

Identification and evaluation of antimicrobial resistance of enterococci isolated from raw ewes' and cows' milk collected in western Sicily: a preliminary investigation

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Abstract

The present work was carried out to investigate the Antimicrobial Resistance (AMR) of enterococci isolated from raw ewes' and cows' milk. The samples were collected from eighteen semi-extensive dairy sheep and cow farms throughout western Sicily. Plate counts, carried out on Rapid *Enterococcus* Agar commonly used to detect food enterococci, revealed a maximal enterococcal concentration of approximately 4.58 Log Colony Forming Unit (CFU)/mL. Colonies were isolated and differentiated based on genetic analysis by Randomly Amplified Polymorphic DNA (RAPD)-PCR. Thirty-eight different strains were identified. Analysis by a species-specific multiplex PCR assay grouped the strains into three *Enterococcus* species such as *Enterococcus durans*, *Enterococcus faecalis* and *Enterococcus faecium*. The 38 strains were also investigated for their antimicrobial resistance by a phenotypic approach. All 38 *Enterococcus* displayed resistance to at least one or more of the antimicrobials tested confirmed that the dairy enterococci could be a vector for the dissemination of antimicrobial resistance. This work showed that enterococci with AMR traits are commonly present in semi-extensive dairy sheep and cow farms of western Sicily pointed out the relevance of informing dairy makers and veterinary regarding the antimicrobial use in order to mitigate problems of public health and veterinary medicine.

Introduction

Enterococci are a group of Lactic Acid Bacteria (LAB) that includes pathogenic, spoilage, and pro-technological microorganisms. They are widely distributed in nature, as they are present in several foods, in particular in those of animal origin (Franz, Holzapfel, and Stiles 1999; Gaglio *et al.*, 2016a). These bacteria are also an integral component of humans and animals' gastrointestinal microflora (Manu *et al.*, 2003) and their presence in food, especially in dairy production, is a consequence of fecal contamination (Franciosi, Settanni, Cologna, Cavazza, and Poznanski, 2011).

Enterococci represent a part of the common LAB community present in milk and they were found in wooden equipment, animal rennet and in different typology of traditional cheeses (Cruciata, Gaglio, Todaro, and Settanni, 2019). Enterococci play an important role during the production of cheese, contributing to the development of the aromatic and organoleptic characteristics due to their proteolytic and lipolytic activities (Giraffa, 2002), and to extend their shelf life (Foulquiè Moreno, Sarantinopoulos, Tsakalidou, and De Vuyst, 2006). Different enterococci have been reported to produce bacteriocin-like inhibitory substance able to inhibit, *in vitro* and *in vivo*, pathogenic bacteria such as *Listeria monocytogenes* (Macaluso, Fiorenza, Gaglio, Mancuso, and Scatassa, 2016; Scatassa *et al.*, 2017), to degrade biogenic amines (Guarcello *et al.*, 2016a) and to protect against infections by promoting the maturation of the host's immune system (Fernández *et al.*, 2012). To this purpose, some of them are being used as components of cheese adjunct cultures (Guarcello *et al.*, 2016b). On the other hand, in the last decades enterococci, unlike other group of LAB, are not more considered Generally Recognized As Safe (GRAS), an essential condition for food production and moreover they are not included in the Quality Presumption of Safety (QPS) list of the European Food Safety Authority (EFSA BIOHAZ Panel, 2016). However, up until today, there are not evidence about the transmission of enterococci infection due to the consumption of food containing enterococci (Gaglio *et al.*, 2016b). The ability of these bacteria to determine human infections is mainly imputable to virulence and Antimicrobial Resistance (AMR) traits. Regarding AMR, enterococci are intrinsically resistant to many antimicrobial agents and show the ability to transfer AMR to other microorganisms through mechanisms of plasmid conjugation that might lead to the generation of multiple antibiotic resis-

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tance genes (Giraffa, 2002). As a matter of fact, the enterococci present in dairy products can be a possible intermediate vehicle for the transmission of antimicrobial resistance both to other commensal strains of the human gastrointestinal tract belonging to the same species than to different genera and pathogenic microorganisms (Guzman Prieto *et al.*, 2016). Normally, in Mediterranean regions the extensive and semi-extensive breeding systems the animals are mainly raised on native pastures, forage crops or stubbles, and receive as supplements commercial concentrates, grains, hay or silages in periods of limited availability of green grass (Sitzia *et al.*, 2015). This type of management, if well applied, allows to reduce stress factors and the presence of diseases with consequent reduced use of antimicrobial agents.

