International Journal of Food Microbiology 236 (2016) 107–114

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses

Raimondo Gaglio [a,b], Natacha Couto [b], Cátia Marques [b], Maria de Fatima Silva Lopes [c], Giancarlo Moschetti [a], Constança Pomba [b], Luca Settanni [a,*]

[a] Dipartimento Scienze Agrarie e Forestali, Università di Palermo, Viale delle Scienze 4, 90128 Palermo, Italy
[b] Laboratory of Antimicrobial and Biocide Resistance, Faculty of Veterinary Medicine, University of Lisbon (FMV-UTL), Lisboa, Portugal
[c] ITQB-Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal

ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 12 July 2016
Accepted 13 July 2016
Available online 15 July 2016

Keywords:
Animal rennet
Antimicrobial resistance
Enterococcus
Raw milk
Traditional cheese
Virulence
Wooden vat

ABSTRACT

Forty enterococci isolated along the production chains of three traditional cheeses (PDO Pecorino Siciliano, PDO Vastedda della Valle del Belìce, and Caciocavallo Palermitano) made in Sicily (southern Italy) were studied for the assessment of their antibiotic resistance and virulence by a combined phenotypic/genotypic approach. A total of 31 *Enterococcus* displayed resistance to at least one or more of the antimicrobials tested. The strains exhibited high percentages of resistance to erythromycin (52.5%), ciprofloxacin (35.0%), quinupristin–dalfopristin (20.0%), tetracycline (17.5%), and high-level streptomycin (5.0%). The presence of *tet*(M), *cat*(pC221), and *aad*E genes for resistance to tetracycline, chloramphenicol, and streptomycin, respectively, was registered in all strains with resistance phenotype. The *erm*(B) gene was not detected in any erythromycin-resistant strain. The *Enterococcus* strains were further tested by PCR for the presence of virulence genes, namely, *gel*E, *asa1*, *efaA*, *ace*, and *esp*. Twenty strains were positive for all virulence genes tested. Among the enterococci isolated from final cheeses, three strains (representing 33.3% of total cheese strains) were sensible to all antimicrobials tested and did not carry any virulence factor. Although this study confirmed that the majority of dairy enterococci are vectors for the dissemination of antimicrobial resistance and virulence genes, only two strains showed a high resistance to aminoglycosides, commonly administered to combat enterococci responsible for human infections. Furthermore, the presence of the strains *E. casseliflavus* FMAC163, *E. durans* FMAC134B, and *E. faecium* PON94 without risk determinants, found at dominating levels over the *Enterococcus* populations in the processed products, stimulates further investigations for their future applications in cheese making. All strains devoid of the undesired traits were isolated from stretched cheeses. Thus, this cheese typology represents an interesting environment to deepen the studies on the risk/benefit role of enterococci in fermented foods for their qualified presumption of safety (QPS) assessment.

1. Introduction

Enterococci belong to the group of lactic acid bacteria (LAB). The genus *Enterococcus* includes pathogenic, spoilage, and pro-technological bacteria. Members of this group are ubiquitous microorganisms that often occur at large numbers in foods, especially those of animal origin (Francesca et al., 2013; Franz et al., 1999; Giraffa and Sisto, 1997; Hugas et al., 2003). The presence of these bacteria in dairy products is usually associated with inadequate hygiene practices as a consequence of fecal contamination (Franciosi et al., 2009a; Suzzi et al., 2000). However, the Commission Regulation (EC) No 1441/2007 of 5 December 2007 allows derogation from Regulation (EC) No 2073/2005 of 15 November 2005 'on microbiological criteria for foodstuffs' declaring that enterococci in food are not always due to fecal contamination and sets no limit for their presence in foods (Commission Regulation, 2007). Enterococci play several positive roles during the fermentation of cheese and meat products; they are defining in the development of the organoleptic characteristics that the food acquire with ripening (Centeno et al., 1996; Cocolin et al., 2007; Foulquié Moreno et al., 2006; Giraffa and Sisto, 1997) and contribute to extend their shelf life. To this purpose, *Enterococcus* of dairy origin have been reported to produce bacteriocins able to inhibit food spoilage and/or pathogenic bacteria (Foulquié Moreno et al., 2006). Different enterococci are being used as components of cheese adjunct cultures (Settanni et al., 2013) or as probiotics (Franz et al., 2011; Giraffa, 2002).

On the other hand, enterococci have assumed a major importance in clinical microbiology because they are intrinsically resistant to many antimicrobial agents and show the ability to acquire, accumulate, and transfer chromosomal elements encoding virulence traits or

* Corresponding author.
E-mail address: luca.settanni@unipa.it (L. Settanni).

http://dx.doi.org/10.1016/j.ijfoodmicro.2016.07.020
0168-1605/

antimicrobial resistance genes (Klibi et al., 2006; Pesavento et al., 2014; Silva et al., 2010). Some studies have reported the detection of antimicrobial resistance and virulence factors of enterococci in retail foods including cheeses (Hammad et al., 2015; Koluman et al., 2009).

The most frequent species belonging to the *Enterococcus* genus found in dairy products are *Enterococcus faecium* and *Enterococcus faecalis* (Aarestrup et al., 2002) as well as *Enterococcus casseliflavus*, *Enterococcus durans*, and *Enterococcus gallinarum* (Franciosi et al., 2009a; Gaglio et al., 2014a; Settanni et al., 2012). *Enterococcus faecium* and *E. faecalis* might represent a public health issue for their resistance to cephalosporins, lincosamides, penicillins, and low levels of aminoglycosides (Hammad et al., 2015). Enterococci isolated from the dairy products also express a similar virulence gene profile as those associated with human infections (Gelsomino et al., 2003; Semedo et al., 2003).

Enterococci are commonly present in raw milk (Franciosi et al., 2009b) and this highlights the importance to focus the attention also on the raw materials used in cheese making and the equipment that contaminate the bulk milk. Traditional Sicilian cheeses are often manufactured with raw milk coagulated with artisanal animal rennet in wooden equipment without the addition of starter cultures (Settanni and Moschetti, 2014). Some of these cheeses are produced applying the stretching technology consisting of two distinct steps, the first leading to a plastic curd and the second to the scalding of the acidified curd to be molded into the final shape. The stretching phase at high temperatures contributes to the safety of the resulting products (Gaglio et al., 2014b). So far, *Enterococcus* isolated from stretched cheeses, typical of the Mediterranean countries, have not been investigated deeply for their antibiotic resistance and virulence.

As a matter of fact, the enterococci present in cheese can be a possible intermediate vehicle for the transmission of multidrug resistance and/or virulent strains able to persist in the human intestinal tract (Jamet et al., 2012; Kayser, 2003; Novais et al., 2005). For these reasons, the present work was performed to evaluate the antimicrobial resistance and virulence of a collection of *Enterococcus* spp. isolated from different Sicilian dairy environments, including raw milk, animal rennet, fresh and aged cheeses, and the wooden equipment used for milk transformation. In order to investigate the possible role of the cheese making technology of the *Enterococcus* selection, several strains from stretched cheeses were included in this study.

