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IDENTIFICATION AND SAFETY ASSESSMENT OF ENTEROCOCCI ISOLATED FROM A SARDINIAN EWE'S RAW MILK PDO CHEESE (FIORE SARDO)

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ABSTRACT

The aim of this study was to identify enterococcal microflora and to assess some safety aspects (presence of antibiotic resistance traits and virulence determinants) of *Enterococcus* species colonizing Sardinian traditional ewe's milk dairy products. Fiore Sardo cheese was chosen as 'model' to carry out this study.

Thus, 322 enterococci isolated from 21 artisanally produced Fiore Sardo cheese samples were genotipically characterised at species level by species-specific PCR and 16S rDNA sequencing, and at strain level by cluster analysis of rep-$(GTG)_5$ PCR, RAPD M13 PCR, and PFGE patterns. Five enterococcal species, two dominant (*E. faecium* and *E. faecalis*) and three less-abundance (*E. durans, E. hirae,* and *E. gallinarum*) were found. A high biodiversity at strain level was found for the dominant species.

The incidences of resistance to 14 antibiotics was phenotypically tested. The presence of genes encoding for tetracycline and erythromycin resistance and for 8 virulence factors was investigated. Different results were obtained especially depending on the medium of origin (with or without antibiotics) of isolates. The incidence of antibiotic resistance and virulence determinants genes was higher for *E. faecalis* isolates.

The results presented in this work were consistent with those reported in literature for food enterococcal isolates suggesting that their presence in Sardinian dairy foods does not represent a threat to human health.

1. INTRODUCTION

1.1 The genus *Enterococcus*: taxonomy and main characteristics.

Enterococci are a complex, diverse, and important group of bacteria. Their identification has always been problematic. In the past, the species now included in the genus *Enterococcus* were classified as streptococci of faecal origin. In 1906, the name *Streptococcus faecalis* was used by Andrews and Horder to identify an organism of faecal origin that clotted milk and fermented mannitol and lactose but not raffinose. Orla-Jensen (1919) described a second organism of this group, *Streptococcus faecium*, which differed from the fermentation patterns of *S. faecalis*. A third species, *Streptococcus durans*, proposed by Sherman and Wing (1935, 1937), was similar to *S. faecium* but had less fermentation activity. The term 'enterococcal group' was used for the first time by Sherman (1937, 1938) to describe streptococci that grew at 10 °C and 45 °C, in broth with pH adjusted to 9.6 and in broth containing 6.5% NaCl, and which survived heating to 60 °C for 30 min.

In 1970, Kalina suggested that *S. faecalis*, *S. faecium* and their subspecies be named *Enterococcus*. In 1984, Schleifer and Kilpper-Balz provided genetic evidence that *S. faecalis* and *S. faecium* were sufficiently different from the other members of the genus to merit a separate genus. Since then, it has been generally accepted that genus *Enterococcus* is valid.

The criteria for inclusion into the *Enterococcus* genus are a combination of DNA-DNA reassociation values, 16S rRNA gene sequencing, WCP (Whole Chromosome Paint), and conventional phenotypic tests. Numerous enterococcal isolates, especially from an environmental source, often remain unidentified when their identification is based on phenotypic traits alone. The problem with the taxonomy of enterococci is generally that they are an heterogeneous group of Gram-positive cocci sharing many characteristics with the genera *Streptococcus* and *Lactococcus*. But this is also the reason why food-associated enterococci are considered to belong to the 'lactic' microflora.

The number of species included in the genus *Enterococcus* fluctuates from time to time, as individual species are moved into other genera, or new taxa are discovered. Several new enterococcal species have been identified since the recognition of *Enterococcus* as a separate genus as a result of improvements in identification methods, combined with a growing interest in the role of these microorganisms as opportunistic pathogens.

To date, 39 species of enterococci, namely *E. aquimarinus*, *E. asini*, *E. avium*, *E. caccae*, *E. camelliae*, *E. canintestini*, *E. canis*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. devriesei*, *E. dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *E. flavescens*, *E. gallinarum*, *E. gilvus*, *E. haemoperoxidus*, *E. hermanniensis*, *E. hirae*, *E. italicus* (syn. *E. saccharominimus*), *E. malodoratus*, *E. moraviensis*, *E. mundtii*, *E. pallens*, *E. phoeniculicola*, *E. porcinus*, *E. pseudoavium*, *E. raffinosus*, *E. ratti*, *E. saccharolyticus*, *E. seriolicida*, *E. silesiacus*, *E. solitarius*, *E. sulfureus*, *E. termitis*, *E. thailandicus* and *E. villorum*, have been added to the genus *Enterococcus* on the basis

of phylogenetic evidence strengthened by 16S rRNA DNA sequencing and/or DNA–DNA hybridization studies. *E. sanguinicola* was proposed as sp. nov. by Carvalho *et al*. at the end of 2008, but, as yet, it has no standing in nomenclature (Euzéby, 2009).

The majority of *Enterococcus* species can be distinguished from other Gram-positive, catalase-negative cocci by their ability to grow from 10 °C to 45 °C (with an optimum growth temperature of 35 °C), survive heating at 60 °C for 30 min, tolerate 6.5% NaCl and 40 % bile and grow between pH 4.0 and 9.6. Most of them can hydrolyse aesculin in the presence of 40% bile salts. Their DNA G+C content ranges from 37 to 45 mol% (Schleifer and Kilpper-Balz, 1984).

Exceptions to some of these characteristics sometimes occur.

Indeed, *E. dispar*, *E. sulfureus*, *E. malodoratus* and *E. moraviensis*, do not grow at 45 °C, and *E. cecorum* and *E. columbae* do not grow at 10 °C while *E. avium*, *E. italicus (*syn. *saccharominimus)*, *E. cecorum* and *E. columbae* grow poorly or not at all in the presence of 6.5% NaCl.

Other species of the genus (*E. mundtii* and *E. casseliflavus*) are yellow-pigmented, while *E. gallinarum*, *E. casseliflavus* and *E. flavescens* are motile (Facklam *et al*., 2002; Folquié Moreno *et al*., 2006).

Enterococcal species possess the Lancefield group D antigen with the exception of *E. cecorum*, *E. columbae*, *E. dispar*, *E. pseudoavium*, *E. saccharolyticus* and *E. sulfurous.*

Enterococci do not contain cytochrome enzymes, but they occasionally produce a pseudocatalase and appear catalase-positive with a weak effervescence. Almost all

strains are homofermentative with lactic acid as the end product of glucose fermentation, and gas is not produced.

Enterococci exhibit, in general, low milk acidifying ability, but contribute to the ripening, texture and aroma development of fermented foods by means of their lipolytic, esterolytic and proteolytic activities. They are able to metabolize citrate producing metabolic end products such as diacetyl, acetaldehyde, acetoin, and 2,3-butanediol, which significantly influence the aroma and flavour of these products.

On the other hand, as a result of the degradation of casein, enterococci can produce undesirable bitter-tasting peptides that can lead to off-flavours (Franz *et al*. 2003; De Vuyst *et al*., 2003, Giraffa, 2003; Folquié Moreno *et al*., 2006; Bhardwaj *et al*., 2008).

Different enterococcal species can produce a large variety of bacteriocins (enterocins) active against a wide spectrum of spoilage and/or food-borne pathogen bacteria as *Propionibacterium, Pediococcus, Bacillus* spp., *Listeria monocytogenes., Clostridium botulinum, Clostridium butyricum, Clostridium perfringens, Staphylococcus aureus* and *Vibrio cholerae* (Franz *et al*., 2003; De Vuyst *et al*., 2003; Giraffa, 2003; Folquié Moreno *et al*., 2006; Cocolin *et al*. 2007, Bhardwaj *et al*., 2008; Choho *et al*., 2008).

Given their commensal status, enterococci are also used as probiotics for humans or farm animals (Franz *et al*., 1999; Tannok and Cook, 2002; Giraffa, 2003; Folquié Moreno *et al*., 2006; Ogier and Serror, 2008).

1.2 Enterococci in dairy products

The food chain and especially dairy foods have clearly been established as an important source of enterococci in the human environment.

The *Enterococcus* genus is naturally present in many traditional Mediterranean cheeses produced in Italy, Spain, Greece, France and Portugal from raw or pasteurized goats', ewes', water-buffalos' or cows' milk. Levels of enterococci in different cheese curds range from 10^4 to 10^6 CFU g^{-1} and in the fully ripened cheeses from 10 to 10^7 CFU g^{-1} (Franz *et al*. 1999 and 2003; Folquié Moreno, 2006; Pisano *et al*., 2007; Ogier and Serror, 2008; Comunian *et al*., 2009b). Ordinarily, *E. faecium* and *E. faecalis* are the most frequently encountered species. Other less represented species are *E. durans*, *E. hirae* and *E. gallinarum* (Giraffa *et al*., 1997; Franz *et al*., 1999; Suzzi *et al*., 2000; Andrighetto *et al*., 2001; Gelsomino *et al*., 2001; Sarantinopoulos *et al*., 2001; Aarestrup *et al*., 2002; Giraffa *et al*., 2003; Cosentino *et al*., 2004; Pisano *et al*., 2007).

Folquié Moreno *et al.* (2006) reported an exemplifying list of scientific articles on Mediterranean cheeses in which enterococci have been studied (**Table 1**).

In the case of Fiore Sardo PDO cheese, from which the enterococci object of this study were isolated, the list should be completed with the research papers by Ledda *et al*. (1994), Cogan *et al.* (1997), Mannu *et al*. (2003 and 2006), Consentino *et al*. (2004), Pisano *et al.* (2006), Pisano *et al.* (2007), Mangia *et al*. (2008) and Comunian *et al.* (2009b).

Considering the ancient traditions of these cheeses, it may be assumed that the local population of Mediterranean regions have consumed enterococci for centuries.

Table 1. European cheeses where enterococci have been studied (in: Folquié Moreno *et al.*, 2006. *Int. J. Food Microbiol.*, **106**: 1–24).

Origin	Cheese	Type of milk	Reference
French	Comté	Cow	Bouton et al., 1998
French	Roquefort	Cow	Devoyod, 1969
French	Venaco	Ewe/goat	Casalta and Zennaro, 1997
Greek	farmhouse cheese	Ewe	Gomez et al., 2000
Greek	Feta	Ewe	Litopolou-Tzanetaki et al., 1993
Greek	Feta and Teleme	Ewe	Tzanetakis and Litopoulou-Tzanetaki, 1992
Greek	Kefalotyri	Ewe	Litopolou-Tzanetaki, 1990
Greek	Orinotyri	Ewe	Prodromou et al., 2001
Greek	Pichtogalo Chanion	Ewe/goat	Papageorgiou et al., 1998
Irish	Cheddar	Cow	Gelsomino et al., 2001
Italian	Buffalo Mozzarella	Buffalo	Parente et al., 1989
Italian	Fiore Sardo	Sheep	Ledda et al., 1994
Italian	Fontina	Cow	Battistotti et al., 1977
Italian	Montasio	Cow	Basso et al., 1994
Italian	Monte Veronese	Cow	Torriani et al., 1998
Italian	Mozzarella	Cow	Morea et al., 1999
Italian	Pecorino Sardo	Ewe	Mannu et al., 1999; Mannu and Paba, 2002
Italian	Semicotto Caprino	Goat	Suzzi et al., 2000
Portuguese	Serra da Estrela	Ewe	Macedo et al., 1995
Portuguese	Serra da Estrela	Ewe	Tavaria and Malcata, 1998
Spanish	Armada	Goat	Tornadijo et al., 1995
Spanish	Arzúar	Cow	Centeno et al., 1995
Spanish	Cebreiro	Cow	Centeno et al., 1996, 1999
Spanish	Cebreiro	Cow	Menéndez et al., 1998
Spanish	Majorero	Goat	Fontecha et al., 1990
Spanish	La Serena	Ewe	Fernandez del Pozo et al., 1988
Spanish	Manchego	Ewe	Ramos et al., 1981
Spanish	Roncal-Idiazabal	Ewe	Arizcun et al., 1997
Spanish	San Simón	Cow	García et al., 2002
Spanish	Tenerife goat cheese	Goat	Zárate et al., 1997
Spanish	Tetilla	Cow	Menéndez et al., 2001

Contrary to other lactic acid bacteria, enterococci are not always considered as GRAS (Generally Recognized As Safe) and their programmed application is currently being questioned, though present evidence does not suggest enterococci be regarded as food

borne pathogens (Ogier and Serror, 2008). Moreover, their presence in fermented foods appears unavoidable because of their ability to survive in hostile conditions, resulting from their heat resistance, wide range of growth temperatures, tolerance to different pH and salt concentrations, and their wide spread existence in the environment (water, soil, vegetables, insects) (Deibel and Silliker, 1963; Mundt, 1986, Folquié Moreno *et al*., 2006).

Although, at present, enterococci are considered as a normal existence of the food microflora (Franz *et al.*, 1999), they are also used as indicator organisms of faecal contamination of food and poor hygiene in production processes. Actually, their existence is not necessarily associated with warm-blooded animals faecal contamination. In a study on artisanal raw milk Cheddar cheese produced at farm-level in Ireland, Gelsomino *et al.* (2001, 2002) demonstrated that cows' faeces were not the source of enterococci in the cheese. The same enterococcal species and clones were isolated from family member faeces, bulk tanks, milking equipment, milk and cheese, but not from cows' faeces.

Production of biogenic amines can be an undesirable activity of enterococci in fermented dairy products when enterococci are present in high number. Food intoxication caused by ingestion of biogenic amines determines a number of symptoms of increasing complexity which include headache, vomiting, increase of blood pressure and even allergic reactions of strong intensity (Giraffa, 2002; Karovacova and Kohajdova, 2005; Martuscelli *et al*., 2005; Bhardwaj *et al*., 2008).

The principal concern over presence of enterococci in foods is their pathogenic potential based on horizontal transfer of genes for factors associated with virulence and antibiotic resistance (Franz *et al*., 1999).

Nevertheless, different authors highlighted the importance of their contribution to flavour development during cheese ripening and the benefit due to their ability of producing bacteriocins to preserve dairy products from spoilage and food borne pathogen bacteria, and positively considered their use as starter or probiotic adjunct cultures (Franz *et al*., 1999; Sarantinopoulous *et al*., 2001; Tannock and Cook, 2002; Giraffa, 2002; Giraffa *et al*., 2003; Franz *et al*., 2003; Folquié Moreno, 2006; Ogier and Serror., 2008).

All things considered, enterococci play an important role in the manufacture of cheeses typical of Mediterranean countries, and they have a major impact on this part of the dairy production. However, there is still some discussion concerning the risk and/or their beneficial potential.

1.3 Enterococci in disease

Prior to the identification of multiple antibiotic-resistant strains in the late 1970s, enterococci were considered harmless bacteria, with the exception of those causing endocarditis (Kayser, 2003). Currently, they are considered as emerging pathogens of humans. Harmless in healthy individuals, enterococcal clinical isolates become pathogenic mainly in patients in intensive care units, with severe underlying diseases

or impaired immune systems, or in elderly people (De Vuyst *et al*., 2003; Drahovská *et al*., 2004; Bhardwaj *et al*., 2008; Ogier and Serror, 2008). Furthermore, other important risk factors for acquiring nosocomial enterococcal infections are a long hospital stay, renal insufficiency, neutropenia, transplantation (especially liver and bone marrow transplantation), and the use of urinary or vascular catheters (Kayser, 2003).

In recent decades, enterococci have become of major importance in community-acquired and nosocomial infections and superinfections, such as endocarditis, bacteremia, urinary tract, neonatal, central nervous system, intra-abdominal and pelvic infections (Franz *et al*., 1999; Giraffa, 2002; Folquié Moreno *et al*., 2006), even though, in some cases, such as in polymicrobial intra-abdominal infections, it is not easy to establish the role of enterococci because they are associated with other pathogens.

They are the third most common cause of nosocomial blood infections in the United States and the fourth in Europe (Ogier and Serror, 2008). *E. faecalis* clearly predominates among enterococci isolated from human infections (up to 80 %), while strains of *E. faecium* are associated with the majority of the remaining (20 % of clinical isolates). Other enterococcal species, including *E. avium*, *E.casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. raffinosus*, and *E. solitarius*, are infrequent causes of human infection (Jett *et al*., 1994; Franz *et al*., 1999; Giraffa, 2002; Franz *et al*., 2003; Ogier and Serror, 2008). Recent studies indicate that the proportion of *E. faecium* infections has increased, mainly owing to an increasing number of antibiotic resistant *E. faecium* isolates (Ogier and Serror,

2008). Other enterococcal species such as *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. mundtii* or *E. raffinosus* have been less frequently incriminated in enterococcal infections (Bhardwaj *et al*., 2008; Ogier and Serror, 2008).

The use of antibiotics to which enterococci possess a natural resistance or an only intermediate susceptibility (for instance, preceding antibiotic therapy for other infectious diseases) is an important factor in enterococcal superinfection (Kayser, 2003). Antiobiotic resistance of enterococci and gene transfer mechanisms, which often involve antibiotic resistance, are discussed below; however, antibiotic resistance alone cannot explain the virulence of these bacteria, so other virulence determinants concerning the pathogenicity of enterococci will be also discussed below (Franz *et al*., 1999; Giraffa, 2002).

1.4 Antibiotic susceptibility

1.4.1 Mechanisms of action of antibiotics

How they act differs for various antimicrobial agents (Neu, 1992; Neu and Gootz http://gbs.utmb.edu/microbook/ch11; Kak and Chow, 2002).

They interfere with specific processes that are essential for growth and/or division of the bacterial cell.

Antibiotics can act by interfering with:

- cell wall synthesis (e.g. β-lactams as penicillins and cephalosporins, vancomycin);
- plasma membrane integrity (e.g. polymyxins);
- nucleic acid synthesis (e.g. quinolones and rifampin);
- ribosomal function (e.g. aminoglycosides, tetracycline, chloramphenicol, erythromycin, and clindamycin);
- folate synthesis (e.g. sulfonamides and trimethoprim).

In particular, the mechanisms of action of the antimicrobial agents that, in this study, were tested against enterococci isolated from Fiore Sardo PDO cheese, are discussed below.

1) *Inhibitors of bacterial cell wall synthesis*

β-lactams (penicillin and ampicillin): inhibit polymerization and attachment of new peptidoglycan to cell wall.

Glycopeptides (vancomycin): drugs that interrupt cell wall synthesis by forming a complex with cell wall substrates.

2) *No antibiotics acting on plasma membrane integrity were considered in this study*.

3) *Inhibitors of nucleic acid synthesis*

Quinolones (Fluoroquinolones such as ciprofloxacin, gatifloxacin and levofloxacin): inhibit bacterial DNA replication by preventing DNA gyrase synthesis during bacterial chromosome replication.

Rifamycin (rifampin): inhibits DNA-directed RNA polymerase and interferes specifically with the initiation of the process of transcription of the DNA to RNA thus preventing translation to proteins. However, it has no effect once polymerization has begun.

4) *Inhibitors of ribosome function*

Aminoglycosides (streptomycin, gentamicin, kanamycin) and Tetracyclines* (tetracycline): inhibit 30S units causing the ribosome to misread the genetic code and consequently preventing the elongation of the peptide chain.

Lincosamides (clindamycin*), Macrolides (erythromycin*), Streptogramin (quinupristin/dalfopristin) and Oxazolidinones (linezolid): inhibit 50S units and thus the protein synthesis.

*These agents may be bactericidal or bateriostatics depending on the concentration at the site of infection and the susceptibility of the organism involved.

5) *No antibiotics acting on folate synthesis were considered in this study.*

1.4.2 Antibiotic resistance mechanisms of bacterial cells

Bacterial cells have developed different resistance mechanisms against the action of various antimicrobial agents (Neu, 1992; Neu and Gootz http://gbs.utmb.edu/microbook/ch11; Kak and Chow, 2002).

These mechanisms may involve:

1) changes in the receptor (target) for the drug (modification to insensitivity to inhibitor, reduction in physiologic importance of target, synthesis of new target enzyme that duplicates function of inhibited target),
2) decreased entry of the drug (efflux of more drug than enters cell, failure of modified drug to enter cell),
3) metabolic inactivation of the drug (destruction of the agent, modification of the agent so it fails to bind to target),
4) failure to convert an inactive precursor agent to its active form.

Bacteria can possess one or all of these mechanisms simultaneously as the result of a chromosomal change due to a DNA mutation, or the presence of extrachromosomal DNA that was acquired from another bacterial cell.

In particular, the mechanisms of resistance that enterococci can carry out against the antimicrobial agents tested in this study are reported below.

1) *Resistance due to altered receptors*

Differently from other bacterial groups, enterococci can withstand β-lactams (such as penicillin and ampicillin) because their penicillin binding proteins (PBPs) have a low affinity for penicillins.

In the case of Glycopeptides (such as vancomycin), VRE (Vancomycin Resistant Enterococci) have acquired genetic elements encoding special cell wall-enzymes which change the structure of D-Ala-D-Ala chain so that it does not bind vancomycin and allows normal peptydoglican polymerization to occur in presence of the drug.

Macrolides (as erythromicin) and Lincosamides (as clindamycin) resistance is due to methylation of two adenine nucleotides in the 23S component of 50S RNA and results from induction of an enzyme that is normally repressed. The methylated RNA has less affinity than the unmethylated one for this type of drugs.

Two genes formerly known as *sat*A and *sat*G, now renamed *vat*D and *vat*E, respectively, encode an aminoacids sequence that is closely related to streptogramin acetyltransferases conferring resistance against Streptogramin A and B (quinupristin / dalfopristin) on *E. faecium*.

Bacteria can withstand Rifamycin (as rifampin) due to an alteration of one aminoacid in DNA-directed RNA polymerase which cause a reduced binding of this drug. This kind of resistance occurs at low level in any bacterial population, thus resistance develops by natural selection during a therapy.

Fluoroquinolones (as ciprofloxacin, gatifloxacin and levofloxacin) resistance can be due to a chromosomal mutation that causes an aminoacid substitution in DNA gyrase A subunit that in its turn cause a reduction in the outer membrane permeability.

Oxazolidinones (as linezolid) resistance is due to its target site modification. Mutations identified in various species are associated with G to U substitution in the peptidyl transferase center of 23S rRNA at position 2576 and result in reduced affinity of linezolid to the 50S subunit.

2) *Resistance due to decreased entry of a drug*

There are two major mechanisms of tetracyclines resistance in enterococci: a) active efflux of the drug across the cell membrane by synthesis of large proteins with 14 predicted transmembrane domains, and b) ribosomal protection by alteration of the ribosomal conformation to prevent binding of tetracyclines. A third mechanism is the enzymatic modification of the drug.

Resistance genes can be located on conjugative plasmids or on the chromosome. In this study, the presence of several of tetracycline resistance genes (*tet*L, *tet*M, *tet*S, *tet*W) were investigated.

The *tet*L gene, encodes for large proteins that cause resistance by active efflux of tetracycline out of the cytoplasm.

The *tet*M is the most common gene among enterococci. It is usually carried by conjugative transposons (such as those of Tn*916* and Tn*1545* family), like *tet*S and *tet*W genes, and encodes proteins that confer resistance by ribosomal protection.

Enterococci possess a natural low level of resistance to aminoglycosides (streptomycin, gentamicin, kanamycin) by modifying the compound outside the cell and thus limiting transport of the altered drugs across the cell membrane. The aminoglycoside-modifying enzymes can be phosphotransferases (APHs), coenzyme A-dependent acetyltransferases (AACs), or ATP-dependent nucleotidyltransferases (ANTs).

3) *Resistance due to destruction or inactivation of a drug*

The resistance to β-lactams mediated by penicillinase enzymes, is one of the best–known mechanisms of bacterial resistance, but it was only rarely found in enterococci (in 1983 a strain of *E. faecalis* producing a β-lactamase identical to that produced by *S. aureus* was reported by Murray and Medersky-Samoraj).

1.4.3 Occurrence of antibiotic resistance in enterococci

Enterococci are frequently associated with important antimicrobial resistance markers.

As reported above, some strains are used for the manufacture of fermented foods whereas others are the cause of serious animal and human infections, especially in nosocomial environment in people belonging to the YOPI-group (Young, Older, Pregnant and Immunocompromised) of vulnerable consumers as defined by Mossel and Struijck (1993) and Mossel *et al.* (1998).

Since the 1980s, when *Enterococcus* have been identified as the third or fourth most prevalent genus among nosocomial pathogens, an increasing number of enterococci isolates resistant to different classes of antibiotic is being reported (Bertrand *et al*., 2000; Facklam *et al*., 2002; Leclercq, 2009).

Antibiotic resistance and nosocomial infection are mutually reinforcing phenomena because resistance allows enterococci to survive in the hospital environment, and the hospital provides the opportunity for dissemination of resistant organisms (Franz *et al*., 1999).

Because of they exist in a wide range of environments (gastrointestinal tract of humans and other animals, soil and water, and foods) and are able to survive in different conditions, they may play a significant role in the dissemination of antimicrobial resistance genes also in different environments (Teuber *et al*., 1999; Cocconcelli *et al*., 2003; Salyers *et al*., 2004; Rizzotti *et al*., 2005 and 2009). Thus, it is important to assess their potential to bear resistances and to transfer them.

First of all, it is necessary to determine the minimum inhibitory concentration (MIC) for a large range of antibiotics. The detection of the MIC above the breakpoint (BP) levels, for one or more antimicrobials, requires further investigations. Indeed, when a strain shows resistance to a specific antibiotic, while the species normally does not, it is considered to be an 'acquired resistance', with a degree of risk of transfer generally greater than that associated with intrinsic resistance (Becquet, 2003; Ammor *et al*., 2007). When the majority of the strains show resistance to an antibiotic it is likely to be an intrinsic, non-transferable resistance.

Enterococci can show both 'natural' (intrinsic) or 'acquired' antibiotic resistance. Furthermore, spontaneous mutations in their 'own' DNA leading to antibiotic resistance can also occur.

Enterococci (especially *E. faecium*) are intrinsically resistant to a broad range of antimicrobial agents such as β–lactams, monobactams, cephalosporins, sulphonamides, polimyxins, low levels of aminoglycosides, lincosamides and quinolones, vancomycin in *E. gallinarum* and *E. casseliflavus/flavescens* (low level), and streptogramins in *E. faecalis* (Franz *et al*., 1999; Franz *et al*., 2003; Kak and Chow, 2002; Klare *et al*., 2003; Kaçmaz and Aksoy, 2005; Folquié Moreno *et al*., 2006; Ogier and Serror, 2008).

If, on the one hand, their intrinsic resistances abilities in themselves are of minor concern, on the other hand, those very abilities may have enabled them to futher acquire genes encoding resistance against other drugs as chloramphenicol and erythromycin, and high-level resistance to aminoglycosides, penicillins, clindamycin, tetracycline, fluoroquinolones and vancomycin, making the treatment of infections difficult (Franz *et al*., 1999; Mundy *et al*., 2000; Klare *et al*., 2003).

However, a combination of cell-wall-active antibiotics (e.g. penicillins) with aminoglycosides (e.g. streptomycin, kanamycin and gentamicin) could act synergistically and indeed have been used successfully in the treatment of enterococcal infections. But this is not always a viable option. Indeed, clinical isolates resistant to these antimicrobial agents, alone or combined, were first detected in the early 1970s (Franz *et al*., 1999).

A major concern is the emergence of vancomycin restistant enterococci (VRE). Vancomycin is a glycopeptide considered as the last resort antibiotic, used to treat serious infections due to resistant Gram-positive bacteria, and given exclusively in a clinical environment when all others fail. As reported above, enterococci vancomycin resistance can be intrinsic (*van*C in *E. gallinarum*), but it is more commonly acquired (*van*A, *van*B, *van*D, *van*E, *van*G) (Ogier and Serror, 2008). VanA and VanB phenotypes are of highest clinical importance and most frequently found in *E. faecalis* and *E. faecium*. Vancomycin was previously used for the treatment of enterococcal infections with strains exhibiting a high level β-lactams resistance, or in cases of β-lactams caused allergy. Unfortunately, many VRE are also highly resistant to all standard anti-enterococcal antimicrobials leaving few treatment options (Franz *et al*., 1999).

The transfer of antibiotic resistance genes from enterococcal strains to other enterococci is well documented, and among them, glycopeptides resistance genes transfer is of more concern. The genes transfer mechanisms may involve both conjugative and non-conjugative plasmids as well as conjugative transposons (Franz *et al*., 1999).

A number of studies have attempted to compare the resistance spectra of different enterococci according to their human, animal or food origins. Antibiotic resistant enterococci are isolated from foods. However, resistance to the clinically relevant antibiotics such as ampicillin, penicillin, gentamycin and vancomycin occurs in very few food isolates (Franz *et al*., 2001; Lopes *et al*., 2003; Mannu *et al*., 2003; Peters *et*

al., 2003; Cosentino *et al*., 2004; Čanžek Majhenič *et al*., 2005; Lopes *et al*., 2005; Folquié Moreno *et al*., 2006; Ogier and Serror, 2008). Concerning the other antimicrobial compounds, the occurrence of antibiotic resistance among dairy isolates seems to vary somewhat between studies, and is often strain- and region dependent (Ogier and Serror, 2008).

Generally, chloramphenicol, tetracycline and erythromycin resistances are a major concern for enterococci colonising dairy products (64, 45 and 32% of resistance, respectively) (Ogier and Serror, 2008).

Tetracycline resistance is one of the most frequently acquired resistances among enterococci of food origin (Peters *et al*., 2003) and the *tet*M is the most common gene that confers them resistance by encoding proteins that alter the ribosomes conformation to prevent binding of Tetracyclines (Kak and Chow, 2002; Agersø *et al*., 2008). Other genes, also detected among environmental, veterinary and clinical isolates, are *tet*K and *tet*L (which encode proteins that cause resistance by active efflux), *tet*O, *tet*S and *tet*W (as *tet*M by ribosomal protection), *tet*U (unknown mechanism) (Kak and Chow, 2002; Gevers *et al*., 2003; Macovei *et al*., 2006). Often Tet^R isolates show co-resistance to erythromycin and/or chloramphenicol. Therefore, it was supposed that these genes could be associated with transposons (mainly of Tn*916* and Tn*1545* family) carrying multiple antibiotic resistance genes (Huys *et al*., 2004), and Tet^R isolates may provide a suitable molecular basis for the further selection of multiple resistances. It is noteworthy that successful transfer of resistances to clinical enterococcal strains is significantly higher than to isolates from food (Klein, 2003).

The most frequent acquired erythromycin resistance mechanism in enterococci is mediated by genes (usually *erm*B and rarely *erm*A and *erm*C) encoding an erm methylase that modifies the 23S rRNA. A revised nomenclature designates the *erm*B gene to include enterococcal resistance genes previously known as *erm*AM and *erm*AMR (Jensen *et al*., 1999; Roberts *et al*., 1999; Teuber *et al*., 1999; Kak and Chow, 2002; Poeta *et al*., 2005; Stovcik *et al*., 2008).

Other erythromycin resistance genes are the *mef*A and *msr*A. The first gene encodes an efflux protein that pumps macrolides out of the cell, and it appears to be harboured on a conjugative element and mediates a low level of erythromycin resistance. The *msr*(A) gene confers resistance to both Macrolides and Streptogramin B antibiotics *via* an ATP-binding transporter protein and has been detected in *E. faecium* clinical isolates (Kak and Chow, 2002).

However, compared to antibiotic resistance in pathogenic bacteria, relatively few studies have investigated acquired antibiotic resistance in non-pathogenic bacteria, so few reliable data are available to develop a quantitative risk assessment. Commensal bacteria as enterococci may act as a reservoir for antibiotic resistance genes found in human pathogens, and the main threat is that they can transfer resistance genes to more pathogenic species (as *Staphylococcus aureus* and *Listeria monocytogenes*). Transfer from *E. faecalis* to *S. aureus* in a cutaneous mouse model (Noble *et al*., 1992) and in vitro transfer to *Listeria* spp. has been reported (Biavasco *et al*., 1996).

Enterococci can reach humans mainly via the food chain, but once established in a human population, they can be spread in various ways. It is important to consider that bacteria isolated from a human source may not necessarily have originated directly

from animals shedding them or from contaminated food animal products (Gevers, 2002). Several studies proved the transfer of plasmids between human and animal bacteria, but in most cases it is impossible to trace in which direction the transfers have taken place. It is definitely unquestionable that the risk of acquisition of resistant bacteria from animals is higher for humans who stay in close contact to animals or animal products, such as farmers, abattoir workers and most of all for veterinarians who work in an environment where a high selective pressure in favour of antibiotic resistant bacteria, resulting from the use of antimicrobials, is common.

Therefore, more studies aimed at carefully monitoring the spread of resistant strains in the environment and in foods should be carried out in order to avoid the opportunity of transferring resistance genes from enterococci to pathogens responsible of serious infections.

1.5 Use of antibiotics in animal husbandry

There are three different applications for antimicrobial use in animals: 1) therapy, 2) prophylaxis and 3) growth promotion.

1.5.1 Therapy

Therapeutic use of antimicrobial agents is intended to control an existing bacterial infection. Antimicrobial agents are usually used for treatment of subclinical or clinical mastitis during lactation, enteric and pulmonary infections, skin and organ

abscesses. Individual animal treatment is commonly performed in dairy cows, calves and swine whereas, for food-producing animals which are kept in larger groups, group treatment is preferably performed. Moreover, veterinary intervention in such large animal groups often occurs when the first animal shows symptoms of the disease, because it is thought that it is economically more advantageous to medicate the entire animal group at an early stage to reduce the number of sick or dead animals. With such treatment regimes, the antimicrobials are commonly administered as feed additives or via drinking water (Gevers, 2002; Samanidou and Evaggelopoulou, 2008). The antimicrobial agents that currently are used to treat or prevent bacterial infections in animals are essentially the same classes of compounds that are used in human medicine.

1.5.2 Prophylaxis

Prophylaxis is a solely preventive measure. Its application can be to both individual animals and to groups of animals. For instance, the prophylactic intramammary administration of antimicrobials at therapeutic levels at the end of the lactation period is used to prevent mastitis. However, prophylactic animals' treatment is criticized for providing the basis of selection of resistance (Gevers, 2002).

1.5.3 Growth promotion

Shortly after the introduction of the therapeutic use of antibiotics, the growth-promoting effect of these products on farm animals was discovered. Several antibiotics have been used as growth promoters, by the addition of small quantities of

antibiotics to the diet, during the animals' early life, for at least five decades (Gevers, 2002; Dibner and Richards, 2005; Castanon, 2007; Samanidou and Evaggelopoulou, 2008). The mechanisms of growth promotion are still not fully elucidated. Four hypotheses have been proposed to explain their action: (1) nutrients may be protected against bacterial destruction; (2) nutrients may be more effectively absorbed, because of a thinning of the small intestinal barrier due to the absence of GI microflora; (3) production of toxins by intestinal bacteria may be reduced and (4) subclinical intestinal infections may be reduced (Butaye *et al*., 2003; Samanidou and Evaggelopoulou, 2008). However, different classes of antibiotic and the animal species involved may imply different mechanisms (Dibner and Richards, 2005; Samanidou and Evaggelopoulou, 2008). The use of antibiotics as growth promoters has become widespread, in the beginning even without any restrictions. The widespread use of antibiotics as growth promoters was first criticized in UK by the end of the 1960s, which resulted in the 'Swann Report' (Swann, 1969). Although the economic benefits of using antibiotics, whether for therapeutic, prophylactic or growth promoting purposes were clearly highlighted, much concern was expressed about the possible induction of antibiotic resistance among bacteria of human and animal origin and the subsequent loss of effectiveness in the treatment of human bacterial disease (Gevers, 2002; Butaye *et al*., 2003; Samanidou and Evaggelopoulou, 2008). However, in the end the Swann Committee on the use of antibiotics in animal husbandry and veterinary medicine, under pressure from the Farming Industry, accepted that in the UK the use of antibiotics for growth promotion should be restricted to antibiotics which make a significant economic difference in the raising of

livestock, have little or no application as therapeutic agents in humans or animals, and do not impair the efficacy of a prescribed therapeutic drug through the development of resistant strains. The Swann report also formed the basis for European wide legislation in Directive 70/524 (1970), in which a list of allowable additives with their maximum and minimum dosages, withdrawal period from slaughter and animal species in which the product may be used was published (Gevers, 2002; Butaye *et al*., 2003; Dibner and Richards, 2005; Castanon, 2007; Samanidou and Evaggelopoulou, 2008). Worldwide differences in the use and regulations of antibiotics are large. The first nation to eliminate the use of antimicrobials for growth promotion was Sweden in 1986. Avoparcin was banned both in Denmark and Norway in 1995 in response to concerns that its use contributed to the creation of an animal reservoir of glycopeptides-resistant enterococci. In the same year, the Norwegian animal feed industry voluntarily terminated all use of AGPs (Animal Growth Promoters). Shortly after, also the Danish animal feed industry voluntarily discontinued the use of all AGPs and, during the period 1998–1999, the AGPs were gradually phased out in Denmark (Samanidou and Evaggelopoulou, 2008). The EC legislation was amended on several occasions, and since 1997 several antibiotics have been forbidden. Then, from 2001 there were only four AGPs permitted in the EU: avilamycin, monensin (for cattle), flavophospholipol (flavomycin) and salinomycin. However, by January 1st, 2006 EU had banned the use of all antibiotics as growth promoters. After this date, antimicrobials should only be allowed in feed as coccidiostats (Castanon, 2007; Samanidou and Evaggelopoulou, 2008). Although by 2006 the use of antimicrobial agents for growth promotion was no longer approved in the EU, their inappropriate

use for food animals continues in North America and Australia. In contrast to EU, the United States of America allows 19 different antibiotics to be used for growth promotion including some, e.g. penicillin and streptomycin, that are used for treating people in the USA.

1.6 Virulence factors

Enterococci have been associated with high mortality rates (12-68 %) in patients with bacteremia, but they are often part of a polymicrobic bacteremia, thus making it difficult to determine their independent contribution to morbidity and mortality of the patients (Oprea and Zervos, 2007).

In order to cause infection, enterococci must:

a) colonize the host tissue,

b) resiste host specific and unspecific defense mechanisms,

c) produce pathological changes.

They do this expressing virulence traits associated with adhesion to host tissue, translocation, invasion and abscess formation, modulation of host inflammatory responses, secretion of toxic products, and evasion of immune responses (Jett *et al*., 1994; Franz *et al*., 1999, Eaton and Gasson, 2001).

Virulence of *Enterococcus* spp. may be linked to particular species, either *E. faecalis* and, to a lesser extent, *E. faecium* being the most relevant members of this genus with regard to clinical aspects (Eaton and Gasson, 2001; Franz *et al*., 2001; Giraffa, 2002;

Kayser, 2003; Ogier and Serror, 2008; Hällgren *et al*., 2009). Occasionally infections are due to *E. gallinarium*, *E. raffinosus*, *E. casseliflavus*, *E. avium*, *E. pseudoavium*, *E. malodoratus*, *E. mundtii*, *E. durans*, *E. hirae* or *E. solitarius*. Additional species such as *E. cecorum*, *E. columbae*, *E. saccharolyticus*, *E. dispar*, *E. sulfureus*, *E. seriolicida*, and *E. flavescens* have been proposed as additions to this list (Mundy *et al*., 2000).

Numerous reports are present in literature on the distribution of *E. faecalis* and *E. faecium* virulence traits among isolates from different sources (including clinical, commensal, environmental and food isolates) showing an incidence varying a lot between studies (Huycke *et al*., 1995; Eaton and Gasson, 2001; Franz *et al*., 2001; Gilmore *et al*., 2002; Dupré *et al*., 2003; Mannu *et al*., 2003; Semedo *et al*., 2003; Creti *et al*., 2004; Drahovská *et al*., 2004; Lepage *et al*., 2006; Abriouel *et al*., 2008; Cariolato *et al*., 2008; Gomes *et al*., 2008; McGowan-Spicer *et al*., 2008; Pangallo *et al*., 2008; Valenzuela *et al*., 2008 and 2009).

The detection of virulence genes may point to a virulence potential in food strains, but foodborne enterococcal infections have never been reported. Furthermore, the presence of virulence genes does not mean that they are functional, as, for instance, various enterococcal isolates carrying *gel*E failed to produce gelatinase (Eaton and Gasson, 2001; Lepage *et al*., 2006; Ogier and Serror, 2008). Thus, it is likely that expression of virulence factors may vary according to the ecological niche, and virulence potential may increase in special cases such as elderly and immunocompromised patients.

Generally, clinical enterococci contain more virulence determinants than do enterococci recovered from food, but the latter rather than being a direct cause of infection, may contribute to spread virulence genes. Indeed, enterococci are known for their ability to exchange genetic information by conjugation, and these processes take commonly place in the gastrointestinal tract. Many enterococcal virulence traits, such as haemolysin-cytolysin production, the adhesion ability and the antibiotic resistance, have been shown to be transmissible by gene transfer mechanisms. Often, the same plasmid may encode a sex pheromone response and either antibiotic resistance or haemolysin production genes (Franz *et al*., 2001; Giraffa, 2002).