With this in mind, the present work was carried out in order to evaluate the antimicrobial resistance of enterococci isolated from different samples of ewes' and cows' milk collected from different Sicilian semi-extensive dairy sheep and cow farms. Enterococci population in the milk samples were enumerated, isolated, identified and evaluated for antimicrobial resistance traits.

Materials and Methods

Sample collection

Eighteen bulk milk samples of cow (n=3) ewe (n=15) were collected from semi-extensive dairy sheep and cow farms throughout western Sicily (Agrigento, Palermo and Trapani provinces) from December 2018 to May to 2019 (Table 1). The eighteen farms chosen randomly were characterized by medium herds of 200-250 milking ewe's and 20-30 milking cows. The sheep reared were of the Valle del Belice breed, while the cows belonged of the Simmental, Brown and Frisian breeds. Just after sampling, all samples were placed into a portable fridge and transferred to the Laboratory of Centro Latte e Lotta alle Mastiti (Istituto Zooprofilattico Sperimentale della Sicilia Adelmo Mirri, Palermo, Italy) where they were immediately subjected to microbiological investigations. The bulk milk samples from each farm were collected in duplicate at two-week intervals.

Microbiological analysis and isolation

All 18 samples were serially diluted in physiological solution (0.9% w/v NaCl). Cells suspensions were then inoculated on Rapid *Enterococcus* Agar (REA) purchased from Bio-Rad (Hercules, CA, USA) and aerobically incubated for 48h at 37°C. Plate counts were carried out in duplicate.

Gram-positive and catalase-negative bacterial cultures, presumptively considered enterococci, were obtained by randomly picking between five and ten colonies from the highest dilution plates. The isolates were purified by successive sub-culturing, and stored in Medium 17 (M17) broth media (Oxoid, Milan, Italy) containing 20% glycerol (v/v) at - 80°C until further analysis.

Genotypic differentiation and identification

Genomic DNAs of presumptive enterococci isolates cultures were extracted using the InstaGene Matrix kit (Bio-Rad) following the manufacturer's instructions. Cells were harvested after overnight growth in MRS (Man Rogosa e Sharpe) broths at 37°C and washed in distilled H₂O. The crude cell extracts were used as templates for PCRs.

Strains were differentiated by Random Amplification of Polymorphic DNA (RAPD)-PCR analysis in a 30 µl reaction volume with the primers M13, AB111 and AB106 used singly as reported by Gaglio *et al.* (2017).

The RAPD patterns were analysed

using Gelcompare II software version 6.5 (Applied-Maths, Sint, Marten-Latem, Belgium).

The unequivocal identification of the strains belonging to the *Enterococcus* genus was obtained by applying the multiplex PCR assay on the *sodA* gene as described by Jackson, Fedorka-Cray, and Barrett (2004) with primers DU1, DU2, FL1, FL2, FM1 and FM2 specific for *Enterococcus durans*, *Enterococcus faecalis* and *Enterococcus faecium*, CA1, CA2, MU1, MU2, HI1 and HI2 specific for *Enterococcus casseliflavus*, *Enterococcus mundtii* and *Enterococcus hirae*.

Antimicrobial susceptibility test

The different enterococci strains were characterized for their susceptibility to different antimicrobial compounds commonly used for the treatment of human and animal infections (Gaglio *et al.*, 2016b) by the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017). The inocula were prepared by micro-dilution method suspending colonies in 5 ml of physiological solution (0.9 % NaCl, w/v) until reaching a standard density of 0.5 McFarland. The cell suspensions were swabbed for confluent growth onto Mueller Hinton agar with defibrinated 5% sheep blood and 6 mm filter paper discs were placed on the surface of the test medium and incubated aerobically at 37°C for 18-24h.

Fourteen antimicrobial compounds belonged to 11 families: penicillins [peni-

cillin G (P- 10 UI) 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)]; phenicol [chloramphenicol (C-30 µg)]; streptogramins [quinupristin/dalfopristin (QD-15 µg)]; oxazolidinones [linezolid (LZD-30 µg)]; aminoglycosides [high level gentamicin (CN-120 µg) and streptomycin (S-10 µg)]; sulphonamides [sulphamethoxazole/trimethoprim (SXT- 25 µg)]; riphampicine [rifampicin (RD- 30 µg)] were tested.

After incubation, each strain was classified as Susceptible (S), Intermediate (I) or Resistant (R) based on the inhibition zone diameters according to the CLSI guidelines (CLSI, 2017). All antibiotics were purchased from Oxoid.