2. Materials and methods

2.1. Enterococcus strains

In this study, a collection of 40 enterococci isolated along the production chains of traditional cheeses made in Sicily (southern Italy) and belonging to the culture collection of the Agricultural Microbiology laboratory of the Department of Agricultural and Forest Science—University of Palermo (Palermo, Italy), was analyzed. The 40 enterococci, identified by PCR method, represent 40 different strains collected from different dairy environments, including the wooden equipment, raw milk, animal rennet used for milk curdling, fresh and ripened cheeses (Table 1). All strains were grown on M17 (Oxoid, Milan, Italy) at 37 °C for 24 h.

2.2. Antimicrobial susceptibility

The 40 *Enterococcus* strains were tested for their antimicrobial susceptibility by the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). The inocula were prepared by suspending colonies in 5 mL of physiological solution (0.85% NaCl, w/v) until a density of 0.5 McFarland standard was reached. The cell suspensions were swabbed for confluent growth onto Mueller Hinton agar (Oxoid, Hampshire, UK). Twelve antimicrobial compounds commonly used for the treatment of human and animal infections were tested. The antimicrobial belonged to different families: penicillins [penicillin (P—10 units) and ampicillin (AMP—10 μg)]; glycopeptides [vancomycin (VA—30 μg)]; macrolides [erythromycin (E—15 μg)]; tetracyclines [tetracycline (TE—30 μg)]; fluoroquinolone [ciprofloxacin (CIP—5 μg) and levofloxacin (LEV—5 μg)]; phenicols [chloramphenicol (C—30 μg)]; streptogramins [quinupristin–dalfopristin (QD—15 μg)]; oxazolidinones [linezolid (L—30 μg)]; and aminoglycosides [high-level gentamicin (CN—120 μg) and high-level streptomycin (STR—300 μg)].

After incubation at 37 °C for 18 h, the inhibition halos were measured and the strains classified as resistant (R), intermediate resistant (IR), or susceptible (S) according to the CLSI (CLSI, 2015).

The minimum inhibitory concentration (MIC) was determined for each IR or R strain on a given antimicrobial. MICs were determined by the broth microdilution method according to the CLSI (CLSI, 2015). *Enterococcus faecalis* ATCC 29212 was used as quality control strain.

All antimicrobial compounds were purchased from Oxoid.

2.3. Phenotype method for gelatinase and hemolysin production

Gelatinase production was determined by depositing a drop of each *Enterococcus* culture on a plate containing Gelatin Agar as described by Lopes et al. (2006). Hemolytic activity was assessed by streaking the cultures onto Columbia blood agar supplemented with 5% (v/v) horse blood (Becton Dickinson) and incubated at 37 °C for 24–48 h, under anaerobic condition (Gaspar et al., 2009).

The hemolytic reactions were classified as total or β-hemolysis (clear zone of hydrolysis around the colonies), partial or α-hemolysis (green halo around the colonies) and absent or γ-hemolysis.

Each test was performed in duplicate.

2.4. DNA extraction and molecular approach

The DNA for molecular analyses was extracted following the methodology described by Ruzauskas et al. (2015). The presence of antimicrobial resistance genes was investigated on the IR and R strains by PCR. The genes investigated were *erm*(A), *erm*(B), *erm*(C) for resistance to macrolide, lincosamides, and streptogramins B; *msr*(A) and *mph*(C) for resistance to macrolide and streptogramins B; *tet*(K), *tet*(M) for resistance to tetracycline; $cat_{(pC221)}$ for resistance to chloramphenicol; *aad*A and *aad*E for resistance to streptomycin; *van*A and *van*B for resistance to vancomycin.

The presence of the genes involved in the expression of virulence traits for aggregation *gelE* (gelatinase), *asa1* (aggregation substance), *efaA* (endocarditis antigen), *ace* (adhesion of collagen), and *esp* (enterococcal surface protein) was also investigated by PCR.

The primers used for PCRs are reported in Table 2.

2.5. Statistical and explorative multivariate analyses

An explorative multivariate analysis was employed to investigate the relationship among strains. A hierarchical cluster analysis (HCA) (joining, tree clustering) was carried out for grouping the strains according to their dissimilarity, measured by Euclidean distances, whereas cluster aggregation was based on the Ward's method (Martorana et al., 2015; Todeschini, 1998).

The input matrix used for HCA consisted of phenotypical (antimicrobial resistance, MIC, gelatinase, and hemolysis activities) and genotypic (antimicrobial resistance and virulence genes) characteristics of strains.

Statistical data processing and graphic construction were achieved by using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Antimicrobial susceptibility and MIC determination

The prevalence of antimicrobial resistance with regards to species and source of isolation of the 40 strains is shown in Table 3. The

Table 1
Origin of the *Enterococcus* strains used in this study.

Strain	Species	Origin	Reference
PON82	*E. gallinarum*	PDO Vastedda della valle del Belìce cheese[a]	Gaglio et al. (2014a)
PON85	*E. faecalis*	PDO Vastedda della valle del Belìce cheese[a]	Gaglio et al. (2014a)
PON94	*E. faecium*	PDO Vastedda della valle del Belìce cheese[a]	Gaglio et al. (2014a)
PON111	*E. faecium*	PDO Vastedda della valle del Belice cheese[a]	Gaglio et al. (2014a)
PSL68	*E. faecium*	PDO Pecorino Siciliano cheese[b]	Todaro et al. (2011)
MOB6	*E. faecalis*	Wooden vat surfaces	Settanni et al. (2012)
FMA8	*E. faecalis*	Bovine bulk milk	Settanni et al. (2012)
FMA288	*E. gallinarum*	Wooden vat surfaces	Settanni et al. (2012)
FMA444	*E. faecalis*	Bovine bulk milk	Settanni et al. (2012)
FMA463	*E. faecalis*	Bovine bulk milk	Settanni et al. (2012)
FMA604	*E. faecalis*	Bovine bulk milk	Settanni et al. (2012)
FMA713	*E. faecalis*	Bovine bulk milk	Settanni et al. (2012)
FMA721	*E. faecalis*	Wooden vat surfaces	Settanni et al. (2012)
CGLBL109	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL115	*E. faecalis*	Animal rennet	Cruciata et al. (2014)
CGLBL118	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL139	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL146	*E. faecalis*	Animal rennet	Cruciata et al. (2014)
CGLBL186	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL188	*E. faecalis*	Animal rennet	Cruciata et al. (2014)
CGLBL203	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL204	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL213	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL221	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL225	*E. faecium*	Animal rennet	Cruciata et al. (2014)
CGLBL274	*E. faecium*	Animal rennet	Cruciata et al. (2014)
FMAC98	*E. casseliflavus*	Caciocavallo Palermitano cheese[c]	Di Grigoli et al. (2015)
FMAC134B	*E. durans*	Caciocavallo Palermitano cheese[c]	Di Grigoli et al. (2015)
FMAC163	*E. casseliflavus*	Caciocavallo Palermitano cheese[c]	Di Grigoli et al. (2015)
FMAC219	*E. faecalis*	Caciocavallo Palermitano cheese[c]	Di Grigoli et al. (2015)
WVS1	*E. faecium*	Wooden vat surfaces (cows' cheese)	Scatassa et al. (2015)
WVS31	*E. faecium*	Wooden vat surfaces (cows' cheese)	Scatassa et al. (2015)
WVS53	*E. faecalis*	Wooden vat surfaces (cows' cheese)	Scatassa et al. (2015)
WVS231	*E. faecium*	Wooden vat surfaces (cows' cheese)	Scatassa et al. (2015)
WVS296	*E. faecalis*	Wooden vat surfaces (cows' cheese)	Scatassa et al. (2015)
WVS356	*E. faecalis*	Wooden vat surfaces (ewes' cheese)	Scatassa et al. (2015)
WVS388	*E. faecium*	Wooden vat surfaces (ewes' cheese)	Scatassa et al. (2015)
WVS426	*E. faecalis*	Wooden vat surfaces (ewes' cheese)	Scatassa et al. (2015)
WVS439	*E. faecium*	Wooden vat surfaces (ewes' cheese)	Scatassa et al. (2015)
WVS442	*E. faecalis*	Wooden vat surfaces (cows' cheese)	Scatassa et al. (2015)