Thus, monitoring virulence traits presence in food isolates is necessary because of the potential for their dissemination to the consumer.

There is not one sole factor responsible for virulence. About a dozen putative virulence factors have been identified in enterococci of different sources (Eaton and Gasson, 2001; Mundy *et al*., 2000; Gilmore *et al*., 2002; Giraffa, 2002; Semedo *et al*., 2003; Foulquié Moreno *et al*., 2006; Ogier and Serror, 2008). The best described virulence traits are adherence factors on the one hand, and certain secreted products on the other (Kayser, 2003).

In the present work, the presence of eight genes for virulence factors (AS, aggregation substance; Esp, extracellular surface protein; Ace, accessory colonization factor; EfaAfs and EfaAfm *E. faecalis* antigen A; Cyl*A*, activation of Cytolysin; Gel*E*, gelatinase; cpd, pheromone determinant) among Fiore Sardo cheese Ery^R and Tet^R *E. faecium* and *E. faecalis* were investigated. Thus, these traits will be briefly described below.

1.6.1 Enterococcal adhesins

The aggregation substance (AS) is a surface-localized protein encoded by pheromone-responsive, self-transmissible plasmids that mediate binding of donor bacterial cells with plasmid-free recipients, allowing efficient conjugal transfer in a liquid environment (Jett *et al*., 1994; Franz *et al*., 1999; Gilmore *et al*., 2002; Folquié Moreno *et al*., 2006; Bhardwaj *et al*., 2008). The enhancement of enterococcal virulence by aggregation substance appears to occur at multiple levels. The aggregation protein is involved in cell aggregation, adherence to eukaryotic cells, and conjugation. It is capable of promoting adherence of enterococci to epithelial cell surface, a necessary first step in colonization, and of promoting internalization and survival within intestinal macrophages and PMNs. Furthermore, it has a role in the dissemination of plasmid-encoded virulence factors, such as the enterococcal cytolisin (see below) and antibiotic resistance determinants, within the species. Finally, aggregation substance and cytolysin may act synergistically to enhance virulence by facilitating achievement of a quorum and activating the quorum-sensing mode of Cytolysin regulation, resulting in tissue damage and potentially deeper tissue invasion (Franz *et al*., 1999; Mundy *et al*., 2000; Gilmore *et al*., 2002; Folquié Moreno *et al*., 2006). Up to now, AS was exclusively found in *E. faecalis* strains, with a high incidence also among food isolates (Eaton and Gasson, 2001; Franz *et al*., 2001; Folquié Moreno *et al*., 2006).

In addition to aggregation substance, enterococci express a large-molecular weight, cell-surface-localized protein termed Esp, a chromosomally encoded 'enterococcal surface protein'. Esp was originally found in *E. faecalis* and its incidence was shown to be higher among clinical strains than in isolates from healthy individuals. This protein is able to promote the colonisation and persistence in animal tissues (Shankar *et al*., 2001; Franz *et al*., 2003). The presence of Esp also increases cell hydrophobicity, adherence to abiotic surfaces (such as plastic material), and biofilm formation in vitro (Hällgren *et al*., 2009). Indeed, Esp exhibits characteristics of surface protein receptors designated 'Microbial Surface Components Recognising Adhesive Matrix Molecules' (MSCRAMMs) that mediate binding to extracellular matrix proteins (Toledo-Arana *et al*., 2001). The ability to form biofilms increases antibiotic resistance and is important in infections involving catheters and in the dairy industry where enterococci adhere to stainless steel and may contaminate equipment (Gelsomino *et al*., 2002; Waar *et al*., 2002). On the basis of the proximity of *esp* gene to the Cytolysin operon and its role in generating microenvironments where a quorum of bacteria would accumulate (biofilms), it can be hypothesized that Esp may function synergistically with the Cytolysin in a manner analogous to the previously stated synergy between aggregation substance and Cytolysin (Gilmore *et al*., 2002).

Genes enconding for AS, Esp protein and Cytolysin have been shown to be part of a pathogenicity island (PAI) in *E. faecalis* and *E. faecium* (Eaton and Gasson, 2002; Gilmore *et al*., 2002; Shankar *et al*., 2002; Leavis *et al*., 2004; Shankar *et al*., 2006; Ogier and Serror, 2008).

Ace (adhesin to collagen from *E. faecalis*) is another enterococcal adhesin that is similar to MSCRAMMs of other Gram-positive bacteria, particularly to the collagen-binding protein Cna of *S. aureus* (a collagen adhesin shown to be important for endocarditis caused by this species) (Rich *et al*., 1999). Ace not only binds to collagen (types I and IV) but also to laminin and fibrinogen (Rich *et al*., 1999; Nallapareddy *et al*., 2000a, b). Nallapareddy *et al*. (2000b) showed that Ace was expressed by enterococci during human infections, and it is often associated to adherence of bacteria involved in endocarditis to the collagen-rich heart valves (Nallapareddy *et al*., 2008; Gilmore *et al*., 2000). There is diversity in *ace* gene sequence among different *E. faecalis* isolates coming from various geographical regions as well as from various clinical sources (urine, feces, and blood, including endocartis) (Nallapareddy *et al*., 2000b). A considerably variability in *E. faecalis* adherence characteristics has been observed, while no appreciable adherence was detected with *E. faecium* strains till recently (Gilmore *et al*., 2002) when Nallapareddy *et al.* (2008) demonstrated that Acm (an homolgue of Cna like Ace), previously found in *E. faecium* (Nallapareddy *et al*., 2003) contributes to the pathogenesis of this species.

A gene encoding an ~37-kDa dominant antigen of *E. faecalis* (named EfaA for *E. faecalis* endocarditis antigen A) was previously identifed by Lowe *et al.*, 1995 using serum from a patient with *E. faecalis* endocarditis. The translated protein sequence was found to show homology to members of a protein family, designated LraI

(Jenkinson, 1994) that has since then been identified in a number of streptococcal species (Singh *et al*., 1998).

EfaAfs and EfaAfm are the cell adhesins expressed in serum by *E. faecalis* and *E. faecium* respectively. These virulence determinants are commonly found in both species, but differing degrees of homology among *efaA* genes in the different enterococcal species were demonstrated by Eaton and Gasson (2001). The *efaAfm* gene identified in *E. faecium* DNA libraries and its deduced amino acid sequence showed 73% similarity to EfaA of *E. faecalis* (Singh *et al*., 1998), and PCR amplification products with primers designed for *efaA* antigen gene from *E. faecalis* can be obtained for some *E. faecium* strains. Until recently, only the *efaAfs* gene has been shown to influence pathogenicity in animal models (Eaton and Gasson, 2001; Pimentel *et al*., 2007) and has been the most investigated also among *E. faecium* isolates (Dupré *et al*., 2003; Mannu *et al*., 2003; Billström *et al*., 2008).

1.6.2 Secreted factors

The Cytolysin is a haemolytic protein toxin whose operon is mostly carried on a plasmid or is occasionally integrated into the bacterial chromosome (Mundy *et al*., 2000; Gilmore *et al*., 2002; Kayser, 2003). It occurs in some strains of *E. faecalis* and is distantly related to streptolysin S and also to a class of bacteriocins known as lantibiotics (Mundy *et al*., 2000; Gilmore *et al*., 2002). The Cytolysin causes rupture of a variety of target membranes, including bacterial cells (bacteriocin), erythrocytes, and other eukaryotic cells. The production of cytolysin has also been shown to significantly worsen the severity of endocarditis and endophthalmitis in animal

models as well as to contribute to the severity of enterococcal disease in humans (Vankerckhoven *et al*., 2004). Cytolysin genes are carried on a plasmid or are integrated into the bacterial chromosome. The Cytolysin operon is a complex determinant encoding five genetic markers: *cyl*L, *cyl*S, *cyl*M, *cyl*B and *cyl*A (Jett *et al*., 1994). The first two genes encode for two precursor proteins that require the CylM protein to be transformed to a secretable form. CylB helps the secretion of CylL and CylS proteins. These two proteins remain inactive until the enzyme encoded by the gene *cyl*A removes six aminoacids from each amino terminus. In several infection models, Cytolysin has been found to contribute to toxicity and lethality (Kayser, 2003).

Gelatinase is an extracellular zinc-endopeptidase, one of the two secreted proteases (the other is serine) described for *E. faecalis* (Gilmore *et al*., 2002). GelE is able to hydrolyse gelatine, collagen, casein, haemoglobin, and other small biologically active peptides (Jett *et al*., 1994). Although bacterial proteases mainly function to provide peptide nutrients to organisms, they may also cause damage to host tissues and thus constitute virulence factors:

1) causing indirect degradation of host connective tissues;
2) deregulating critical host processes to facilitate microbial invasion and survival in host environments;
3) deregulating key components of the host immune system by degrading either immnoglobulins or complement pathways;

4) degrading specific host proteins resulting in production of toxins;

5) activating viruses either directly or indirectly;

6) mediating direct degradation of host connective tissues or tissue proteins;

or in some other manner acting directly as virulence factor in animal experiments (Gilmore *et al*. 2002).

The presence of Gelatinase production among food *E. faecalis* strains is high (Eaton and Gasson, 2001; Franz *et al*., 2001), and Su *et al.* in 1991 reported the sequence of the protease gene, *gel*E. However, even when *gel*E gene is present, a negative phenotype can be shown (Eaton and Gasson, 2001).

1.6.3 Sex pheromones

Some enterococci possess a plasmid collection mechanism which is based on production of chromosomally encoded 'sex pheromones' (cpd, cob, ccf, cad). Sex pheromones are small linear peptides, 7 to 8 amino acids in length, secreted by enterococci that promote conjugative transfer of plasmid DNA between strains. These peptides are referred to as pheromones because they elicit a specific mating response from plasmid carrying donor cells. Typically, multiple pheromones are secreted simultaneously by a given strain. In addition to pheromones, each pheromone-responsive plasmid encodes a secreted peptide that acts as a competitive inhibitor of its corresponding pheromone (Jett *et al*., 1994). When pheromones bind to receptors on the cell surface of strains that contain plasmid DNA, this signal is transduced and leads to induction of the aggregation substance (AS) gene. When this gene is

expressed, AS mediates the formation of cell clumps by binding to a complementary receptor termed binding substance, that allows the highly efficient transfer of the pheromone plasmid on which the AS gene is encoded (Clewell, 1993; Dunny *et al*., 1995) . The pheromones, however, not only have a role in transfer of plasmid DNA, but also serve as chemo-attractive substances for human neutrophils and induce inflammation and superoxide production (Clewell *et al*., 2002; Bhardwaj *et al*, 2008).

1.7 Fiore Sardo PDO cheese

Fiore Sardo cheese was chosen as 'model' to carry out this study on enterococcal microflora of Sardinian ovine cheeses because it was supposed that it is the maximum expression of the microbial biodiversity of dairy products typical of the Sardinia island. As a matter of fact, it has a tight link with its area of production (including all the island) strengthened also by its ancient traditional production technology (Scintu and Piredda, 2007) which confers on it specific microbiological, chemical and sensory characteristics.

Fiore Sardo is a Protected Designation of Origin (PDO) (Commission Regulation EC N. 1263/96) hard cheese still produced by households in rural areas of Sardinia from ewes raw milk, using lamb rennet paste, and usually without any addition of starter culture. Therefore, the fermentation and ripening processes of this naturally souring product are entirely performed by the indigenous microflora present in the milk and coming from the environment during milking and cheese manufacture.

The adaptation of the structure of dairy plants required for the hygienic norms prescribed by the EU, the need to approach a wider market other than local, the use of milking machines, and the automation of some passages of the transformation technology could reduce the useful microflora coming from environmental contaminations. Nevertheless, the product specification of Fiore Sardo cheese allows the employment of natural and native starter cultures whenever it is necessary to guarantee the correct acidification of the curd and to inhibit the development of undesirable bacteria.

For this reason, in the last few years some small technological innovations have been introduced to the traditional cheese-making of Fiore Sardo cheese (Comunian *et al.*, 2009b).

The traditional method requires the use of equipment like tinned copper vats and wood tools, and breaking the curd up and forming it into moulds are completely manual operations. The use of a mini steel polyvalent cheese vat, the automation of all cheese-making operations, and the addition of a natural whey starter culture are the technological innovations introduced in cheese production, staying however inside the PDO Regulations.

Various studies have been carried out in the past to characterize the microbial composition of the main Sardinian cheeses produced with ewes and goats milk to understand more about how they are made, their microbiological and chemical characteristics, and to improve their quality, especially of Fiore Sardo cheese (Bottazzi *et al.*, 1978; Pettinau *et al.*, 1978; Ledda *et al.*, 1994; Piredda *et al.*, 1996; Mannu *et al.*, 2000; Mannu *et al.*, 2006; Pisano *et al.*, 2006 and 2007; Pirisi *et al.*,

2007; Mangia *et al*., 2008). Such studies showed that lactococci, facultatively heterofermentative lactobacilli (FHL), always associated with enterococci, are the main microbial groups colonizing this cheese. Occasionally, *Streptococcus thermophilus* was also isolated (Pisano *et al*., 2006). Recently, a study was carried out (Comunian *et al*., 2009b) to determine if there are meaningful differences, at various times of ripening, in the microbial composition between cheeses produced at the same farm by traditional and innovative methods, using a shortened, reliable, sensitive, matrix-independent PCR-Culture-Technique (PCR-CT). The search plan findings confirmed that the introduction of small technological innovations, in particular the addition of a natural whey starter culture, the use of tools that make the phases of curd cutting and shaping easier, does not change the characteristics of Fiore Sardo PDO cheese from a microbiological point of view. In fact, the high variability in the microbial counts found for all the species investigated among the experimental productions cannot be explained by the changes made in the manufacturing technology, as the same variability was found in traditionally made cheeses. Indeed, it would appear that microbial contamination depends on accidental factors occurring during milking and manufacturing and are linked to the environmental variations throughout the production season, such as temperatures, type of feeding, lactation stage of the flock, number of milking animals (Barron *et al*., 2001). This is what we would expect from a situation in which traditional manufacture is strongly linked to the seasonality of the milk production.

On the other hand, the applied innovations do reduce the workload and production time, which allow an increase in production without losing the microbiological,

chemical (Addis *et al*., 2007) and sensory (Addis *et al*., 2009) characteristics peculiar to this cheese.

So far, few studies have been carried out concerning the presence of antibiotic resistant strains in Fiore Sardo cheese. Mannu *et al*., in 2003, and Cosentino *et al*., in 2004, investigated the problem of the incidence of virulence determinants and antibiotic resistance among enterococci colonizing this cheese.

Mannu *et al.* (2003) compared dairy *E. faecium* with strains of animal and clinical origin regarding these aspects in order to exclude the presence of such pathogenic traits in *E. faecium*, which are part of the dominant microbiota of ewe's milk cheeses. When one considers that the high levels of enterococci in milk, particularly in ewe's milk, are probably due also to faecal contamination from the animal, then investigating *E. faecium* from ovine faeces is also important.

The results of this study clearly indicate that *E. faecium* strains isolated from both cheese and sheep faeces are less pathogenic than those isolated from clinical samples. A similar pattern of resistance to antibiotics was observed in both dairy and animal strains. It was also found that there was difference in the kind of virulence determinants present in dairy and clinical isolates, while no virulence traits were found in sheep faeces strains. The results of this study suggested that *E. faecium* from traditional Sardinian raw milk cheeses should not be considered to be the a source of untreatable nosocomial enterococcal infections in humans in the island of Sardinia.

Cosentino *et al*., (2004) genetically and technologically characterized different dairy enterococcal species (*E. faecium, E. durans, E. faecalis*). As regards their safety and health features, gelatinase activity was observed in two strains of *E. faecalis* and one

strain of *E. durans*. β-haemolysis on horses' blood was never detected in any of the strains, independently of species. Most strains produced tyramine from tyrosine but none decarboxylated lysine, histidine or ornithine. Overall, a wide spectrum of resistance was observed. Almost all strains were resistant to the aminoglycosides, gentamycin, kanamycin, streptomycin, neomycin, and to the semisynthetic penicillin, oxacillin, but resistance to vancomycin was not widespread among the strains: only one *E. faecium* and one *E. durans* strain were found to be vancomycin resistant. The results showed a low incidence of some potentially pathogenic traits of health concern.

A recent study on *Lactobacillus paracasei* (one of the FHL species constituting the dominant microflora of Fiore Sardo), isolated from Fiore Sardo PDO cheese and from some typical and PDO Italian dairy and meat products (i.e. Water Buffalo Mozzarella PDO cheese, Gorgonzola PDO cheese, Pannerone cheese, and Salame Piacentino PDO), highlighted that the susceptibility to tetracycline and erythromycin and the presence of resistance genes against these drugs in the phenotypically resistant isolates could be related to the isolation environment (e.g. geographical area, and dairy farm) (Comunian *et al*, 2009a). Indeed, the majority of the *Lb. paracasei* strains susceptible to these two antibiotics originated from Fiore Sardo, a cheese produced where no systematic use of antibiotics as growth promoters was carried out over the years in animal husbandry. Conversely, the highest number of resistant strains was observed in dairy and meat products produced in areas where more intensive animal husbandry practices have been applied.

These outcomes are very important as traditional dairy foods, such as Fiore Sardo PDO cheese, are a significant element of Sardinian cultural heritage and their production and sale are critical economic contributors to the region.

Increasing the possibilities for these products through improved competitiveness is a key part of sustainable rural development and can contribute to increase economic growth and social welfare. Therefore, modernisation of some aspects of the traditional cheese making, while ensuring that the product remains of the highest quality, is required.

The results obtained in the present research work, extended to almost the fifty percent of the dairy farms producing Fiore Sardo PDO cheese, could constitute an important resource to improve and defend this product that continues to be a source of pride and identity for those who engage in this traditionally and culturally, as well as socially, important activity.

1.8 Aims of this study

The main objectives of this study were to identify, at both species and strain level, enterococcal microflora and to assess some safety aspects (presence of antibiotic resistance traits and virulence determinants) of *Enterococcus* species colonizing Fiore Sardo PDO cheese, since this *genus* is present, sometimes in high numbers, at different stages of ripening of various ewes' milk cheeses.

The scientific community does not unanimously agree on the opportunity and safety of the presence of enterococci in food, as they are still considered as emerging pathogens in nosocomial infection. Furthermore, even though at present, there is not evidence suggesting enterococci as food borne pathogens, they are well known to be potential vehicles of horizontal transfer of antibiotic resistance and virulence genetic determinants.

For these reasons, resistance to fourteen antibiotics, including tetracycline and erythromycin, two of the most widely used in both clinical and animal therapy whose resistance genes are known to be transferable among enterococci and other commensal and pathogen bacteria, were investigated. The genetic basis of virulence determinants and tetracycline and/or erythromycin resistance in the phenotypically resistant isolates were also investigated.

Other important objectives of the research were to evaluate the biodiversity of enterococcal microflora and complement previous studies on Fiore Sardo PDO cheese microbiota, surveying as high a number as possible of farms producing this cheese in the whole production area.

2. MATERIALS AND METHODS

2.1 Cognitive surveying

A cognitive surveying was conducted over the whole production area of Fiore Sardo PDO among 21 randomly selected typical sheep dairy farms (almost 50% of the total producing farms), located in different dairy areas of Sardinia.

During the visit to the farms, the shepherds were interviewed and information was obtained regarding:

- geographical location (plain, hill or mountain);
- number of animals of the flock;
- type of milking (mechanical or manual);
- cheese manufacture process;
- amount of milk daily used for cheese-making;
- cheese ripening conditions;
- prophylaxis, pathologies and therapies linked with the use of antibiotics.

2.2 Cheese manufactures and sampling

Twenty-one samples coming from one batch of cheese manufactured at each farm were collected, from March to June, at 3.5 months after the production, which is the minimal ripening period for marketing according to the product specification. The cheeses were manufactured by the shepherds using the traditional artisanal technique, during the autumnal and winter season of production, from November to March.

2.3 Microbiological analysis

2.3.1 Plate counts

Samples of cheese were prepared according to the FIL-IDF standard 122C (1996). An aliquot of 20 g of each sample was homogenised with 180 ml of citrate solution in a Stomacher blender (Seward Medical, London, UK). Ten-fold dilutions of the homogenates were prepared in sterile peptone water (1 g/l). Then, 100 µl of each dilution were plated in duplicate on Slanetz and Bartley agar medium (SBA, Oxoid, Unipath Ltd., Basingstoke, UK) supplemented or not with tetracycline (16 µg/ml) (Sigma-Aldrich, Steinheim, Germany), erythromycin (4 µg/ml) (Sigma-Aldrich), kanamycin (1024 µg/ml) (Sigma-Aldrich) or vancomycin (8 µg/ml) (Sigma-Aldrich), for the isolation both of sensitive and antibiotic resistant strains. The antibiotic concentrations took into account the microbiological breakpoint (BP) levels reported

for *Enterococcus* spp. by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (2006), except for the kanamycin (BP not available).

The plates were incubated at 42 °C in aerobiosis for 48 hours.

2.3.2 Isolation, growth, storage conditions and basic phenotypic tests

For each batch of cheese, up to 10 colonies were randomly picked up from each SBA plates with the lowest countable number. The isolates were stored at -80 °C in BHI broth (Oxoid) containing 50 % of sterile glycerol, before being purified.

Pure cultures were obtained by streaking three times the isolates on SBA supplemented or not with tetracycline, erythromycin, kanamycin or vancomycin according to their medium of origin and at the same concentration used for the primary isolation plates.

The media used for plating or isolation and cultivation are briefly described below.

Slanetz Bartley Agar (SBA)

It is a selective medium used for isolation and enumeration of enterococci in food.

Sodium Azide inhibits the growth of Gram-negative bacteria and staphylococci. Enterococci reduce triphenyltetrazolium chloride (TTC) to formazan and grow forming red-pink colonies.

Brain Heart Infusion Broth (BHI)

BHI broth is a general-purpose liquid medium used for the cultivation of a wide variety of microorganisms, including bacteria, yeasts and moulds. It is a nutritious, buffered culture medium that contains infusions of brain and heart tissue and

peptones to supply protein and other nutrients necessary to support the growth of fastidious and non fastidious microorganisms.

After checking for morphology, Gram stain and catalase reaction tests were performed before submitting the pure isolates to molecular identification.

2.3.3 Identification of isolates at species level

Pure, Gram-positive, catalase negative isolates were submitted to species identification.

The majority of isolates were identified by species-specific PCR. The isolates which did not give any expected amplification product using species-specific primers were amplified with primers specific for *Enterococcus* genus and, in case of positive result, submitted to16S rDNA amplification and sequencing.

PCR primers for species and genus identification (Jackson *et al.*, 2004; Ke *et al.*, 1999) and universal primers P0/P6, *Escherichia coli* position 27f and 1497r respectively (Grifoni *et al.*, 1995), used for the amplification of 16S rDNA to be sequenced are reported in **Table 2.**

Template for PCR was prepared by suspending some bacterial colonies from a pure culture of each isolate in 100 µl of sterile deionised water. The cells suspensions were treated in a microwave oven for 15 min at 700 W (in order to avoid evaporation and dryness problems, a beaker containing 200 ml of distilled water was put in to the microwave oven together with the samples). Five microliters of each lysate were used directly to perform PCR.

Table 2. Primers used in this study for species identification and 16S rDNA amplification

Target	Primer	Sequence (5'-3')	Product Size (bp)	Reference
E. avium	AV1 AV2	GCT GCG ATT GAA AAA TAT CCG AAG CCA ATG ATC GGT GTT TTT	368	Jackson *et al*., 2004
E. casseliflavus	CA1 CA2	TCC TGA ATT AGG TGA AAA AAC GCT AGT TTA CCG TCT TTA ACG	288	Jackson *et al*., 2004
E. durans	DU1 DU2	CCT ACT GAT ATT AAG ACA GCG TAA TCC TAA GAT AGG TGT TTG	295	Jackson *et al*., 2004
E. faecalis	FL1 FL2	ACT TAT GTG ACT AAC TTA ACC TAA TGG TGA ATC TTG GTT TGG	360	Jackson *et al*., 2004
E. faecium	FM1 FM2	GAA AAA ACA ATA GAA GAA TTA T TGC TTT TTT GAA TTC TTC TT T A	215	Jackson *et al*., 2004
E. gallinarum	GA1 GA2	TTACTTGCTGATTTTGATTCG TGAATTCTTCTTTGAAATCAG	173	Jackson *et al*., 2004
E. hirae	HI1 HI2	CTTTCTGATATGGATGCTGTC TAAATTCTTCCTTAAATGTTG	187	Jackson *et al*., 2004
E. malodoratus	MA1 MA2	GTAACGAACTTGAATGAAGTG TTGATCGCACCTGTTGGTTTT	134	Jackson *et al*., 2004
E. mundtii	MU1 MU2	CAGACATGGATGCTATTCCATCT GCCATGATTTTCCAGAAGAAT	98	Jackson *et al*., 2004
Enterococcus genus	Ent1 Ent2	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	Ke *et al*., 1999
16S rDNA	P0 P6	GAG AGT TTG ATC CTG GCT CAG CTA CGG CTA CCT TGT TAC GA	1468	Grifoni *et al*., 1995

PCRs protocols

PCRs were performed in a final volume of 25 µl, consisting of 20 µl of PCR mixture and 5 µl of DNA template, in a Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany), both for species- or genus-specific PCR and for 16S rDNA amplification.

The reaction mixture for enterococci species-specific PCRs consisted of 3 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix (Euroclone, Milan, Italy), 2.5 µl 10X Reaction Buffer (Euroclone), 3.5 U Taq (EuroClone), 0.2 M of each primer (Sigma Genosys, Steinheim, Germany), and 5 µl of DNA suspension.

In this work, each species-specific primer pair was used separately instead of performing a multiplex PCR as developed by Jackson *et al.* (2004).

Following an initial denaturation at 95 °C for 4 min, products were amplified by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C (*E. durans, E. faecalis, E. faecium, E. gallinarum, E. avium,* and *E. hirae*) or 60 °C (*E. gilvus* and *E. mundtii*) for 1 min, and elongation at 72°C for 1 mi Amplification was followed by a final extension at 72 °C for 7 min.

For the *Enterococcus* genus-specific-primers Ent1/Ent2, the reaction mixture consisted in: 2.5 mM $MgCl_2$, 0.25 Mm each desoxynucleoside triphosphate (EuroClone), 2.5 µl 10 X Reaction Buffer (EuroClone), 1 U of Taq DNA polymerase (EuroClone), 0.3 mM concentration of each primer (Sigma Genosys), and 5 µl of DNA suspension. The PCR conditions were programmed as follows: an initial denaturation consisting of 3 min at 94 °C followed by 35 cycles consisting of: 30 s denaturation at 94 °C, 30 s annealing at 58 °C, and 60 s extension at 72 °C.

Ten microliters of PCR product was electrophoresed on a 2% 1X Tris-acetate-EDTA agarose gel at 100V and then stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen, Paisley, UK) was used as the standard.

Enterococcus faecium DSM 20477^T and *E. faecalis* DSM 20478^T obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (Braunschweig, Germany), and *E. avium* LMG 10744^T, *E. casseliflavus* LMG 10745^T, *E. malodoratus* LMG 10747^T, *E. mundtii* LMG 4838^T, *E. durans* LMG 10746^T, *E. hirae* LMG 6399^T, and *E. gallinarum* LMG 13129^T obtained from BCCM/LMG Bacteria Collection (Gent, Belgium) were used as positive controls in species- and genus-specific PCRs.

The PCR mixture for 16S rDNA amplification consisted of 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix (Euroclone), 2.5 µl 10X Reaction Buffer (Euroclone), 1.25 U Taq (EuroClone), 0.93 µM each primer (P0 and P6) (Sigma Genosys).

Three microliters of 16S rDNA amplification product were quantified loading them on a 2% 1X Tris-acetate-EDTA agarose gel and electrophoresing at 100V. Gel staining was successively performed in ethidium bromide solution (0.5 µg ml^{-1}). Then, DNA was purified with Montage® PCR Centrifugal Filter Devices (Microcone, Millipore, Billerica, USA). This purification system allows sample clean-up of salts, primers and unicomporated dNTPs with no solvents or chemicals required. After quantification, PCR products were sent to a commercial facility service for sequencing (BMR Genomics, Padova, Italy). The primer P0 was used for sequencing.

The partial 16S rDNA sequences obtained were edited with the software Chromas version 1.43 (Griffith University, Brisbane, Qld, Australia) and aligned with those present in GenBank with the BLAST (Altschul *et al.*, 1997) search program (http://www.ncbi.nml.nih.gov/). The identities of the isolates were determined on the highest score basis.

2.3.4 Strain typing

In the present work, different molecular typing techniques, rep-$(GTG)_5$ PCR (Versalovic *et al.*, 1994; Gevers *et al.*, 2001), RAPD-PCR with primer M13 (Huey and Hall, 1989), and PFGE with SmaI restriction enzyme (Mannu *et al.*, 1999; Graves and Swaminathan 2001), have been applied in order to assess the strain composition of the natural enterococcal population found in artisanal Fiore Sardo PDO cheese. All the isolates were submitted to rep-$(GTG)_5$ PCR and RAPD-PCR. Isolates belonging to *E. faecium* species were also characterised by Pulsed field Gel Electrophoresis (PFGE).

DNA preparation protocol for rep-PCR $(GTG)_5$ and RAPD-PCR with primer M13

The isolates were grown overnight in BHI broth at 37 °C.

DNA was prepared by FTA® Technology (Whatman International Ltd., Maidstone, UK). This technology uses cellulose cards impregnated with a patented chemical formula that lyses cell membranes and denatures proteins on contact. FTA works by lysis of cells releasing the nucleic acid within the matrix of the card, where it is entrapped among the cellulose fibres.

The detailed DNA extraction procedure is reported above.

1. Spot 5 µl of an overnight culture on FTA Card.
2. Dry by air at least 1 hour.
3. Take a sample disc from the dried spot with a micro-punch 2mm diameter.
4. Place disc in a PCR amplification tube.
5. Add 180 µl of FTA Purification Reagent® to PCR tube.
6. Incubate for 5 min at room temperature giving tube moderate manual mixing to disrupt the debris and aid in washing.
7. Remove and discard all used FTA Purification Reagent with a pipette.
8. Repeat steps 5-7 for a total of 2 washes with FTA Purification Reagent.
9. Add 180 µl of TE^{-1} Buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8).
10. Incubate for 5 min. at room temperature.
11. Remove and discard all used TE^{-1} Buffer with a pipette.
12. Repeat steps 9-11 for a total of 2 washes with TE^{-1} Buffer.
13. Ensure that all the liquid has been removed before performing the analysis.
14. Use the washed and eventually dried disc directly to perform PCR.

2.3.4.1 rep-$(GTG)_5$ PCR

PCR reaction was performed in a total volume of 25 µl consisting of 24 µl MegaMix™ Labogen and 1 µl GTG_5 primer 50 mM, using a washed FTA® disc directly as template. An Eppendorf Mastercycler® (Eppendorf) was utilized for the

PCR reaction with cycling parameters of: 7 min at 95 °C, followed by 30 cycles of 30 sec at 95 °C, 1 min at 40 °C, 8 min at 65 °C, and a 16 min final extension at 65 ºC.

Rep-PCR products were separated by electrophoresis on 1.8 % (w/v) agarose gel in Tris-acetate buffer at 90V (Volthour 222) and then stained in ethidium bromide solution (0.5 µg ml^{-1}); the ladder 1kb plus by Invitrogen was used as molecular marker.

Isolates were subjected to rep-PCR analysis at least twice.

Digital photos of gels were taken with Kodak DC 120 Camera (Kodak Digital Science 1D LE 3.0 Software).

2.3.4.2 RAPD-PCR with primer M13

PCR reactions were performed in a total volume of 25 µl consisting of 24 µl MegaMix™ Labogen and 1 µl of M13 primer (5'-GAG GGT GGC GGT TCT-3'), using a washed FTA® disc directly as template. An Eppendorf Mastercycler® (Eppendorf) was utilized for the PCR reaction with cycling parameters of: 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 20 sec at 45 °C, 2 min at 72 °C, and a 10 min final extension at 72 ºC.

Rep-PCR profiles were visualized after electrophoresis on 2 % (w/v) agarose gel in Tris-acetate buffer at 90V, then stained in ethidium bromide solution (0.5 µg ml^{-1}); the ladder 1kb plus by Invitrogen was used as molecular marker.

Isolates were subjected to RAPD-PCR analysis at least twice.

Digital photos of gels were taken with Kodak DC 120 Camera (Kodak Digital Science 1D LE 3.0 Software).

2.3.4.3 Pulsed Field Gel Electrophoresis (PFGE)

A standardized and fast molecular PFGE protocol described for *Listeria monocytogenes* by Graves and Swaminathan (2001) was adapted to enterococci strain typing. Bacteria were grown on brain heart infusion agar plates at 37 °C for 16–18 h. Cells were removed from the plate into a 2 ml sterile NaCl (0.85%) solution (bioMérieux sa, Marcy l'Etoile, France) using a sterile cottonswab and the cell density adjusted up to achieve a cell concentration of 5 McFarland (= 1.5 x 10^9 UFC/ml). The standardized cell suspension (240 µl) was transferred to 1.5 ml microcentrifuge tubes, centrifuged at 13,000 rpm for 5 min. The pellet was resuspended in 240 µl of TE (Tris 10 mM pH8, EDTA 1 mM pH 8). Sixty microliters of 10 mg/ml lysozyme solution was added and mixed with the cells by pipetting up and down. The mixture was incubated in a waterbath at 37 °C for 10 min. An equal volume (300 µl) of molten 1.2% LM agarose (EuroClone), 1% sodium dodecyl sulphate (International Biotechnologies, Inc., New Haven, USA), 0.2 mg/ml Proteinase K (Sigma-Aldrich) prepared in sterile distilled water and maintained at 53–56 °C was added to the 300 µl of cell suspension and mixed by gently pipetting up and down several times. The mixture (600 µl) was dispensed into sample reusable plug molds and allowed to cool for 5 min. The agarose plugs were transferred to 50 ml polypropylene conical tubes containing 4 ml of lysis buffer [50 mM Tris (Sigma-Aldrich) pH 8.0, 50 mM EDTA (J.T. Baker, Mallinckrodt, The Netherlands), pH 8.0, 1% sodium lauroyl sarcosine (Sigma-Aldrich), 0.15mg/ml Proteinase K], incubated for 2 h at 50–54°C. After proteolysis, the lysis buffer solution was removed and the plugs were washed twice with 15 ml of preheated (50 °C) sterile distilled water for 10 min each followed by

four washes with 15 ml of preheated (50 °C) TE buffer for 15 min each in the orbital water bath shaker (50 °C) at 200 rpm. After the final TE wash, the plugs were sliced (2–2.5 mm slices) using a sterile lancet and prepared for restriction digestion or stored in 1.5 ml TE at 4°C until ready for restriction. Digestion with SmaI restriction endonuclease (Promega Italia, Milan, Italy) was performed according to the supplier's instructions. Plugs were transferred in sterile 2 ml Eppendorf tubes containg 25 µl of 10X RE buffer, 40 U of SmaI and sterile mQ water was added up to a final reaction volume of 250 µl. Then, the plugs were incubated. Restriction was done at 37 °C for 5 hours.

DNA fragments were separated in 0.8% (w/v) GellyPhor® PFGE agarose (EuroClone) gel in 0.5 X TBE buffer. Electrophoresis was performed in a CHEF-DRIII (Bio-Rad, Hemel Hempstead, UK), containing 0.5 X TBE buffer equilibrated at 12 °C, at a constant voltage of 6 V cm^{-1}, with different pulse times for a total running time of 16 h: 1-20 s for 5 h; 1-5 s for 5 h; 10-40 s for 6 h. The gels were then stained in ethidium bromide (0.5 µg ml^{-1}). Lambda ladder PFG marker (New England Biolabs Inc., Ipswich, MA, USA) and Low Range PFG Marker were used as molecular size standard. Isolates were subjected to PFGE analysis at least twice.

Digital photos of gels were taken with Kodak DC 120 Camera (Kodak Digital Science 1D LE 3.0 Software).

2.3.4.4 Cluster analysis

The digital photos of fingerprints were analysed by the BioNumerics® V 4.5 software package (Applied Maths, Gent, Belgium).

Patterns generated with both rep-PCR $(GTG)_5$ and RAPD M13 were analysed both separately and using the composite data set comparison tool of Bionumerics. The similarity among profiles was calculated using the Pearson correlation coefficient. Dice coefficient was used for PFGE profiles. Dendrograms were obtained by Unweighted Pair group Method using Arithmetic averages (UPGMA) (Vauterin and Vauterin, 1992).

For *E. faecium* isolates the composite data set comparison tool was also used to analyse the PFGE patterns together with rep-PCR and RAPD M13 profiles.

2.3.4.5 Determination of the typability, reproducibility, similarity index and Discriminatory Power/Diversity Index.

Typability (ability to assign a type to the isolates tested by it) of the strain typing techniques used in this study was calculated as the percentage of typable isolates over the total number of the isolates analysed (van Belkum *et al*., 2007).

To assess the reproducibility (ability of a typing method to assign the same type to an isolate tested on independent occasions, separated in time and/or place) (van Belkum *et al*., 2007) of each strain typing technique, control strains *Enterococcus faecium* DSM 20477^T and *E. faecalis* DSM 20478^T obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (Braunschweig, Germany), *E. durans* LMG 10746^T, *E. hirae* LMG 6399^T, and *E. gallinarum* LMG 13129^T, obtained from BCCM/LMG Bacteria Collection (Gent, Belgium), were subjected to molecular characterization using RAPD-PCR, rep-PCR, and PFGE (only DSM 20477). Each analysis was performed in quadruple for each strain. Cluster analysis was performed

as described above. For each species and each typing technique, the percentage of similarity among the four separately obtained banding patterns of the same strain coming from different cultures, and amplified or submitted to restriction reaction in different days, was considered as the reproducibility index of each technique for each species. Then, this percentage was used as threshold to discriminate different strains. Moreover, each PCR and restriction reactions were controlled for reproducibility by inclusion of the reference strains belonging to the species that was analyzed among isolates.

Where there was a combined analysis of rep-PCR and RAPD patterns, or rep-PCR, RAPD and PFGE patterns, an average threshold similarity index was calculated on the basis of those previously calculated for each technique, in order to discriminate different strains.

The Discriminatory Power (D.P.) of the typing techniques used in this study was calculated by means of Sympson's index of diversity (D) (Simpson, 1949) as modified by Hunter and Gaston (1988) (D.I.):

$$D.I. = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

,

where N is the total number of isolates in the sample population, S is the total number of types described, and n_j is the number of isolates belonging to the j^{th} type.

The same index was used also to evaluate the percentage of biodiversity among enterococci population studied.

2.4 Antibiotic resistance

2.4.1 MIC determination

Minimum Inhibitory Concentrations (MICs) of the most used antibiotics in veterinary and human medicine, covering several of the known chemical and functional classes, were determined using the Sensitre® Microbiology Systems (Trek Diagnostic Systems, Magellan Biosciences, Cleveland, Ohio, USA). This is a ready-to-use micro-titre plate version of the classic broth dilution method. The plates, chosen among those available on the market on the basis of their content in most used antibiotics against *Enterococcus* spp., contained different concentrations of the following lyophilised antibiotics: ampicillin, cefazolin, ceftriaxone, ciprofloxacin, clarithromycin, clindamycin, erythromycin, gatifloxacin, gentamicin, levofloxacin, linezolid oxacillin + 2 % NaCl, penicillin, quinupristin/dalfopristin, rifampin, streptomycin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin. Antimicrobial agents were tested at twofold serial dilutions, and the lowest concentration that inhibits the visible growth of an organism was regarded as the MIC.

The micro-titre plate layout and the tested antibiotics concentrations are reported in **Figure 1**.

Fig. 1. List and concentrations (µg/ml) of antibiotics tested, and micro-titre plate layout.

