AML), Ampicillin (AMP), Cefotaxime (CTX), Ciprofloxacin (CIP), Chloramphenicol (C), Clindamycin (DA), Erythromycin (E), Imipenem (IPM), Gentamicin (CN), Kanamycin (K), Nalidixin (NA), Neomycin (N), Norfloxacin (NOR), Penicillin (P), Streptomycin (S), Sulphamethoxazol/Trimethoprim (SXT), Tetracycline (TE), Trimethoprim (W) and Vancomycin (VA) Oxoid, Basingstoke, UK. All plates were held for 24 hours for accurate detection of resistance in vancomycin (CLSI document M100-S30, 2020).

All strains that exhibited resistance to Ampicillin (5) were further analyzed in Tryptic Soy Broth (Liofilchem, Roseto degli Abruzzi, Italy) by two-fold dilutions of the antibiotic from 20μg/ml to 1.25μg/ml to determine the Minimum Inhibitory Concentrations (MICs). After inoculation, the cultures were incubated at 37^{O}C for 24h. Detected MICs were evaluated according to CLSI document M100-S30 (2020) and EFSA FEEDAP Panel (2018). The vancomycin resistance of the selective strains was determined using the method of the antimicrobial substance at MRS agar containing Vancomycin (Sigma, USA) at20μg/ml final concentration.

MOLECULAR IDENTIFICATION

DNA extraction

DNA isolation was performed with the Pure Link TM Genomic DNA Extraction Kit (Life Sciences-Thermo Fisher Scientific, USA) using5 ml of overnight-grown bacterial cells.

Multiplex PCR

Identification of the isolates to genus and species level was done by multiplex PCR as described by Psomas et al. (2023).

Sequencing

The *16SrRNA* gene of the 12 *Enterococci* strains was amplified as described by Psomas et al. (2023). The PCR products were sequenced on an ABI3500 genetic analyzer. Each strain was identified by comparing the DNA sequence obtained with those in the NCBI database (Psomas et al., 2023). Furthermore, the obtained *16SrRNA* gene sequences have been submitted to NCBI and received the GenBank accession numbers showed at Table 3.

RESULTS

Technological Characterization

In total, 25 colonies of enterococci from mountain areas and 25 from low land areas were selected from the cheesemaking trials. The macroscopic identification was based on color, size, and shape. This was followed by Gram staining and the selection of Gram+ grains which were tested for catalase production and sporulation ability. Candidate enterococci were screened by biochemical tests according to Sharpe's criteria (Harrigan et al., 1976; Fusco et al., 2019). This was followed by the control of acid production in milk at 32 ^{O}C and 42 ^{O}C, the ability to form

Table 1. Technological properties of enterococci strains: Acidification ability (Clot and Aroma), Proteolysis and Lipolysis (TRB).

STAGE OF ISOLATION	MEDIA	STRAIN	ACIDIFICATION ABILITY CLOT		AROMA		PROTEOLYSIS		LIPOLYSIS (TRB)
			32^{O}C	42^{O}C	32^{O}C	42^{O}C	37^{O}C	7^{O}C	
			48h	48h	48h	48h	24h/48h	10 days	
MILK	KAA	128.2	+	+	yes	yes	-	-	+
	KAA	128.5	+	+	yes	yes	+	+	+
CURD	KAA	141.4	+	+	yes	yes	-	-	+
	RO	141.1	+	+	yes	yes	-	-	+
BEFORE SALTING	KAA	135.2	+	+	yes	yes	+	+	+
30 DAYS	KAA	160.1	+	+	yes	yes	+	+	+
	KAA	160.2	+	+	yes	yes	-	+	+
60 DAYS	KAA	169.2	+	+	yes	yes	+	+	-
	KAA	169.5	+	+	yes	yes	+	+	-
90 DAYS	KAA	177.3	+	+	yes	yes	+	+	-
	M17	177.4	+	+	yes	yes	+	-	-
	KAA	179.1	+	+	yes	yes	-	-	+

(+): presence, (-): absence

a clot, serum excretion during coagulation and finally the production and evaluation of aroma (Routray & Mishra, 2011; Georgieva et al., 2014). Aroma was assessed by a panel of six participants (Fusco et al., 2019) and all strains were characterized as producing pleasant aromas. Based on the desired technological characteristics, 12 strains were selected for identification and further study with molecular techniques (multiplex PCR and Sanger sequencing). Of the 12 strains, 2 were from raw milk, 2 from curd, 1 from curd before salting, 2 from 30-day-ripened cheese, 2 from 60-day-ripened cheese, and 3 from 90-day-ripened cheese (Table 1).