[a] Fresh raw ewes' milk cheese.
[b] Ripened raw ewes' milk cheese.
[c] Ripened raw cows' milk cheese.

frequency of resistance resulted as follows: 21 strains for erythromycin, 14 strains for ciprofloxacin, 8 strains for quinupristin-dalfopristin, 7 strains for tetracycline, 2 strains for streptomycin, and 1 strain for chloramphenicol. No resistance was observed for penicillin, ampicillin, vancomycin, levofloxacin, linezolid, and high level of gentamicin. A total of 31 *Enterococcus* out of 40 strains displayed resistance to at least one antimicrobial compound. Three strains exhibited a multidrug-resistant phenotype (resistance to at least three antimicrobials). In particular, *E. faecalis* FMAC219 was resistant to chloramphenicol/quinupristin–dalfopristin/streptomycin, *E. faecium* CGLBL118 to erythromycin/tetracycline/ciprofloxacin, and *E. faecalis* CGLBL188 to erythromycin/ciprofloxacin/quinupristin–dalfopristin. Resistance to erythromycin was detected in only one *E. faecalis* (WWS442) strain and four *E. faecium* (PSL68, WVS231, WVS439, and CGLBL274) strains. Resistance to quinupristin–dalfopristin was detected in *E. faecalis* FMA463, FMA604, FMA721, and CGLBL146. *E. faecalis* FMA444, WVS356, and CGLBL115 showed resistance to tetracycline. The resistance profiles of enterococci are shown in Table 4.

On the base of the preliminary results obtained by the disk diffusion assay, a total of 27 enterococci characterized for their R or IR behavior were subjected to the MIC determination by microdilution assay (Table 4). MIC values confirmed the classification resulted from the disk diffusion assay, because the strains classified as resistant by the first technique were also found to be resistant by MIC.

3.2. Detection of antimicrobial resistance gene by PCR

All R and IR enterococci were screened for the presence of the antimicrobial resistance genes most commonly reported in enterococci and the results are reported in Table 4.

All seven strains resistant to tetracycline in the phenotypic assay revealed the presence of *tet*(M) gene. None of these strains carried the *tet*(K) gene. The *aad*E gene was found in both strains showing high-level resistance to streptomycin, while the $cat_{(pC221)}$ gene, associated with chloramphenicol resistance, was found in *E. faecalis* FMAC219. None of the erythromycin resistance genes tested was detected. Although none of the strains showed resistance to vancomycin, the presence of the genes associated with the resistance to this antimicrobial was investigated, but they were all negative.

3.3. Virulence activity and related genes

The results of gelatinase and hemolytic activity assay of the 40 enterococci of dairy origin analyzed in this study are reported in Table 4. Only six *E. faecalis* (FMA8, FMA463, FMA713, FMAC219, WVS356, and WVS426) strains showed a positive gelatinase reaction, while β-hemolytic activity was barely detected in two strains of *E. faecalis* (FMA288 and FMA444), two strains of *E. gallinarum* (PON82 and FMA288), and in one strain of *E. casseliflavus* (FMAC98).

Table 2
Primers used for PCR reactions carried out in this study.

Gene	Primer name	Oligonucleotide sequence (5′–3′)	Reference
erm(A)	Tn554-1	AAGCGGTAAAACCCCTCTGAG	Jensen et al. (2002)
	Tn554-2	TCAAAGCCTGTCGGAATTGG	
erm(B)	Erm(B)-1	CATTTAACGACGAAACTGGC	Jensen et al. (2002)
	Erm(B)-2	GGAACATCTGTGGTATGGCG	
erm(C)	Erm(C)-1	ATCTTTGAAATCGGCTCAGG	Jensen et al. (2002)
	Erm(C)-2	CAAACCCGTATTCCACGATT	
msr(A)	msrA-F	GCTTAACATGGATGTGG	Perreten et al. (2005)
	msrA-R	GATTGTCCTGTTAATTCCC	
mph(C)	mphC-F	CATTGAATGAATCGGGAC	Perreten et al. (2005)
	mphC-R	TTCATACGCCGATTCTCC	
tet(K)	tetK-1	TTAGGTGAAGGGTTAGGTCC	Aarestrup et al. (2000)
	tetK-2	GCAAACTCATTCCAGAAGCA	
tet(M)	tetM-F	GTTAAATAGTGTTCTTGGAG	Aarestrup et al. (2000)
	tetM-R	CTAAGATATGGCTCTAACAA	
cat(pC221)	catpC221-F	ATTTATGCAATTATGGAAGTTG	Schnellmann et al. (2006)
	catpC221-R	TGAAGCATGGTAACCATCAC	
*aad*A	AadAf	GCAGCGCAATGACATTCTTG	Sáenz et al. (2004)
	AadAr	ATCCTTCGGCGCGATTTTG	
*aad*E	ant(6)-IF	CGGGAGAATGGGAGACTTTG	Kobayashi et al. (2001)
	ant(6)-IR	CTGTGGCTCCACAATCTGAT	
*Van*A	VanAf	GGAAAACGACAATTGCTATT	DANMAP (2008)
	VanAr	GTACAATGCGGCCGTTA	
*Van*B	VanBf	ATCGGCCTACATTCTTACA	DANMAP (2008)
	VanBr	AGCGTTTAGTTCTTCCGT	
gelE	GEL 11	TATGACAATGCTTTTTGGGAT	Vankerckhoven et al. (2004)
	GEL 12	AGATGCACCCGAAATAATATA	
ace	ace1f	GAATTGAGCAAAAGTTCAATCG	Martín-Platero et al. (2009)
	ace1r	GTCTGTCTTTTCACTTGTTTC	
asa1	asa1f	CCAGCCAACTATGGCGGAATC	Creti et al. (2004)
	asa1r	CCTGTCGCAAGATCGACTGTA	
efaA	EFA-AF	GCCAATTGGGACAGACCCTC	Creti et al. (2004)
	EFA-AR	CGCCTTCTGTTCCTTCTTTGGC	
esp	esp-F	TTGCTAATGCTAGTCCACGACC	Eaton and Gasson (2001)
	esp.-R	GCGTCAACACTTGCATTGCCGAA	

Twenty enterococci had virulence genes. All *E. faecalis* strains were positive to at least one virulence factor. The presence of multiple virulence factors was detected more frequently in *E. faecalis* than in *E. faecium*. Specifically, the *gelE* gene was detected in 15 strains (37.5%), the *asa1* in 19 (47.5%), the *efaA* in 10 (25.0%), the *ace* in 11 (27.5%), and the *esp* in 8 (20.0%). Nineteen enterococci had multiple virulence factors. *E. faecium* CGLBL203 possessed only the *gelE* gene. Moreover, nine of the *gelE* positive strains did not express gelatinase activity.