Antibiotics		Concentrations
AMP	Ampicillin	0.12 - 16
FAZ	Cefazolin	2 - 16
AXO	Ceftriaxone	8 - 64
CIP	Ciprofloxacin	0.5 - 2
CLA	Clarithromycin	1 - 8
CLI	Clindamycin	0.5 - 4
ERY	Erythromycin	0.25 -8
GAT	Gatifloxacin	1 - 8
GEN	Gentamicin	2 -16, 500
LEVO	Levofloxacin	0.25 - 8
LZD	Linezolid	0.5 - 8
OXAT	Oxacillin+2%NaCl	0.25 - 8
PEN	Penicillin	0.06 - 8
SYN	Quinupristin/Dalfopristin	0.12 - 4
RIF	Rifampin	0.5 - 4
STR	Streptomycin	1000
TET	Tetracycline	2 - 16
SXT	Trimethoprim/sulfamethoxazole	0.5/9.5 - 4/76
VAN	Vancomycin	1 - 32

POS Positive Control
NEG Negative Control

GPN2F - Gram-Positive MIC Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	ERY 0.25	ERY 0.5	ERY 1	ERY 2	ERY 4	ERY 8	CLA 1	CLA 2	CLA 4	CLA 8	GEN 500	STR 1000
B	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	SYN 0.12	SYN 0.25	SYN 0.5	SYN 1	SYN 2	SYN 4
C	CLI 0.5	CLI 1	CLI 2	CLI 4	FAZ 2	FAZ 4	FAZ 8	FAZ 16	TET 2	TET 4	TET 8	TET 16
D	AMP 0.12	AMP 0.25	AMP 0.5	AMP 1	AMP 2	AMP 4	AMP 8	AMP 16	GEN 2	GEN 4	GEN 8	GEN 16
E	RIF 0.5	LEVO 0.25	LEVO 0.5	LEVO 1	LEVO 2	LEVO 4	LEVO 8	LZD 0.5	LZD 1	LZD 2	LZD 4	LZD 8
F	RIF 1	PEN 0.06	PEN 0.12	PEN 0.25	PEN 0.5	PEN 1	PEN 2	PEN 4	PEN 8	CIP 0.5	CIP 1	CIP 2
G	RIF 2	SXT 1/19	SXT 2/38	SXT 4/76	AXO 8	AXO 16	AXO 32	AXO 64	GAT 1	GAT 2	GAT 4	GAT 8
H	RIF 4	SXT 0.5/9.5	OXA+ 0.25	OXA+ 0.5	OXA+ 1	OXA+ 2	OXA+ 4	OXA+ 8	NEG	POS	POS	POS

Each isolate to be tested was plated on to BHI agar medium and overnight aerobically incubated at 35 ± 2 °C. Several colonies were picked up from the agar plate and emulsified in 2 ml sterile NaCl (0.85%) solution (bioMérieux) up to achieve a cell concentration of 0.5 McFarland (= 10^8 UFC/ml). As recommended by CLSI, the inoculum standardization was based on determination of cell density by spectrometry (Densimat - bioMérieux). One hundred microliters of cell suspension (10^8 CFU/ml) were added to 10 ml of cation adjusted Sensitre® Muller-Hinton broth (MAIM S.L., Barcelona, Spain), obtaining a concentration of 1 x 10^6 CFU/ml. After vortexing, the cell suspension was transferred in a sterile multi-channel solution basin. In order to obtain an inoculum of 5 x 10^5 CFU/ml per well, as stated by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (2006), 50 µl were poured in each well.

Fifty microliters of sterile Sensitre® Mueller-Hinton broth were inoculated into a single well and used as negative control. The plates were covered with the adhesive seal, and aerobically incubated at 35 ± 2 °C for 16-20 hours before visual reading of growth. For vancomycin MIC determination plates were read after 24 hours of incubation.

In order to monitor the precision, repeatability and accuracy of susceptibility tests, *Enterococcus faecealis* ATCC® 29212, a strain with known sensitivity to the antimicrobial agents to be tested, was weekly used as Quality Control (QC).

For the majority of the antimicrobial tested, the MICs and the level of resistance were determined according to the recommendations of the CLSI (2006).

In the lack of CLSI interpretive criteria, EFSA breakpoints were used to consider strains as resistant (**Table 3**).

No microbiological breakpoints for *Enterococcus* spp. were available for 5 (cefazolin, ceftriaxone, clarithromycin, oxacillin + 2% NaCl, and trimethoprim/sulfamethoxazole) out of the 19 antibiotics contained in the Sensitre® ready-to-use micro-titre plates. Therefore, MICs results obtained for these antibiotic will be not reported.

Table 3. CLSI (2006) and EFSA (2008) *Enterococcus* antibiotic resistance interpretive criteria (Breakpoint µg/ml; S = Susceptible, I = Intermediate, R = Resistant).

Antibiotic	**Breakpoints *µg/ml***		
	S	**I**	**R**
Ampicillin[1]	≤ 8	-	≥ 16
Ciprofloxacin[1]	≤ 1	2	≥ 4
Erythromycin[1]	≤ 0.5	1 - 4	≥ 8
Gatifloxacin[1]	≤ 2	4	≥ 8
Gentamicin (high-level) [1]	<500		≥ 500
Levofloxacin[1]	≤ 2	4	≥ 8
Linezolid[1]	≤ 2	4	≥ 8
Penicillin[1]	≤ 8	-	≥ 16
Quinupristin-dalfopristin [1]	≤ 1	2	≥ 4
Rifampin[1]	≤ 1	2	≥ 4
Streptomycin (high-level) [1]	< 512	-	≥ 1000
Tetracycline[1]	≤ 4	8	≥ 16
Vancomycin[1]	≤ 4	8 -16	≥ 32
Clindamycin[2]	-	-	> 4
Cefazolin[3]	-	-	-
Ceftriaxone [3]	-	-	-
Clarithromycin[3]	-	-	-
Oxacillin + 2% NaCl [3]	-	-	-
Trimethoprim/Sulfamethoxazole [3]	-	-	-

[1] CLSI M100-S16 (2006) Table 2D. M7-A6-MIC Testing section.

[2] No CLSI *Enterococcus* interpretive criteria available for this antimicrobial. EFSA (2008) microbiological breakpoints (µg/ml) are reported. Strains with MICs higher than the above breakpoints are considered as resistant

[3] Neither CLSI nor EFSA *Enterococcus* interpretive criteria (breakpoints µg/ml) are available for the genus *Enterococcus*.

2.4.2 Presence of antibiotic resistance genes

For *E. faecium* and *E. faecalis* strains resistant to erythromycin, tetracycline or vancomycin on the basis of the antibiotic susceptibility testing, the possible presence of genes responsible for resistance to those antibiotics (*ermA, ermB, ermC, tetL, tetM, tetS, tetW, vanA and vanB*) was investigated by PCR using primers and protocols previously described in literature (Dutka-Malen *et al.*, 1995; Olsvik *et al.*, 1995; Jensen *et al.*, 1999; Gevers *et al.*, 2003; Aminov *et al.*, 2001) (**Table 4**). The presence of Vancomycin resistance genes was also investigated for the only *E. gallinarum* isolate. The DNA was extracted as previously described for the identification of isolates at species level (**Paragraph 2.3.3**).

PCRs were performed in a final volume of 25 µl, consisting of 20 µl of PCR mixture and 5 µl of DNA template, in a Eppendorf Mastercycler® (Eppendorf). The composition of the PCR mixtures, the thermal cycles used for the amplification of each resistance gene, and the electrophoresis conditions used for visualizing the PCR products are reported below.

Positive control strains were kindly supplied by the Microbiology Institute, Università Cattolica del Sacro Cuore, Piacenza (Italy).

Table 4. Primers used in this study for PCR detection of genes associated with resistance to tetracycline, erythromycin and vancomycin, and their products size.

Target	Primer	Sequence	Product Size (bp)	Reference
*erm*A	Tn554-2	TCAAAGCCTGTCGGAATTGG	560	Jensen *et al*., 1999
	Tn554-1	AAGCGGTAAACCCCTCTGAG		
*erm*B	ErmB-1	CATTTAACGACGAAACTGGC	425	Jensen *et al*., 1999
	ErmB-2	GGAACATCTGTGGTATGGCG		
*erm*C	ErmC-1	ATCTTTGAAATCGGCTCAGG	295	Jensen *et al*., 1999
	ErmC-2	CAAACCCGTATTCCACGATT		
*tet*L	TetL FW 3	GTMGTTGCGCGCTATATTCC	696	Gevers *et al*., 2003
	TetL RV 3	GTGAAMGRWAGCCCACCTAA		
*tet*M	TetM F	GAACTCGAACAAGAGGAAAGC	740	Olsvik *et al*., 1995
	TetM R	ATGGAAGCCCAGAAAGGAT		
*tet*S	TetS FW	GAAAGCTTACTATACAGTAGC	169	Aminov *et al*., 2001
	TetS RV	AGGAGTATCTACAATATTTAC		
*tet*W	TetW FW	GAGAGCCTGCTATATGCCAGC	168	Aminov *et al*., 2001
	TetW RV	GGGCGTATCCACAATGTTAAC		
*van*A	A1	GGGAAAACGACAATTGC	732	Dutka-Malen *et al*., 1995
	A2	GTACAATGCGGCCGTTA		
*van*B	B1	ATGGGAAGCCGATAGTC	635	Dutka-Malen *et al*., 1995
	B2	GATTTCGTTCCTCGACC		
*van*C-1	C1	GGTATCAAGGAAACCTC	822	Dutka-Malen *et al*., 1995
	C2	CTTCCGCCATCATAGCT		

PCR protocols for resistance genes detection

ermA, _ermB_ and _ermC_ (Jensen _et al._, 1999)

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 1 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 94°C for 5 min followed by 30 cycles of DNA denaturation at 94°C for 45 s, primer annealing 58°C for 30s, and DNA extension at 72°C for 45 s. After the last cycle, the reaction was terminated by incubation at 72°C for 7 min, and the products were stored at 4°C.

Electrophoresis conditions: the PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 1.2 % agarose gel, and the gels were stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

tetL (Gevers _et al._, 2003)

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 2 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 1 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 94°C for 5 min followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing 55°C for 1 min, and DNA extension at 72°C for 2 min. After the last cycle, the reaction was terminated by incubation at 72°C for 10 min, and the products were stored at 4°C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 1% agarose gel, and the gels were stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

***tet*M (Olsvik *et al*. 1995)**

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 2 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 1 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 94 °C for 5 min followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing 60 °C for 30 s, and DNA extension at 72°C for 1 min. After the last cycle, the reaction was terminated by incubation at 72°C for 5 min, and the products were stored at 4 °C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 1 % agarose gel, and the gels were stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

***tet*S (Aminov *et al*., 2001)**

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 1.5 mM $MgCl_2$, 0.1 mM (each) deoxynucleoside triphosphate, 1 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 94°C for 2 min followed by 25 cycles of DNA denaturation at 94°C for 30 s, primer annealing 55°C for 2 min, and DNA extension at 72°C for 30 s. After the last cycle, the reaction was terminated by incubation at 72°C for 7 min, and the products were stored at 4°C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 2.5% agarose gel, and the gels were stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

***tet*W (Aminov *et al*., 2001)**

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 1.5 mM $MgCl_2$, 0.1 mM (each) deoxynucleoside triphosphate, 1 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 94 °C for 5 min followed by 25 cycles of DNA denaturation at 94°C for 30 s, primer annealing 64 °C for 2 min, and DNA extension at 72°C for 30 s. After the last cycle, the reaction was terminated by incubation at 72°C for 7 min, and the products were stored at 4°C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 2.5% agarose gel, and the gels were stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

***van*A, *van*B and *van*C (Dutka-Malen *et al*., 1995)**

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 7 mM $MgCl_2$, 0.5 mM (each) deoxynucleoside triphosphate, 2 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 94 °C for 2 min followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing 54 °C for 1 min, and DNA extension at 72°C for 1 min. After the last cycle, the reaction was terminated by incubation at 72°C for 10 min, and the products were stored at 4°C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 1.5 % agarose gel, and the gels were stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

2.5 Presence of virulence determinants genes

Isolates belonging to *E. faecium* and *E. faecalis* species showing resistance to erythromycin and tetracycline were also characterised for the presence of several virulence determinants. The DNA extraction for the detection of gene coding for virulence factors was performed as previously described for the identification of isolates at species level (**Paragraph 2.3.3**). Specific primers for the following eight virulence genes were used: *agg*A, *ace*, *gel*E, *esp*, *efa*A*fm*, *efa*A*fs*, *cpd* and *cyl*A. The list of the primers used and their amplification products are reported in **Table 5**.

Table 5. Primers used for PCR detection of gene coding for virulence factors and their products size.

Target	Primer	Sequence	Product Size (bp)	Reference
agg	TE3 TE4	AAGAAAAAGTAGACCAAC AACGGCAAGACAAGTAAATA	1553	Eaton and Gasson, 2001
gelE	TE9 TE10	AGTTCATGTCTATTTTCTTCAC CTTCATTATTTACACGTTTG	403	Eaton and Gasson, 2001
esp	TE34 TE36	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	933	Eaton and Gasson, 2001
cpd	TE51 TE52	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	782	Eaton and Gasson, 2001
efaAfm	TE37 TE38	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	735	Eaton and Gasson, 2001
efaA	efaA1 F efaA2 R	CGTGAGAAAGAAATGGAGGA CTACTAACACGTCACGAATG	499	Mannu *et al.*, 2003
ace	ACE1 F ACE2 R	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	320	Mannu *et al.*, 2003
cylA	CYT I CYT IIb	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	Vankerckhoven *et al.*, 2004

PCRs were performed in a final volume of 25 µl, consisting of 20 µl of PCR mixture and 5 µl of DNA template, in a Eppendorf Mastercycler® (Eppendorf). The composition of the PCR mixtures, the thermal cycles used for the amplification of each resistance gene, and the electrophoresis conditions used for visualizing the PCR products are reported below.

Strains used in previous studies (Mannu *et al*., 2003) and belonging to the Collection of microorganisms of the DiRPA's Microbiology Laboratory of AGRIS Sardegna were included as positive controls in PCRs.

PCR protocols for virulence genes detection

***agg*, *gel*E, *esp*, *efaAfm* and *cpd* (Eaton and Gasson, 2001)**

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 1 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and and 5µl of cell template.

PCR program: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing (52° C for *agg*, 56 °C for *gel*E, 55 °C for *esp* and *cpd*, 58 °C for *efaAfm*) for 45 sec, and elongation at 72°C for 45 s. After the last cycle, the reaction was terminated by incubation at 72°C for 7 min, and the products were stored at 4°C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 0.8 % agarose gel for *agg* and *esp* genes and 1.5 % agarose gel for *gel*E gene. The gels were stained in ethidium bromide solution (0.5 µg

ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

***ace* and *efa*A (Mannu *et al.*, 2003)**

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 2 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 94 °C for 1 min followed by 45 cycles (30 for *efa*A primers) of DNA denaturation at 94°C for 1 min, primer annealing 56 °C for 1 min, and DNA extension at 72°C for 1 min. After the last cycle, the reaction was terminated by incubation at 72°C for 10 min, and the products were stored at 4°C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 2 % agarose gel then stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

***cyl*A (Vankerckhoven *et al.*, 2004)**

PCR mixture: 2.5 µl of 10X reaction buffer (EuroClone), 2.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 2 U of Taq DNA polymerase (EuroClone), 0.5 µM of each primer and 5µl of cell template.

PCR program: an initial denaturation step at 95 °C for 15 min followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing 56 °C for 1 min, and DNA

extension at 72°C for 1 min. After the last cycle, the reaction was terminated by incubation at 72°C for 10 min, and the products were stored at 4°C.

Electrophoresis conditions: The PCR products (5 µl) were analyzed by electrophoresis in TAE buffer on 1.5 % agarose gel then stained in ethidium bromide solution (0.5 µg ml^{-1}). DNA molecular weight marker 1kb plus (Invitrogen) was used as molecular mass standard.

3. RESULTS AND DISCUSSION

3.1 Cognitive surveying

Before starting the study on the enterococcal microflora of Fiore Sardo PDO cheese, because, as mentioned in the introduction, this is a product closely related to its production area, a cognitive survey was carried out to relate our results to the production conditions of Sardinia.

In **Figure 2** the geographical distribution of the 21 randomly selected typical sheep dairy farms producing Fiore Sardo PDO is shown. The main information obtained through the cognitive surveying is reported below.

Sixty-seven percent of the dairy farms is located on the hills, 24 % on the plain, and 9% on the mountain areas of the island.

On average, there are 600-700 dairy sheep *per* farm with the actual range being between 400 and 1000. Only in 1 out of the 21 dairy farms surveyed ewes are hand-milked.

The production season starts in November/December and ends in June. All the dairy farms have a mini dairy plant with separate rooms for the phases of cheese-making, salting, smoking and ripening.

Most of the dairy farms use a tinned copper vat to warm up and coagulate the milk. Several of them have a mini steel polyvalent cheese vat. Nevertheless, breaking the curd up and forming it into moulds are generally completely manual operations, and

the shepherds use a polyvalent cheese vat exclusively to warm up the milk. Only two producers use it for the mechanization of all cheese-making operations.

At each farm, around 400-500 litres (range between 160 and 700) *per* day are used for making cheese.

In accordance with its PDO regulation, Fiore Sardo cheese must ripen for at least 3.5 months. Eighty percent of the producers allow the cheeses to ripen at room temperature, either at the dairy farm or in cellars at their house. Only 20% use ripening chambers with controlled temperature.

Because one of the main objectives of this study was to assess the antibiotic resistance among enterococci of Fiore Sardo cheese, during the survey of each farm, particular attention was paied to clarify for what purpose, and in what measure antibiotics were used. Unfortunately, the shepherds were able to provide only limited information on this regard. From August to October, when the milk/cheese production is suspended, prophylaxis treatments are carried out to prevent the most common pathologies (mastitis and lameness) occurring in dairy sheep. Mastitis is commonly diagnosed in all the dairy farms in 1-5% of sheep; lameness in 25% of dairy farms, in 1-2% of sheep. Tetracycline, oxytetracycline and ampicillin are the most frequently used antibiotics to treat mastitis, while oxytetracycline chlorohydrate and copper sulphate are commonly used to treat lameness.

Fig. 2. Geographic distribution of the 21 surveyed farms of production.

3.2 Microbiological analysis

3.2.1 Plate counts, isolation and basic phenotypic tests

The plate counts varied considerably depending on the antibiotic added to the medium utilized, and were quite different when no antibiotic was added.

Indeed, the highest number of colonies grew in SBA without any addition of antibiotic (6.03 ± 1.07 Log CFU/g), while the lowest number (0.10 ± 0.46 Log CFU/g) was counted in SBA + 8 µg/ml of vancomycin (**Table 6**).

Table 6. Log CFU/g counted in the different culture media utilized in this study.

Culture media	N. of farms with counts ≥ 1 log CFU/g	Log CFU/g			
		Min	Max	Average	sd
SBA	21	4.12	7.65	6.03	± 1.07
SBA + 4 µg/ml Ery	7	0.00	6.05	1.29	± 2.00
SBA + 1024 µg/ml Kan	14	0.00	6.15	2.43	± 1.95
SBA + 16 µg/ml Tet	15	0.00	5.85	2.78	± 1.96
SBA + 8 µg/ml Van	1	0.00	2.11	0.10	± 0.46

Differences in bacterial counts also occurred depending on the farm from which the cheese samples were collected (**Fig. 3**). Furthermore, no bacterial growth was detected for some cheese samples depending on which antibiotic was added to the culture medium.

Tetracycline prevented the growth of bacteria from cheese samples coming from farms 1, 9, 12, 13, 19 and 20, while kanamycin achieved the same result in farms 1, 4, 8, 12, 13, 14 and 15.

In SBA + 4 µg/ml erythromycin, bacteria from cheese samples from only seven farms (4, 5, 6, 10, 11, 16 and 18) were able to grow, while vancomycin allowed the bacterial growth only from cheese samples coming from farm 10.

For antibiotic-containing media, when growth occurred it was equal to 3.87 ± 1.28, 3.65 ± 1.03, 3.89 ± 0.94 and 2.11 ± 0.00 Log CFU/g for erythromycin, kanamycin, tetracycline and vancomycin, respectively.

Briefly, presumptive enterococci were present in all Fiore Sardo cheese samples analysed, as from all farms high levels of them were counted in SBA without antibiotic addition, but they did not grow in every antibiotic-containing medium. Furthermore, when growth was observed in antibiotic-containing media, it occurred for cheese samples coming from a small number of farms.

These results are very important since they mean that Fiore Sardo presumptive enterococcal microflora is quite sensitive to the four antibiotics used for this study. In particular, vancomycin and erythromycin, crucial drugs used in human and veterinary medicine, were able to prevent growth of enterococcal microflora from almost all, or from a large majority of the cheese samples analysed, while kanamycin and tetracycline were effective in 33.3 and 28.6 % of samples, respectively.

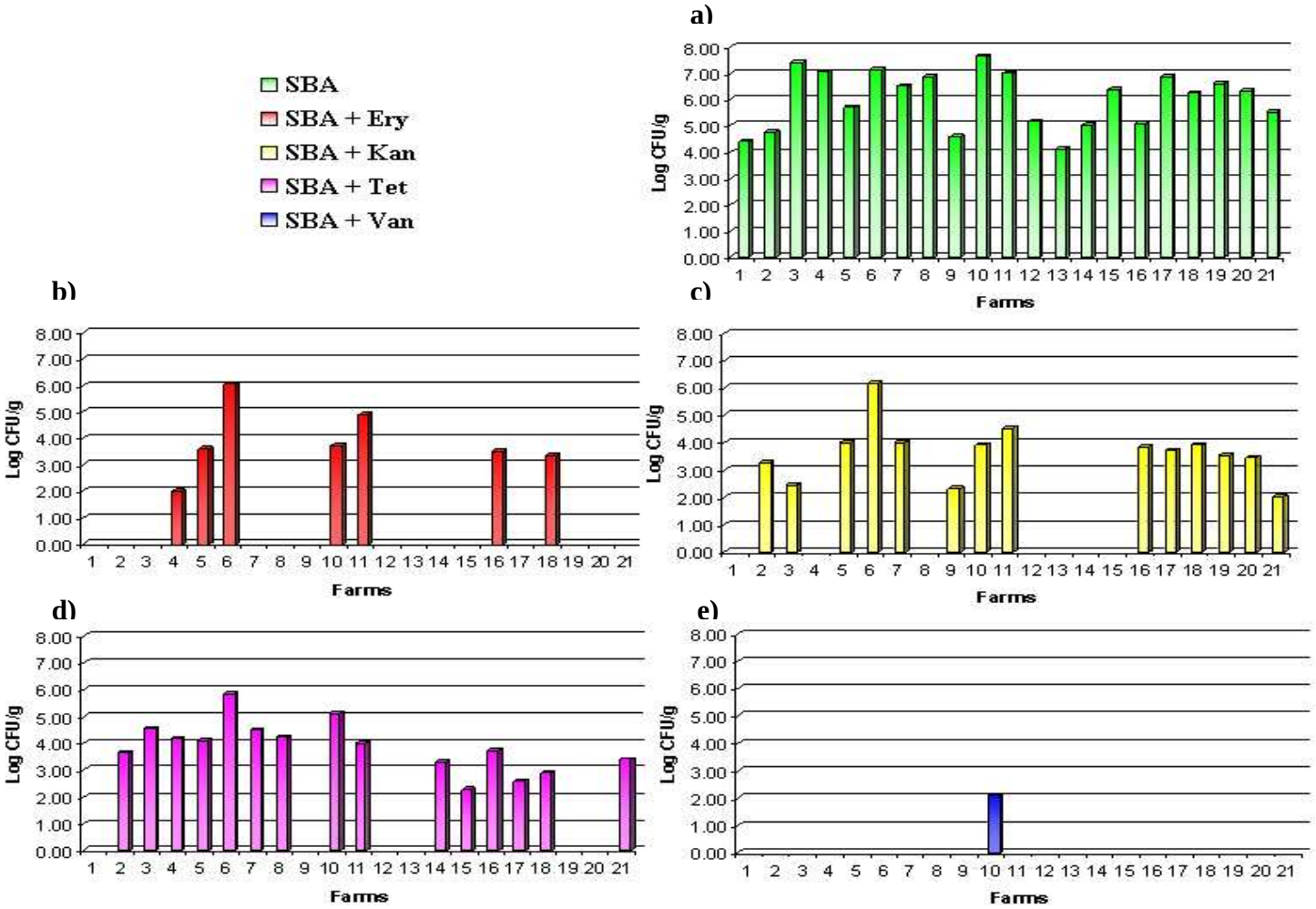


Fig. 3. Log CFU/g counted *per* each farm in the different culture media used in this study. **a**) SBA, **b**) SBA + erythromycin; **c**) SBA + kanamycin; **d**) SBA + tetracycline; **e**) SBA + vancomycin.

In total, 328 isolates were collected from all the media and purified.

All the isolates were Gram positive and catalase negative.

Most isolates (122, 37%) were obtained from SBA without antibiotic supplementation. The supplementation of the culture medium with tetracycline and kanamycin obtained around the same amount of isolates (85, 26%, and 72, 22%, respectively), while from SBA plus erytrhomycin a lower number of isolates (43, 13%) were obtained. Only 6 (2%) colonies from SBA plus vancomycin were isolated (**Fig. 4**).

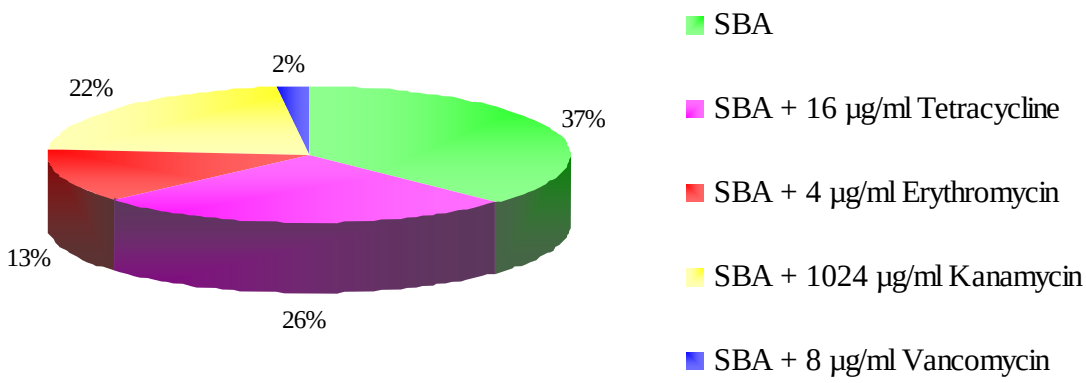


Fig. 4 Percentage of colonies isolated from SBA medium supplemented or not with antibiotic.

3.2.2 Identification of isolates at species level

The six isolates coming from SBA + 8 µg/ml vancomycin, after purification, were not identified as enterococci, so no further investigations were made of them.

Among the remaining 322 isolates belonging to *Enterococcus* genus, five species were identified (*E. faecium*, *E. faecalis*, *E. durans*, *E. hirae*, and *E. gallinarum*). The distribution of the isolates among the species, on the basis of their medium of origin, is reported in **Figure 5**.

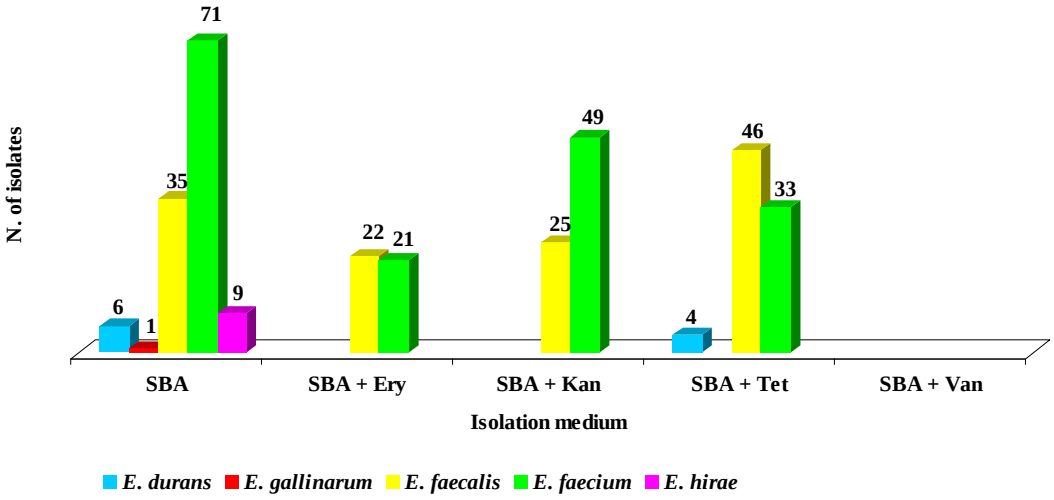


Fig. 5. Distribution of species *per* medium of isolation.

All the species were isolated in their greatest number, or in some cases exclusively (*E. gallinarum* and *E. hirae*), from SBA without any addition of antibiotics, except *E.*

faecalis that was isolated in highest number from SBA + 16 µg/ml of tetracycline, suggesting a greater tetracycline resistance than the other species.

E. faecium and *E. faecalis* were the dominant species (92 % of isolates), and were able to grow in all media used (except that supplemented with 8 µg/ml of vancomycin).

On the whole, the number of *E. faecium* isolates (174, 53 %) was higher than *E. faecalis* (128, 39 %). When comparing SBA without any antibiotics, and SBA supplemented with 1024 µg/ml of kanamycin, *E. faecium* isolates were twice *E. faecalis* isolates. But *E. faecalis* was the species most frequently isolated when 16 µg/ml of tetracycline were added to SBA medium.

Not taking into account SBA + vancomycin, the lowest number of isolates, all belonging to *E. faecalis* and *E. faecium* species (almost in the same number), was obtained from SBA + 4 µg/ml of erythromycin.

E. durans, *E. hirae* and *E. gallinarum* were only occasionally isolated (10, 3 %; 9, 3 % and 1, 0.3 % respectively) mainly from SBA medium without antibiotic addition, but four isolates belonging to the species *E. durans* were isolated in presence of 16 µg/ml of tetracycline.

The distribution of the isolates belonging to the five species detected at each farm, depending on the isolation medium is shown in **Figure 6**.

Among the two dominant species, only *E. faecium* was isolated from the cheeses sampled at all the farms, while *E. faecalis* was not isolated in 6 out of the 21 sample-farms (1, 5, 7, 9, 13 and 20).

The other three species were detected at farms as follows: *E. durans* at farms 10, 14, 15 and 20; *E. hirae* at farms 3, 5, 11, 12 and 15; *E. gallinarum* only at farm 19.

A number of *E. durans* and *E. hirae* isolates (5 each) comparable to the number of isolates belonging to the dominant species detected at each farm, was isolated from farms 20 and 3, respectively. Therefore, in some farms these two species seem to be the co-dominant enterococcal microflora beside *E. faecium* and/or *E. faecalis*.

Our results are in agreement with those reported in literature, since, as already mentioned in the Introduction (see **Paragraph 1.2 Enterococci in dairy products**) enterococci are detected in the same range of counts in many Mediterranean dairy products (Franz *et al*. 1999 and 2003, Folquié Moreno, 2006, Pisano *et al*., 2007; Ogier and Serror, 2008, Comunian *et al*., 2009b).

Particularly, the five species that were detected in this study are the most frequently isolated, and among them *E. faecium* and *E. faecalis* are the dominant ones (Giraffa *et al*., 1997; Franz *et al*., 1999; Suzzi *et al*., 2000; Andrighetto *et al*., 2001; Gelsomino *et al*., 2001; Sarantinopoulos *et al*., 2001; Aarestrup *et al*., 2002; Giraffa *et al*., 2003; Cosentino *et al*., 2004; Pisano *et al*., 2007).

Farm	No of isolates	*E. durans*					*E. gallinarum*					*E. faecalis*					*E. faecium*					*E. hirae*				
		NA[a]	Ery	Kan	Tet	Van	Na[a]	Ery	Kan	Tet	Van	Na[a]	Ery	Kan	Tet	Van	Na[a]	Ery	Kan	Tet	Van	Na[a]	Ery	Kan	Tet	Van
1	6																6									
2	17											5			5		1		6							
3	16														4		1		2	4		5				
4	10											5			3		1	1								
5	29																5	8	8	7		1				
6	33											1	10	10	3		5			4						
7	20																6		8	6						
8	11														6		5									
9	6																4		2							
10	24				3							2	3	3	3		4	3	3							
11	24											3	3	6	6		2	3				1				
12	6											2					3					1				
13	5																5									
14	12	1										1			6		4									
15	8				1							1					4			1		1				
16	25											3					4	6	6	6						
17	16											4					2		5	5						
18	24											4	6	6	4		2		2							
19	6						1					2					3									
20	12	5															1		6							
21	12											2			6		3		1							

[a]: No Antibiotic

Fig. 6. Number of isolates belonging to the five different species detected at each farm, depending on the medium used.

3.2.3 Strain typing

The typability of all the strain typing techniques resulted equal to 100%, since a DNA profile for each of the isolates was obtained by means of using either one technique by itself or a combination of them.

In **Figures 7 and 8** the dendrograms obtained from clusters analysis of rep-$(GTG)_5$ and RAPD M13 profiles of *E. faecium* (DSM 20477^T), *E. faecalis* (DSM 20478 ^{T}), *E. durans* (LMG 10746^T), *E. hirae* (LMG 6399^T) and *E. gallinarum* (LMG 13129^T) reference strains are shown.

For each species, the percentage of similarity among the four repetitions of the same reference strain is highlited in the dendrograms. These percentages correspond also to the reproducibility of the technique for each species, and were chosen as threshold similarity indexes to discriminate different strains among *E. faecium*, *E. faecalis*, *E. durans*, and *E. hirae* isolates. For the species *E. gallinarum* this index was determinated even though only one isolate was detected and performing strain typing was not necessary.

The *E. hirae* LMG 6399 rep-$(GTG)_5$ pattern turned out the most repeatable (87.3 % of minimum similarity among the patterns), while *E. durans* LMG 10746 pattern was the least repeatable (80.2 %).

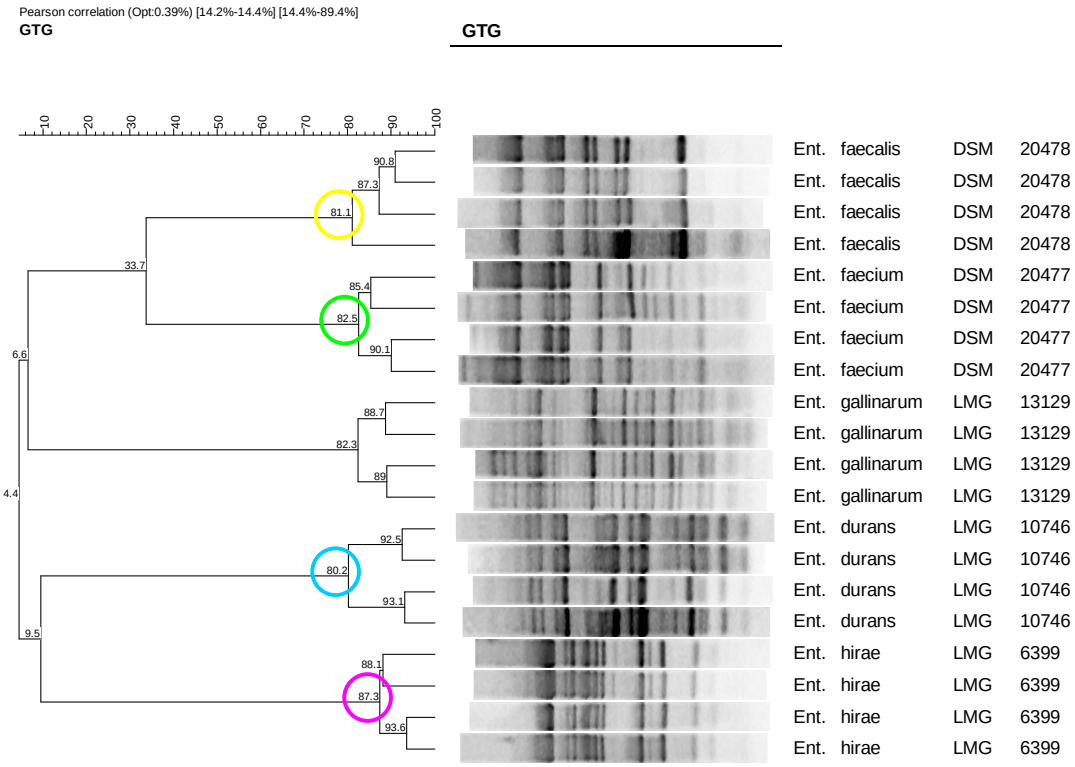


Fig. 7. Cluster analysis of the rep-$(GTG)_5$ profiles obtained amplifying four times each reference strain representing the five species detected in this study. The similarity indexes chosen as threshold in order to perform strain typing are highlighted by coloured circles.

The minimum similarity percentage among repetitions of rep-$(GTG)_5$ patterns of *E. faecium* DSM 20477 and *E. faecalis* DSM 20478 reference strains was 82.5 and 81.1%, respectively.

As regards RAPD M13, this technique came out more repeatable than rep-$(GTG)_5$ for *E. faecium*, *E. faecalis* and *E. hirae* species: 90.8, 91.0 and 90.6 % of minimum similarity among the four patterns obtained from the same reference strain,

respectively. Only for the *E. durans* reference strain RAPD M13 pattern was less repeatable (76.2 %) than rep-$(GTG)_5$ (80.2%).

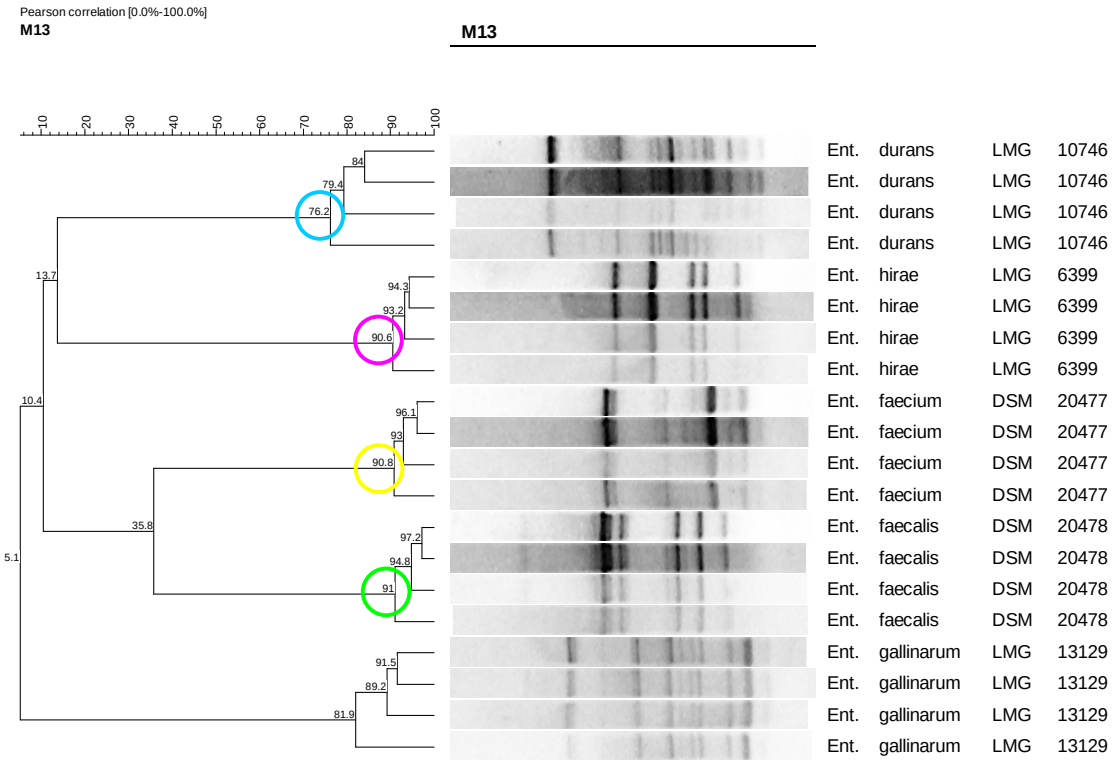


Fig. 8. Cluster analysis of the RAPD M13 profiles obtained amplifying four times each reference strain representing the five species detected in this study. The similarity indexes chosen as threshold in order to perform strain typing are highlighted by coloured circles.

When a combined analysis of rep-PCR and RAPD patterns was carried out, because the Bionumerics composite data set comparison tool averages out the similarity matrices of single experiments, the average threshold similarity indexes calculated on

the basis of those previously determined for each species and each technique were used (*E. faecium* 86.7, *E. faecalis* 86.1, *E. durans* 78.2 and *E. hirae* 89.0%) in order to discriminate different strains.

Strain typing by means of PFGE patterns was performed only for *E. faecium* isolates, the most frequently isolated species. In this case the threshold similarity index calculated among the four digestion repetitions of the same reference strain (DSM 20477), loaded on different gels, using the Dice correlation coefficient, was 100% (**Fig. 9**).

In order to perform the combined analysis of rep-PCR, RAPD and PFGE patterns, it was necessary to estimate the PFGE patterns similarity threshold (92.4%) among reference strain repetitions, also using the Pearson correlation coefficient (used to analyse the DNA profiles obtained by means of the other two typing techniques) (**Fig. 10**).

Then, the average threshold of similarity among the three techniques was calculated (88.57%).