The 6 strains that showed proteolytic activity in both temperatures were isolated from raw milk from curd before salting, 30-day-ripened cheese and 90-day-ripened cheese The 4 strains that did not exhibit proteolytic activity strains were isolated from milk curd and 90-day-ripened cheese. Finally, one strain (KAA 160.2) of *Enterococcus faecalis* showed proteolytic activity at 7^{o}C (10 days), but not at 37^{o}C (24h/48h) and one strain of *Enterococcus faecium* showed proteolytic activity at 37^{o}C, but not at 7^{o}C. These strains were isolated from 30-day-ripened-cheese and 90-day-ripened-cheese, respectively.

In terms of their lipolytic activity, 8 strains were positive in the tributyrin test, i.e., they had the ability to break down tributyrin into butyric acid and glycerol originated from raw milk, curd before salting, 30-day-ripened cheese and 90-day-ripened cheese. The negative strains were isolated from 60-day-ripened and 90-day-ripened cheese.

Phenotypic Identification

As far as enterococci were concerned, when examined under the microscope, none of the strains was observed to form tetrads, a morphology characteristic of the genera *Tetragenococcus* and *Pediococcus* (Schillinger U. and Lücke F., 1987).

All isolates were tested for gas production from glucose, growth at both temperatures (10^{o}C, 45^{o}C), pH 4.4, 6.5% NaCl, 18% NaCl, bile esculin, hydrolyze esculin and vancomycin-modified MRS medium.

Isolates from the KAA agar medium didn't produce gas from glucose and showed a positive growth test at 10^{o}C and 45^{o}C. All isolates were unable to grow in pH 4.4 and in the presence of 18% NaCl, while they were able to grow in the presence of 6.5% NaCl and hydrolyze esculin.

According to Schillinger U. and Lücke F. (1987), all the tested strains belong to the genus *Enterococcus*. From the 12 *Enterococcus spp*. strains four have been identified as *E. faecalis*, seven as *E. faecium* and one as *E. hirae*, isolated from different samples: milk, curd, curd before salting, 15 days, 30 days, 60 days and 90 days (Table 2).

As for the haemolycin test five strains (KAA 141.1, RO 141.1, KAA 172.4, M17 177.4 and KAA 179.1) a clear zone of hydrolysis around the colonies was observed (b haemolysis).

Table 2. Identification of isolated enterococci after biochemical and molecular analysis.

STAGE OF ISOLATION	MEDIA	STRAIN	PHENOTYPIC BIOCHEMICAL RESULTS	PCR RESULTS	SEQ RESULTS	GENBANK ACCESION NUMBERS
MILK	**KAA**	**128.2**	***E. faecalis***	***E. faecalis***	***E. faecalis***	OR098611
	KAA	**128.5**	***Enterococcus spp.***	***E.hirae***	***E.hirae***	**OR098612**
CURD	**KAA**	**141.4**	***E. faecalis***	***E. faecalis***	***E. faecalis***	OR098619
	RO	**141.1**	***Enterococcus spp.***	***E. faecium***	***E. faecium***	OR098622
BEFORE SALTING	**KAA**	**135.2**	***Enterococcus spp.***	***E. faecium***	***E. faecium***	OR098613
30	**KAA**	**160.1**	***Enterococcus spp.***	***E. faecium***	***E. faecium***	OR098614
DAYS	**KAA**	**160.2**	***Enterococcus spp.***	***E. faecalis***	***E. faecalis***	OR098615
60	KAA	169.2	***Enterococcus spp.***	***E. faecium***	***E. faecium***	OR098616
DAYS	**KAA**	**169.5**	***Enterococcus spp.***	***E. faecium***	***E. faecium***	OR098617
90	**KAA**	**177.3**	***Enterococcus spp.***	***E. faecium***	***E. faecium***	OR098618
DAYS	**M17**	**177.4**	***Enterococcus spp.***	***E. faecium***	***E. faecium***	OR098621
	KAA	**179.1**	***E. faecalis***	***E. faecalis***	***E. faecalis***	OR098620

Antibiotic resistance

All isolates were screened for resistance/sensitivity to selected 19 antibiotics (Amoxycillin (AML), Ampicillin (AMP), Cefotaxine (CTX), Ciprofloxacin (CIP), Chloramphenicol (C), Clindamycin (DA), Erythromycin (E), Imipenem (IPM), Gentamicin (CN), Kanamycin (K), Nalidixin (NA),Neomycin (N), Norfloxacin (NOR), Penicillin (P), Streptomycin (S), Suphamethoxazole/ Trimethoprim (SXT), Tetracycline (TE), Trimethoprim (W) and Vancomycin (VA) using a Disc Diffusion Method. MICs verified the sensitivity/resistance of the Disk Diffusion Method. Finally, as for the ampicillin resistance and according to the safety rules of EFSA all strains were safe (EFSA FEEDAP Panel, 2018).

The analytical results are shown in Table 3.