3.4. Multivariate statistical analysis

HCA classified the strains in accordance to their mutual dissimilarity and relationship (Fig. 1) by using a total of 35 variables, including susceptibility to antimicrobials, presence of genes for antibiotic resistance and virulence, gelatinase activity, hemolysis type, and MICs. The 40 strains were clearly separated into two main groups (mega-clusters A and B). The mega-cluster B included all strains resistant to antibiotics and positive for virulence factors. The components of this cluster were all strains from milk, the majority of those from the wooden vats, some from animal rennets, and only two strains from cheeses. The mega-cluster A included only two β-hemolytic strains (*E. casseliflavus* FMAC98 and *E. gallinarum* PON82) and 19 non-virulent enterococci. Specifically, most of rennet strains, some from wooden vats, and two strains from cheese were antibiotic resistant but not virulent, two cheese strains were virulent but antibiotic sensible, and, interestingly, three strains (*E. casseliflavus* FMAC163, *E. durans* FMAC134B, and *E. faecium* PON94) were susceptible to antibiotics and negative for the presence of virulence factors. Thus, the risk factors of the enterococci studied were correlated to the source of isolation.

4. Discussion

Enterococci are omnipresent in several traditional fermented foods. These bacteria are responsible for typicality, but they are also involved in safety issues, mainly associated with antimicrobial resistance and virulence characters. Thus, members of *Enterococcus* genus are bacteria with a contrasting role in cheese for their risk/benefit aspects. In this study, a collection of 40 strains of dairy origin was characterized phenotypically and genotypically in order to explore their safety aspects. Contrarily to previous studies (Martín-Platero et al., 2009; Nieto-Arribas et al., 2011; Morandi et al., 2015) carried out on the characterization of the antibiotic resistance and virulence determinants of cheese *Enterococcus*, our collection was composed of strains isolated along the entire production chain of three traditional raw milk cheeses. In this manner, we investigated not only the enterococci from final (fresh and ripened) cheeses but also those from raw materials (bulk milks and animal rennets) and from the contaminating sources (wooden surfaces of the vats used from milk transformation). To our knowledge, this is the first work aimed to analyze the antibiotic resistance and the virulence of *Enterococcus* from stretched (cows' and ewes' milk) cheeses.

Enterococci, especially *E. faecium* and *E. faecalis*, are commonly present in milk products from different countries (Nieto-Arribas et al., 2011; Suzzi et al., 2000). They are also often detected on the wooden equipment used for traditional cheese making (Scatassa et al., 2015) and in the animal rennet necessary for milk curdling during the production of these kind of cheeses (Cruciata et al., 2014), but the scientific knowledge lacks of information on the antibiotic resistance and virulence of *Enterococcus* colonizing the wooden vat biofilms and transferred to cheese by the animal rennets.

The members of the *Enterococcus* genus generally possess a broad spectrum of natural antimicrobial resistances including resistance to cephalosporins, polymyxines, low concentrations of aminoglycosides,

Table 3
Percentage of antimicrobial resistance of the enterococcal isolates (reported in brackets) from different dairy environments.

Antibiotics[a]	Bulk milk	Animal rennet			Wooden vat surfaces				PDO Vastedda della valle del Belice cheese				PDO Pecorino Siciliano cheese	Caciocavallo Palermitano cheese			Total strains
	E. faecalis (5)	*E. faecium* (10)	*E. faecalis* (3)	Total (13)	*E. faecium* (5)	*E. faecalis* (7)	*E. gallinarum* (1)	Total (13)	*E. faecium* (2)	*E. faecalis* (1)	*E. gallinarum* (1)	Total (4)	*E. faecium* (1)	*E. faecalis* (1)	*E. durans* (1)	*E. casseliflavus* (2)	Total (4)	(40)
P	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AMP	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
E	0.0	100.0 (10)	33.3 (1)	84.6 (11)	100.0 (5)	42.9 (3)	0.0	61.5 (8)	50.0 (1)	0.0	0.0	25.0 (1)	100.0 (1)	0.0	0.0	0.0	0.0	52.5 (21)
TE	20.0 (1)	10.0 (1)	33.3 (1)	15.4 (2)	0.0	42.9 (3)	0.0	23.1 (3)	0.0	100.0 (1)	0.0	25.0 (1)	0.0	0.0	0.0	0.0	0.0	17.5 (8)
CIP	0.0	90.0 (9)	33.3 (1)	76.9 (10)	60.0 (3)	0.0	0.0	23.1 (3)	50.0 (1)	0.0	0.0	25.0 (1)	0.0	0.0	0.0	0.0	0.0	35.0 (14)
LEV	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0 (1)	0.0	0.0	0.0	2.5 (1)
QD	40.0 (2)	0.0	66.7 (2)	15.4 (2)	0.0	28.6 (2)	0.0	15.4 (2)	0.0	100.0 (1)	0.0	25.0 (1)	0.0	100.0 (1)	0.0	0.0	25.0 (1)	20.0 (8)
L	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
STR	0.0	0.0	0.0	0.0	0.0	14.3 (1)	0.0	7.7 (1)	0.0	0.0	0.0	0.0	0.0	100.0 (1)	0.0	0.0	25.0 (1)	5.0 (2)

[a] P, penicillin; AMP, ampicillin; VA, vancomycin; E, erythromycin; TE, tetracycline; CIP, ciprofloxacin; LEV, levofloxacin; C, chloramphenicol; QD, quinupristin-dalfopristin; L, linezolid; CN, gentamicin; STR, streptomycin.

clindamycin, fluoroquinolones, streptogramines (*E. faecalis*), and trimethoprim–sulfamethoxazole (Arias et al., 2010; Delgado et al., 2007). Previous studies (Jamet et al., 2012; Macovei and Zurek, 2007; Teuber et al., 1999) conducted on the distribution of antimicrobial resistance of *Enterococcus* spp. in cheeses reported very high levels of antimicrobial resistances among these bacteria. From this perspective, there is still a strong necessity to deepen the knowledge of *Enterococcus* strains present in raw milk cheeses produced with different technologies. This could be useful to support their qualified presumption of safety (QPS), since no nosocomial infection due to the consumption of dairy products containing enterococci has been registered so far.