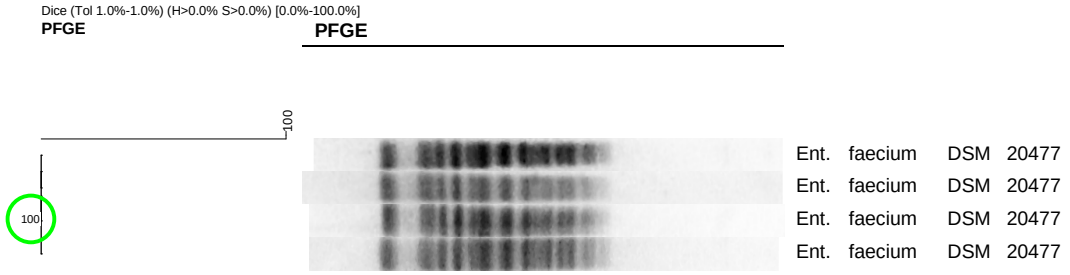


Fig. 9. Cluster analysis (using Dice correlation coefficient) of the PFGE profiles obtained digesting four times the DSM 20477 *E. faecium* reference strain. The similarity index is highlighted by a coloured circle.

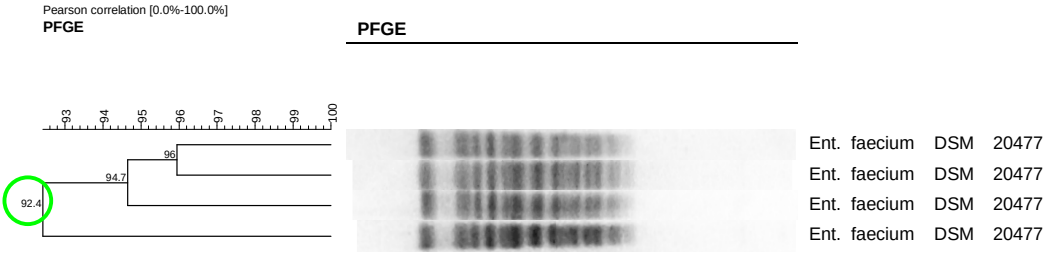


Fig. 10. Cluster analysis (using Pearson correlation coefficient) of the PFGE profiles obtained digesting four times the DSM 20477 *E. faecium* reference strain. The similarity index is highlighted by a coloured circle.

The number of clusters and the Diversity Index (D.I.) (also corresponding to the Discrimination Power - D.P. - of each technique) calculated for each species after cluster analysis of their rep-PCR, RAPD and PFGE patterns (these last only for *E. faecium* isolates), one by one or combined are summarized in **Table 7**.

Table 7. Results of cluster analyses performed on rep-$(GTG)_5$ PCR, RAPD M13 and PFGE patterns.

		E. faecium	*E. faecalis*	*E. durans*	*E. hirae*	*E. gallinarum*	Total
	N. of isolates	174	128	10	9	1	322
Rep-PCR $(GTG)_5$	N. of clusters	59	13	3	5	1	81
	% of similarity threshold[a]	82.5	81.1	80.2	87.3	-	-
	Diversity Index / Discriminatory power	0.952	0.781	0.511	0.722	-	-
RAPD M13	N. of clusters	100	**58**[c]	**6**[c]	8	1	**173**
	% of similarity threshold[a]	90.8	91.00	76.2	90.6	-	-
	Diversity Index / Discriminatory power	0.989	**0.963**[c]	**0.889**[c]	0.972	-	-
Rep-PCR $(GTG)_5$ + RAPD M13	N. of clusters	109	49	4	**8**[cd]	1	171
	% of similarity threshold[a]	86.65	86.65	78.2	88.95	-	-
	Diversity Index / Discriminatory power	0.9916	0.953	0.733	**0.972**[cd]	-	-
PFGE	N. of clusters	**129**[c]	-	-	-	-	-
	% of similarity threshold[b]	100	-	-	-	-	-
	Diversity Index / Discriminatory power	**0.995**[c]	-	-	-	-	-
Rep-PCR $(GTG)_5$ + RAPD M13 + PFGE	N. of clusters	118	-	-	-	-	-
	% of similarity threshold[a]	88.57	-	-	-	-	-
	Diversity Index / Discriminatory power	0.9924	-	-	-	-	-

-: not performed

[a]: Correspond to the reproducibility of the technique and was calculated by Pearson correlation coefficient.

[b]: Correspond to the reproducibility of the technique and was calculated by Dice correlation coefficient.

[c]: best result obtained

[d]: no clusters with isolates coming from different farms

For greater convenience, dendrograms obtained by Bionumerics software cluster analysis of isolates are shown at the end of this paragraph (**3.2.3 Strain typing**) (**Figs 20-33**).

In each figure, the species, the identification code (AREN#; EEN#; KEN#, and TEN# corresponding to isolates from SBA, SBA + erythromycin, SBA + kanamycin, and SBA + tetracycline, respectively) and the number of the farm of origin of each isolate are reported beside the patterns image. Coloured rectangles highlight isolates belonging to the same biotype.

As already reported in literature (Švec *et al*., 2005) in a study conducted on 30 enterococci species, the use of rep-$(GTG)_5$ fingerprinting alone came out to be not suitable to discriminate isolates at strain level, as a low number of biotypes is obtained and isolates from different farms revealed analogous fingerprint patterns, while they were clearly distinguishable by the other typing technique used in this study.

The Diversity Index should ideally be 1.00. Even though Hunter and Gaston (1988) stated that a D.I. > 0.90 would seem satisfactory to interpret with confidence typing results, van Belkum *et al*. (2007) deem it should be at least in the order of 0.95 for a typing system to be considered more or less 'ideal'.

As regards *E. faecium*, as was expected, PFGE achieved the highest performance among the typing techniques used. PFGE patterns cluster analysis made it possible to distinguish 129 biotypes with a very high D.I./D.P. (0.995).

Among the 129 PFGE groups, the majority (104) were constituted by a single isolate. The largest PFGE-type group consisted of 6 isolates. Only in this case, isolates coming from different farms (5 from farm 16, and 1 from farm 17) were grouped together (**Figs 11** and **20**).

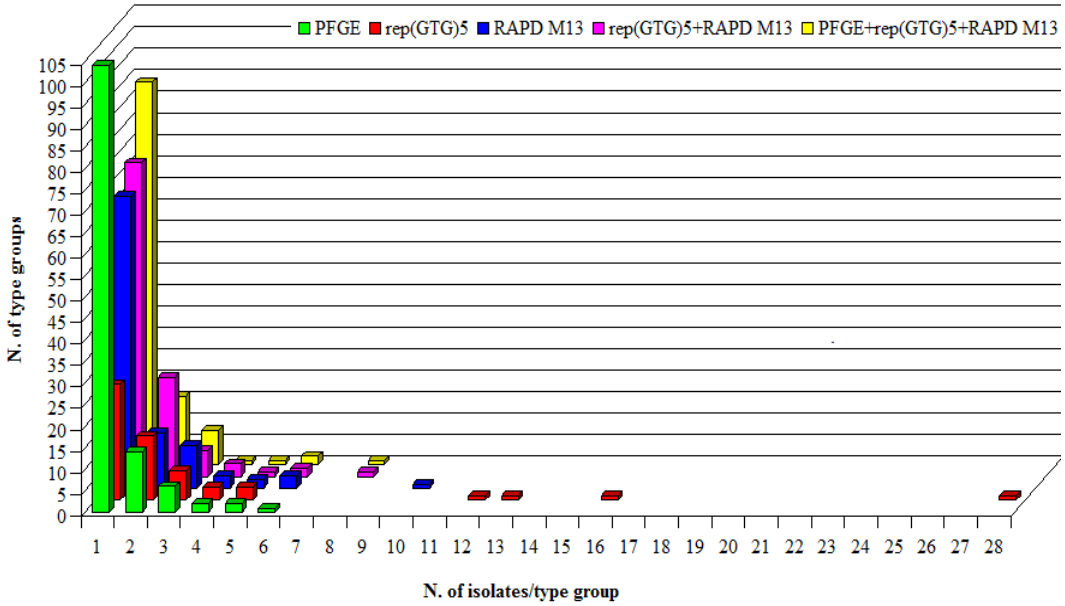


Fig. 11. Numerousness of type groups obtained for *E. faecium* isolates depending on the typing technique used.

Fifty-nine *E. faecium* biotypes were identified by rep-PCR patterns, 100 by RAPD PCR and 109 by a combined analysis of the patterns obtained by the two techniques (see **Table 7**).

In comparison with PFGE, a lower number of biotypes, many of them containing more than one isolate, were obtained by cluster analysis of rep and RAPD patterns singly or combined, even though isolates coming from different farms but sharing the same PFGE-type (see above), were discriminated by these techniques (**Figs 20**, **21** and **22**).

Among the 59 rep-$(GTG)_5$-types, only 27 were constituted by a single isolate, 4 contained more than 10 isolates: the largest group was constituted by 28 isolates (**Fig.**

11). Furthermore, in many cases, isolates coming from different farms were grouped together.

Cluster analysis of RAPD M13 patterns gave rise to a higher number of groups constituted by a single isolate (68), and only 1 by 10 isolates, among 100 groups obtained in total by using this technique. However, a large number of RAPD M13-types were constituted by isolates coming from different farms (**Fig. 22**).

If PFGE, which is considered the gold standard typing technique for all *Enterococcus* species, is not taken into account, best results (D.I./D.P. = 0.9916 and 109 biotypes), were obtained by combining rep-$(GTG)_5$ patterns with RAPD M13 ones (see **Table 7**).

In this way, 73 biotypes constituted by a single isolate, and only 1 by 10 isolates were obtained (see **Fig. 11**). However, also in this case, biotypes with isolates coming from different farms were grouped together (**Fig. 23**).

Better results cannot be obtained by combined analysis of PFGE, rep-$(GTG)_5$ and RAPD M13 patterns (D.I./D.P. = 0.9924, 118 biotypes) in comparison with the analysis of PFGE patterns considered alone (**Fig. 24**).

In order to summarize the results related to *E. faecium* strain typing, in **Figure 12** the D.I./D.P. and the number of biotypes obtained according to the different typing technique chosen are compared.

On the basis of suggestions put forward by Hunter and Gaston (1988) and van Belkum *et al*. (2007), each single technique could be used to perform *E. faecium* strain typing. Moreover, Rep and RAPD techniques can complement each other when

performing a combined analysis of their patterns obtained by two simple amplification reactions, but the better choice is definitely the PFGE technique.

However, an initial screening of isolates could be performed by combining rep-$(GTG)_5$-PCR and RAPD M13 before submitting them to a more expensive and time consuming technique as PFGE. In this way only the isolates belonging to the same rep-$(GTG)_5$/RAPD M13-type group should be processed by PFGE, leaving out single types, thus saving up time and money.

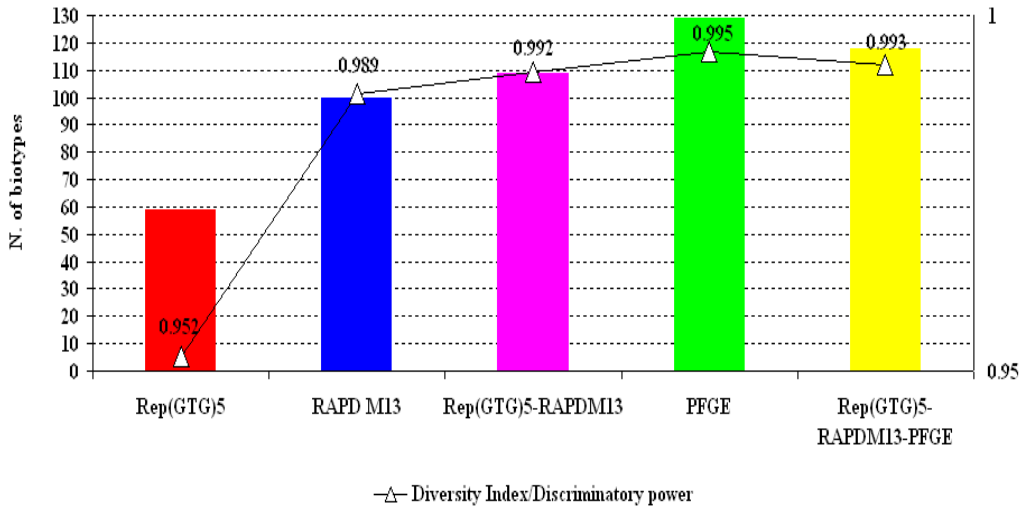


Fig. 12. D.I./D.P. and number of biotypes obtained for each typing technique used in this study for *E. faecium* strain typing.

Since the PFGE technique was not applied, RAPD PCR with M13 primer turned out to be the highest-performance technique for both *E. faecalis* and *E. durans* strain typing.

Indeed, the highest number of different biotypes (58 and 6, respectively for *E. faecalis* and *E. durans*), and then the highest D.I./D.P. (0.963 and 0.889, respectively) were obtained by using this technique (see **Table 7**).

Cluster analysis of *E. faecalis* rep-$(GTG)_5$ patterns gave rise to the lowest number of type-groups (13). Among them, 4 were constituted by a single isolate, and 4 by more than 10 isolates. The largest group was constituted by 41% of isolates (52) (**Fig. 13**). Moreover, a large number of rep-$(GTG)_5$-types were formed of isolates coming from different farms (**Fig. 25**).

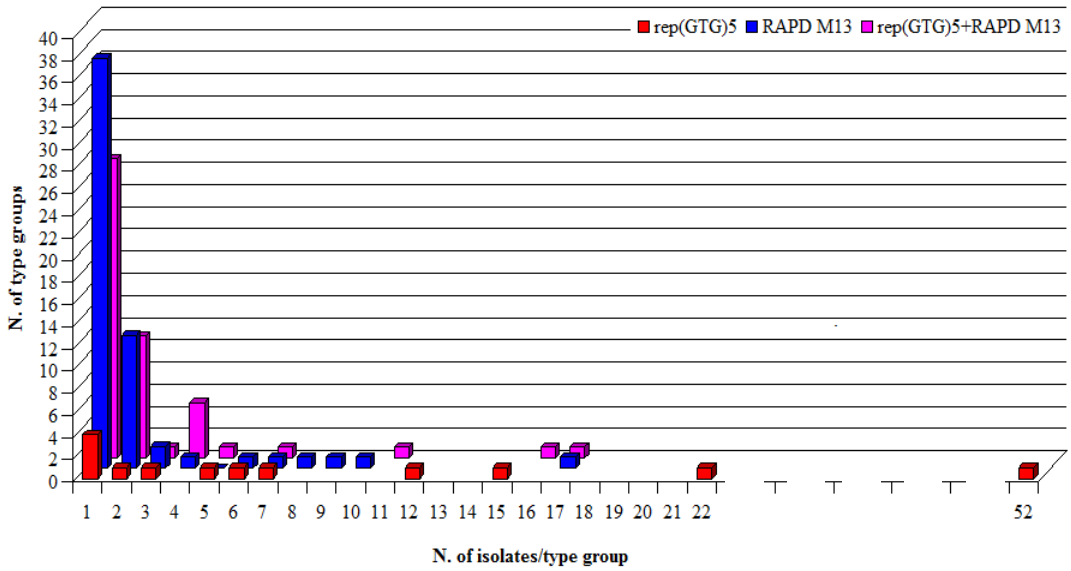


Fig. 13. Numerousness of type groups obtained for *E. faecalis* isolates depending on the typing technique used.

Among the 58 *E. faecalis* RAPD M13-types, 37 were constituted by a single isolate, 19 contained from 2 to 9 isolates, and 2 RAPD M13-types were formed of 10 or more isolates (17 isolates in the largest group) (**Fig. 13**). Furthermore, in many cases, isolates coming from different farms were grouped together (**Fig. 26**).

Better results were not obtained by combined analysis of rep-$(GTG)_5$ and RAPD M13 patterns in comparison with the analysis of *E. faecalis* RAPD M13 patterns considered alone (**Fig. 27**).

In **Figure 14** the comparison of the results (D.I./D.P. and number of clusters) related to *E. faecalis* strain typing according to the different typing technique are summarized.

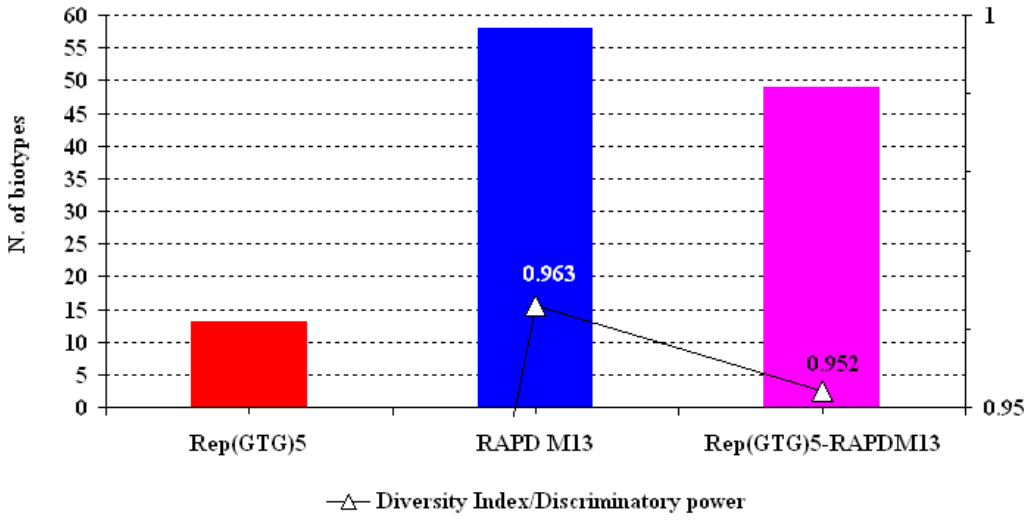


Fig. 14. D.I./D.P. and number of biotypes obtained for each typing technique used in this study for *E. faecalis* strain typing.

The D.I. (0.781) obtained from rep-$(GTG)_5$ profiles analysis was far below the threshold of 0.95 fixed by van Belkum *et al*. (2007), and also under that (0.90) fixed by Hunter and Gaston (1988) in order to interpret with confidence typing results. So this technique alone was not at all effective to perform *E. faecalis* strain typing.

When applied to *E. durans,* RAPD M13 technique was able to distinguish 6 biotypes (3 single profiles), and rep-$(GTG)_5$ only 3 (1 included 7 isolates), while 4 biotypes were obtained combining the two techniques (**Figs 28, 29** and **30**). Furthermore, very low D.I./D.P values (under the threshold of 0.90) came out for this species (see **Table 7**).

The same number of *E. hirae* biotypes (8) and the same D.I. (0.972) were obtained both by cluster analysis of the sole RAPD PCR profiles and of their patterns combination with rep-$(GTG)_5$ ones (see **Table 7**). However, a different distribution of the strains among biotypes was obtained. Indeed, combined analysis provided a clearer distinction between the isolates according to their farm of origin as RAPD M13 patterns cluster analysis grouped together two isolates coming from farm 12 and 15, while rep-$(GTG)_5$/RAPD M13 patterns analysis enabled the separation of these two isolates whilst grouping two isolates coming from the same farm (03) (**Figs 31, 32** and **33**). Therefore, in this case combining the two patterns was the highest performing typing method.

N.B. Both for *E. durans* and *E. hirae* a very low number of isolates (10 and 9, respectively) were analysed, so caution should be used when considering the results of strain typing for this two species.

The D.I. for the entire population of enterococci under study was calculated taking into account the number of strains and their distribution into biotypes obtained by the best technique used for each species. Among the 322 enterococci isolated, a total of 202 strains, belonging to 5 species, were discriminated (129 *E. faecium* by PFGE; 58 *E. faecalis* and 6 *E. durans* by RAPD M13; 8 *E. hirae* by combining rep-$(GTG)_5$ and RAPD M13; 1 *E. gallinarum*) with a D.I. equal to 0.995.

It must be stressed that this is a very high biodiversity value, and it is probably an underestimate since a higher number of biotypes could be obtained by typing all isolates by means of PFGE technique.

In order to evaluate the erythromycin, kanamycin and tetracycline ability of inhibiting the grow of Fiore Sardo cheese enterococci, the Diversity Indexes among *E. faecium* and *E. faecalis* isolated from SBA, SBA + erythromycin, SBA + kanamycin, and SBA + tetracycline were separately calculated using for each species the highest performance technique. However, as PFGE technique was applied exclusively to *E. faecium* isolates, the biodiversity of the two species was compared by using results obtained by RAPD M13 technique.

Due to the small number of isolates, it was not possible to evaluate this aspect for *E. durans, E. hirae,* and *E. gallinarum*.

The number of biotypes and the biodiversity (indicated as Diversity Index) calculated for each isolation medium are reported in **Table 8**.

Table 8. Number of biotypes and biodiversity of *E. faecalis* and *E. faecium* according to the medium of isolation.

	E. faecalis			*E. faecium*				
		RAPD M13			RAPD M13		PFGE	
	N. of isolates	N. of biotypes	D.I.	N. of isolates	N. of biotypes	D.I.	N. of biotypes	D.I.
SBA	35	26	0.982	71	52	0.987	62	0.996
SBA+Ery	22	8	0.836	21	9	0.519	10	0.891
SBA+Kan	25	7	0.793	47	21	0.950	27	0.963
SBA+Tet	46	31	0.967	35	23	0.960	30	0.993
TOTAL	128	72[a]		174	105[b]		129	

[a]: the total number of *E. faecalis* RAPD M13-types is 58. The excess of 14 is due to the repeated isolation of the same biotype from different media (n. 11 from two, and n. 3 from three media).

[b]: the total number of *E. faecium* RAPD M13-types is 100. The excess of 5 is due to the repeated isolation of the same biotype from different media (n.5 from two media).

SBA was the medium for which the highest level of biodiversity was found both for *E. faecium* and *E. faecalis* isolates, since no selective factors were added.

In order to better evaluate the selective power of the different antibiotic towards the two dominant species, Diversity Indexes obtained by mean of the same typing technique were compared (0.987 and 0.982 for *E. faecium* and *E. faecalis*, respectively; 0.996 for *E. faecium* by PFGE).

Lower levels of biodiversity were calculated for isolates from SBA + tetracycline (D.I. 0.960 and 0.967, for *E. faecium* and *E. faecalis*, respectively; 0.993 for *E. faecium* by PFGE), suggesting a certain sensitivity of both species to this drug. Therefore, the higher number of *E. faecalis* isolated from this medium in comparison with SBA does not indicate a high level of resistance of this species to tetracycline, since the lower level of biodiversity found suggests that the same resistant strains were isolated more times.

Very low levels of biodiversity were obtained both for *E. faecium* and *E. faecalis* isolated from SBA + erytrhomycin (D.I. 0.519 and 0.836, respectively; 0.891 for *E. faecium* by PFGE). It was the lowest D.I. calculated for *E. faecium*, while for *E. faecalis*, the lowest D.I. was obtained among isolates from SBA + kanamycin (0.793).

As mentioned above, lower D.I. values are likely related to lower resistance of the isolates to the drugs added to the cultivation media. From this point of view, the very low D.I. calculated for *E. faecium* isolates coming from SBA + erythromycin, and *E. faecalis* isolates coming from SBA + kanamycin could be interpreted as a higher sensitivity of the first species to erythromycin, and of the second to kanamycin.

On the other hand, in order to evaluate and evaluate the biodiversity of the isolates belonging to different species, the D.P. of the different techniques used for strain typing should also be taken into account. Indeed, if Diversity Indexes relative to *E. faecium* PFGE-types are considered instead of RAPD M13 ones, higher values are obtained.

In **Figures 15, 16** and **17** Diversity Indexes calculated for *E. faecium* and *E. faecalis* and for each isolation medium were visually compared.

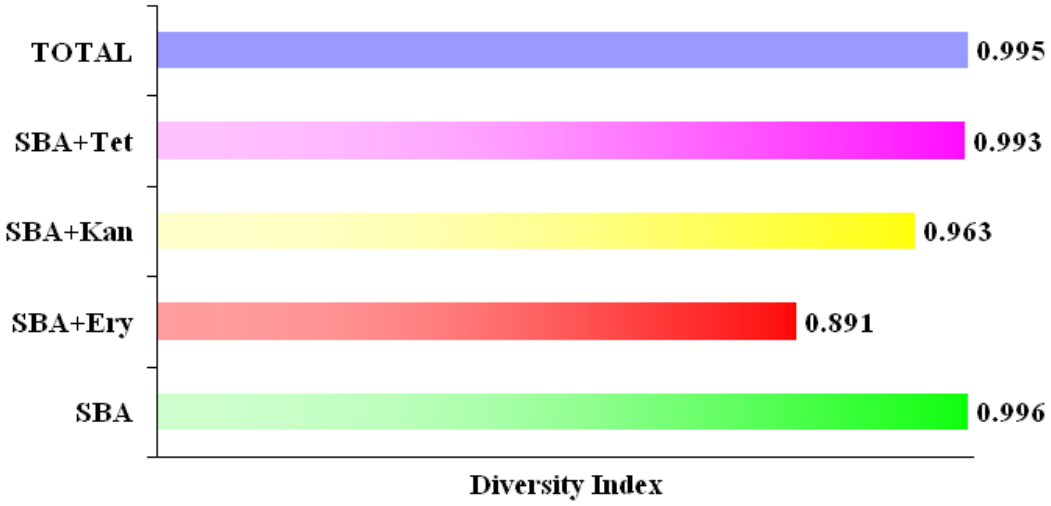


Fig. 15. D.I. calculated among *E. faecium* PFGE-types according to the medium of origin.

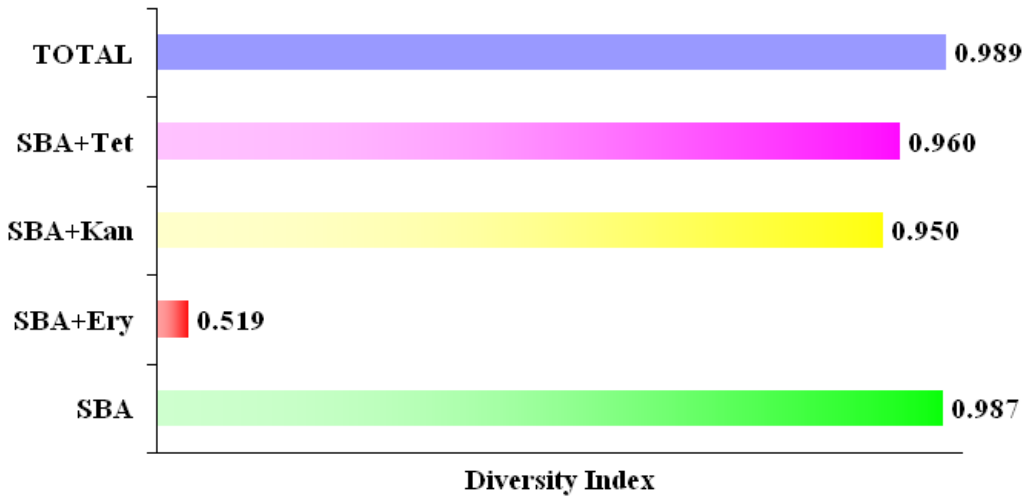


Fig. 16. D.I. calculated among *E. faecium* RAPD M13-types according to the medium of origin.

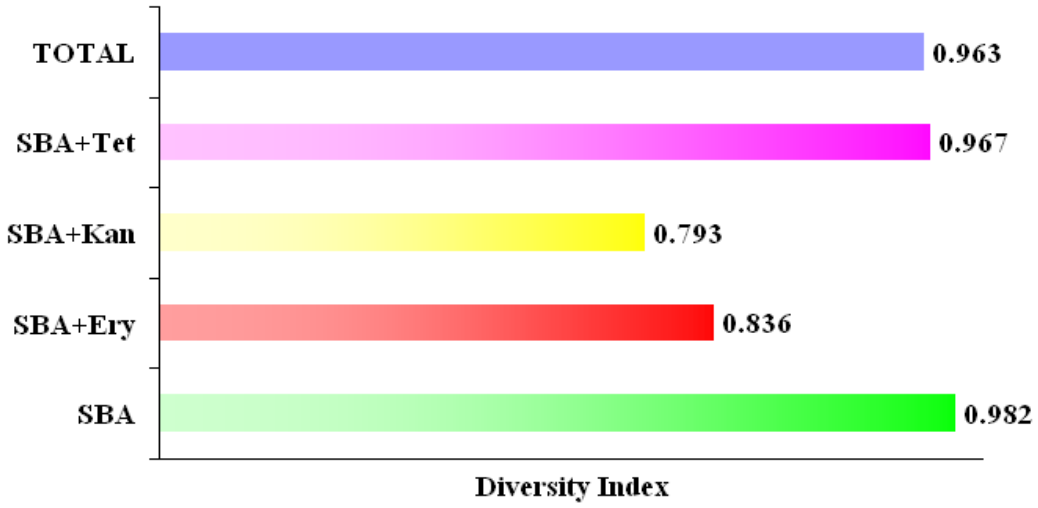


Fig. 17. D.I. calculated among *E. faecalis* RAPD M13-types according to the medium of origin.

In order to evaluate if some strains are exclusively of a certain medium or not, the distribution of *E. faecalis* isolates into RAPD M13-types according to the media of isolation was considered (**Fig. 18**).

Among 58 biotypes, 11 were constituted by isolates coming from two or three different media, while 21 were formed exclusively of isolates from SBA, 21 from SBA + tetracycline, 3 from SBA + erithromycin, and 2 from SBA + kanamycin.

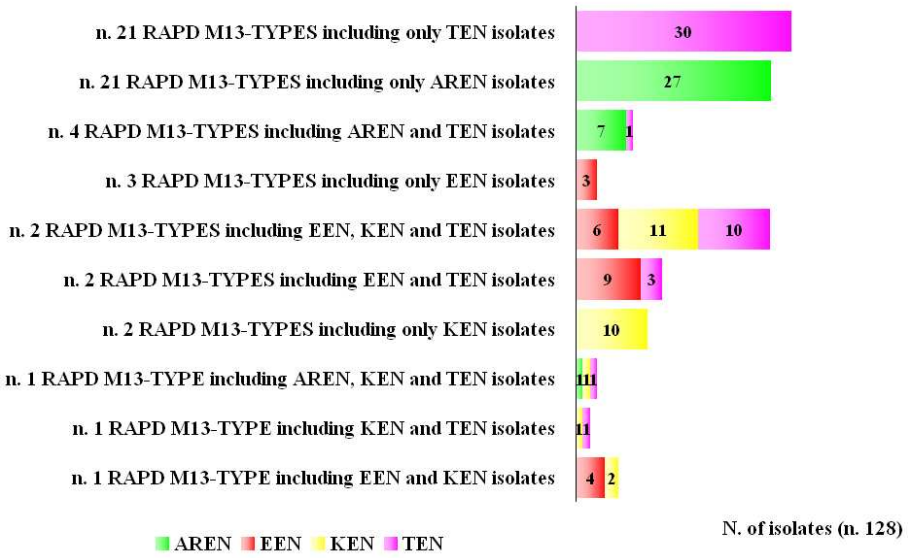


Fig. 18. Distribution of *E. faecalis* isolates into 58 RAPD M13-types according to the media of isolation.

As regards *E. faecium*, only 5 RAPD M13-types were constituted by isolates coming from two different media, while the remaining 95 consisted of isolates coming from the same medium (**Fig. 19**), while if PFGE-types are considered, no biotype was made up of isolates coming from different media (data not shown).

As previously stated about the D.I. values, the D.P. of each strain typing technique should be taken into account to better evaluate if resistant strains are spread among dominant strains growing in a non-selective for antibiotic resistance medium.

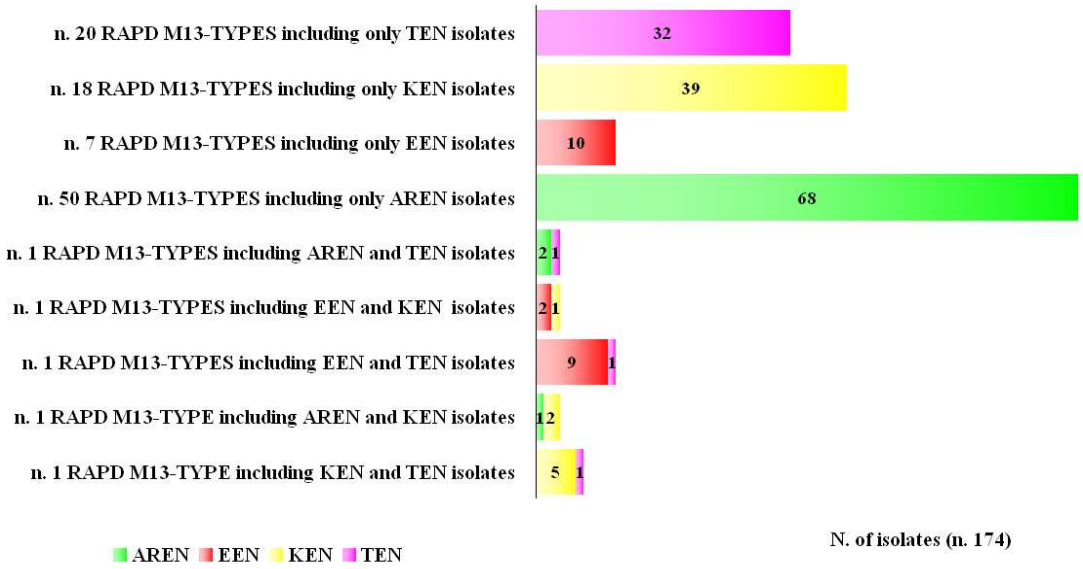


Fig. 19. Distribution of *E. faecium* isolates into 100 RAPD M13-types according to the media of isolation.

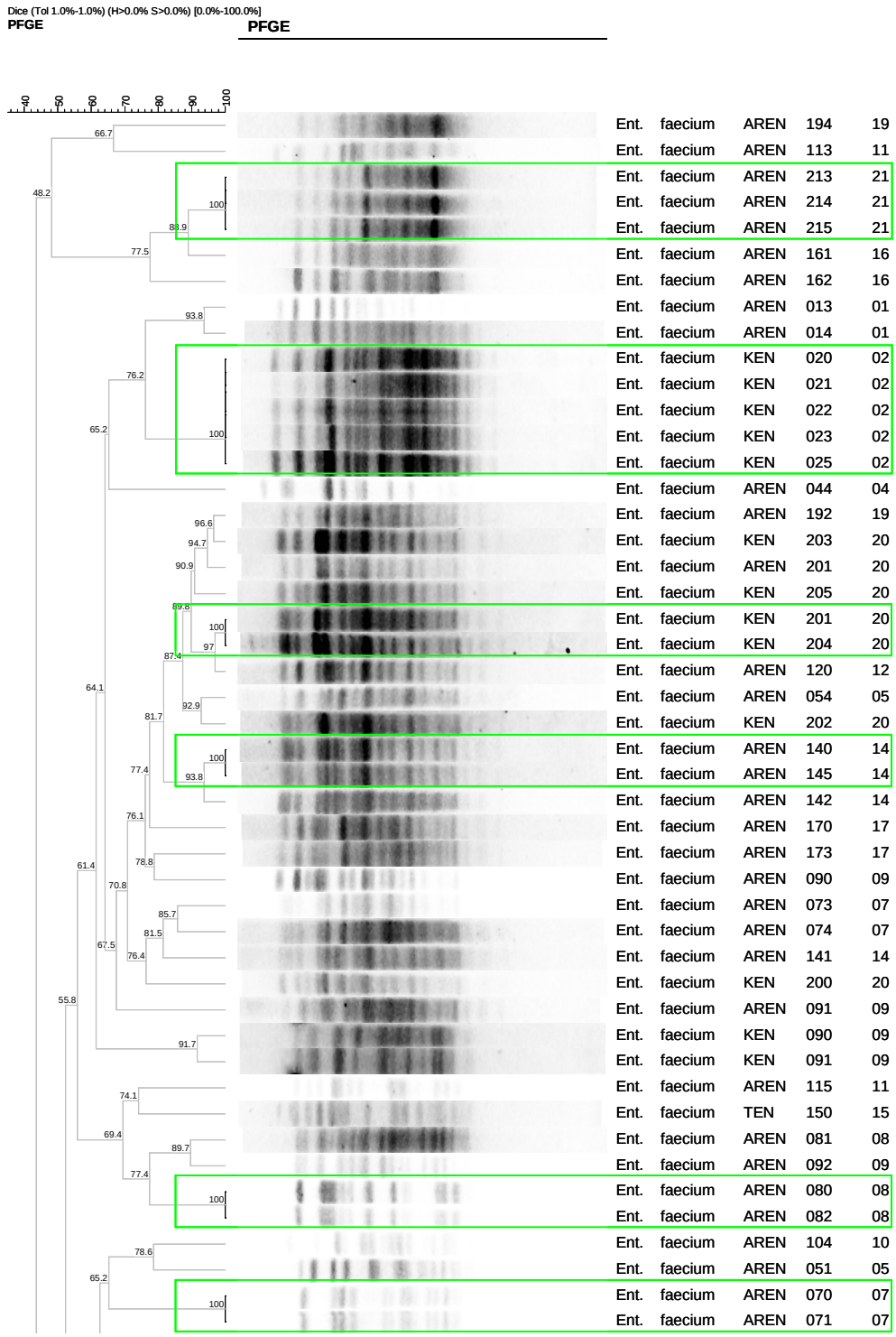


Fig 20. Dendrogram obtained by Bionumerics cluster analysis of *E. faecium* PFGE patterns. *Continued on following 3 pages*.

