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

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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

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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