Amoxycillin (AML), Ampicillin (AMP), Cefotaxine (CTX), Ciprofloxacin (CIP), Chloramphenicol (C), Clindamycin (DA), Erythromycin (E), Imipenem (IPM), Gentamicin (CN), Kanamycin (K), Nalidixin (NA), Neomycin (N), Norfloxacin (NOR), Penicillin (P), Streptomycin (S), Suphamethoxazole/ Trimethoprim (SXT), Tetracycline (TE), Trimethoprim (W), Vancomycin (VA), Kanamycin-Esculin-Azide Agar medium (KAA), Rogosa Agar medium (RO)

According to the Disc Diffusion Method, one strain of *E. faecium* (8.3%) and one strain of *E. faecalis* (8.3%) were resistant to Vancomycin (10μg/ml) and all other strains (83.3%) were sensitive. The above strains analyzed by MIC in Vancomycin and only strain *E. faecalis* was resistant up to 20μg/ml.

In general, six strains (50%) appeared resistance to antibiotics. The predominant antibiotic resistance was in Ampicillin with four strains of *E. faecium* (75%) being resistant. Two strains of *E. faecium* (28.6%) and one of *E. faecalis* (25%) were resistant to Erythromycin and one strain of *E. faecalis* (25%) was resistant to Imipenem. As for the multi-resistant two strains of *E. faecium* (28.6%) appeared resistance to two antibiotics (AMP, E) and one strain *E. faecalis* (25%) resistance to IMP, VA.

As for the sensitivity that could be based on deletions or/and silent genes, one strain of *E. faecalis* (25%) and one of *E. faecium* (14.3%) were sensitive in four antibiotics AML, CIP, C, SXT and CIP, C, N, SXT, respectively. Moreover, two strains of *E. faecium* (16.7%), one strain of *E. faecalis* (25%) and the unique strain of *E. hirae* were sensitive in two antibiotics. Finally, three strains of *E. faecium* (42.9%) and two strains of *E. faecalis* (50%) were sensitive to one antibiotic.

Multiplex PCR- Sequencing

The multiplex PCR identified the 12 strains shown at Table 2 to genus and species level that was verified with the sequencing of their *16SrRNA* gene, presenting a very high homology with the deposited sequences in NCBI database. The aforementioned results are in accordance with the phenotypic identification. All 12 strains were identified as *Enterococcus spp*. and more specifically, four strains have been identified as *E. faecalis*, seven strains as *E. faecium* and one strain as *E. hirae.*

DISCUSSION

As already mentioned, enterococci are included in

Table 3. Differentiation of the strains in antimicrobial sensitivity/resistance.

Strains	Deletions or silent genes	Acquired resistance
128.2KAA, *E. faecalis*	TE	IPM, VA (up to 20μg/ml)
128.5KAA, *E. hirae*	AML, NOR	
135.2KAA, *E. faecium*	NA	AMP
141.4KAA, *E. faecalis*	AML, C	
141.1RO, *E. faecium*	TE	-
160.1KAA, *E. faecium*	-	AMP, E
160.2KAA, *E. faecalis*	AML	E
169.2KAA, *E. faecium*	CTX, N	AMP, E
169.5KAA, *E. faecium*	CIP, C, N, SXT	AMP
177.3KAA, *E. faecium*	AML	
177.4M17, *E. faecium*		VA (up to 10μg/ml)
179.1KAA, *E. faecalis*	AML, CIP, C, SXT	

increased populations among the indigenous species of Lactic Acid Bacteria (LAB), especially in cheeses. It is known that thanks to their glycolytic, proteolytic and lipolytic activity they effectively determine the flavor of cheeses (Gomes et al., 2008). The metabolism of citrates and the production of aromatic compounds in cheese ripening is due to the action of LAB and especially to the action of enterococci (Terzic-Vidojevic et al. 2021). It is obvious that thanks to these technological properties enterococci can be used not only as starter cultures in the food industry but also as bacteria with possible probiotic use. Based on the above, enterococci would be an ideal tool for enhancing the taste and aroma of cheese only if a case-by-case isolates analysis could ensure that they are not carriers of virulence and antimicrobial resistance genes. We have already mentioned that a wide discussion has opened about the cheese ecosystem as a possible reservoir of antibiotic resistance genes that are transferred to human strains in the gastrointestinal tract.

It is precisely this dual aspect of enterococci that represent a challenge for dairy technology and a large field for future research. It is very important to be able to ensure the presence of isolates who will possess the unique technological characteristics and at the same time ensure that they will not pose even the slightest possible risk to public health. Therefore, it is of great importance to analyze enterococcal strains and determine the parameters that will ensure that they are safe for future use in the food industry either as starters or as probiotics. It is promising that no report of enterococcal infection has been found from the use of enterococci as probiotics. However, a case-by-case analysis of enterococcal strains to be used in the food industry is necessary to ensure that they do not carry virulence and antibiotic resistance genes (Erginkaya et al., 2018).