Fig. 20. *Continued*

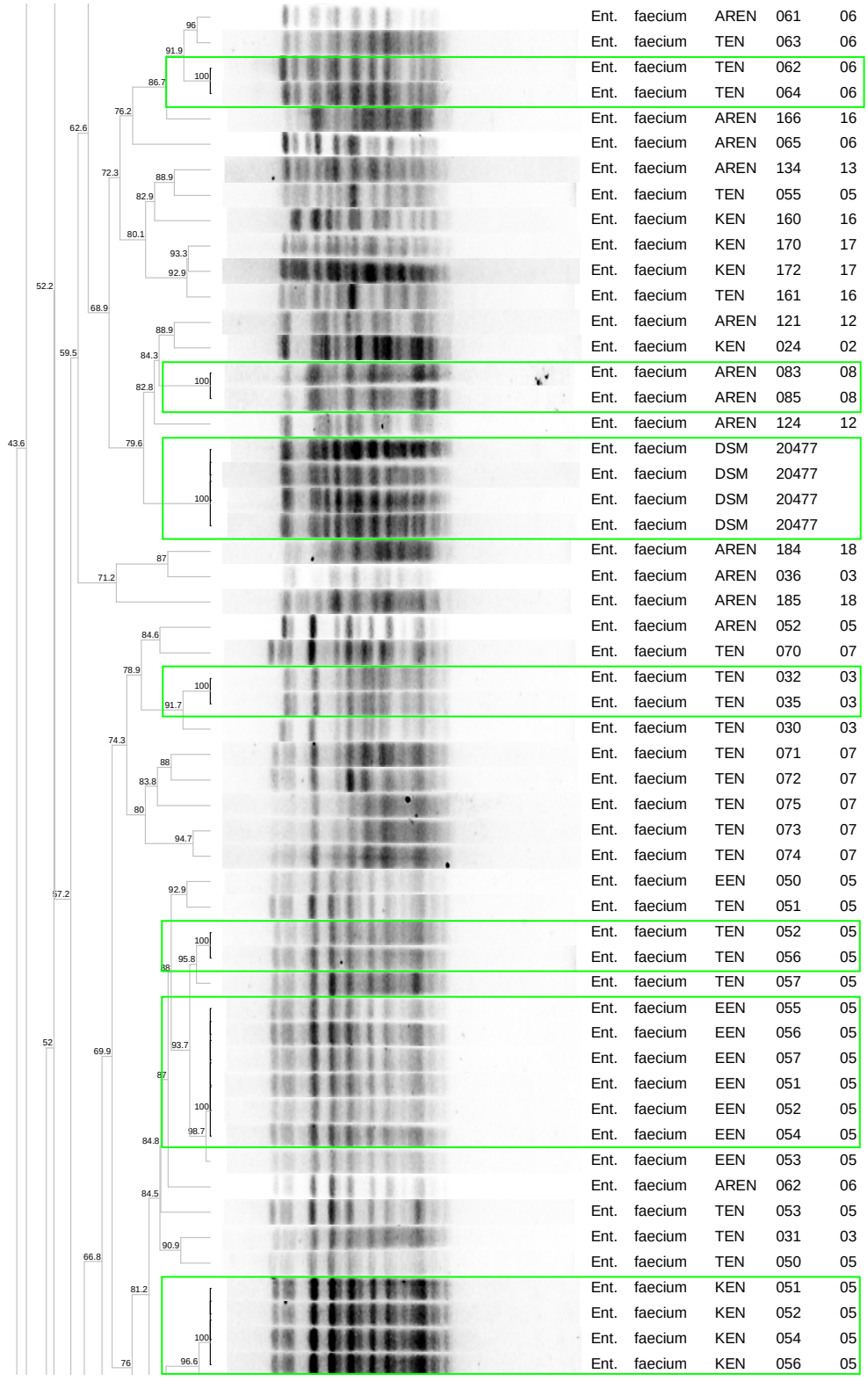


Fig. 20. *Continued*

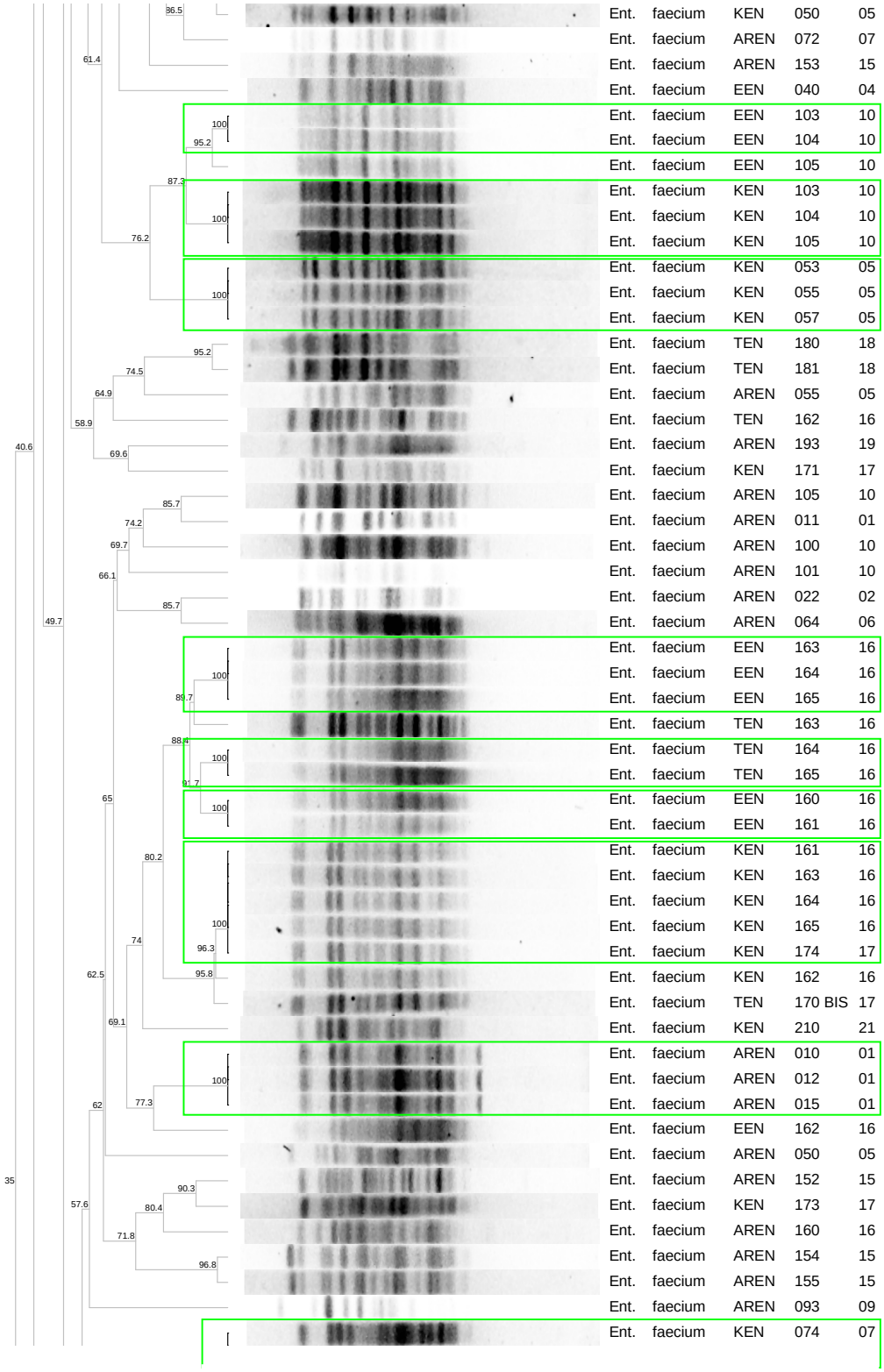
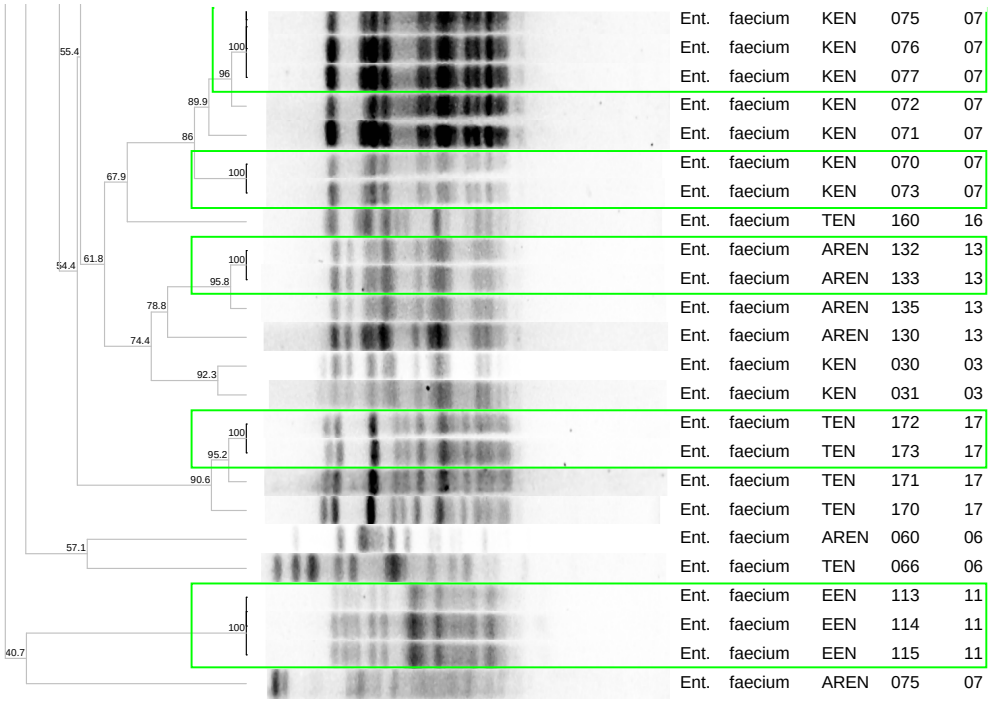


Fig. 20. *Continued*



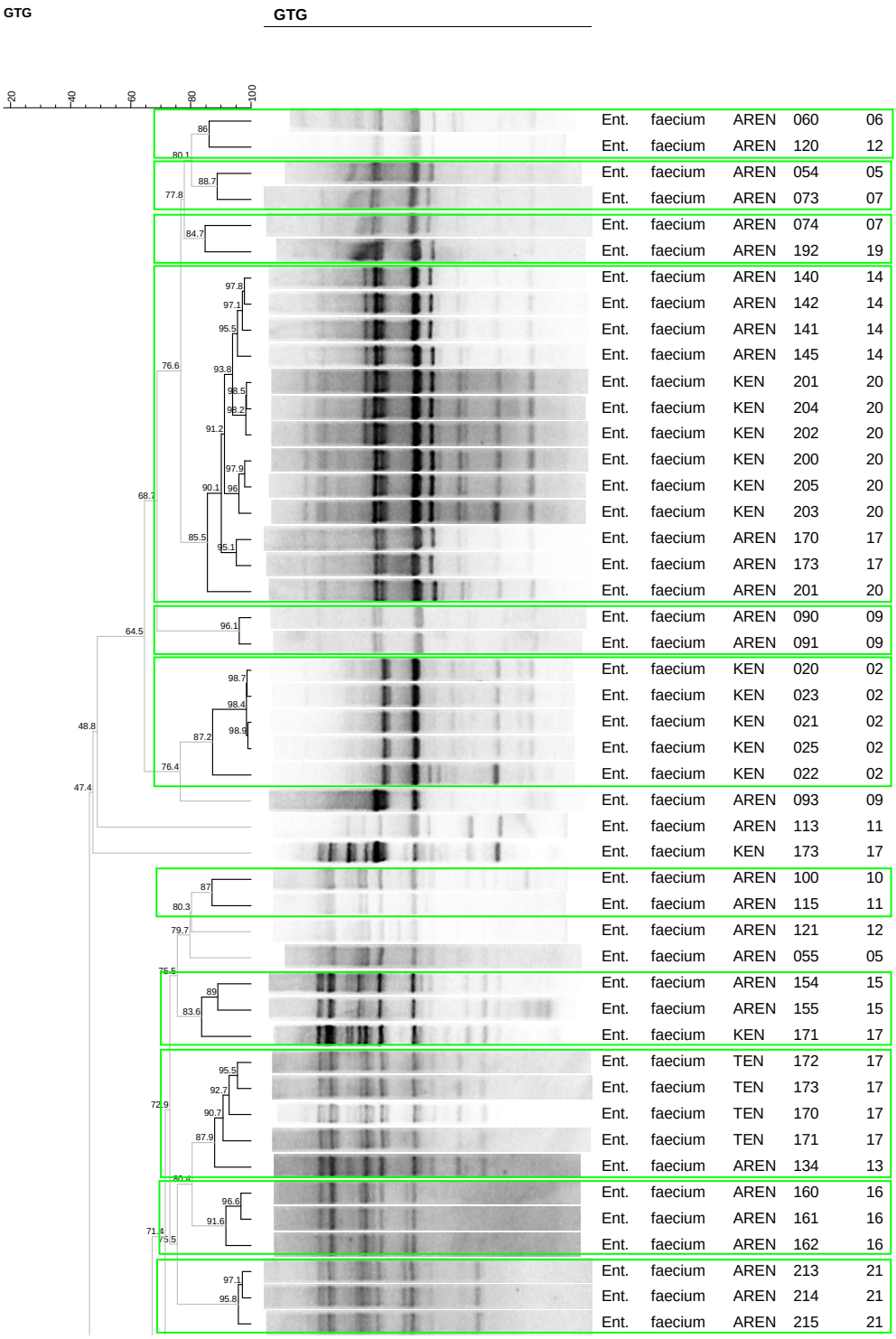


Fig. 21. Dendrogram obtained by Bionumerics cluster analysis of *E. faecium* rep-$(GTG)_5$ patterns. *Continued on following next 3 pages.*

Fig. 21. *Continued*

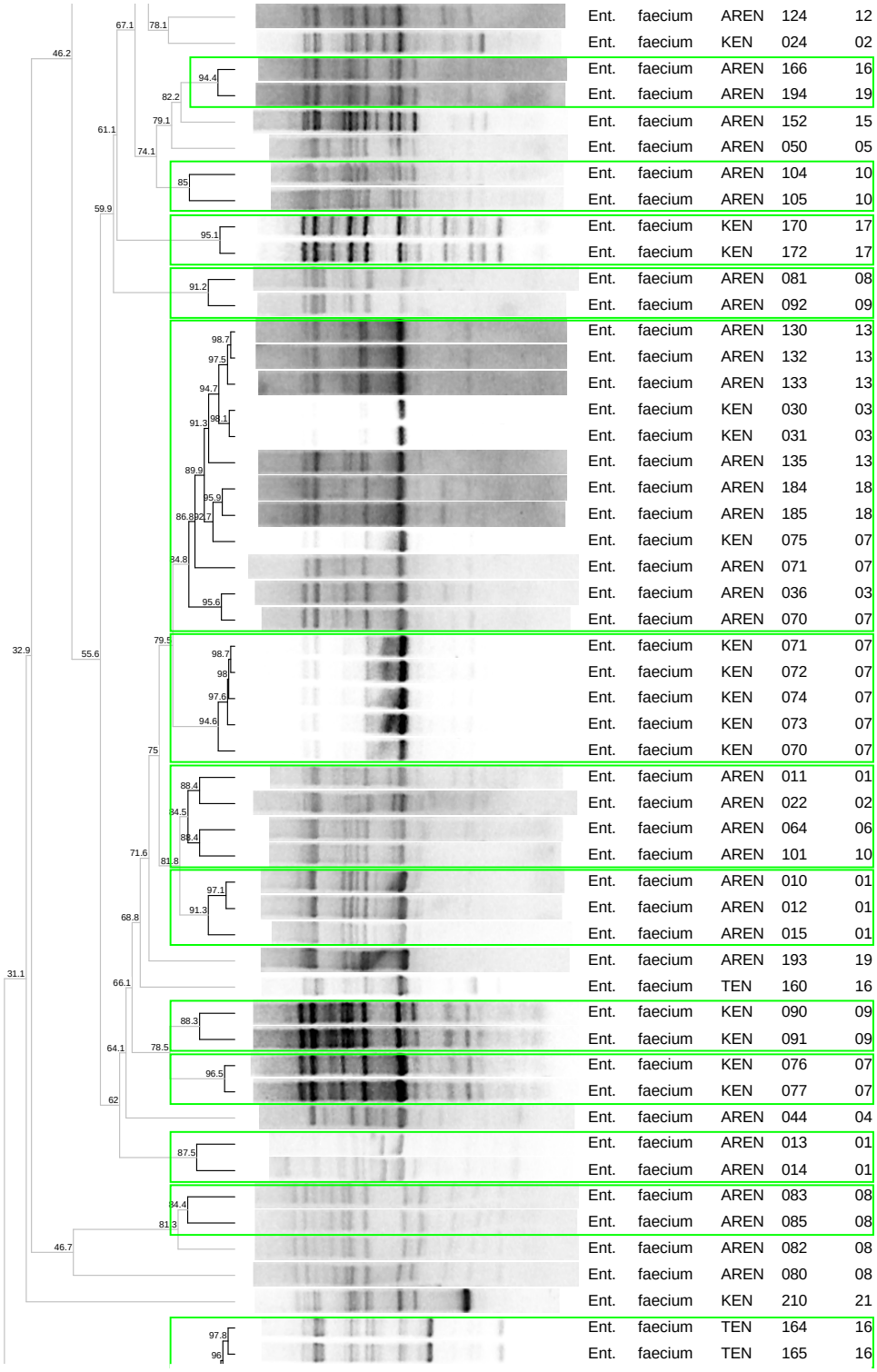


Fig. 21. *Continued*

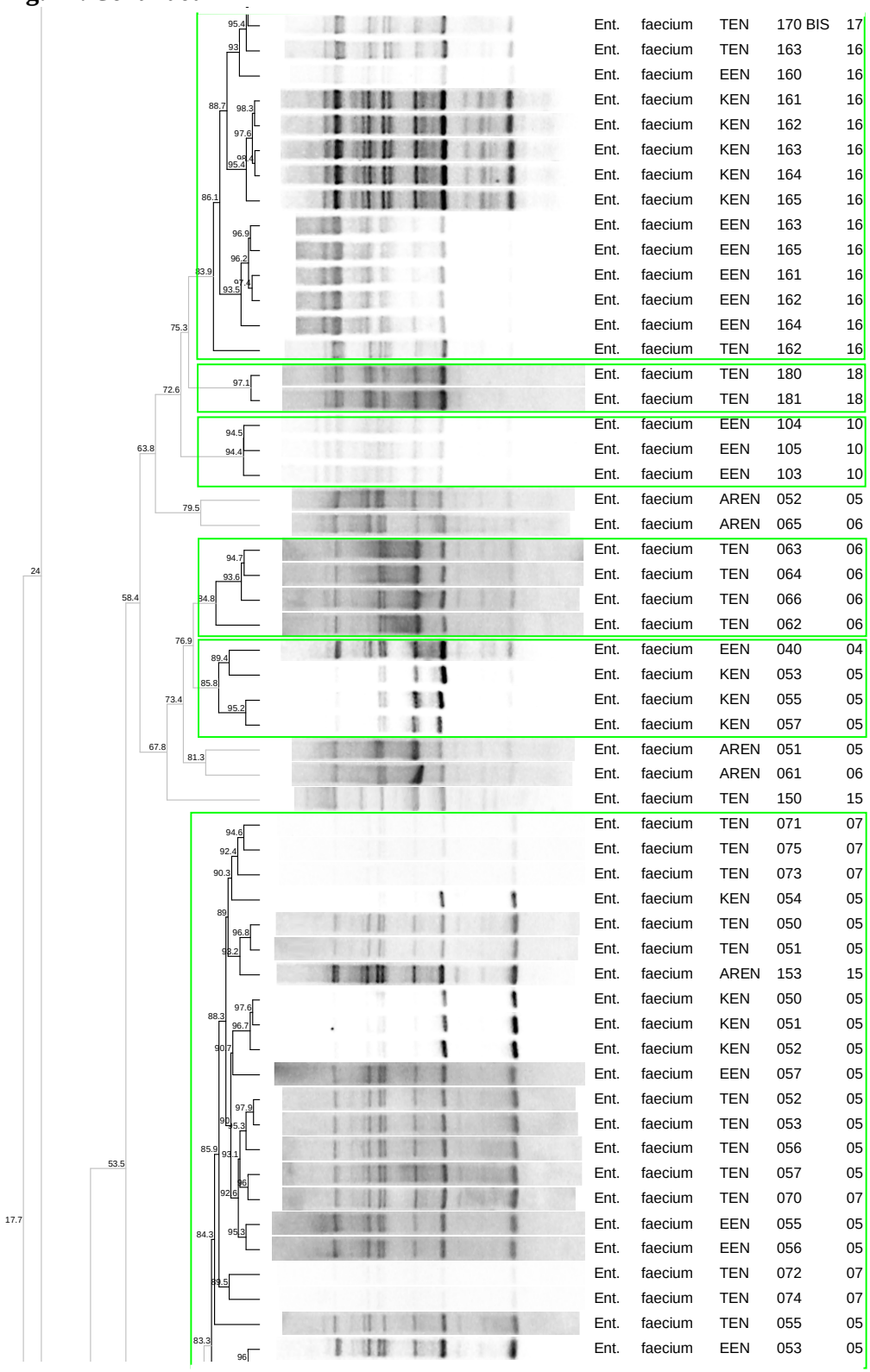
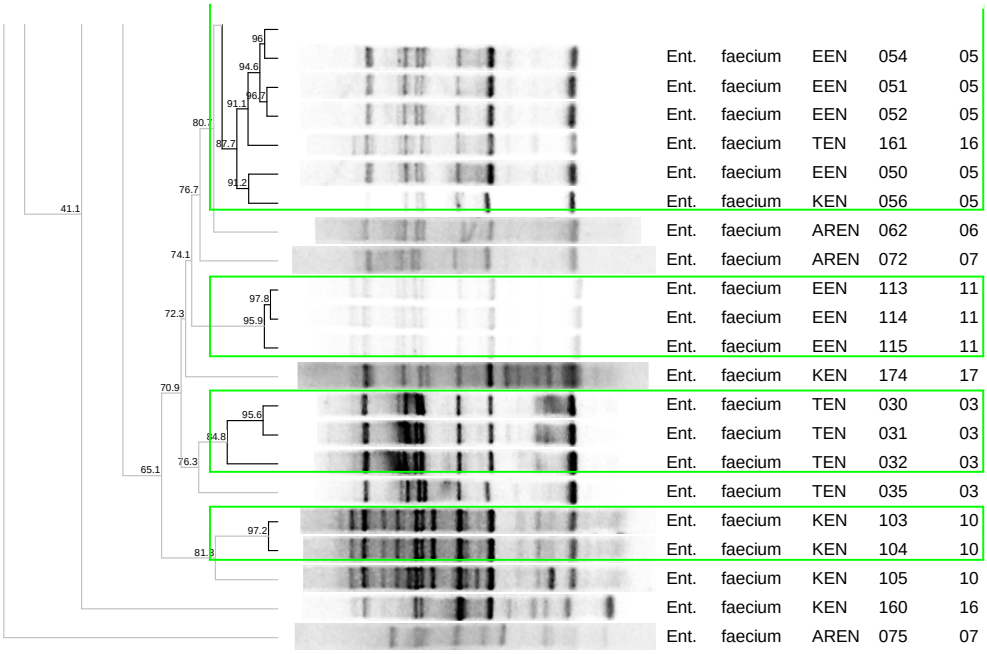


Fig. 21. *Continued*



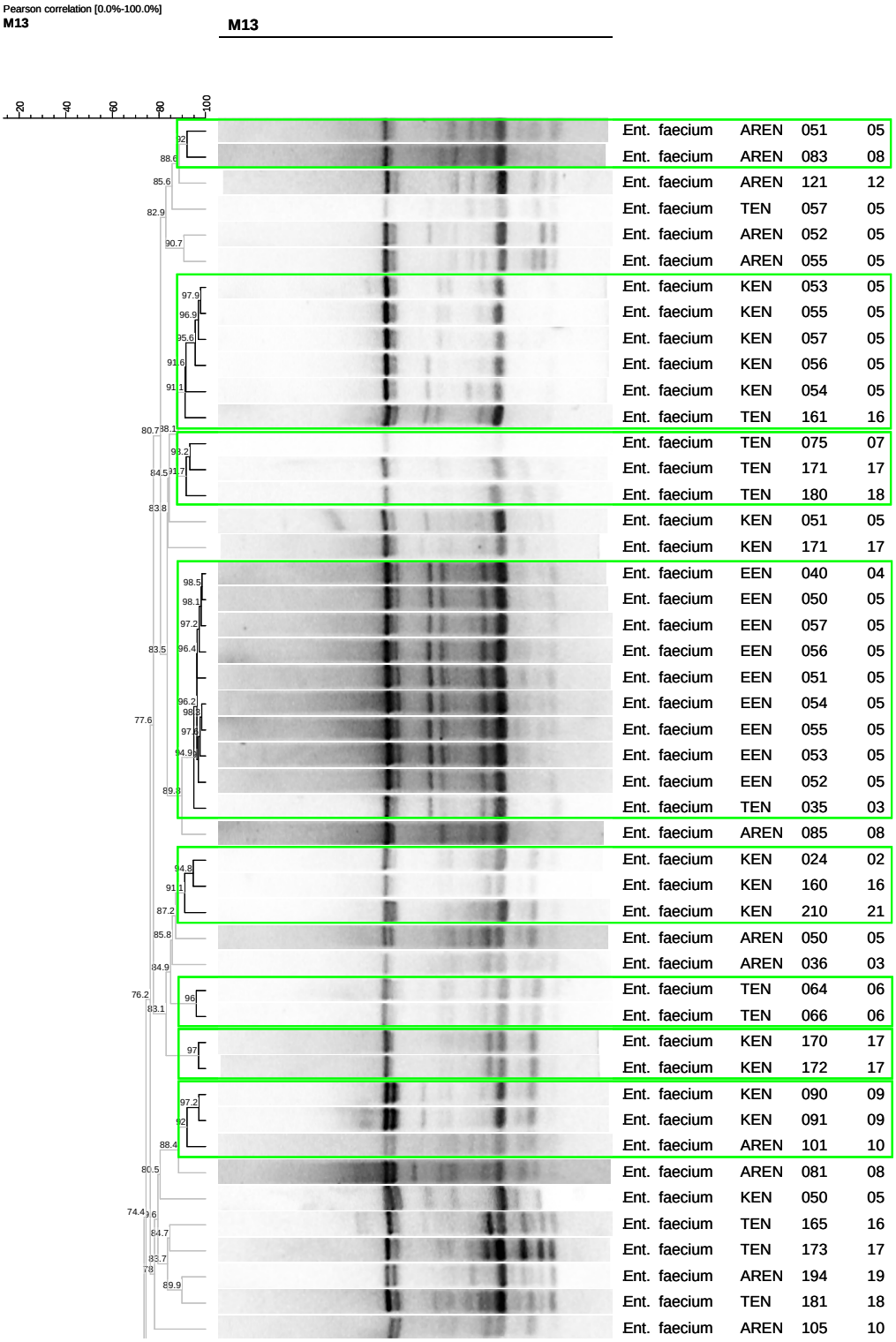


Fig 22. Dendrogram obtained by Bionumerics cluster analysis of *E. faecium* RAPD M13 patterns. *Continued on following 3 pages.*

Fig. 22. *Continued*

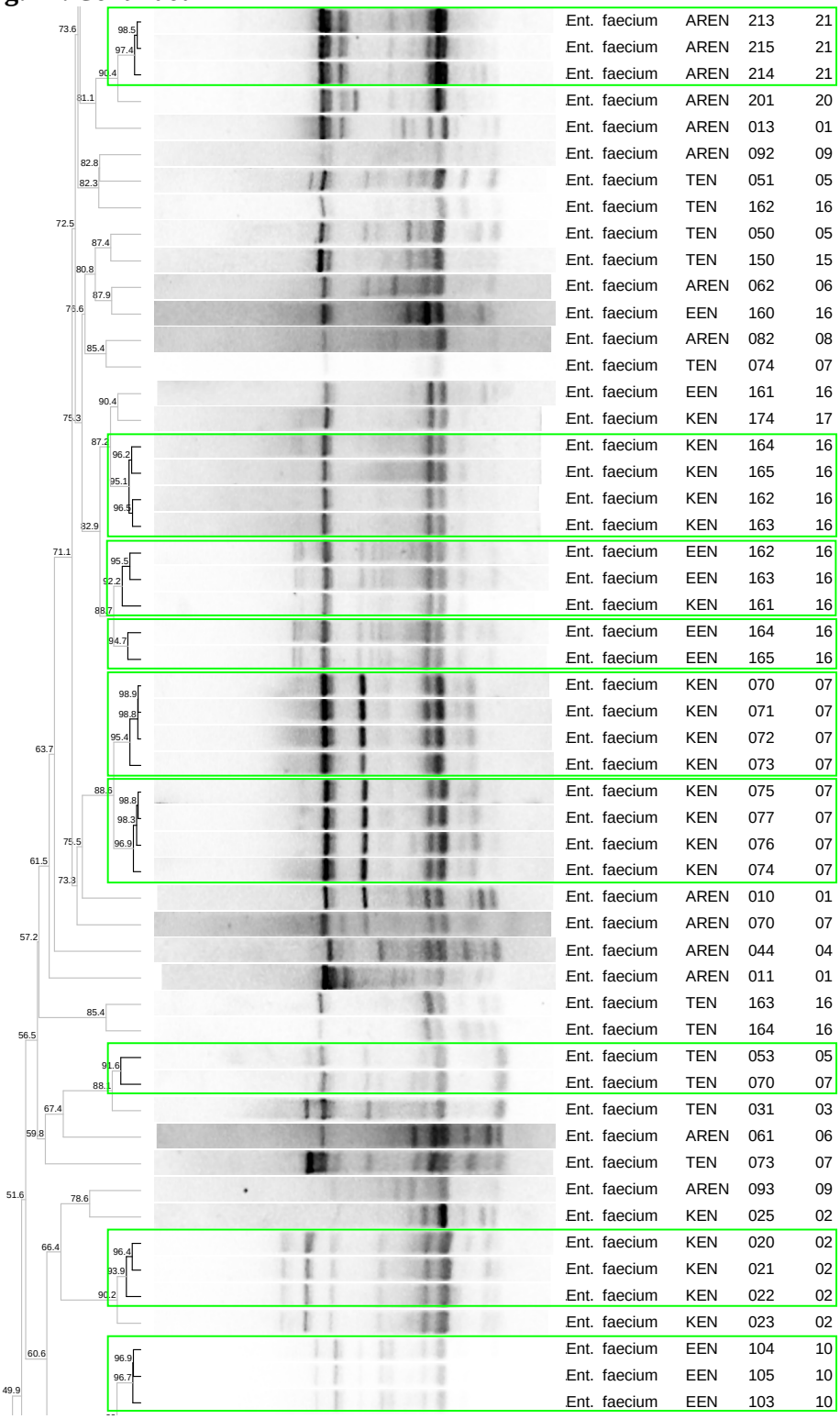


Fig. 22. *Continued*

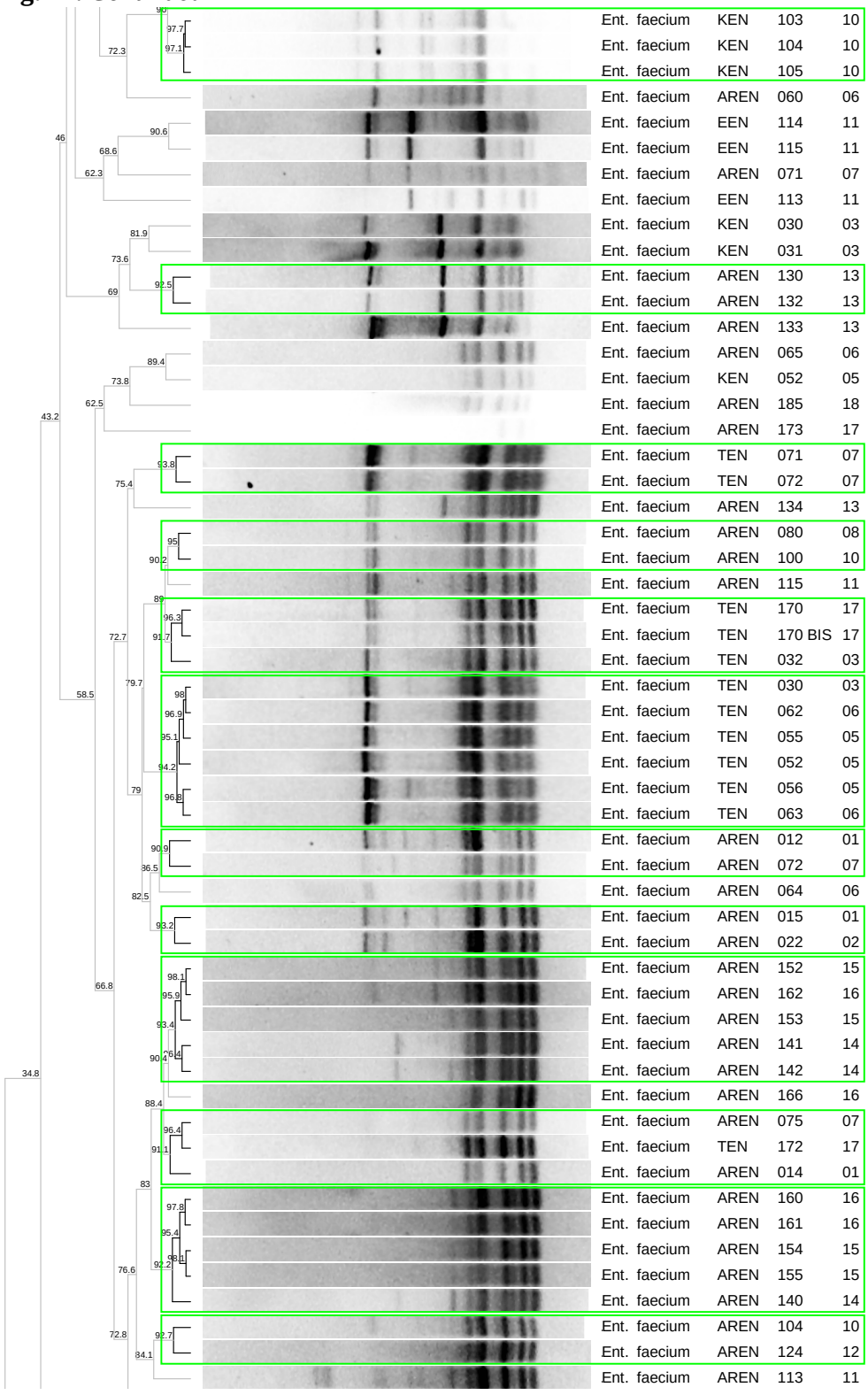
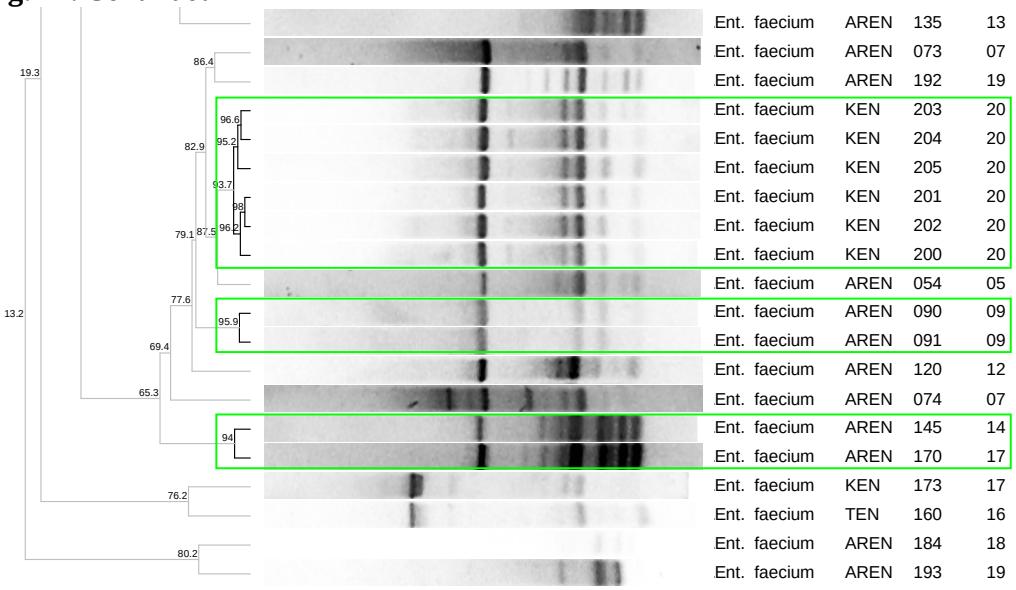


Fig. 22. *Continued*



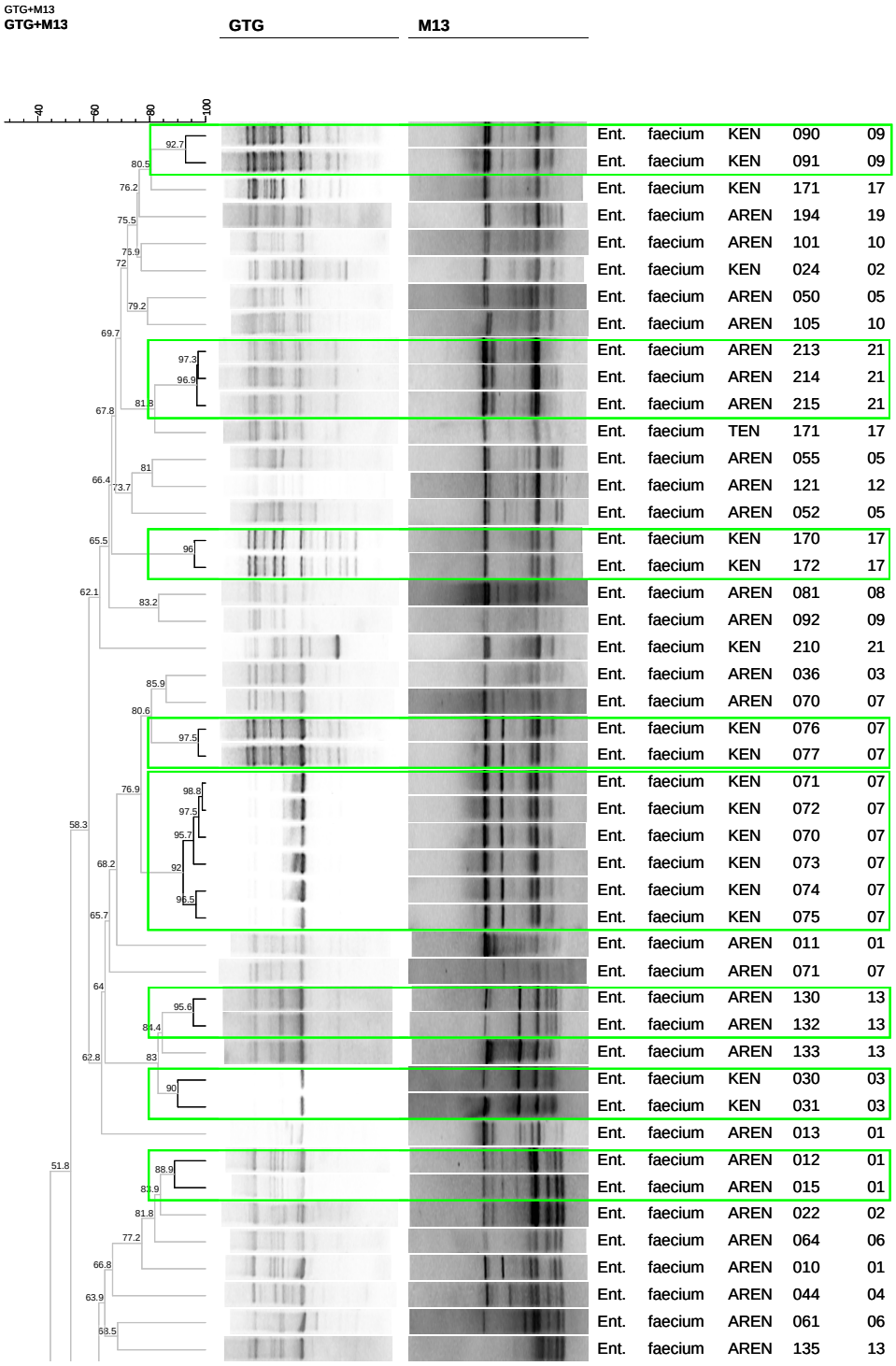


Fig. 23. Dendrogram obtained by Bionumerics cluster analysis of *E. faecium* rep-$(GTG)_5$ and RAPD M13 combined patterns. *Continued on following 3 pages*.