The antimicrobial susceptibility tests showed resistance to erythromycin, ciprofloxacin, quinupristin–dalfopristin, tetracycline, streptomycin, and chloramphenicol at different percentages of resistance. In accordance to the published literature on antimicrobial resistance in enterococci isolated from food (Franz et al., 2001), our results did not show *Enterococcus* strains resistant to penicillins (penicillin and ampicillin). Enterococci have intrinsic low-level resistance to the aminoglycosides because of the limited ability of these agents to penetrate the cell wall (Moellering et al., 1980). Except for *E. faecalis* FMAC219 and WWS53 that were resistant to high-level streptomycin, all the other strains did not show high-level resistance to aminoglycosides. Since these classes of antimicrobial agents (penicillins, glycopeptides, and aminoglycosides) represent the most common therapeutic options for the treatment of enterococcal infections (Chow, 2000), our investigation highlighted the limited role (only 2 strains showing high-level resistance to aminoglycosides) of the strains isolated from the dairy environments sampled in Sicily in the dissemination of resistance to these agents necessary to combat enterococci responsible for human diseases. Interestingly, only one strain (FMAC219) with these characteristics was isolated from cheese.

The genetic investigation of the presence of resistance genes in our strains showed that tetracycline resistance was mainly associated with *tet* genes. These results are in accordance with what was reported by Huys et al. (2004) who analyzed several enterococci of dairy origin. In particular, the seven strains with tetracycline-resistant phenotype carried the *tet*(M) gene, but not the *tet*(K). Similar results were reported by other authors (Huys et al., 2004; Wilcks et al., 2005). The *erm*(B) gene is considered to be the most widespread macrolide resistance gene among enterococci from foods (Teuber et al., 1999). However, this gene and other genes involved in the resistance to this antimicrobial compound were not detected in the strains that were resistant. Thus, a deeper investigation of the genetic cluster responsible for resistance to erythromycin in our enterococci is necessary to detect the determinants involved.

Only one strain was resistant to chloramphenicol and it carried the *cat* gene. The incidence of this gene among food enterococci is generally higher (Hummel et al., 2007) than that evidenced in our study. The *aad*E gene was detected in all strains with high-level resistance to streptomycin, a trait recorded for other *Enterococcus* isolates from foods of animal origin (Aslam et al., 2012). Furthermore, in this study, all strains were also screened for the most common vancomycin resistance genes (*van*A and *van*B). This screening was performed because of the emergence of enterococci resistant to glycopeptides in many developed countries, which is attributed to the overuse of avoparcin as an animal growth promoter (Koluman et al., 2009). However, none of the 40 strains object of this investigation was vancomycin resistant.

In general, besides their innate resistance to antimicrobials, most of the strains included in our study had acquired resistance to at least one of the antimicrobials tested. Enterococci found in milk and derived products can be highly resistant to antibiotics because these agents are commonly used for the treatment of bacterial infection, especially mastitis. However, this phenomenon is less pronounced in extensive farms where animals enjoy welfare conditions than those intensively farmed. The traditional raw milk PDO Pecorino Siciliano, PDO Vastedda della Valle del Belìce, and Caciocavallo Palermitano cheeses that provided

Table 4
Characteristics of the *Enterococcus* strains studied.

Strain	Species	Antimicrobial resistance phenotype[a]	Antimicrobial MIC (μg/ml)					Antimicrobial genes	Gelatinase activity	Type of hemolysis	Virulence factors
			E	CIP	TE	STR	C				
FMAC219	*E. faecalis*	C-QD-STR				≥2000	32	*aad*E, *cat*(pC221)	+	γ	*gelE, asa1, efaA, ace*
CGLBL118	*E. faecium*	E-TE-CIP	4	2	32			*tet*(M)	−	γ	*asa1*
CGLBL188	*E. faecalis*	E-CIP-QD	2	2					−	γ	*gelE, asa1, esp*
WVS296	*E. faecalis*	E-TE	2		32			*tet*(M)	−	γ	*asa1, esp*
PON111	*E. faecium*	E-CIP	2	2					−	γ	
WVS1	*E. faecium*	E-CIP	2	2					−	γ	
WVS31	*E. faecium*	E-CIP	2	2					−	γ	
WVS388	*E. faecium*	E-CIP	2	2					−	γ	
CGLBL109	*E. faecium*	E-CIP	4	2					−	γ	
CGLBL139	*E. faecium*	E-CIP	256	2					−	γ	
CGLBL186	*E. faecium*	E-CIP	2	2					−	γ	
CGLBL203	*E. faecium*	E-CIP	8	2					−	γ	*gelE*
CGLBL204	*E. faecium*	E-CIP	8	2					−	γ	
CGLBL213	*E. faecium*	E-CIP	2	2					−	γ	
CGLBL221	*E. faecium*	E-CIP	8	2					−	γ	
CGLBL225	*E. faecium*	E-CIP	8	2					−	γ	
WVS426	*E. faecalis*	E-QD	8						+	γ	*gelE, asa1, efaA, ace*
PON85	*E. faecalis*	TE-QD			16			*tet*(M)	−	γ	*asa1, esp*
WWS53	*E. faecalis*	TE-STR			16	2000		*tet*(M), *aad*E	−	γ	*gelE, asa1, esp*
WWS442	*E. faecalis*	E	2						−	γ	*gelE, asa1, esp*
PSL68	*E. faecium*	E	16						−	γ	
WVS231	*E. faecium*	E	2						−	γ	
WVS439	*E. faecium*	E	4						−	γ	
CGLBL274	*E. faecium*	E	2						−	γ	
FMA463	*E. faecalis*	QD	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	+	β	*gelE, asa1, efaA, ace*
FMA604	*E. faecalis*	QD	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	γ	*gelE, asa1, efaA, ace*
FMA721	*E. faecalis*	QD	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	γ	*gelE, asa1, efaA, ace*
CGLBL146	*E. faecalis*	QD	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	γ	*gelE, asa1, esp*
FMA444	*E. faecalis*	TE			16			*tet*(M)	−	γ	*gelE, asa1, efaA, ace*
WVS356	*E. faecalis*	TE			16			*tet*(M)	+	β	*gelE, asa1, efaA, ace, esp*
CGLBL115	*E. faecalis*	TE			32			*tet*(M)	−	γ	*asa1, esp*
PON82	*E. gallinarum*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	β	
PON94	*E. faecium*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	γ	
MOB6	*E. faecalis*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	γ	*asa1, efaA, ace*
FMA8	*E. faecalis*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	+	γ	*gelE, asa1, efaA, ace*
FMA288	*E. gallinarum*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	β	*gelE, asa1, ace*
FMA713	*E. faecalis*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	+	γ	*gelE, asa1, efaA, ace*
FMAC98	*E. casseliflavus*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	β	
FMAC134B	*E. durans*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	γ	
FMAC163	*E. casseliflavus*	n.d.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	−	γ	

Abbreviations: n.d., not detected (value < detection limit of method); n.e. (not evaluated).
[a] P, penicillin; AMP, ampicillin; VA, vancomycin; E, erythromycin; TE, tetracycline; CIP, ciprofloxacin; LEV, levofloxacin; C, chloramphenicol; QD, quinupristin-dalfopristin; L, linezolid; CN, gentamicin; STR, streptomycin.

the *Enterococcus* analyzed in this work were all made from milk of animals raised at pasture with a limited antibiotic pressure.