Statistical analyses

Statistical analyses of microbiological counts performed between the eighteen bulk milk samples were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial data were converted to the log scale before statistical elaborations. The post-hoc Tukey's multiple range whereas Student's *t*-test was applied to determine the significance and the difference between means values of eighteen bulk milk samples. A *p*<0.05 was deemed significant.

Table 1. Milk samples.

Sample	City of dairy factory	Province	Type of Milk
M1	Castronovo di Sicilia	Palermo	Bovine
M2	Castronovo di Sicilia	Palermo	Bovine
M3	Cammarata	Agrigento	Bovine
M4	Collesano	Palermo	Ovine
M5	Gangi	Palermo	Ovine
M6	Partinico	Palermo	Ovine
M7	Alimena	Palermo	Ovine
M8	Gangi	Palermo	Ovine
M9	Gibellina	Trapani	Ovine
M10	Menfi	Agrigento	Ovine
M11	Contessa Entellina	Palermo	Ovine
M12	Partanna	Trapani	Ovine
M13	Santa Margherita di Belice	Agrigento	Ovine
M14	Santa Margherita di Belice	Agrigento	Ovine
M15	Salemi	Trapani	Ovine
M16	Gangi	Palermo	Ovine
M17	Gangi	Palermo	Ovine
M18	Alimena	Palermo	Ovine

Results and Discussion

Microbiological analyses, differentiation and identification of enterococci

Figure 1 shows the viable counts of the enterococci harbored on milk samples. The levels of these microorganisms were in the range of 2.40–4.58 showing statistical significant difference ($p < 0.05$) between the samples object of investigation. With the exception of samples M3, M4, M5 and M14 the levels of enterococci detected on REA were at approximately 3–4 Log CFU/mL. Similar levels have been reported for raw ewes' milk used for traditional cheese productions (Gaglio *et al.*, 2019a).

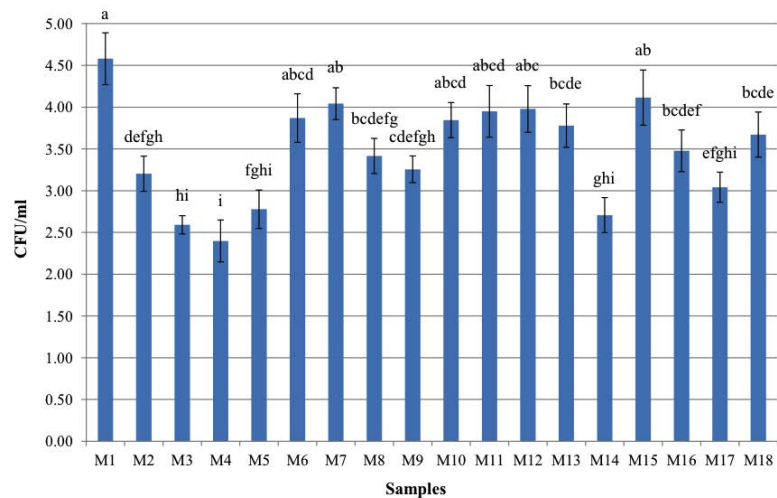
A total of 72 colonies were collected from 18 bulk milk samples. All cultures were inspected microscopically and after Gram and catalase test, 66 coccus-shaped Gram-positive and catalase negative cultures were subjected to RAPD analysis in order to perform the strain typing. As reported in the dendrogram (Figure 2), the combination of the three RAPD patterns of each isolate indicated that the enterococcal community isolated from raw ewes' and cows' milk was composed of 38 different strains. These strains were further analysed by a species-specific multiplex PCR assay, which identified 17 *E. faecalis*, 12 *E. faecium* and 9 *E. durans*. The dendrogram clearly showed that, except for the strain 3246 (*E. durans*) and 3261 (*E. faecium*), all the other strains grouped per species.

All *Enterococcus* species identified are commonly associated with raw milk and cheeses (Franciosi *et al.*, 2011; Gaglio *et al.*, 2019b), including stretched cheeses (Gaglio *et al.*, 2019a) and wooden vats used for cheese making in Italy (Cruciata *et al.*, 2018) and France (Licitra *et al.*, 2007).

Antibiotic susceptibility test

The 38 *Enterococcus* strains were tested for their antimicrobial susceptibility to 14 antimicrobial compounds by the disk diffusion method according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2017). Patterns of antibiotic resistance with regards to species and source of isolation of the 38 strains are reported in Table 2. The results of antibiotic susceptibility test relatively to ampicillin and gentamicin are not reported in table, because no strains was scored resistant. All 38 *Enterococcus* displayed resistance to at least one or more of the antimicrobials tested. In particular, between the 38 strains, five (13.2%) showed multidrug-resistant phenotype (resistance to at least three antibiotics).