Fig. 23. *Continued*

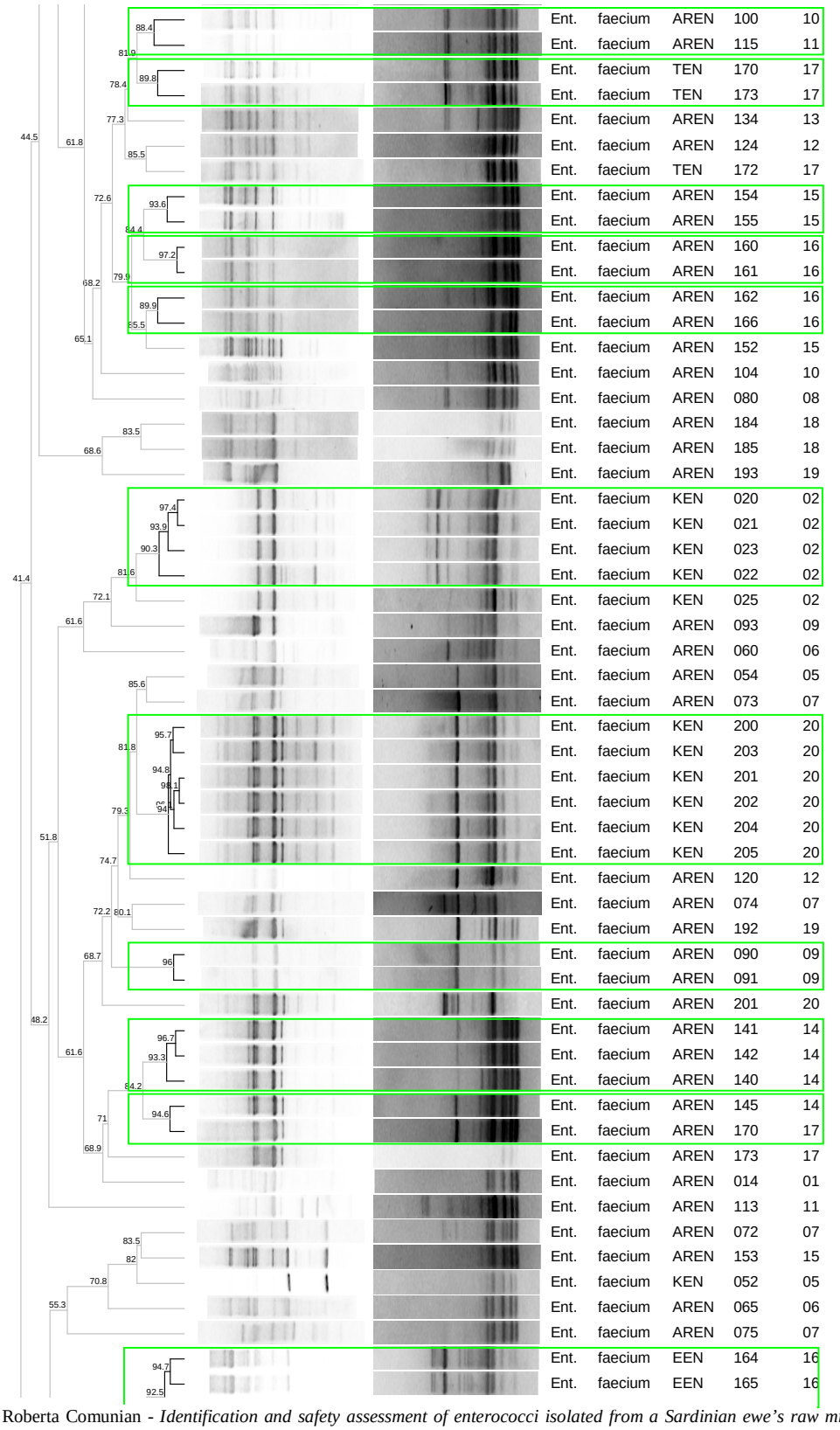


Fig. 23. *Continued*

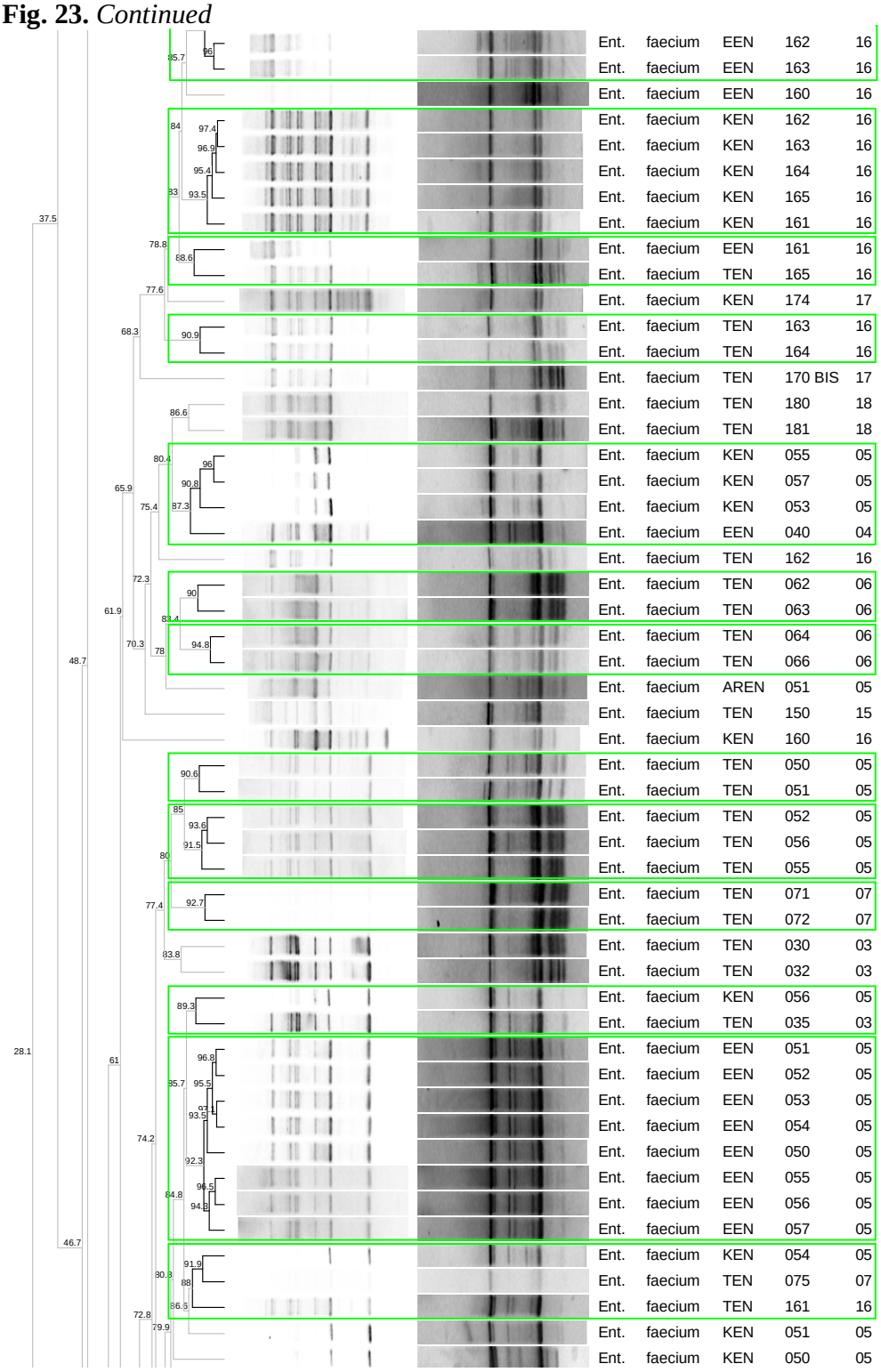
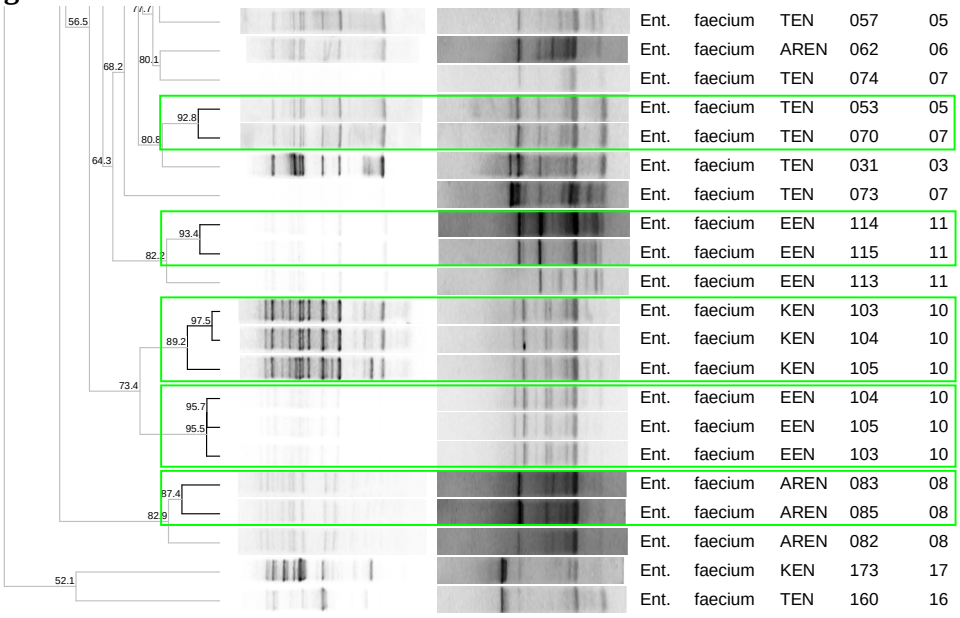


Fig. 23. *Continued*



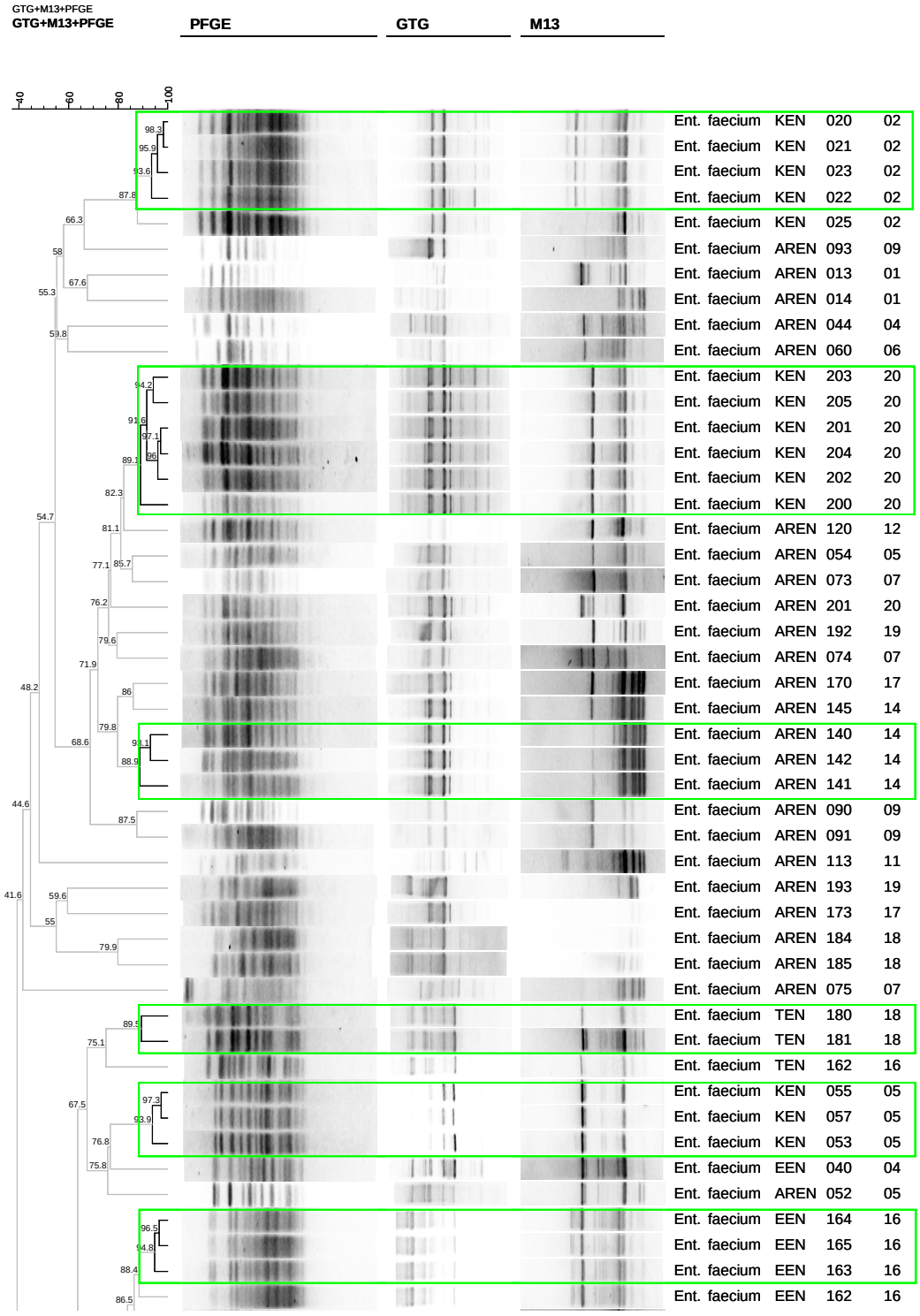


Fig. 24. Dendrogram obtained by Bionumerics cluster analysis of *E. faecium* rep-$(GTG)_5$, RAPD M13, and PFGE combined patterns. *Continued on following 3 pages.*

Fig. 24. *Continued*

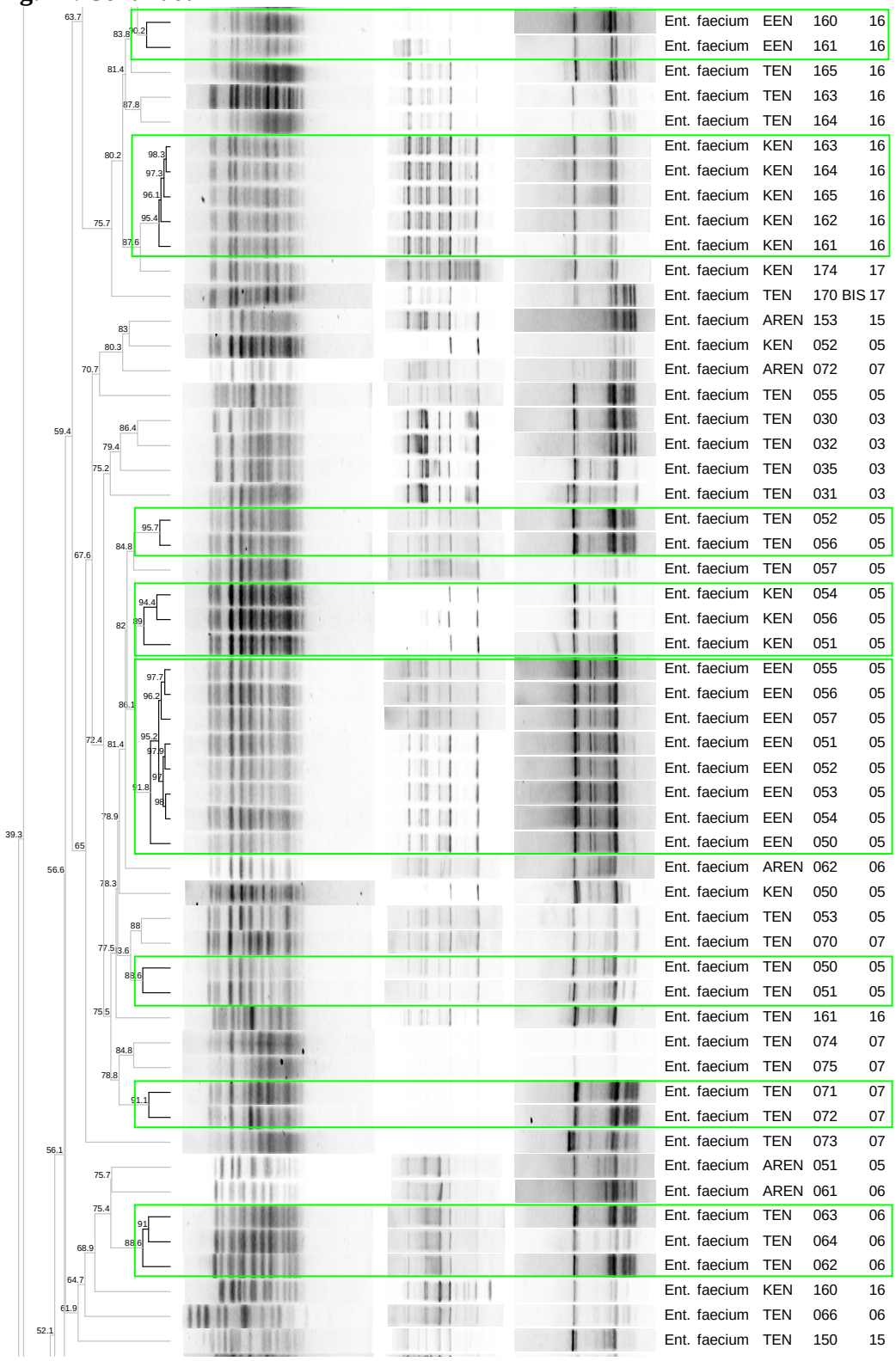


Fig. 24. *Continued*

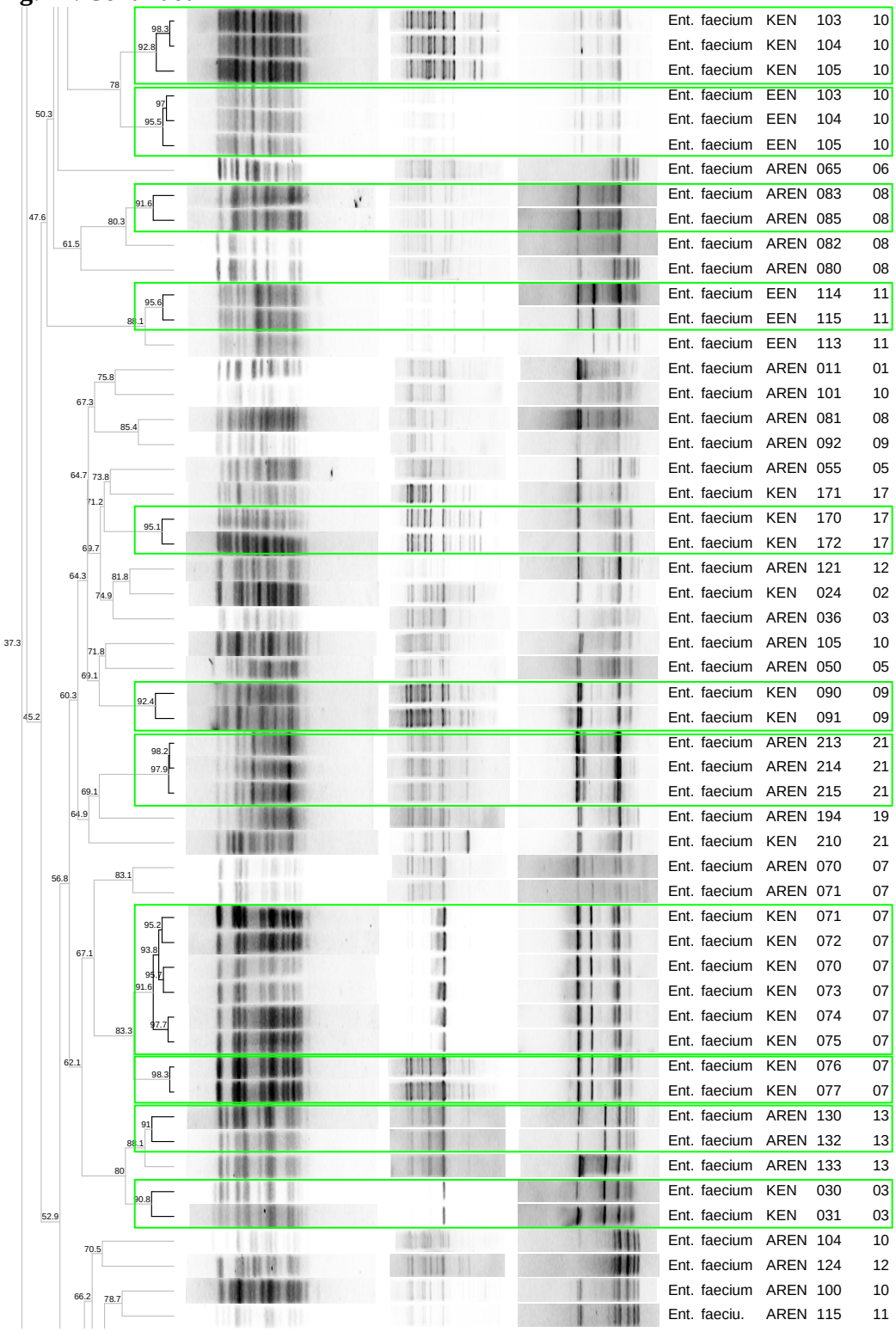
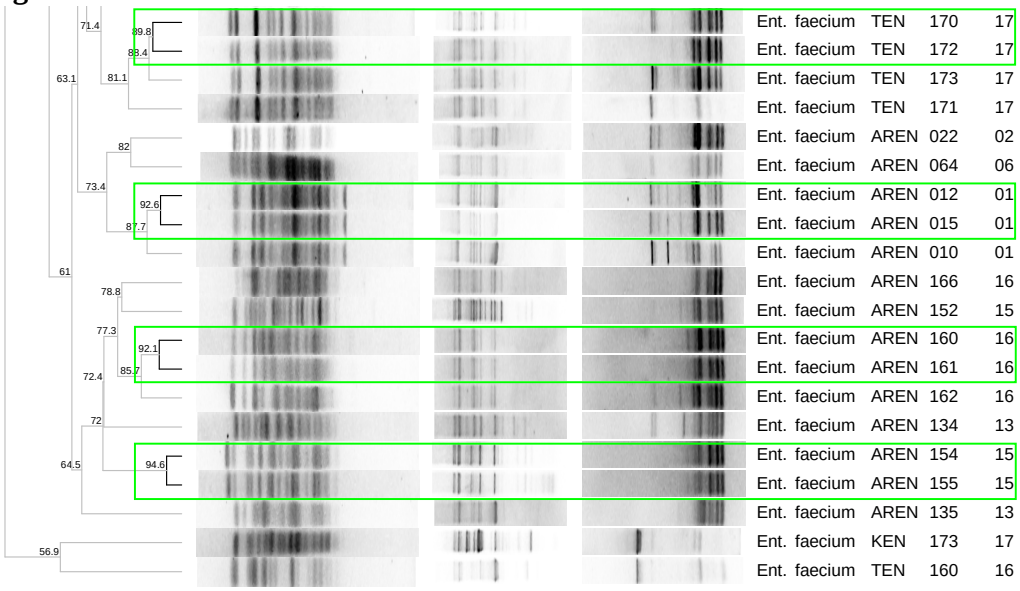


Fig. 24. *Continued*



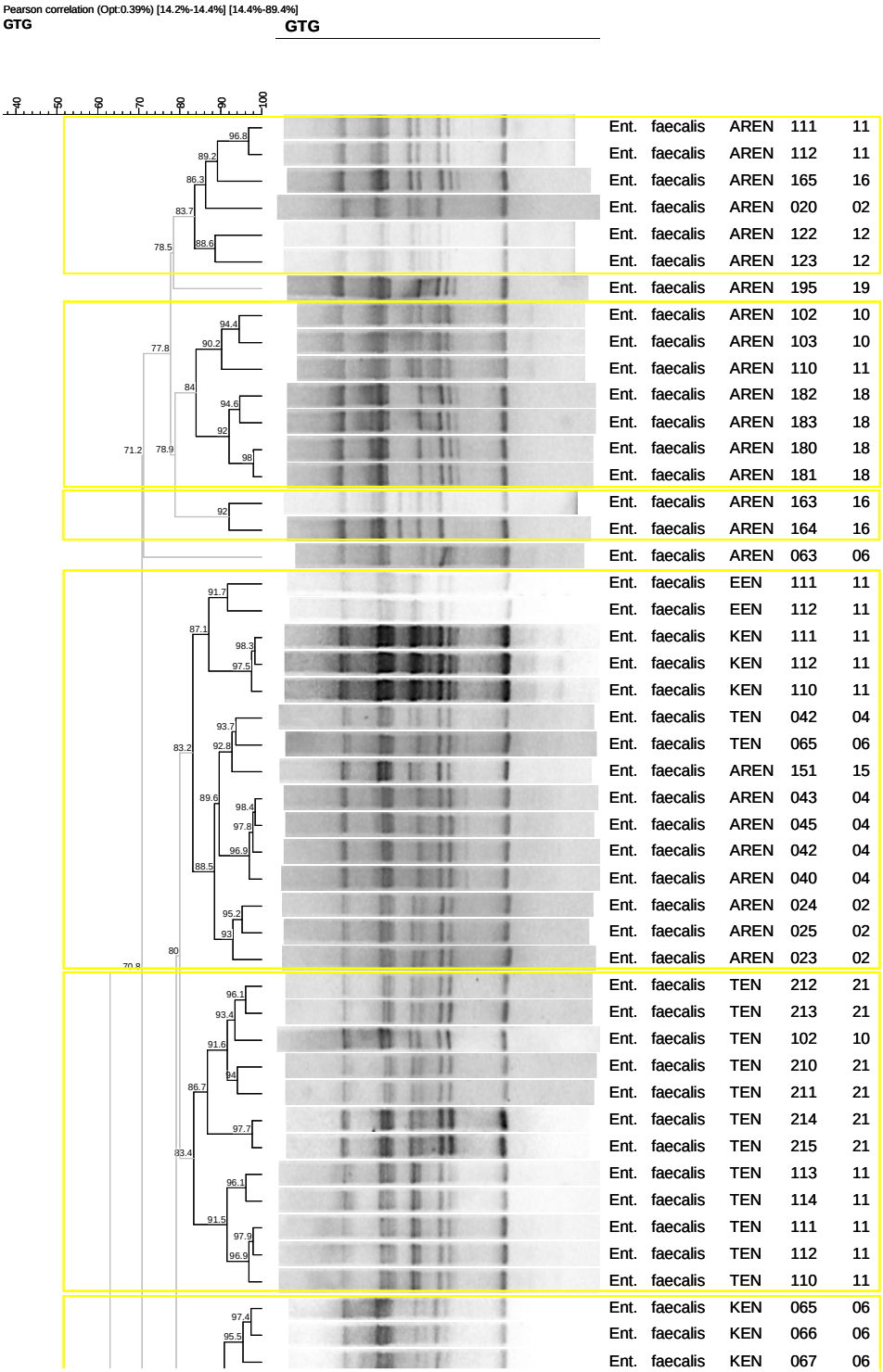


Fig. 25. Dendrogram obtained by Bionumerics cluster analysis of *E. faecalis* rep-$(GTG)_5$ patterns. *Continued on following 2 pages*.

Fig. 25. *Continued*

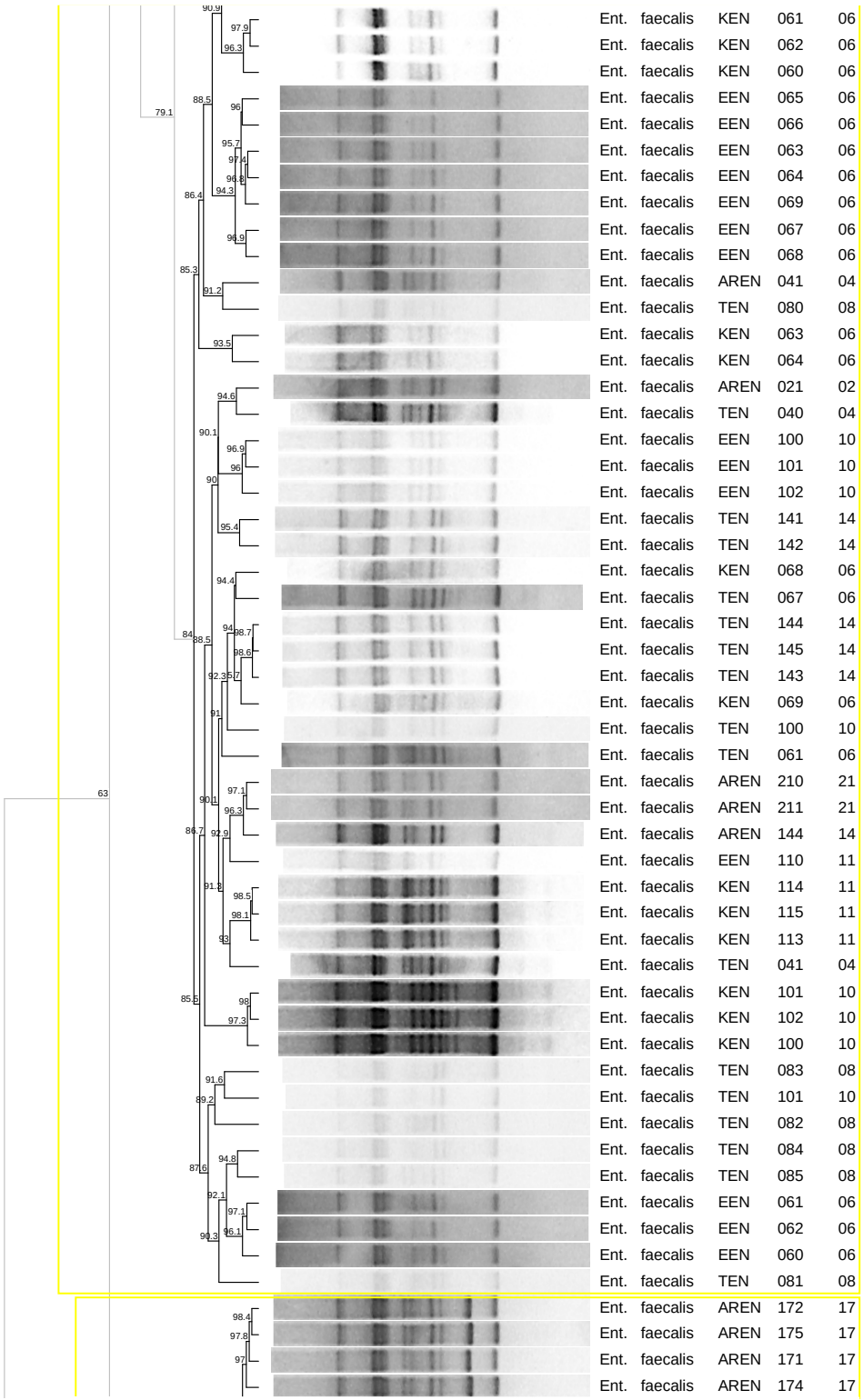
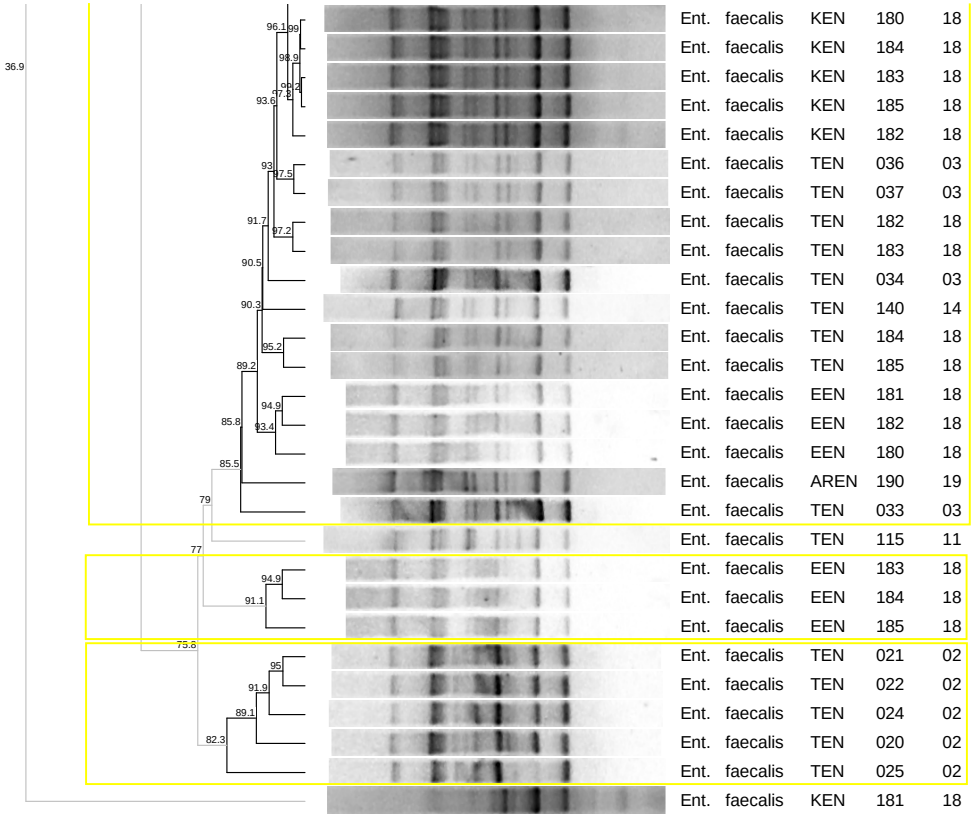


Fig. 25. *Continued*



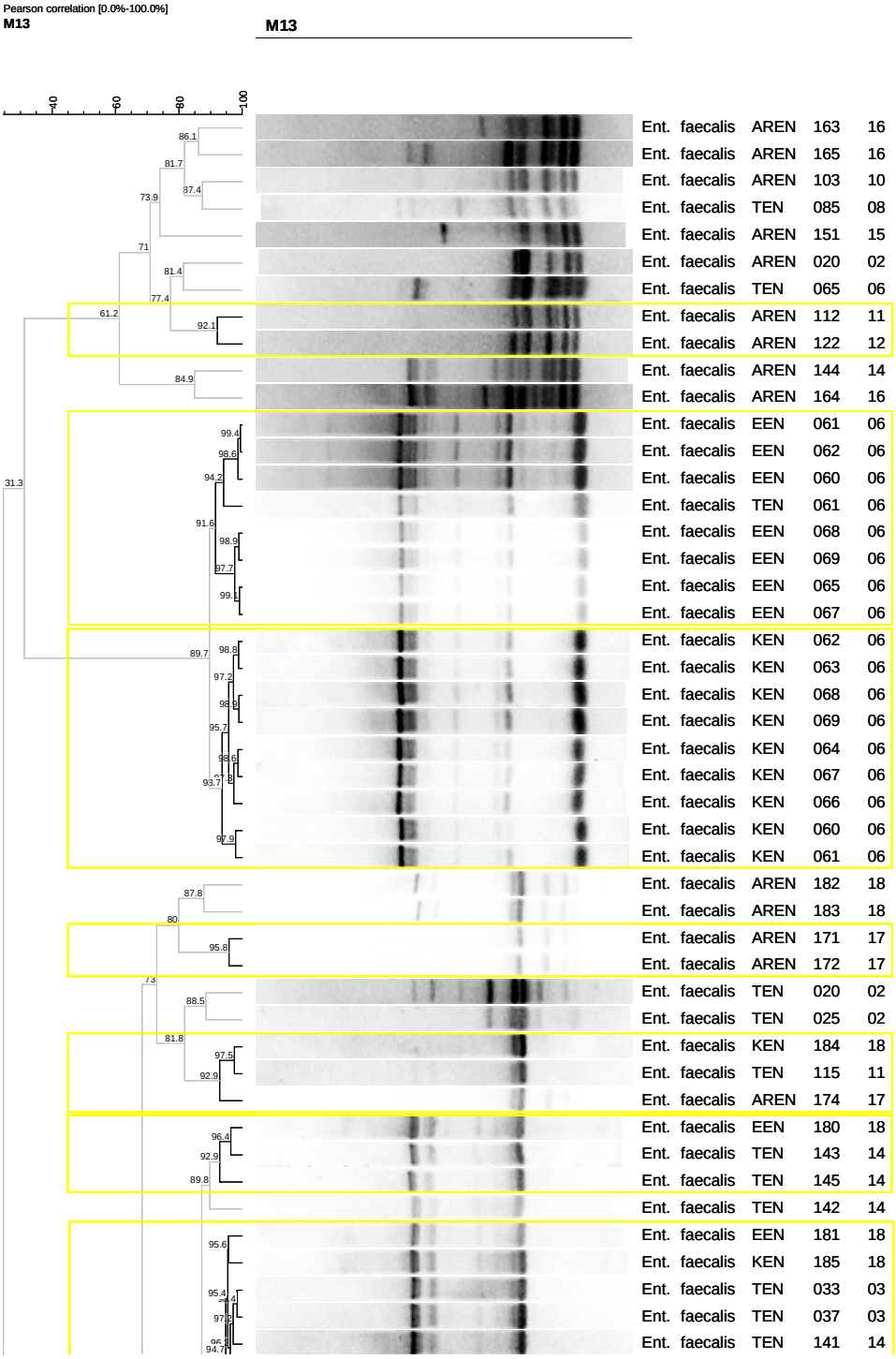


Fig. 26. Dendrogram obtained by Bionumerics cluster analysis of *E. faecalis* RAPD M13 patterns. *Continuend on following 2 pages*.

Fig. 26. *Continued*

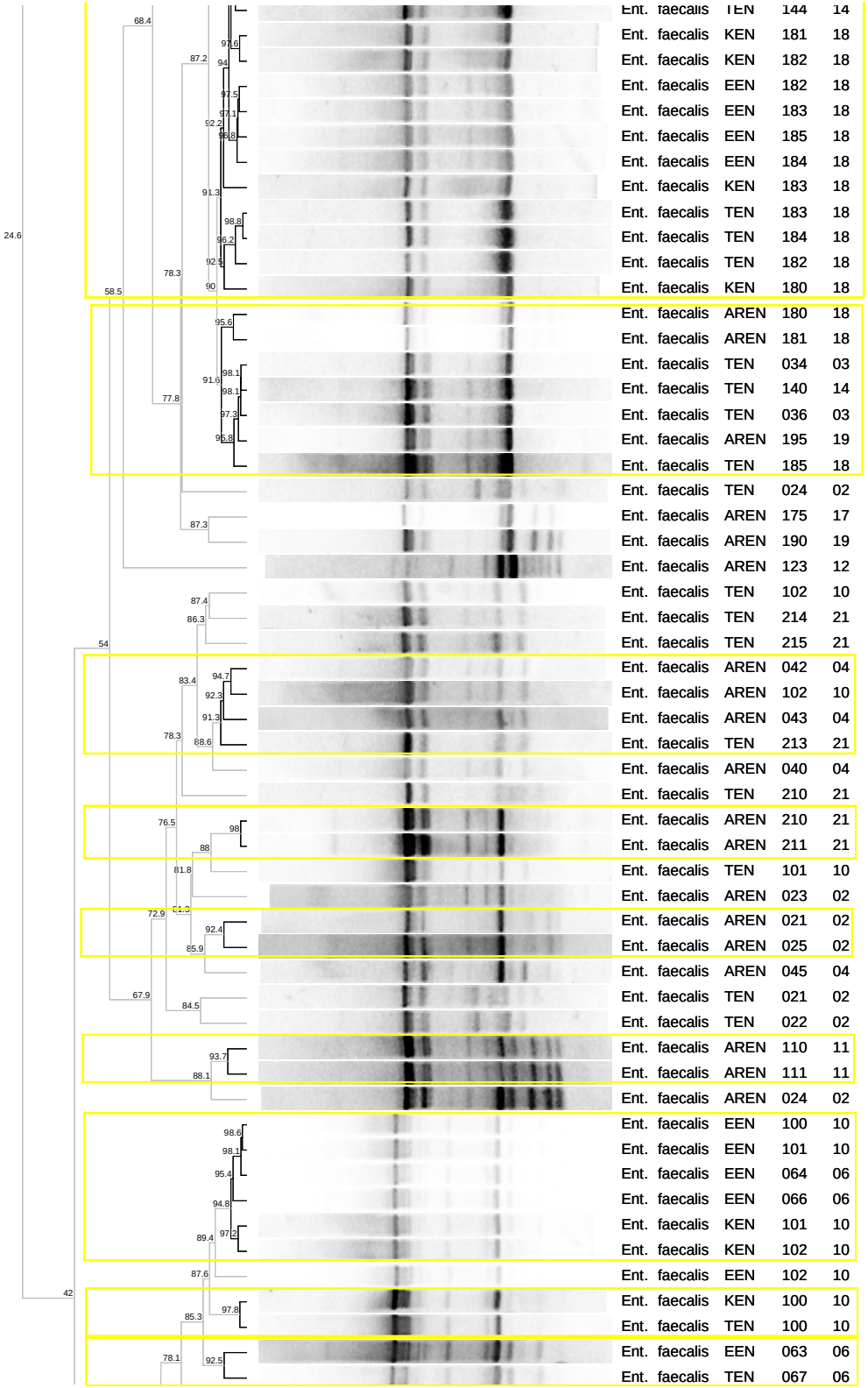
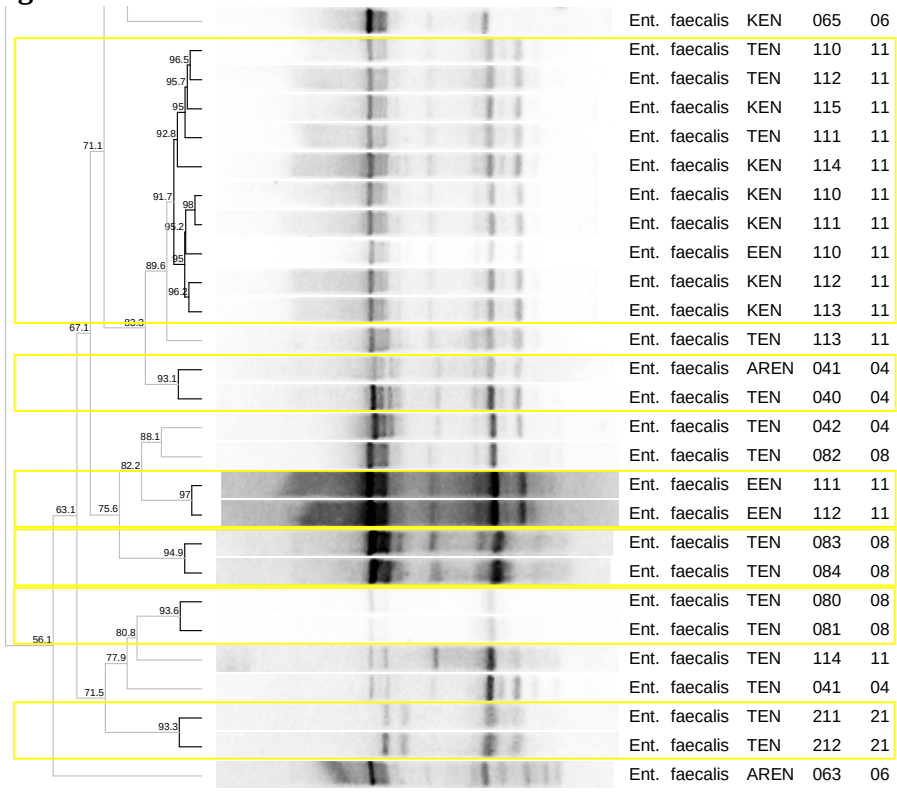