The investigation of the virulence genes evidenced the presence of *esp.*, *asa*1, *efa*A, *ace*, and *gel*E genes. According to Eaton and Gasson (2001), the incidence of these virulence traits was quite low among *E. faecium* strains that are commonly found in dairy productions (Foulquié Moreno et al., 2006). Regarding the current status of this species, the assessment for QPS was performed (EFSA, 2012) and it was concluded that the strains associated to clinical infections could be differentiated from non-pathogenic strains. The safety criteria for *E. faecium* are the susceptibility to ampicillin (MIC ≤ 2 mg/L) and the absence of three genetic markers (IS*16*, hyl_{Efm}, and *esp*) associated with virulence. This is of value for the Panels on Additives and Products or Substances used in Animal Feed (FEEDAP) dealing with the strain-specific notification, but it is too recent knowledge for a QPS recommendation, considering the recent information on the evolution of the epidemiology of *Enterococcus* infections in humans (EFSA, 2013).

The sources of strain isolation indicated a certain correlation between the origin of enterococci and their antibiotic resistance/virulence factors. All strains from milk, the majority of those from wooden vats, some from animal rennets, and only two strains from cheeses showed all risk factors, since they were antibiotic resistant and positive for virulence determinants. Most of rennet strains, some from wooden vats and barely two strains from cheese were antibiotic resistant but not virulent. On the contrary, two cheese strains were virulent but not antibiotic resistant. Interestingly, the strains *E. casseliflavus* FMAC163, *E. durans* FMAC134B, and *E. faecium* PON94, isolated from final cheeses, were susceptible to all 12 antimicrobials tested and did not carry any virulence factors. All antibiotic sensible strains were isolated only from stretched cheeses. The antibiotic sensible strains are probably present in raw materials and/or equipment surfaces at lower levels than the antibiotic resistant ones and, for this reason, not detected by plate count. The production of antibiotics by some bacteria provides them with a competitive advantage over non-resistant bacteria in their environment (Criswell, 2004). On the other hand, the technological parameters encountered during processing, such as stretching, might exert a selection pressure on enterococci and this could explain the dominance of the antibiotic-sensible strains over the *Enterococcus* populations of the

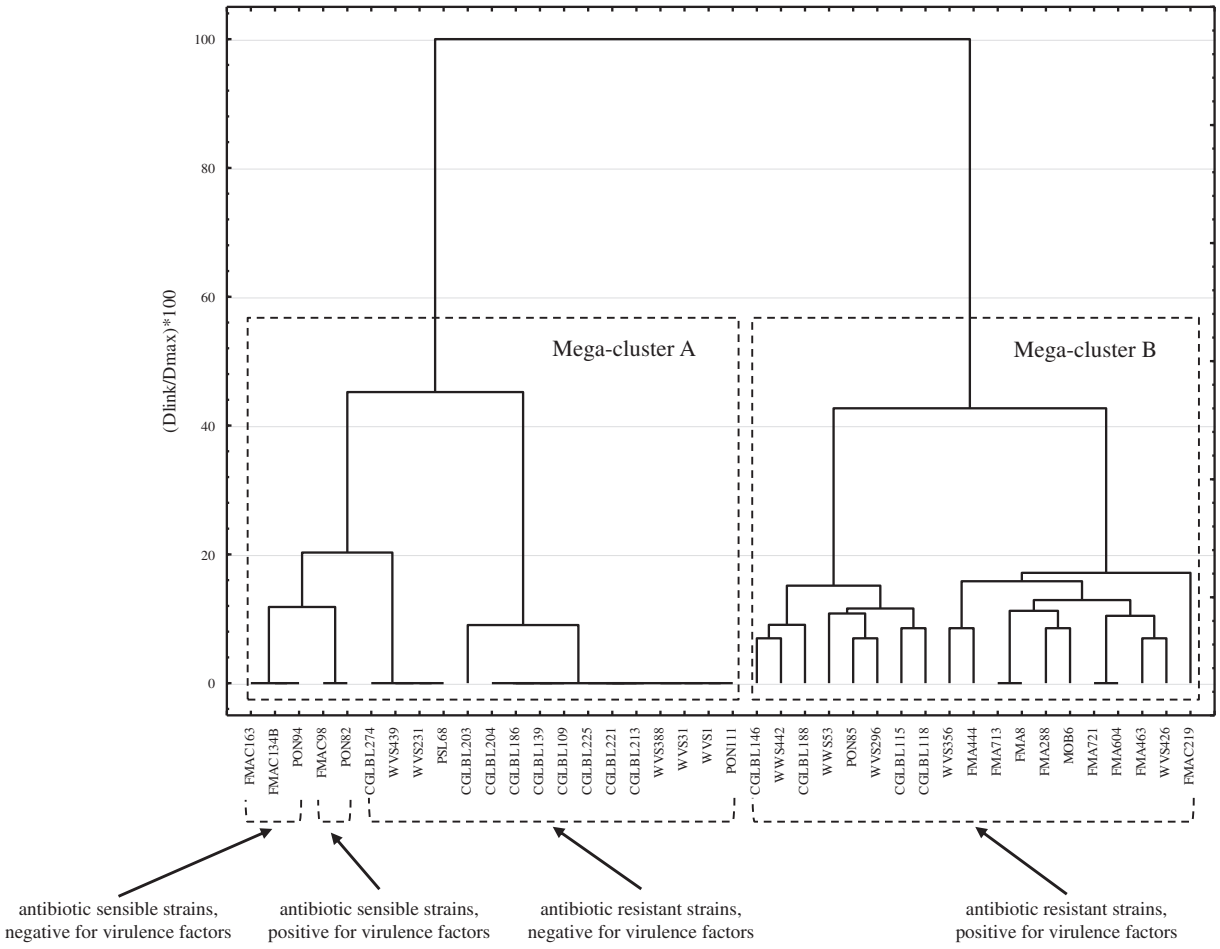

Fig. 1. Dendrogram of *Enterococcus* strains resulting from the HCA based on antimicrobial resistance, MIC, gelatinase production, hemolysis activity, antimicrobial resistance, and virulence genes. The dissimilarity among samples was measured by Euclidean distance, whereas cluster aggregation was achieved by the Ward's method.

stretched cheeses. This hypothesis needs further investigations to state a direct influence of the stretching phase on the selection of antibiotic sensible *Enterococcus*.

This study showed that three *Enterococcus* strains are characterized by the absence of risk factors and, from an application perspective, they are suitable for dairy productions. However, the technological potential of these harmless strains has to be investigated before addition in cheese making to safeguard the final typicality. It is worth noting that the three strains devoid of risk factors were isolated from stretched cheeses, suggesting that the cheeses made by the stretching technology might represent an interesting source of QPS enterococci.