None of the *Enterococcus* species iden-



Results indicate mean values \pm standard deviation of four determinations (carried out in duplicate for two independent sampling). Different superscript letters indicate significant differences between mean values of microbial concentrations according to Tukey's test among the samples for $p < 0.05$

Figure 1. Microbiological concentrations of *Enterococcus* spp. (CFU/mL) in raw milk samples.

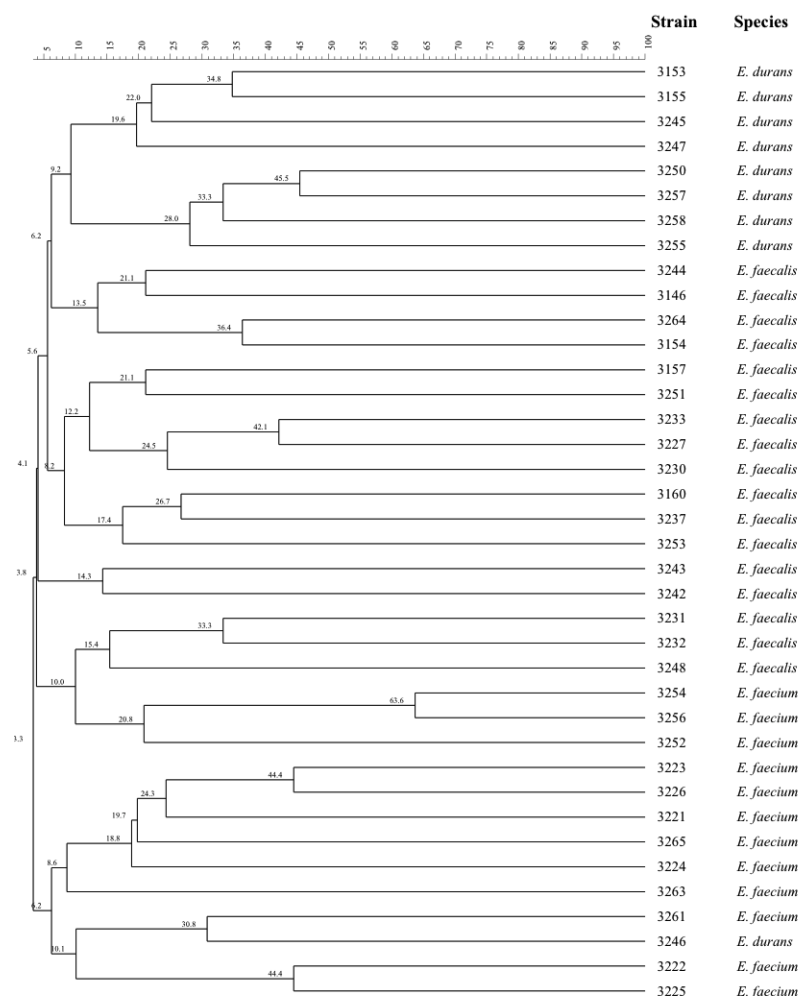


Figure 2. Dendrogram obtained with combined RAPD-PCR patterns of the LAB strains isolated from raw ewes' and cows' milk. Scale bar indicate the percentage of similarity. Abbreviations: *E.*, *Enterococcus*.

tified in this study was susceptible to streptomycin. These results confirmed an intrinsic resistance of enterococci to this antimicrobial agent as previously reported by Çitak, Yucel, and Orhan (2004). In particular, in this study, a considerable percentage of enterococci (89.1%) isolated from Turkish white cheese were resistant to streptomycin at 10 µg. Moreover, the *Enterococcus* strains isolated in this study

exhibited high percentages of resistance to quinupristin/dalfopristin (23.7%) and tetracycline (15.8%) and high percentages of intermediate susceptibility to ciprofloxacin (50%) and erythromycin (53%). These results confirmed as reported by Gaglio *et al.* (2016b) for the resistance to quinupristin/dalfopristin and by Silveti, Morandi, and Brasca (2019) regarding intermediate susceptibility to erythromycin

and ciprofloxacin of different strains of *E. faecalis*.

The high percentage of resistance registered for quinupristin/dalfopristin, was a surprising for the enterococci isolated from raw milk. Indeed, this antimicrobial agent is commonly used for the treatment of human infections (Allington and Rivey, 2001) and its use is not authorized in veterinary medicine. To this purpose, the presence of

Table 2. Antimicrobial resistance of the enterococcal isolates.