Fig. 26. *Continued*



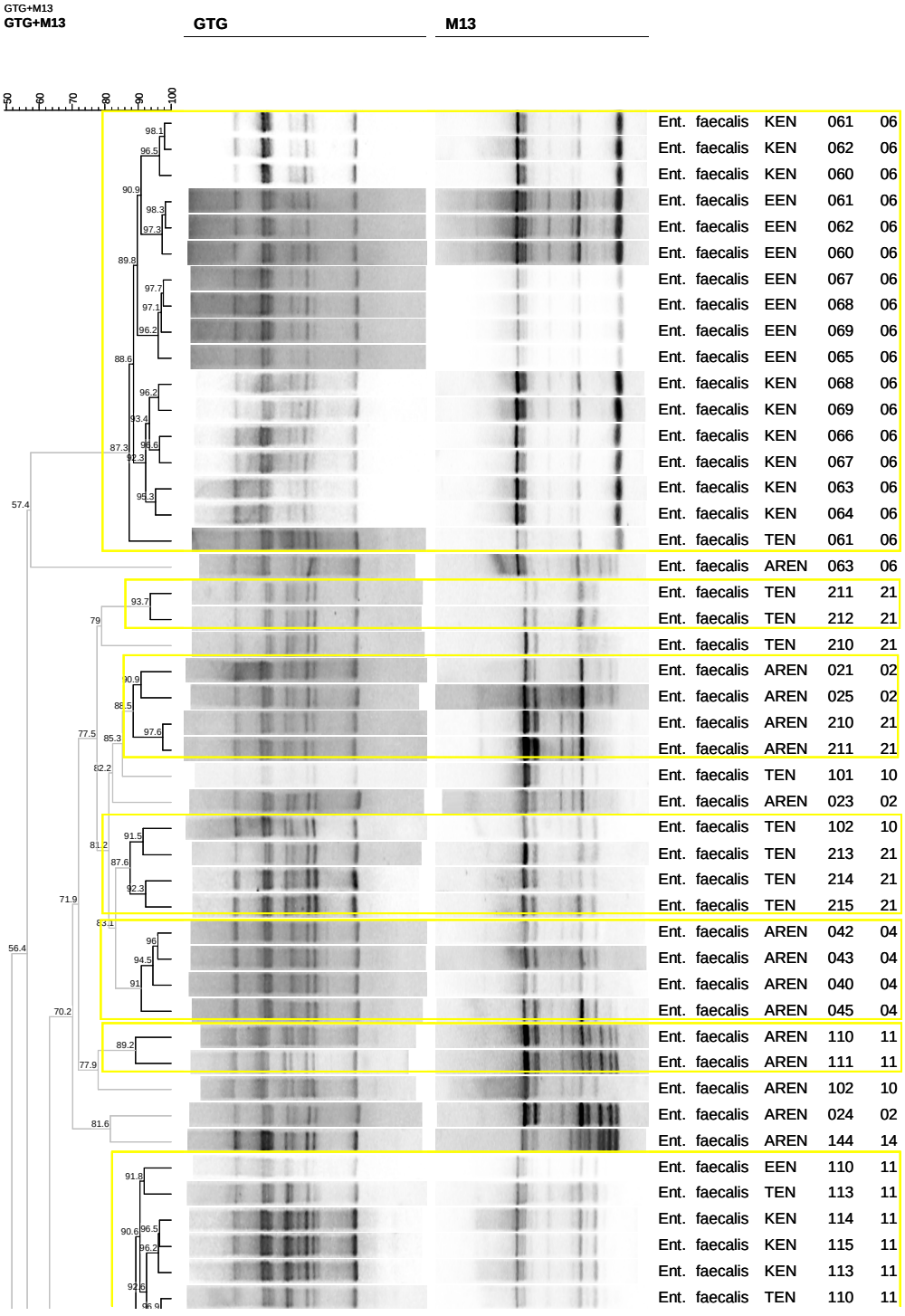


Fig. 27. Dendrogram obtained by Bionumerics cluster analysis of *E. faecalis* rep-$(GTG)_5$ and RAPD M13 combined patterns. *Continued on following 2 pages.*

Fig. 27. *Continued*

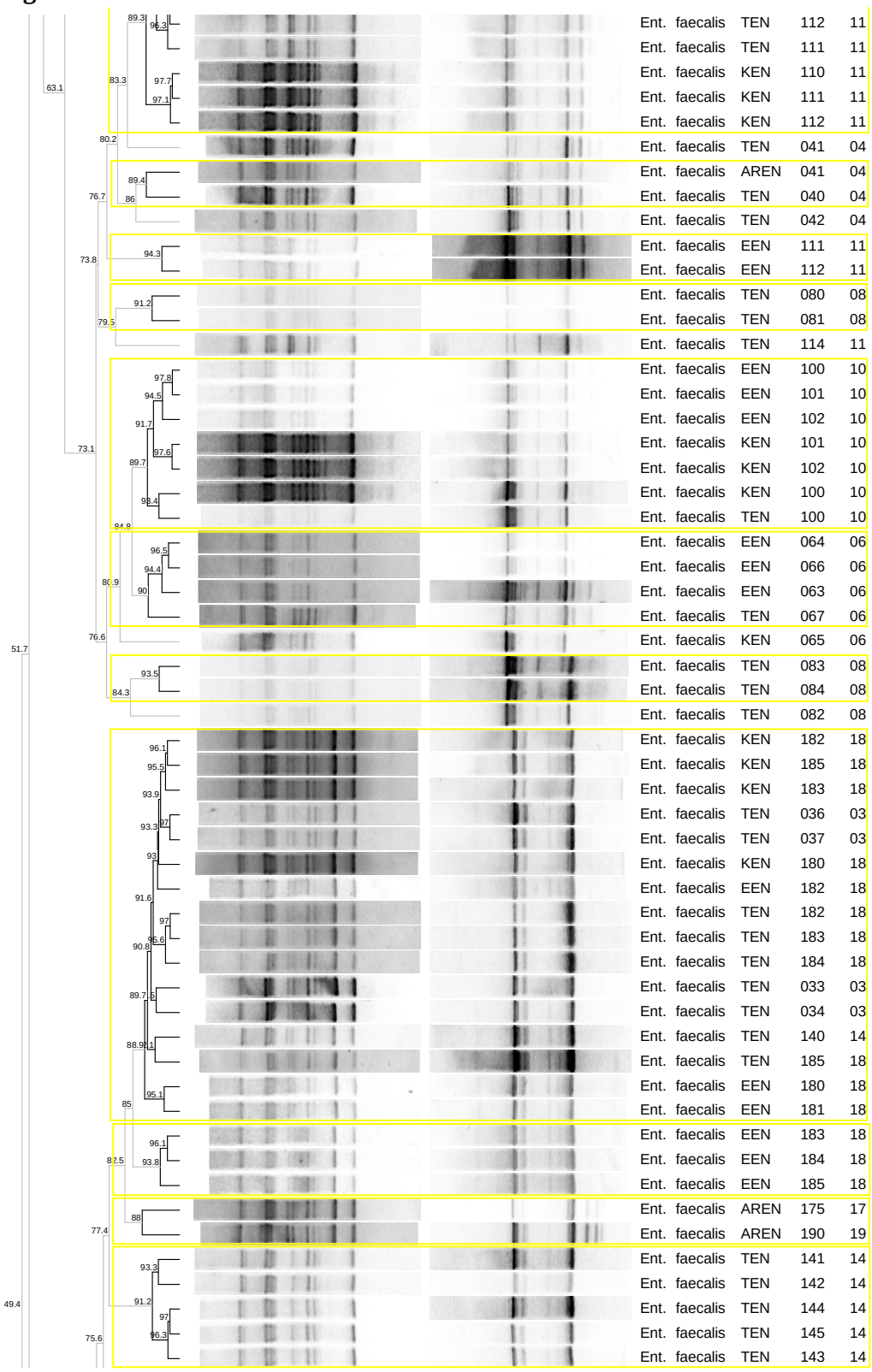
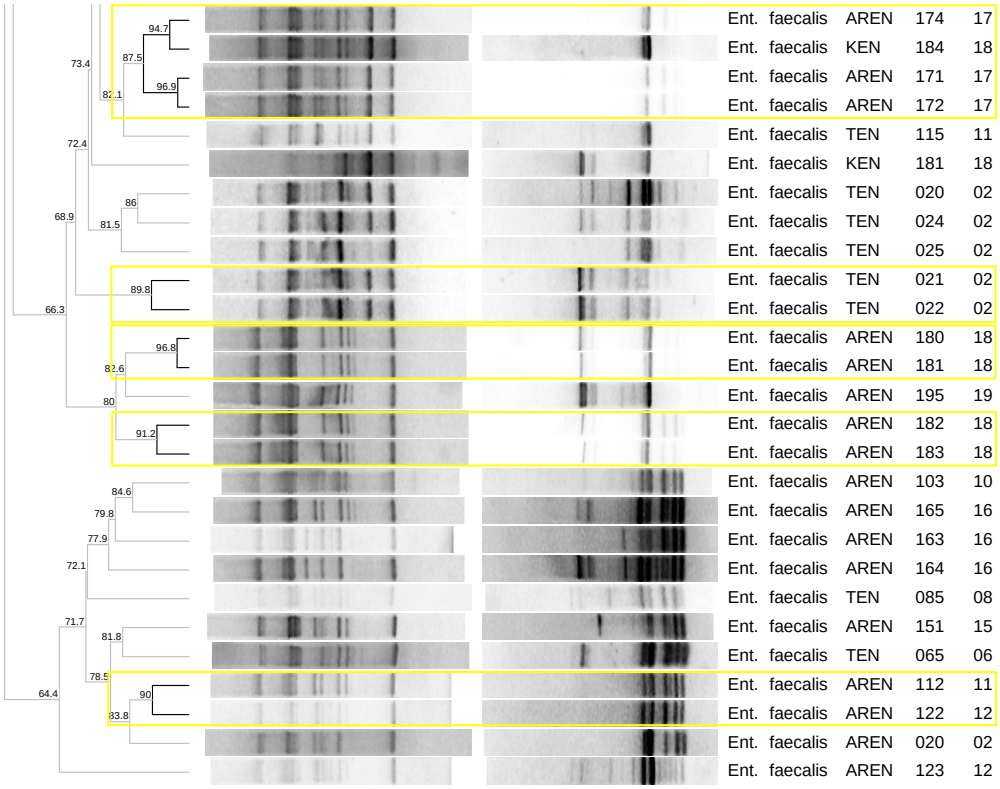


Fig. 27. *Continued*



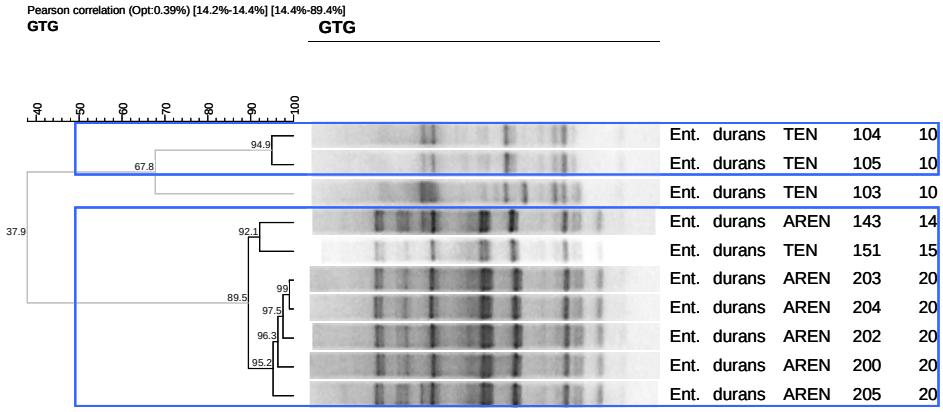


Fig. 28. Dendrogram obtained by Bionumerics cluster analysis of *E. durans* rep-$(GTG)_5$ patterns.

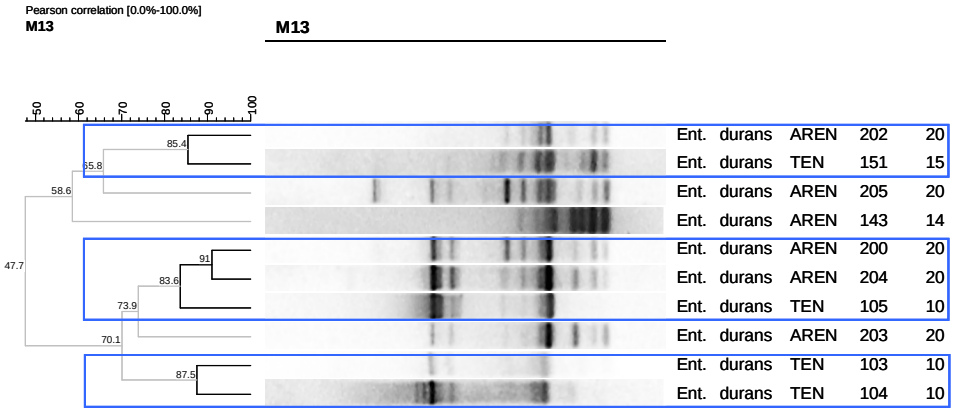


Fig. 29. Dendrogram obtained by Bionumerics cluster analysis of *E. durans* RAPD M13 patterns.

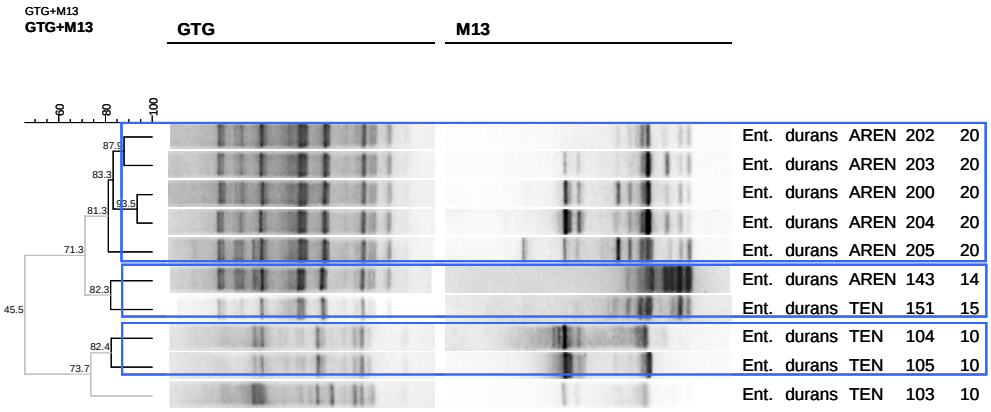


Fig. 30. Dendrogram obtained by Bionumerics cluster analysis of *E. durans* rep-$(GTG)_5$ and RAPD M13 combined patterns.

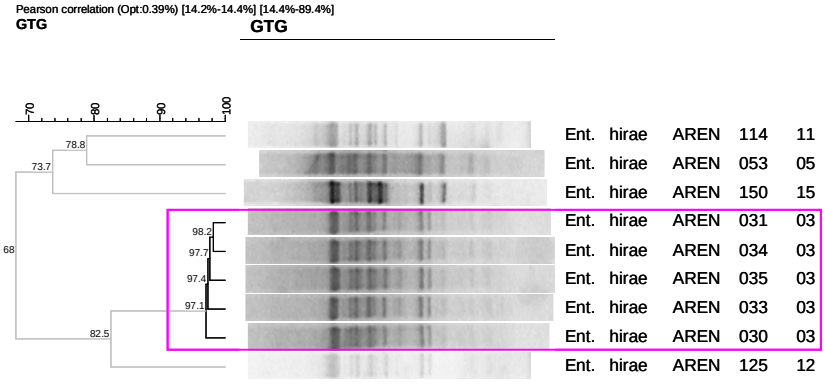


Fig. 31. Dendrogram obtained by Bionumerics cluster analysis of *E. hirae* rep-$(GTG)_5$ patterns.

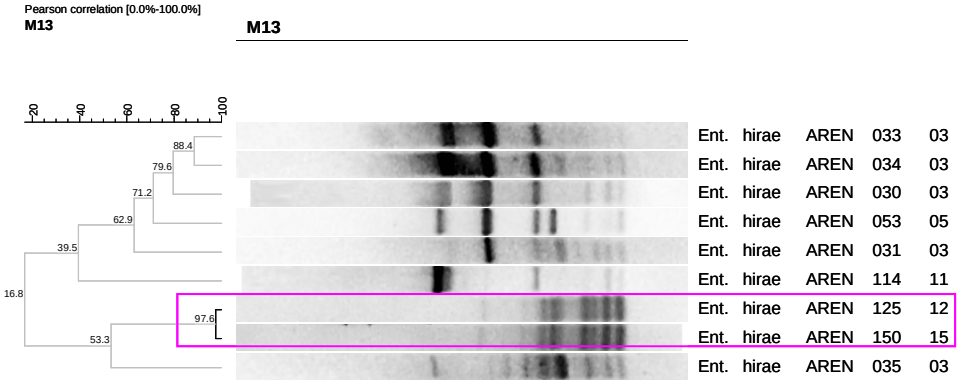


Fig. 32. Dendrogram obtained by Bionumerics cluster analysis of *E. hirae* RAPD M13 patterns.

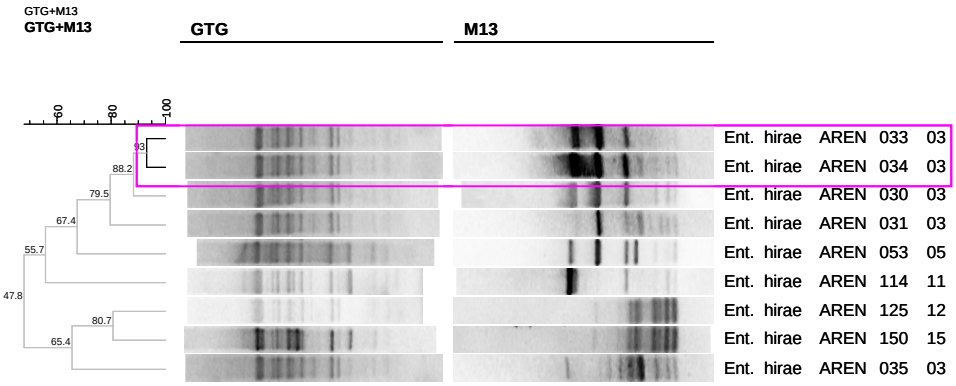


Fig. 33. Dendrogram obtained by Bionumerics cluster analysis of *E. hirae* rep-$(GTG)_5$ and RAPD M13 combined patterns.

3.3 Antibiotic resistance

3.3.1 MIC determination

Since the Discriminatory Powers of the strain typing techniques used, except PFGE for *E. faecium*, were not completely satisfactory, determinating the MIC values for all isolates, instead of one representative isolate *per* biotype, was chosen. In this way, none biotype was erroneously omitted and it was possible to better compare the behaviour of the different species.

For *E. faecium* and *E. faecalis* the results of the MIC tests obtained were charted both not considering the media of origin and taking it into account.

As shown in **Figures 34**(**a** and **b**) and **35**(**a, b**, and **c**), *E. faecium* and *E. faecalis* were resistant to a higher number of antibiotics in comparison to the isolates belonging to the non-dominant species. In these graphs, antibiotics are displayed in order of decreasing percentage of resistant isolates while in the following Figures, in which isolates are divided according to the medium of origin, the antibiotics are shown in alphabetical order. Since most of *E. faecium* isolates belonged to different PFGE-types and the percentages of resistant *E. faecium* PFGE-type were very similar to those of resistant isolates, only data referred to isolates will be shown.

As enterococci intrinsically possess a low level of resistance to Aminoglycosides, the percentage of gentamicin and streptomycin resistant isolates was calculated considering only HLR (High Level Resistant) isolates (MIC gen > 500 µg/ml; MIC str > 1000 µg/ml).

Results are shown and discussed, either by grouping antibiotics belonging to the same class or by reference to classes of antibiotics usually acting in synergy against enterococcal infections.

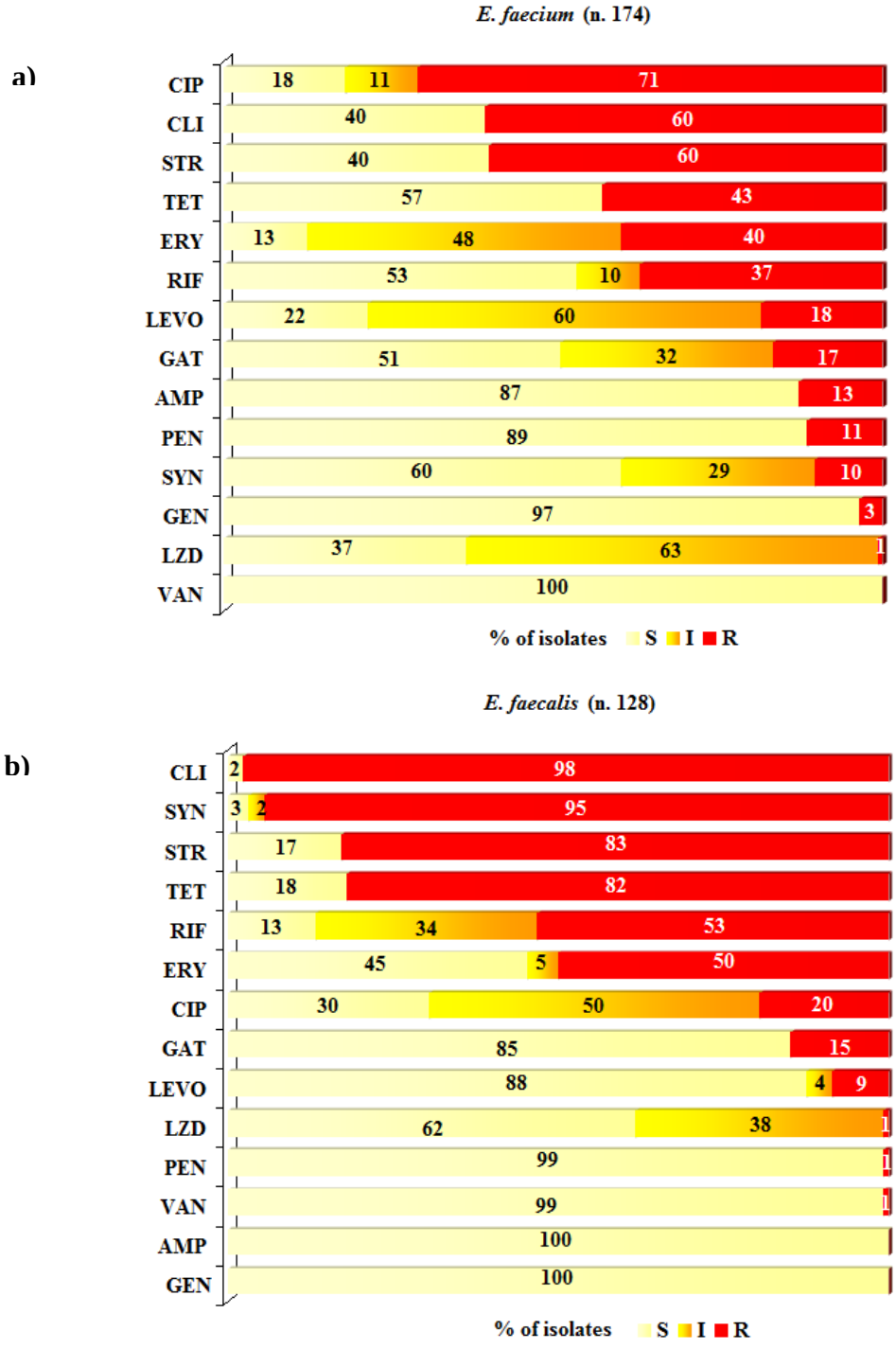
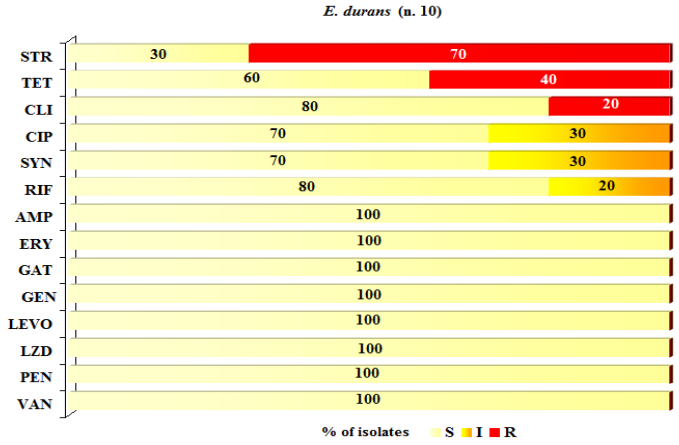
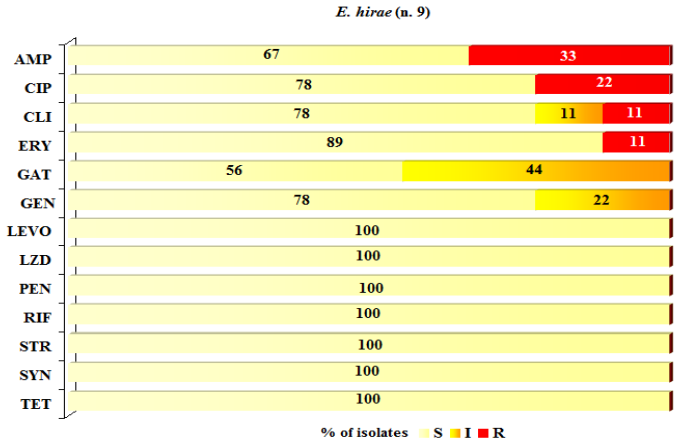


Fig. 34. Percentage of sensitive (S), intermediate (I) and resistant (R) *E. faecium* (**a**) and *E. faecalis* (**b**) isolates coming from all the media used in this study. For gentamicin and streptomycin only HLR isolates were considered.

a)



b)



c)

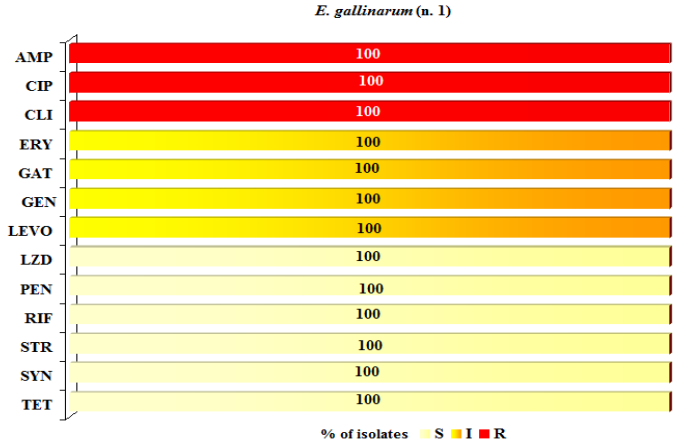


Fig. 35. Percentage of sensitive (S), intermediate (I) and resistant (R) *E. durans* (**a**), *E. hirae* (**b**), and *E. gallinarum* (**c**) isolates coming from all the media used in this study. For gentamicin and streptomycin only HLR isolates were considered.

β-lactams (*ampicillin and penicillin*) **and Aminoglycosides** (*gentamicin and streptomycin*)

Among *E. faecium* isolates 11 and 13% were resistant to penicillin and ampicillin, respectively (**Fig. 34a**), while, among *E. faecalis* isolates only 1% was resistant to penicillin, and none to ampicillin (**Fig. 34b**). All the isolates belonging to the other species detected, with the exception of the *E. gallinarum* isolate, were sensitive to β-lactams (**Fig. 35a, b,** and **c**).

No ampicillin- and/or penicillin-resistant *E. faecium* from SBA without antibiotic addition was isolated.

Most of the resistant *E. faecium* isolates came from SBA + erythromycin (43 and 33 % Pen^R and Amp^R isolates from this medium, respectively) (**Fig. 36**). All the *E. faecalis* Pen^R isolates came from SBA + erythromycin (**Fig. 37**). On the whole, a higher percentage of *E. faecium* isolates came out to be resistant to the β-lactams tested compared with *E. faecalis* (**Fig. 38**).

Enterococci are commonly considered intrinsically resistant to ampicillin and penicillin due to the low affinity of their penicillin-binding proteins (PBPs) for the β-lactams agents (Kak and Chow, 2002). As regards ampicillin, *E. faecium* and *E. faecalis* isolates showed modal MIC values between 0.5 and 1, and between 1 and 2, respectively, while modal MIC values of penicillin ranged between 2 and 4 for both species (**Fig. 39a** and **b**).

These MIC values are largely lower than CLSI breakpoints to define resistant strains (see **Table 3**).

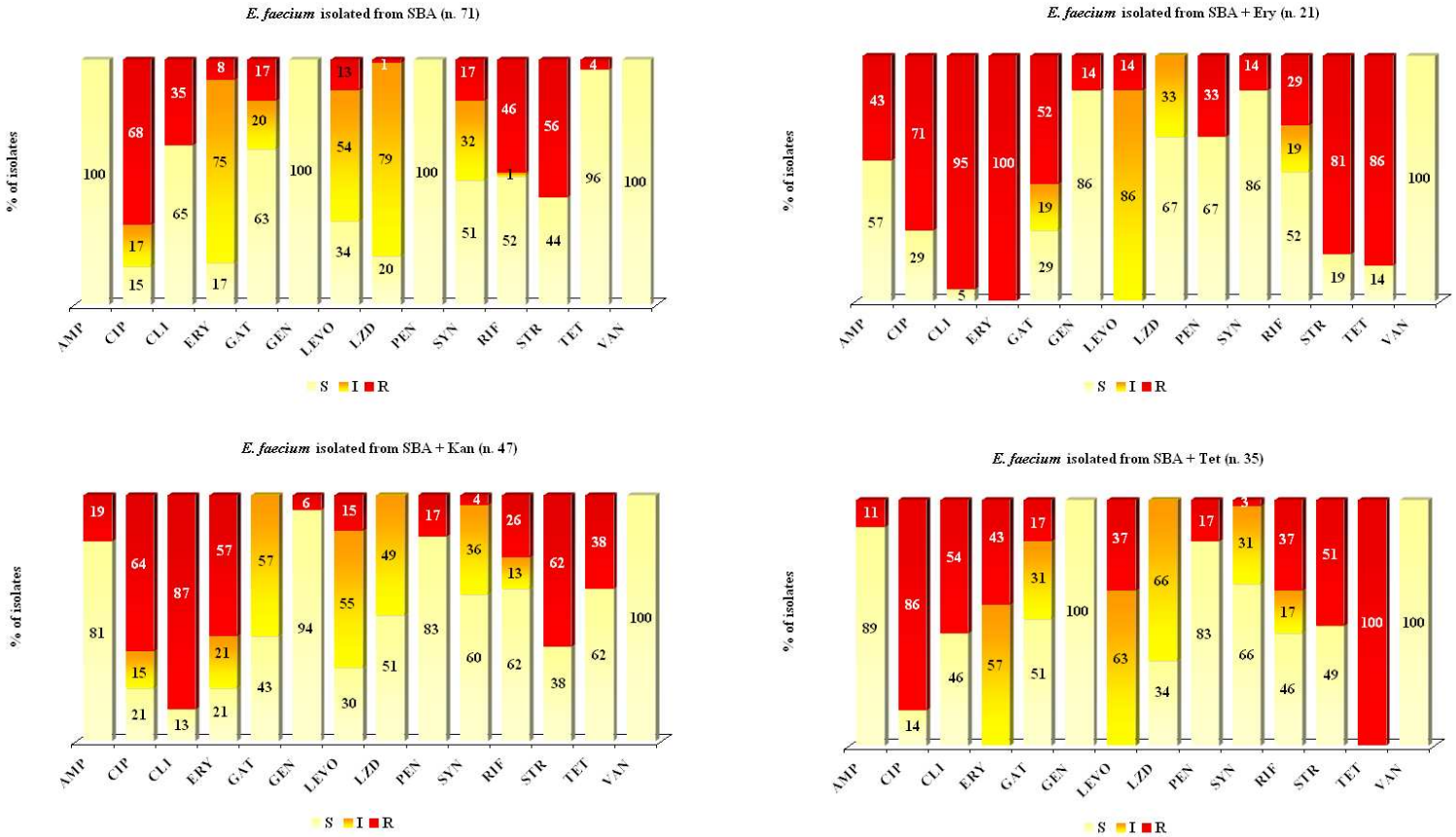


Fig. 36. Percentage of sensitive, intermediate and resistant *E. faecium* isolates depending on the isolation media. For gentamicin and streptomycin only HLR isolates were considered.

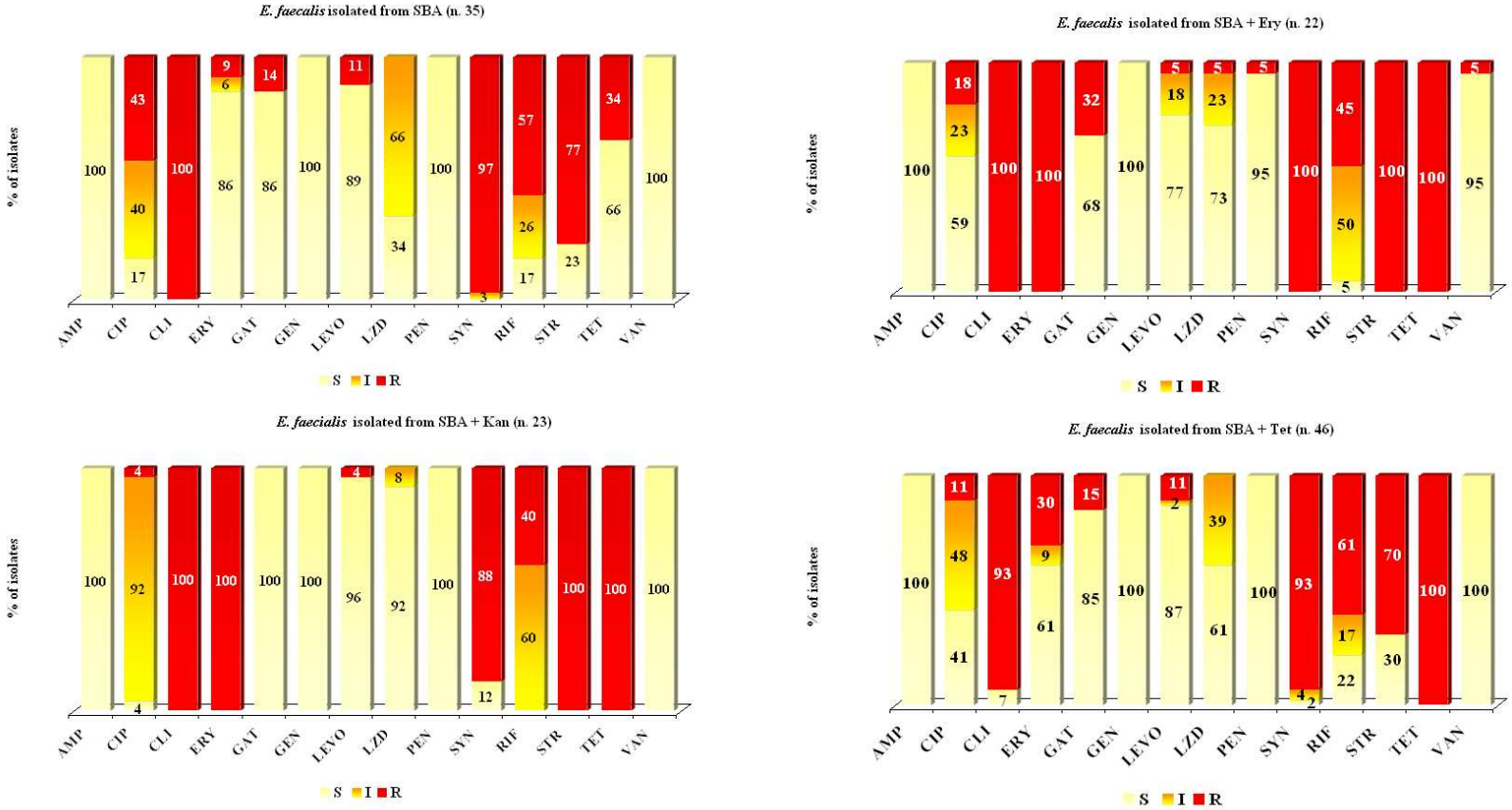
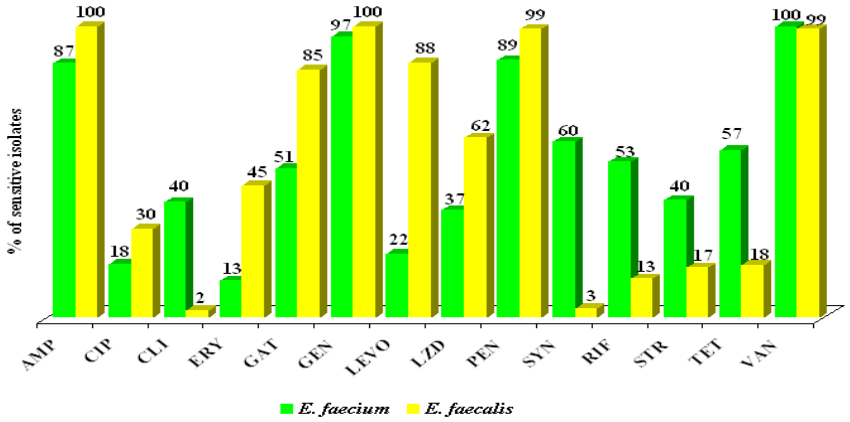
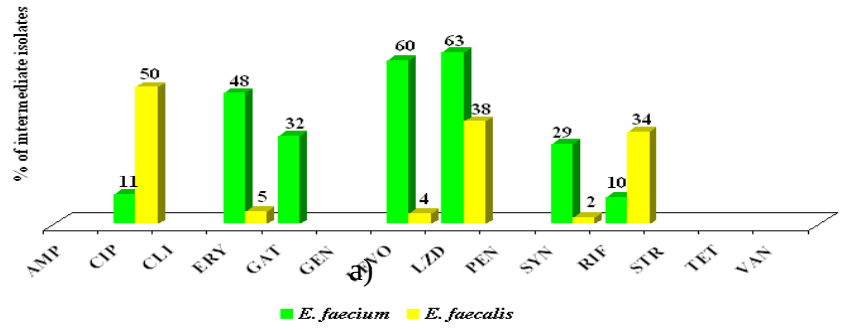


Fig. 37. Percentage of sensitive, intermediate and resistant *E. faecalis* isolates depending on the isolation media. For gentamicin and streptomycin only HLR isolates were considered.

a)



b)



c)

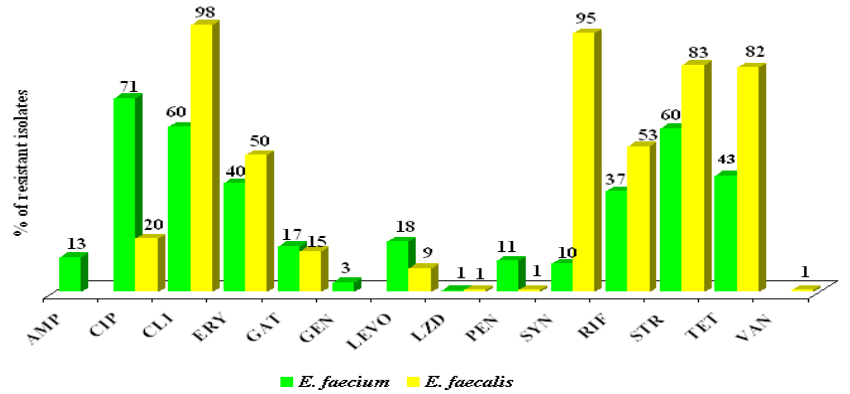


Fig. 38. Comparison among *E. faecium* and *E. faecalis* isolates as concern the percentage of sensitive (a), intermediate (b) and resistant (c) isolates coming from all the media used in this study. For gentamicin and streptomycin only HLR isolates were considered.