5. Conclusions

This study is the first report on the antimicrobial resistance and virulence of enterococci isolated during different steps of production of three traditional Sicilian raw milk cheeses, including raw materials, equipment surfaces and stretched cheeses. The results of the present work confirmed that dairy enterococci might be a potential source for dissemination of antimicrobial resistances and virulence among bacteria. However, the presence of *Enterococcus* in the final cheeses is not generally at levels that trigger a healthy alert, and as demonstrated by this investigation, a considerable percentage (33.3%) of the strains isolated from the final cheeses did not carry any risk factor. For this reason, experiments are being prepared to test, *in vivo*, the ability of the strains *E. casseliflavus* FMAC163, *E. durans* FMAC134B, and *E. faecium* PON94, added as secondary adjuncts cultures, to dominate the indigenous *Enterococcus* populations for the production of safer cheeses without compromising the typicality ascribed to this microbial group. Although this applications needs important and detailed studies, the selection of harmless strains, proven their dairy traits, to be used for the production of safe and typical cheeses would provide relevant insights to support the QPS status of *Enterococcus* from dairy environments and to valorize the traditional products.

References

Aarestrup, F.M., Agerso, Y., Gerner-Smidt, P., Madsen, M., Jensen, L.B., 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn. Microbiol. Infect. Dis. 37, 127–137.

Aarestrup, F.M., Butaye, P., Witte, W., 2002. Nonhuman reservoirs of enterococci. In: Gilmore, M. (Ed.), The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance. American Society for Microbiology, Washington, pp. 55–99.

Arias, C.A., Contreras, G.A., Murray, B.E., 2010. Management of multidrug-resistant enterococcal infections. Clin. Microbiol. Infect. 16, 555–562.

Aslam, M., Diarra, M.S., Checkley, S., Bohaychuk, V., Masson, L., 2012. Characterization of antimicrobial resistance and virulence genes in *Enterococcus* spp. isolated from retail meats in Alberta, Canada. Int. J. Food Microbiol. 156, 222–230.

Centeno, J.A., Menendez, S., Rodriguez-Otero, J.L., 1996. Main microbial flora present in natural starters in Cebreiro raw cow's milk cheese, Northwest Spain. Int. J. Food Microbiol. 33, 307–313.

Chow, J.W., 2000. Aminoglycoside resistance in enterococci. Clin. Infect. Dis. 31, 586–589.

Clinical and Laboratory Standards Institute, 2015. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement (M100-S25). Clinical and Laboratory Standards Institute, Wayne, PA.

Cocolin, L., Foschino, R., Comi, G., Fortina, M.G., 2007. Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goat milk. Food Microbiol. 24, 752–758.

Commission Regulation, 2007. No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union.