Strain	Species	VA	E	P	TE	CIP	LEV	L	STR	C	QD	RD	STX
Cow milk													
3153	<i>E. durans</i>		I			I			R				
3155	<i>E. durans</i>		I			I			R				
3154	<i>E. faecalis</i>	I	I			I			R				
3157	<i>E. faecalis</i>		I		R				R				
3160	<i>E. faecalis</i>	I				I			R				
Sheep milk													
3245	<i>E. durans</i>							I	R				
3246	<i>E. durans</i>	I				I			R		R		
3247	<i>E. durans</i>								R		I		
3250	<i>E. durans</i>				R	I			R		I		
3255	<i>E. durans</i>	I						I	R		I		
3257	<i>E. durans</i>	I	I			I			R		R		R
3258	<i>E. durans</i>		I			I			R		R		
3146	<i>E. faecalis</i>	I	I		R	I			R		R	I	
3227	<i>E. faecalis</i>					I			R		I		
3228	<i>E. faecalis</i>	I	I			I			R			I	
3231	<i>E. faecalis</i>		I			I			R		R		
3233	<i>E. faecalis</i>		I		R	I			R		R		
3235	<i>E. faecalis</i>	I	I			I			R				
3237	<i>E. faecalis</i>	I	I						R		R		
3240	<i>E. faecalis</i>		I			I			I				
3242	<i>E. faecalis</i>		I			I			I		R		
3244	<i>E. faecalis</i>					I			R		I		
3248	<i>E. faecalis</i>		I						R				
3251	<i>E. faecalis</i>		I			I			R				
3253	<i>E. faecalis</i>		I			I			R				
3264	<i>E. faecalis</i>	I	I						R		R	I	
3221	<i>E. faecium</i>								R				
3222	<i>E. faecium</i>								R		I		
3223	<i>E. faecium</i>								R		I		
3224	<i>E. faecium</i>								R				
3225	<i>E. faecium</i>		I						R				
3226	<i>E. faecium</i>								R				
3252	<i>E. faecium</i>								R				
3254	<i>E. faecium</i>	R	I		R				R		I		
3256	<i>E. faecium</i>								R				R
3261	<i>E. faecium</i>	I							R		I		
3263	<i>E. faecium</i>				R				R		I		R
3265	<i>E. faecium</i>								R	I	I		

VA: vancomycin; E: erythromycin; P: penicillin; TE: tetracycline; ciprofloxacin: CIP: ciprofloxacin; LEV: levofloxacin; L: linezolid; STR: streptomycin; C: chloramphenicol; QD: quinupristin-dalfopristin; RD: rifampicin; STX: sulphamethoxazole/trimethoprim. R= resistant; I= intermediate; no letter= susceptible.

these strains in semi-extensive dairy sheep and cow farms could be due to cross-contaminations not associated with the animals. On the contrary, the resistance to tetracycline and sulphonamides is mainly due to the use of this antimicrobial agents in Sicilian farms veterinary practices. Regarding vancomycin, one strains was found resistant while eleven with intermediate susceptibility. The only stain resistant to vancomycin belonged to the specie *E. faecium*. Indeed, according to Russo *et al.* (2018), this resistance trait is common among *E. faecium*. As reported by Kang and co-workers (Kang, Kim, Chon, and Seo, 2017), our results exhibited high percentages (95%) of *Enterococcus* strains susceptible to chloramphenicol. Regarding linezolid just two stains resulted resistant. These result is in accordance with what was reported by Hammad, Hassan, and Shimamoto (2004) who analyzed several enterococci isolated from Egyptian fresh raw milk cheese. In accordance to the published literature on antimicrobial resistance in enterococci isolated from dairy productions (Gaglio *et al.*, 2016b; Silveti *et al.*, 2019), our results showed a higher percentage of *Enterococcus* strains susceptible to penicillins (penicillin) and fluoroquinolones (levofloxacin). Between these antimicrobial compounds, penicillins represents the most common therapeutic options for the treatment of enterococcal infections (Chow, 2000).

Conclusions

This study represents the first investigation on the antimicrobial resistance of enterococci isolated from raw ewes' and cows' milk samples collected from semi-extensive dairy sheep and cow farms of western Sicily.

The results of the present study confirmed that dairy enterococci might be a potential source for dissemination of antimicrobial resistances among bacteria in semi-extensive dairy sheep and cow farms of western Sicily.

This pointed out the relevance of informing dairy makers and veterinary regarding the antimicrobial use in order to mitigate problems of public health and veterinary medicine by reducing the potential impact of transmission of resistant bacteria to humans via the food chain.

However, further studies are being prepared to better characterize the safety of these enterococci in terms of detection of antimicrobial resistance genes, virulence as well as cellular toxicity.

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