In our study, all tested strains produced pleasant aroma, and this could be attributed to the ability of enterococci strains to utilize citrate and pyruvate as the sole carbon source. This is in accordance with similar observations regarding several enterococci strains (Giménez-Pereira, 2005; Graham et al., 2020).Sarantinopoulos et al. (2001) showed that *E. faecalis*, *E. faecium*, and *E. durans* strains could only use citrate and pyruvate as carbon sources (Sarantinopoulos et al., 2001).

The fact that eight out of twelve strains exhibited lipolytic activity confirms the results of other authors (Durlu-Ozkaya et al., 2001; Sarantinopoulos et al., 2001; Graham et al. 2020). More specifically, according to Sarantinopoulos et al. (2001), *E. faecalis* strains had the highest lipolytic activity, followed by *E. durans*, while *E. faecium* strains had the lowest lipolytic activity.

Regarding acid production, all tested strains exhibited milk acidifying ability at 32ºC and 42ºC, as it was shown for enterococci from previous publications (Suzzi et al., 2000; Sarantinopoulos et al., 2001; Giraffa, 2003; Aspri et al., 2017). The above authors also concluded that strains of E. faecalis with food origin were those that produced the most acid. Moreover, the fact that seven strains did not exhibit haemolytic activity is very promising. The findings by Gomes et al. (2008), who discovered that *E. faecalis* strains (38.7%) were the dominating species to cause -hemolysis, were in line with these findings (Gomes et al., 2008).

A very important parameter for the possible use of enterococci in the food industry is the emergence of antibiotic-resistant strains of enterococci. The possibility that antimicrobial resistance genes can be carried by enterococcal strains found in food, mainly in the gastrointestinal tract of humans, raises public health concerns. In several studies over the past twenty years, resistance to antibiotics such as penicillin, ampicillin, streptomycin and vancomycin has been limited (Franz et al., 2003). Although in Greece dairy sheep and goat farming is an important sector and a percentage of 90% of the produced milk is used for cheese production, the usage of antibiotics on the farms even in cases of infections or in preventing measures have not been extensively studied. The most frequently used antibiotics in cases of infections are oxytetracycline, penicillin, streptomycin and amoxicillin (Lianou and Fthenakis, 2022). However, according to the CLSI document M100-S30, 2020, penicillin or ampicillin resistance among enterococci due to β-lactamase production has been reported very rarely. In recent years the appearance of vancomycin-resistant strains and strains with multiple resistance to antibiotics has been noted, causing concern. In general, food-borne VRE (vancomycin-resistant enterococci) have a lower rate of virulence factor than those of medical-origin VRE isolates (Erginkaya et al., 2018). The fact that only one out of twelve strains from the tested dairy enterococci was resistant to vancomycin up to 20μg/ml, and one strain was resistant to vancomycin up to 10μg/ml but not to 20μg/ml is

in accordance with published data on the results of extensive use of antibiotics in livestock production (Terzic-Vidojevic et al., 2021). The resistance in the above antibiotics could be explained as a phenomenon of interactions between animals, or farmers and animals, of the everyday farming activities, between the physical environments. Moreover, Vanden Eng et al. (2003) pointed out that people who generally live in farming regions appeared to have the higher antibiotic consumption.

CONCLUSIONS

It is generally accepted that the use of enterococci in food industry or as probiotic cultures could be a dilemma related to the food safety. We consider it an advantage that enterococci are involved with a positive role in most traditional food fermentations studied so far, and usually most strains are considered opportunistic rather than pathogenic. This is reinforced by the fact that the morbidity of healthy people resulting from enterococcal infections appears to be very low. Also important is the fact that in the food industry many strains have a long history of safe use and large-scale commercial application (Franz et al., 2003; Yerlikaya and Akbulut, 2020; Terzic-Vidojevic et al., 2021).

It is very important that 12 strains of *Enterococcus* spp. were isolated, exhibiting excellent technological properties and limited antibiotic resistance. Two of those were found to be sensitive to vancomycin up to 10μg/ml and nine up to 20μg/ml and therefore are even more promising, in terms of their safety, for their potential use as adjunct cultures and worth further analysis.

In closing, we can conclude that enterococci cannot be characterized as exclusively safe or dangerous for public health. The key answer is the analysis for each strain, and this requires a large number of in-vitro and in-vivo tests so as to ensure that the particular strain of enterococci is correct and at the same time has the appropriate technological properties. Only after meeting these two conditions, it can be used in the production of fermented dairy foods.

CONFLICT OF INTEREST

None declared

ACKNOWLEDGMENTS

In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

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