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R., Baldassarri, L., 2004. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. J. Med. Microbiol. 53, 13–20.
Criswell, D., 2004. The" Evolution" of Antibiotic Resistance. Institute for Creation Research.
Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., De Filippis, F., Mancuso, I., La Storia, A., Moschetti, G., Settanni, L., 2014. Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 80, 2050–2061.
DANMAP, 2008. Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Foods and Humans in Denmark. Appendix 2. pp. 117–125 (http://www.danmap.org/pdfFiles/Danmap_2008.pdf).
Delgado, M., Neto, I., Correia, J.H.D., Pomba, C., 2007. Antimicrobial resistance and evaluation of susceptibility testing among pathogenic enterococci isolated from dogs and cats. Int. J. Antimicrob. Agents 30, 98–100.
Di Grigoli, A., Francesca, N., Gaglio, R., Guarrasi, V., Moschetti, M., Scatassa, M.L., Settanni, L., Bonanno, A., 2015. The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening. Food Microbiol. 46, 81–91.
Eaton, T.J., Gasson, M.J., 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. 67, 1628–1635.
EFSA, 2012. Guidance on the safety assessment of *Enterococcus faecium* in animal nutrition. EFSA J. 10 (5), 2682 (10 pp.).
EFSA, 2013. Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA J. 11 (11), 3449 (108 pp.).
Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106, 1–24.
Francesca, N., Sannino, C., Moschetti, G., Settanni, L., 2013. Microbial characterisation of fermented meat productions from the Sicilian breed "Suino Nero Dei Nebrodi" and antibacterial potential of lactic acid bacteria. Ann. Microbiol. 63, 53–62.
Franciosi, E., Settanni, L., Cavazza, A., Poznanski, E., 2009a. Presence of enterococci in raw cow's milk and "Puzzone di Moena" cheese. J. Food Process. Preserv. 33, 204–217.
Franciosi, E., Settanni, L., Cavazza, A., Poznanski, E., 2009b. Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. Int. Dairy J. 19, 3–11.
Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E., 1999. Enterococci at the crossroads of food safety? Int. J. Food Microbiol. 47, 1–24.
Franz, C.M.A.P., Huch, M., Abriouel, H., Holzapfel, W., Gálvez, A., 2011. Enterococci as probiotics and their implications in food safety. Int. J. Food Microbiol. 151, 125–140.
Franz, C.M.A.P., Muscholl-Silberhorn, A.B., Yousif, N.M.K., Vancanneyt, M., Swings, J., Holzapfel, W.H., 2001. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Appl. Environ. Microbiol. 67, 4385–4389.
Gaglio, R., Francesca, N., Di Gerlando, R., Cruciata, M., Guarcello, R., Portolano, B., Moschetti, G., Settanni, L., 2014a. Identification, typing, and investigation of the dairy characteristics of lactic acid bacteria isolated from "Vastedda della valle del Belìce" cheese. Dairy Sci. Technol. 94, 157–180.
Gaglio, R., Scatassa, M.L., Cruciata, M., Miraglia, V., Corona, O., Di Gerlando, R., Portolano, B., Moschetti, G., Settanni, L., 2014b. *In vivo* application and dynamics of lactic acid bacteria for the four-season production of Vastedda-like cheese. Int. J. Food Microbiol. 177, 37–48.
Gaspar, F.B., Crespo, M.T.B., Lopes, M.F.S., 2009. Proposal for a reliable enterococcal cytolysin production assay avoiding apparent incongruence between phenotype and genotype. J. Med. Microbiol. 58, 1122–1124.
Gelsomino, R., Vancanneyt, M., Cogan, T.M., Swings, J., 2003. Effect of raw-milk cheese consumption on the enterococcal flora of human feces. Appl. Environ. Microbiol. 69, 312–319.
Giraffa, G., 2002. Enterococci from foods. FEMS Microbiol. Rev. 26, 163–171.
Giraffa, G., Sisto, F., 1997. Susceptibility to vancomycin of enterococci isolated from dairy products. Lett. Appl. Microbiol. 25, 335–338.
Hammad, A.M., Hassan, H.A., Shimamoto, T., 2015. Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. in Egyptian fresh raw milk cheese. Food Control 50, 815–820.
Hugas, M., Garriga, M., Aymerich, M.T., 2003. Functionality of enterococci in meat products. Int. J. Food Microbiol. 88, 223–233.
Hummel, A., Holzapfel, W.H., Franz, C.M.A.P., 2007. Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. Syst. Appl. Microbiol. 30, 1–7.
Huys, G., D'Haene, K., Collard, J.M., Swings, J., 2004. Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. Appl. Environ. Microbiol. 70, 1555–1562.
Jamet, E., Akary, E., Poisson, M.A., Chamba, J.F., Bertrand, X., Serror, P., 2012. Prevalence and characterization of antibiotic resistant *Enterococcus faecalis* in French cheeses. Food Microbiol. 31, 191–198.
Jensen, L.B., Hammerum, A.M., Bager, F., Aarestrup, F.M., 2002. Streptogramin resistance among *Enterococcus faecium* isolated from production animals in Denmark in 1997. Microb. Drug Resist. 8, 369–374.
Kayser, F., 2003. Safety aspects of enterococci from the medical point of view. Int. J. Food Microbiol. 88, 255–262.
Klibi, N., Gharbi, S., Masmoudi, A., Ben Slama, K., Poeta, P., Zarazaga, M., Fendri, C., Boudabous, A., Torres, C., 2006. Antibiotic resistance and mechanisms implicated in clinical enterococci in a Tunisian hospital. J. Chemother. 18, 20–26.
Kobayashi, N., Mahbub Alam, M., Nishimoto, Y., Urasawa, S., Uehara, N., Watanabe, N., 2001. Distribution of aminoglycoside resistance genes in recent clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus avium*. Epidemiol. Infect. 126, 197–204.
Koluman, A., Akan, L.S., Çakiroğlu, F.P., 2009. Occurrence and antimicrobial resistance of enterococci in retail foods. Food Control 20, 281–283.
Lopes, M.F.S., Simões, A.P., Tenreiro, R., Marques, J.J., Crespo, M.T., 2006. Activity and expression of a virulence factor, gelatinase, in dairy enterococci. Int. J. Food Microbiol. 112, 208–214.
Macovei, L., Zurek, L., 2007. Influx of enterococci and associated antibiotic resistance and virulence genes from ready-to-eat food to the human digestive tract. Appl. Environ. Microbiol. 73, 6740–6747.
Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M., 2009. Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. Int. J. Food Microbiol. 132, 24–32.
Martorana, A., Alfonzo, A., Settanni, L., Corona, O., La Croce, F., Caruso, T., Moschetti, G., Francesca, N., 2015. An innovative method to produce green table olives based on "pied de cuve" technology. Food Microbiol. 50, 126–140.
Moellering, R.C., Murray, B.E., Schoenbaum, S.C., Adler, J., Wennersten, C.B., 1980. A novel mechanism of resistance to penicillin-gentamicin synergism in *Streptococcus faecalis*. J. Infect. Dis. 141, 81–86.
Morandi, S., Silvetti, T., Miranda Lopez, J.M., Brasca, M., 2015. Antimicrobial activity, antibiotic resistance and the safety of lactic acid bacteria in raw milk Valtellina Casera Cheese. J. Food Saf. 35, 193–205.
Nieto-Arribas, P., Seseña, S., Poveda, J.M., Chicón, R., Cabezas, L., Palop, L., 2011. *Enterococcus* populations in artisanal Manchego cheese: biodiversity, technological and safety aspects. Food Microbiol. 28, 891–899.
Novais, C., Coque, T., Costa, M., Sousa, J., Baquero, F., Peixe, L., 2005. High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. J. Antimicrob. Chemother. 56, 1139–1143.
Perreten, V., Vorlet-Fawer, L., Slickers, P., Ehricht, R., Kuhnert, P., Frey, J., 2005. Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. J. Clin. Microbiol. 43, 2291–2302.
Pesavento, G., Calonico, C., Ducci, B., Magnanini, A., Lo Nostro, A., 2014. Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. Food Microbiol. 41, 1–7.
Ruzauskas, M., Couto, N., Kerziene, S., Siugzdiniene, R., Klimiene, I., Virgailis, M., Pomba, C., 2015. Prevalence, species distribution and antimicrobial resistance patterns of methicillin-resistant staphylococci in Lithuanian pet animals. Acta Vet. Scand. 57, 1–7.
Sáenz, Y., Briñas, L., Domínguez, E., Ruiz, J., Zarazaga, M., Vila, J., Torres, C., 2004. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. Antimicrob. Agents Chemother. 48, 3996–4001.
Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G., 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41.
Schnellmann, C., Gerber, V., Rossano, A., Jaquier, V., Panchaud, Y., Doherr, M.G., Thomann, A., Straub, R., Perreten, V., 2006. Presence of New *mec*A and *mph*(C) Variants Conferring Antibiotic Resistance in *Staphylococcus* spp. Isolated from the Skin of Horses before and after Clinic Admission. J. Clin. Microbiol. 44, 4444–4454.
Semedo, T., Santos, M.A., Lopes, M.F.S., Marques, J.J.F., Crespo, M.T.B., Tenreiro, R., 2003. Virulence factors in food, clinical and reference enterococci: a common trait in the genus? Syst. Appl. Microbiol. 26, 13–22.
Settanni, L., Moschetti, G., 2014. New trends in technology and identity of traditional dairy and fermented meat production processes: preservation of typicality and hygiene. Trends Food Sci. Technol. 37, 51–58.
Settanni, L., Di Grigoli, A., Tornambé, G., Bellina, V., Francesca, N., Moschetti, G., Bonanno, A., 2012. Persistence of wild *Streptococcus thermophilus* strains on wooden vat and during the manufacture of a Caciocavallo type cheese. Int. J. Food Microbiol. 155, 73–81.
Settanni, L., Gaglio, R., Guarcello, R., Francesca, N., Carpino, S., Sannino, C., Todaro, M., 2013. Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: application on a traditional raw ewes' milk cheese. Int. J. Dairy Technol. 32, 126–132.
Silva, N., Igrejas, G., Figueiredo, N., Gonçalves, A., Radhouani, H., Rodrigues, J., Poeta, P., 2010. Molecular characterization of antimicrobial resistance in enterococci and *Escherichia coli* isolates from European wild rabbit (*Oryctolagus cuniculus*). Sci. Total Environ. 408, 4871–4876.
Suzzi, G., Caruso, M., Gardini, F., Lombardi, A., Vannini, L., Guerzoni, M., Andrighetto, C., Lanorte, M.T., 2000. A survey of the enterococci isolated from an artisanal Italian goat's cheese (semicotto caprino). J. Appl. Microbiol. 89, 267–274.
Teuber, M., Meile, L., Schwarz, F., 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Van Leeuwenhoek 76, 115–137.
Todaro, M., Francesca, N., Reale, S., Moschetti, G., Vitale, F., Settanni, L., 2011. Effect of different salting technologies on the chemical and microbiological characteristics of PDO Pecorino Siciliano cheese. Eur. Food Res. Technol. 233, 931–940.
Todeschini, R., 1998. Introduzione alla Chemiometria. EdiSES s.r.l., Naples.
Vankerckhoven, V., van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H., 2004. Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in Enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. J. Clin. Microbiol. 42, 4473–4479.
Wilcks, A., Andersen, S.R., Licht, T.R., 2005. Characterization of transferable tetracycline resistance genes in *Enterococcus faecalis* isolated from raw food. FEMS Microbiol. Lett. 243, 15–19.