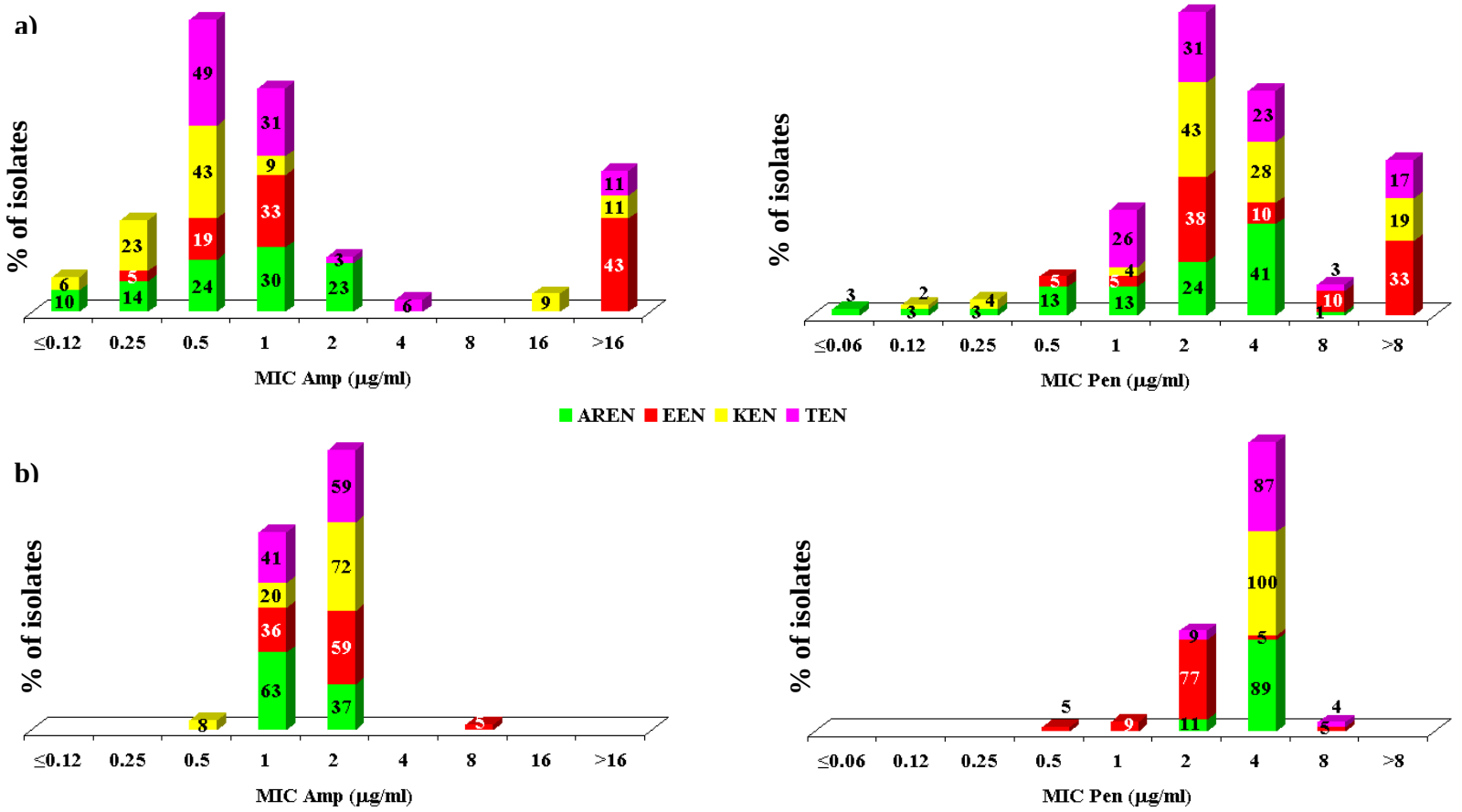


Fig. 39. β-lactams (ampicillin and penicillin) MIC value distribution of (a) *E. faecium* (174) and (b) *E. faecalis* (128) isolates depending on their medium of origin

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Therefore, the results obtained in the present study, like those reported in other recent works carried out on different foods, would seem to show that not all enterococci, including those from food, are intrinsically resistant to β-lactams. Indeed, very low percentages (mostly 0 %) of resistant isolates have been detected both in Fiore Sardo cheese (Mannu *et al*., 2003; Cosentino *et al.*, 2004; present study), and in other cheeses, milk, raw and cooked sausages, ham, meat, vegetables, fruits, and water (Peters *et al*., 2003; Ben Omar *et al*., 2004; Drahovská *et al*., 2004; Čanžek Majhenič *et* al., 2005; Lopes *et al*., 2005; Abriouel *et al*., 2008; Pangallo *et al*., 2008; Valenzuela *et al*., 2008 and 2009; Barbosa *et al.*, 2009). Only a few studies report high percentages (from 45 and >60%) of β-lactams resistant enterococci from food (Franz *et al*., 2001; Bulajić and Mijačević, 2004; Gomes *et al*., 2008).

A different behaviour has been observed among enterococci isolated from clinical specimens. Clinical enterococci exhibit intrinsically reduced susceptibility to penicillin and to the aminopenicillins with MICs 100 times higher than MICs against streptococci (Kayser, 2003). Around 60-80% of clinical *E. faecium* strains (in some hospital over 90%) are resistant to ampicillin (Klare *et al*., 2003), whereas less than 2% of *E. faecalis* isolates possess this resistance (Hällgren *et al*., 2001; Kak and Chow, 2002; Dupré *et al*., 2003; Klare *et al*., 2003; Mannu *et* al., 2003; Simonsen *et al.*, 2003; Drahovská *et al*., 2004; Kaçmaz and Aksoy, 2005; Messi *et al*., 2006; Lambiase *et al*., 2007; Helmi *et al*., 2008).

Penicillins are often successfully used in combination with aminoglycosides (e.g. streptomycin, kanamycin and gentamicin) for treatment of enterococcal infections in human medicine. Indeed, aminoglycosides (inhibitors of ribosome function) would be

ineffective if used as monotherapy against enterococci because of their intrinsic tolerance to low levels of these drugs, but in combination with penicillins (cell-wall active antibiotics) can act synergically (Kak and Chow, 2002). Unfortunately, clinical enterococci isolates resistant to some of these antibiotics combinations have been detected from early 1970s (Franz *et al*., 1999; Peters *et al*., 2003; Kaçmaz and Aksoy, 2005), posing a major problem in treating human clinical infections. The incidence of resistance to gentamicin is generally low in Europe, but some strains isolated from chicken meat exported from China to Europe showed a high level gentamicin resistance coupled with a high level streptomycin resistance (Teuber *et al.*, 1999). Therefore, the import of such kinds of food stuff is of major concern and should be well regulated as there would well be a risk of spreading such resistance to these antibiotics to Europe.

As regards enterococci isolated to carry out this study, only 3 % of *E. faecium* and none of *E. faecalis* were resistant to a high level of gentamicin (HLR Gen), while more than 50 % of isolates belonging to all the species, except *E. hirae* and *E. gallinarum*, showed a high resistance to streptomycin (HLR Str, MIC > 1000 µg/ml) (**Figs 34a** and **b, 35a**, **b** and **c**). Concerning gentamicin, the results are consistent with those reported in literature for food and environmental isolates, while, as already reported by Cosentino *et al.* (2004), the percentage of HLR streptomycin enterococci from Fiore Sardo turned out to be higher than those generally reported for non clinical isolates (Arvanitidou *et al*., 2001; Ben Omar *et al*., 2003; Abriouel *et al*., 2008; Pangallo *et al*., 2008).

However, when percentages were calculated looking at each separate medium of isolation, it would seem that HLR Gen isolates were not dominant among the whole enterococcal population of Fiore Sardo cheese. Thus in the medium without antibiotics, susceptible isolates successfully competed with the resistant ones and prevented their isolation. Indeed, most of HLR Gen *E. faecium* isolates came from SBA + erythromycin (14 % of isolates from this medium) and from SBA + another aminoglycoside drug, kanamycin (6 %), none from SBA or SBA + tetracycline (**Fig. 36**).

HLR Str *E. faecium* were mainly isolated from SBA + erythromycin (81 % of isolates from this medium), but high percentages of HLR Str were also detected among isolates coming from SBA + kanamycin (62 %), SBA (56 %), and SBA + tetracycline (51 %) (**Fig. 36**).

HLR Str *E. faecalis* were mainly isolated from SBA + erythromycin and SBA + kanamycin (100 % of isolates from these media), but very high percentages of HLR Str *E. faecalis* were also isolated from SBA and SBA + tetracycline (77 and 70%, respectively) (**Fig. 37**).

Both *E. faecium* and *E. faecalis* isolates showed gentamicin modal MIC values of 16, so they do not possess intrinsic HLR against gentamicin (MIC ≥ 500) (**Fig. 40a** and **b**).

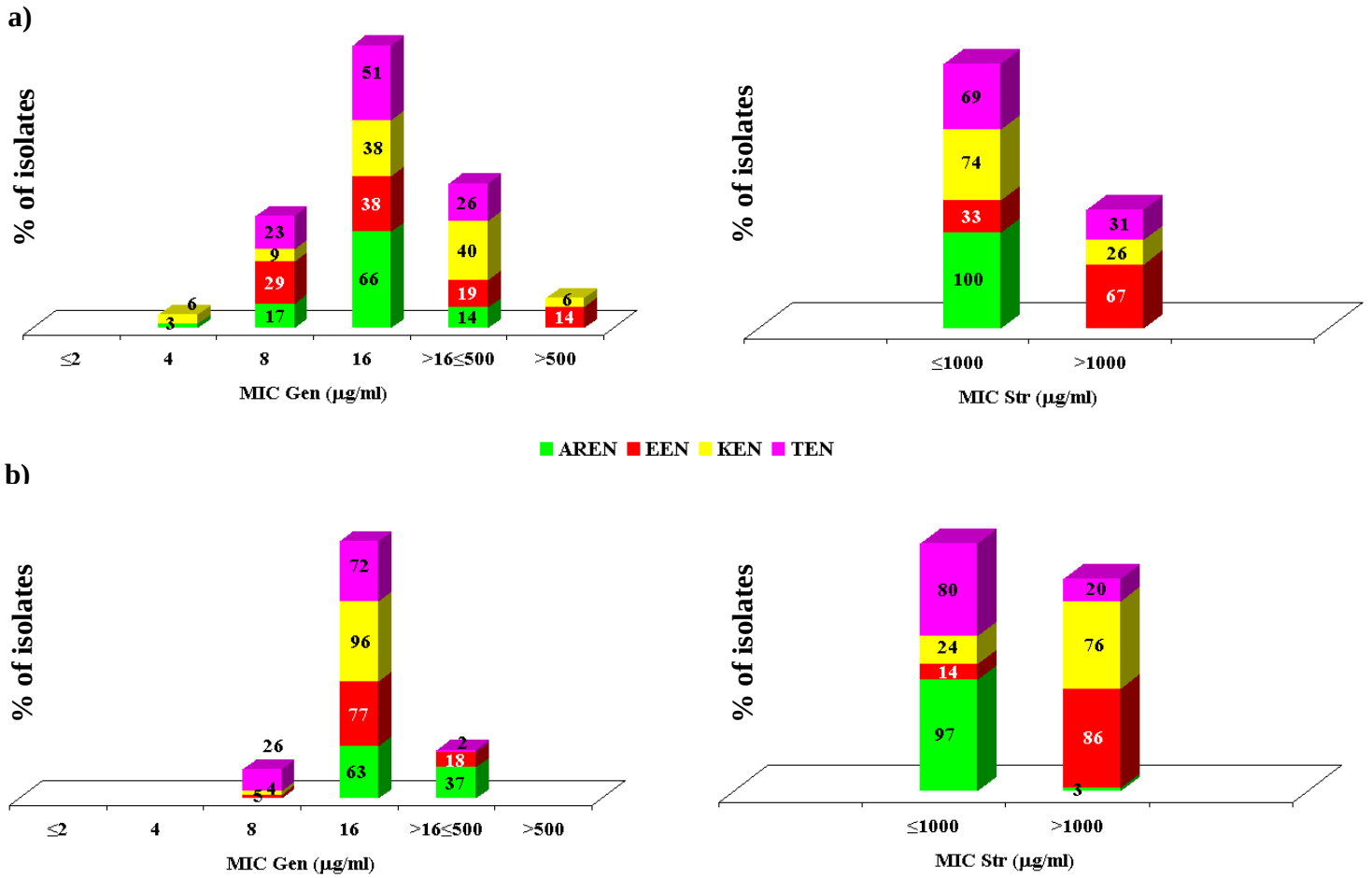


Fig. 40. Aminoglycosides (gentamicin and streptomicin) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

In the case of streptomycin, for *E. faecium* the modal MIC value was ≤ 1000, irrespective from the isolation media, but different results were obtained if each isolation medium was considered separately. In fact, 100 % of *E. faecium* from SBA, the majority of those from SBA + kanamycin and SBA + tetracycline showed MIC values ≤ 1000, while 67 % of *E. faecium* coming from SBA + erythromycin showed a MIC > 1000 (HRL Str) (**Fig. 40a**).

E. faecalis isolates had a slightly different behaviour. Indeed, like most (86 %) of isolates from SBA + erythomycin showed HLR against streptomycin (MIC > 1000), so did the majority of isolates from SBA + kanamycin (76 %) did (**Fig. 40b**).

Even though MIC values of kanamycin were not determined, it is likely that Fiore Sardo cheese also contains enterococci resistant to a high level of this Aminoglycoside, as around 22 % of all the isolates recovered for this study were able to grow in SBA + 1024 μg/ml of kanamycin (49 *E. faecium* and 25 *E. faecalis*).

In general, adding drugs (especially erythromycin) to the isolation medium favoured the selection of *E. faecium* and *E. faecalis* isolates resistant against both β-lactams and aminoglycosides.

Fluoroquinolones *(ciprofloxacin, gatifloxacin and levofloxacin)*

E. durans isolates (10) were susceptible to all the Fluoroquinolones antibiotics tested, while 22 % of *E. hirae* isolates were resistant only to ciprofloxacin. The sole *E. gallinarum* isolate was resistant only to ciprofloxacin (**Fig. 35**).

Among dominant species, 71 % of *E. faecium* and 20% of *E. faecalis* isolates were resistant to ciprofloxacin. Lower percentages of isolates showed resistance against

gatifloxacin and levofloxacin (17 and 18 % of *E. faecium* and 15 and 9% of *E. faecalis*, respectively) (**Fig. 34a** and **b**).

When different media are considered separately, high percentages (ranging between 64 and 86 %) of Cip^R *E. faecium* were isolated from all media (**Fig. 36**). Cip^R *E. faecalis* were isolated from all the media used, but in very different percentages always lower than *E. faecium* (**Fig. 37**). Almost half of *E. faecalis* isolated from SBA without any antibiotic addition was Cip^R, while adding kanamycin to the isolation medium only 4 % of Cip^R *E. faecalis* were collected.

The highest percentage of Gat^R *E. faecium* isolates was found in SBA + erythromycin (52 % of isolates from this medium), while no Gat^R *E. faecium* were isolated from SBA + kanamycin, and 17 % both from SBA, and SBA + tetracycline. As for *E. faecium*, no Gat^R *E. faecalis* were isolated from SBA + kanamycin, while the percentage of gatifloxacin resistant *E. faecalis* isolated from SBA, SBA + tetracycline ranged between 14-15%. From SBA + erythromycin the highest percentage (32 % of isolates from this medium) of Gat^R *E. faecalis* was obtained.

$Levo^R$ *E. faecium* isolates were mainly isolated from SBA + tetracycline (37 % of isolates from this medium).

Among Fluoroquinolones tested, levofloxacin turned out to be the most effective against *E. faecalis* isolated from all the media. Indeed, $Levo^R$ *E. faecalis* ranged between 4 - 5 % of isolates coming from SBA + kanamycin and SBA + erytrhomycin, and they were 11 % of those coming from both SBA and SBA + tetracycline.

In general, *E. faecium* was more resistant than *E. faecalis* to Fluoroquinolones, especially against ciprofloxacin (**Fig. 38**).

E. faecalis ciprofloxacin modal MIC value was 2, while for *E. faecium* it was ≥ 4. Therefore, *E. faecium* population colonizing Fiore Sardo cheese turned out to be resistant to this Fluoroquinolone (**Fig. 41**).

As regards gatifloxacin, both *E. faecium* and *E. faecalis* isolated from Fiore Sardo turned out to be susceptible, showing modal MIC values (= 2 and ≤ 1, respectively) lower than the breakpoint (≥ 8) fixed by CLSI to identify resistant isolates.

Levofloxacin modal MIC values were 4 and 2 for *E. faecium* and *E. faecalis*, respectively. Thus, as with gatifloxacin, lower than corresponding CLSI breakpoint to consider an isolate as resistant (**Fig. 42**).

In general, Fluoroquinolones have a large spectrum of activity against Gram negative and Gram positive microorganisms, but exhibit only a weak effect on enterococci (Peters *et al*., 2003). Barbosa *et al*. (2009) detected 93.4% of ciprofloxacin resistant enterococci among isolates from fermented meat products produced in Portugal. It has been assumed that this could be due to an overwhelming use of Fluoroquinolones both in human and veterinary medicine (Appelbaum and Hunter, 2000). A comparative study was carried out by Abriouel *et al.* (2008) concerning antimicrobial resistance among *E. faecalis* and *E. faecium* isolates from fruits and vegatable foods, and clinical specimens. A lower incidence of antibiotic resistance was found in food isolates than in clinical ones. In particular, in the case of ciprofloxacin, 35.29 and 45.45% of Cip^R *E. faecalis* and *E. faecium* respectively were isolated from food, and 61.53 and 83.33% of Cip^R *E. faecalis* and *E. faecium* respectively from clinical

specimens. As with Fiore Sardo cheese isolates, the percentages of resistant *E. faecium* are higher than those calculated for *E. faecalis*.

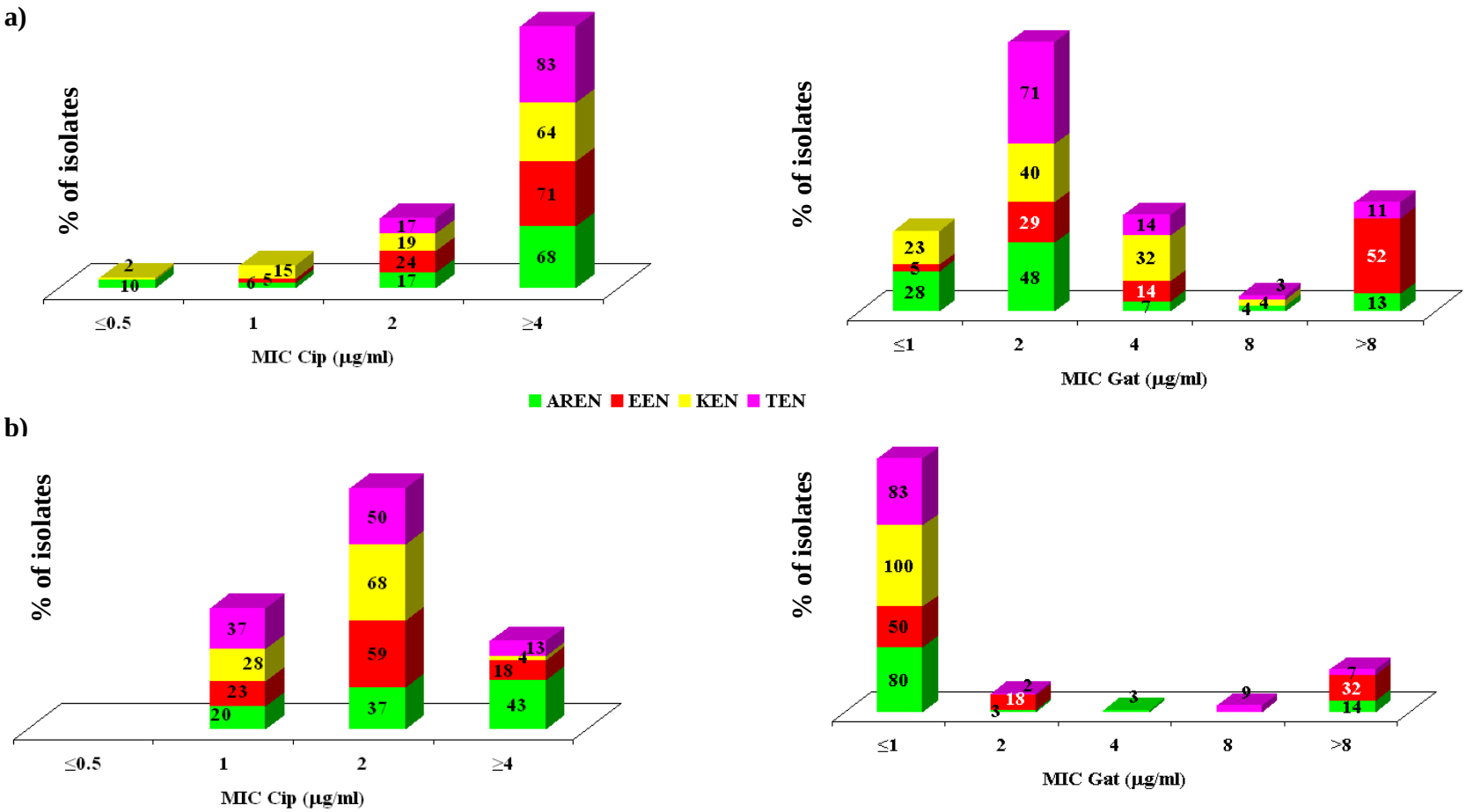


Fig. 41. Fluroquinolones (ciprofloxacin, gatifloxacin and, in the next Figure, levofloxacin) MIC value distribution of (a) *E. faecium* (174) and (b) *E. faecalis* (128) isolates depending on their medium of origin.

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

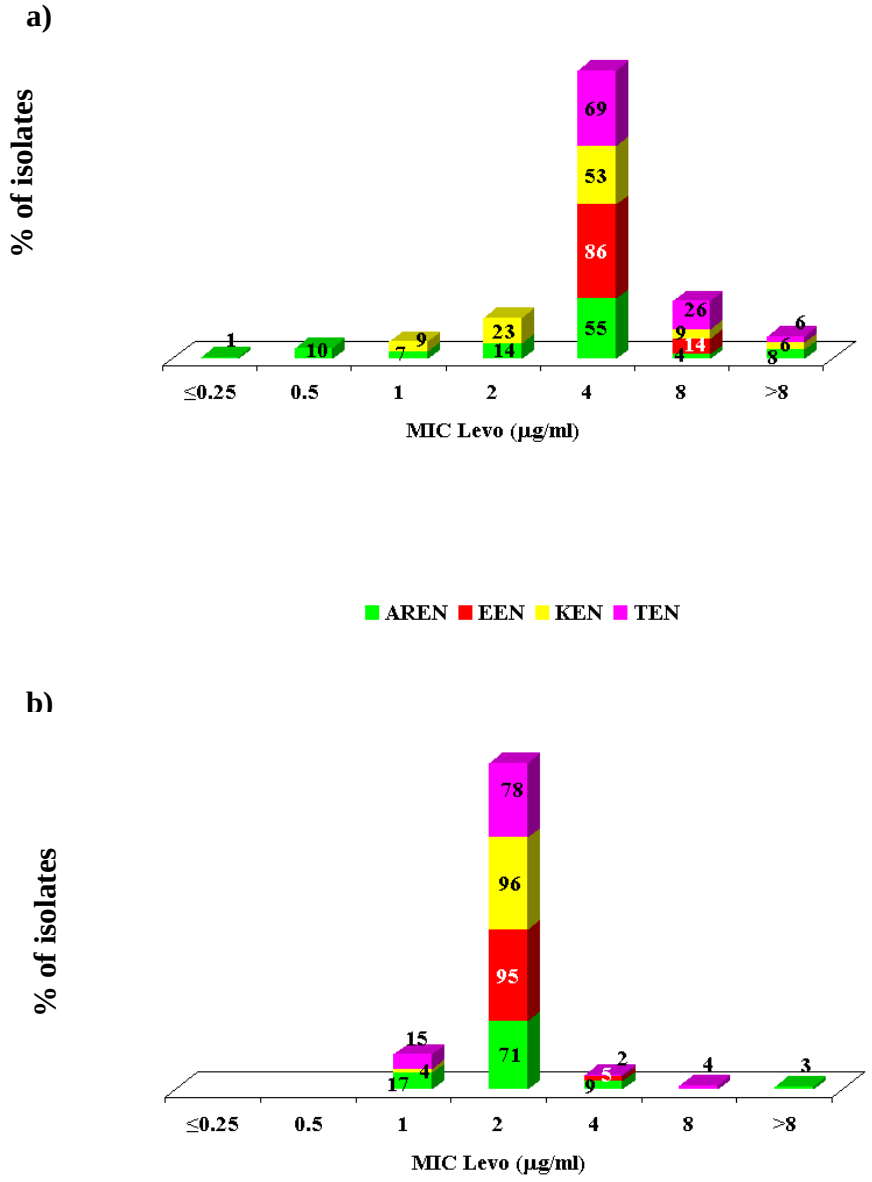


Fig. 42. Fluroquinolones (levofloxacin) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

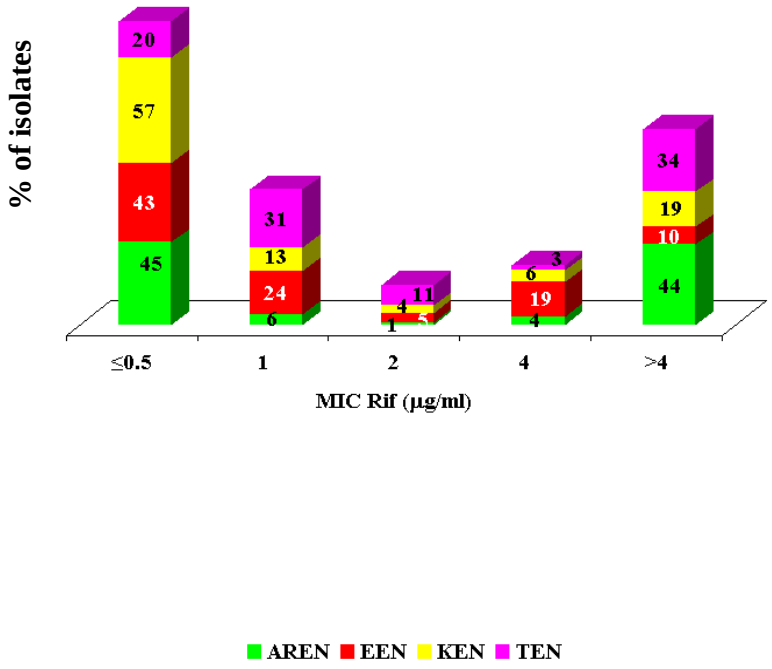
However, in Fiore Sardo, the incidence of Cip^R *E. faecium* was only slightly lower than that found by Abriouel *et al.* (2008) among clinical isolates, while Cip^R *E. faecalis* were very much lower, less than half of those detected by Abriouel *et al.* (2008). The resistance to ciprofloxacin was the most frequently spread among enviromental enterococci isolated by Pangallo *et al.* (2008).

Finally, the incidence of Levo^R enterococci, especially *E. faecalis*, from Fiore Sardo cheese was much lower than those calculated for clinical and also food isolates tested by other authors (Abriouel *et al.*, 2008).

Rifamycin *(rifampin)*

Only isolates belonging to the dominant species showed resistance against rifampin (37 and 53 % of *E. faecium* and *E. faecalis*, respectively) (**Figs 34a** and **b**; **35a, b** and **c**). For both species, resistant isolates came from all the media used in this study, but, on the whole, *E. faecium* turned out to be more susceptible than *E. faecalis* to this drug (**Fig. 38**), displaying a modal MIC value ≤ 0.5, while for *E. faecalis* it ranged between 2 and > 4 (**Fig. 43**). Among food isolates, higher percentages (around 60 % or much higher) of Rif^R *E. faecalis* and *E. faecium* was found by various authors (Giraffa, 2002; Ben Omar *et al.*, 2004; Abriouel *et al.*, 2008; Valenzuela *et al.*, 2008 and 2009; Barbosa *et al.*, 2009) in comparison with those found among Fiore Sardo cheese isolates, generally with a percentage of resistant *E. faecalis* higher than *E. faecium*. Similar or higher proportions (up to 83 %) of rifampin resistant enterococci were detected among clinical isolates (Abriouel *et al.*, 2008; Templer, 2008).

a)



b)

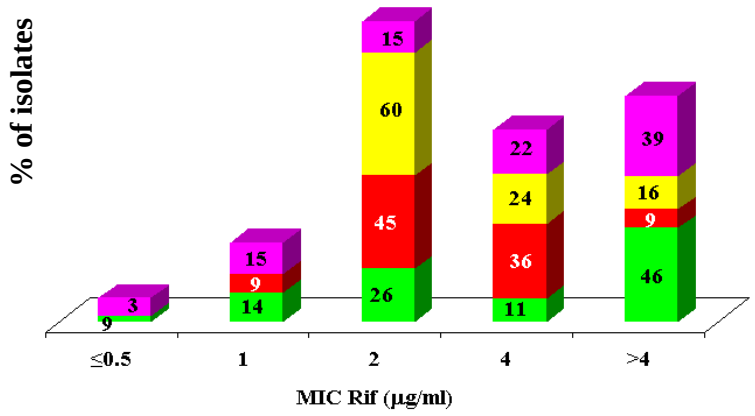


Fig. 43. Rifamycins (rifampin) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Different studies were carried out also on isolates from environmental specimens such as wastewater treatment plants, coastal bathing waters, and rivers (Arvanitidou *et al*., 2001; da Costa *et al*., 2006; Pangallo *et al*., 2008). Very different percentages (from 16 up to > 50%) of enterococci resistant to rifampin were found among isolates from these kinds of sample. As rifampin is almost exclusively used to treat tubercolosis, the resistance rates of enterococci from wastewater might be influenced by a high presence of hospital units in the urban area considered (da Costa *et al*., 2006).

Macrolides *(erythromycin),* ***Lincosamides*** *(clindamycin),* ***Streptogramin A*** *and* ***B*** *(quinupristin/dalfopristin)* ***(MLS)***

The enterococci resistance mechanism to the Macrolide antibiotics results in reduced ribosomal binding to not only erythromycin and other Macrolides, but also to the Lincosamide and Streptogramin B antibiotics (MLS_B) (Kak and Chow, 2002; LeBlanc, 2006).

Among enterococci from Fiore Sardo, 20.8% (67/322), most of them (61) belonging to *E. faecalis* species showed a MLS resistance phenotype (47.7% of the *E. faecalis* isolates) (**Table 9**). Coupled Lincosamides and Streptogramins resistance (LS) was detected with around the same incidence among *E. faecalis* isolates. On the other hand, only 6 out 174 *E. faecium* (3.4% of the isolates of this species) displayed a MLS resistance phenotypic profile, while the association between Macrolides and Lincosamides resistance was the most frequently detected (30.5 % of the *E. faecium*) among the isolates belonging to this species.

Different MLS patterns were reported in literature for enterococci isolated both from food animals and humans (Hayes *et al*., 2005; LeBlanc, 2006).

Table 9. MLS (Macrolides, Lincosamides, Streptogramins) phenotypic profiles of *E. faecalis* and *E. faecium* isolates.

MLS Resistance profile	Number of isolates (%)	
	E. faecalis	*E. faecium*
None	1 (0.8)	69 (39.7)
M	-	-
L	3 (2.3)	34 (19.5)
S	2 (1.6)	-
ML	3 (2.3)	53 (30.5)
MS	-	-
LS	58 (45.3)	12 (6.9)
MLS	61 (47.7)	6 (3.4)
Total	128	174

Almost all *E. faecalis* isolates (98 %), 60 % of *E. faecium*, and the sole *E. gallinarum* isolate were resistant to clindamycin, while the percentage of resistant *E. durans* and *E. hirae* isolates was 20 and 11 %, respectively (**Figs 34a** and **b; 35a**, **b** and **c**).

Cli^R isolates came from all media used in this study, but as regards *E. faecium* a considerably lower percentage was isolated from SBA without antibiotic addition (35 % of isolates from this medium) (**Figs 36** and **37**). *E. faecalis* came out to be more resistant to clindamycin than *E. faecium* (**Fig. 38**). Indeed, almost all the isolates showed MIC > 4, while among *E. faecium* almost half of the isolates from SBA without antibiotic showed very low MIC values (≤ 0.5) (**Fig. 44a** and **b**).

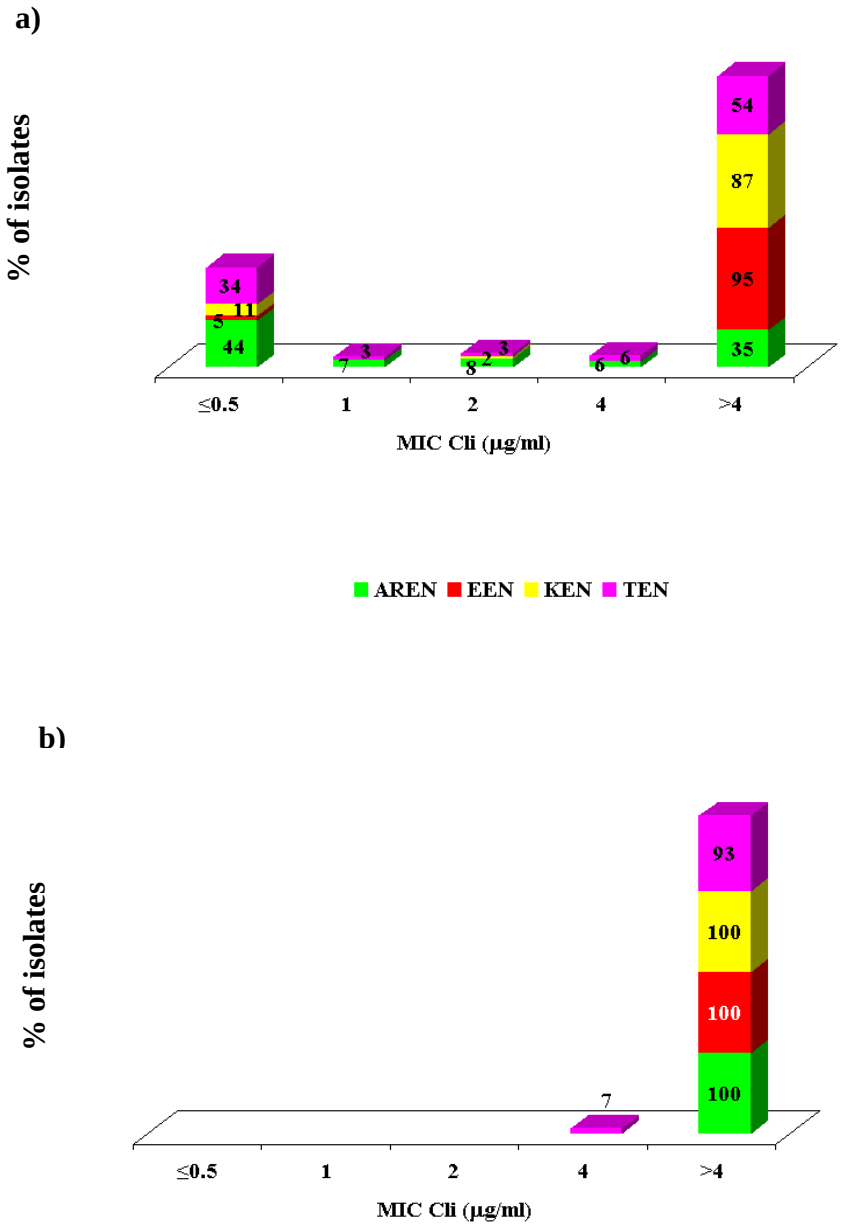


Fig. 44 Lincosamides (clindamycin) MIC value distribution of (a) *E. faecium* (174) and (b) *E. faecalis* (128) isolates depending on their medium of origin

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

The results obtained are not surprising, since intrinsic resistance of enterococci to low levels of Lincosamides is well known (Franz *et al*., 1999 and 2003; Giraffa, 2002; Kayser, 2003; Klare *et al*.,, 2003; Folquié Moreno *et al*., 2006; Bhardwaj *et al*., 2008). As reported in literature, high pecentages of Cli^R enterococci have been isolated both from food and clinical specimens. A high incidence (68.35%) of Lincomycin resistant enterococci from fresh and ripened cheese made from raw and cooked milk was reported by Bulajić and Mijačević (2004). Among 9 antibiotics tested by Čanžek Majhenič and co-workers (2005), clindamycin was the only one against which enterococci from Tolmic cheese turned out to be resistant. *E. faecium* isolated by Lambiase *et al.* (2007) from various biological specimens (blood, respiratory tract, urine) were 100 % resistant against clindamycin.

Concerning Streptogramins (quinupristin/dalfopristin), *E. faecium* and *E. faecalis* showed opposite behaviours. The first species was quite sensitive (only 10 % of Syn^R isolates), while 95 % of *E. faecalis* were resistant to this drugs combination (**Figs 34** and **38**).

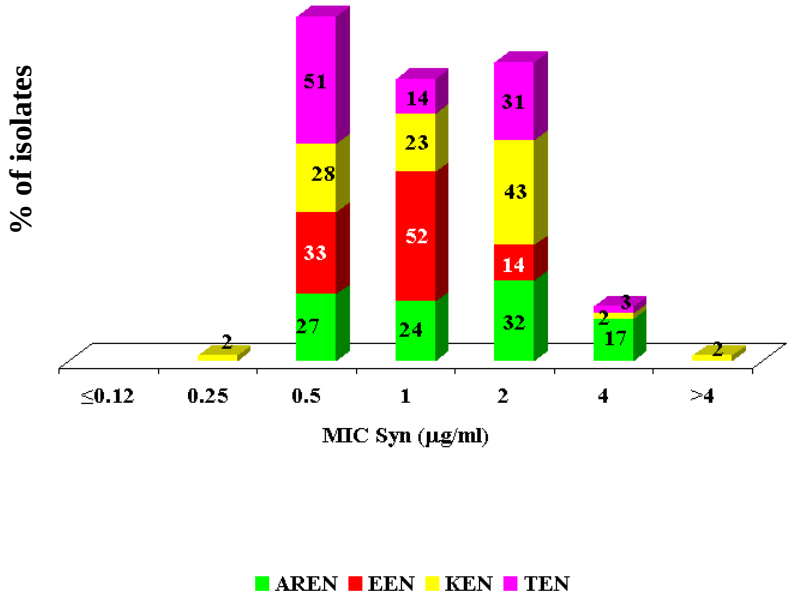
None of non-dominant species isolates turned out resistant (**Fig. 35a, b** and **c**).

Higher percentages of Syn^R *E. faecium* were isolated from SBA and SBA + erythromycin (17 and 14%, respectively) than from SBA + tetracycline and SBA + kanamycin (3 and 4 % of isolates from these media, respectively) (**Fig. 36**).

Syn^R *E. faecalis* were isolated in very high percentage from all the media used (from 88 to 100 % of isolates from each medium) (**Fig. 37**).

Modal quinupristin/dalfopristin MIC values ranged between 0.5 and 2 for *E. faecium*, while it was > 4 for *E. faecalis* (**Fig. 45**).

a)



b)

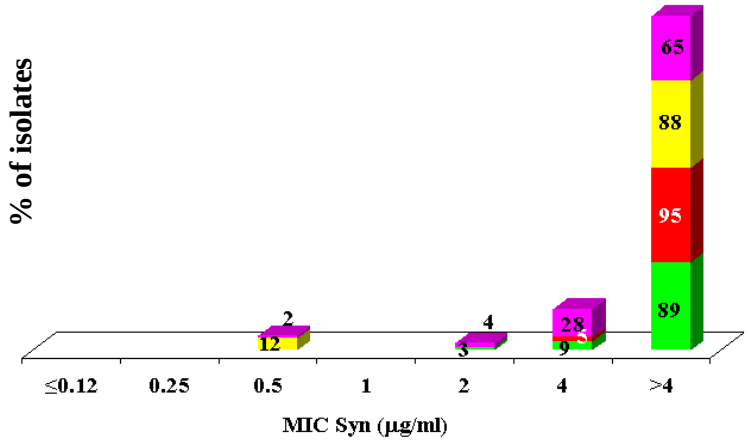


Fig. 45. Streptogramins (quinupristin/dalfopristin: Synercid®) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Our results are in accordance with most of the recent studies where the intrinsic resistance of *E. faecalis* and the susceptibility of *E. faecium* to Streptogramins is reported both for food and clinical enterococci (Hällegren *et al*., 2001; Peters *et al*., 2003; Folquié Moreno *et al*., 2006). Other authors found a low incidence (25 % Ben Omar *et al*., 2004 and 56.52% Valenzuela *et al*., 2008) of Syn^R isolates belonging to *E. faecalis* in Spanish and Moroccan foods, while higher incidence (29 %) of *E. faecium* Syn^R isolates than in Fiore Sardo cheese were reported (Peters *et al*., 2003). Very likely, resistance to quinupristin/dalfopristin is quite common in enterococci as virginiamycin (a combination of two pristinamycins like quinupristin/dalfopristin) had been used as growth promoter in animals fed since 1974 before its ban by the European Union in 1998 (van den Bogaard and Stobberingh, 2000).

Only the two dominant species, *E. faecium* and *E. faecalis*, were resistant to erythromycin, the second species showing a slightly higher percentage of resistant isolates (40 and 50 %, respectively) (**Fig. 34a** and **b; Fig. 35a**, **b** and **c**; **Fig. 38**).

It is important to underline that erythromycin and tetracycline are two of the most widely used antibiotics in animal husbandry and were chosen in this study as the additions to the isolation medium in order to select resistant isolates. It was supposed that resistant strains were not dominant among enterococci colonising Fiore Sardo cheese, but discordant results (incidences ranging between 2.5 and 86.9% of isolates resistant against this antibiotic) were obtained in previous studies using different media for the primary isolation (Mannu *et al*., 2003; Cosentino *et al*., 2004). The medium used by Mannu *et al*. (2003) was SBA, as in this study, while Cosentino *et*

al. (2004) used KF agar, both non containing antibiotics. So, it was crucial to evaluate if differences in the incidences of Ery^R isolates can occur if erythromycin is added to the isolation medium.

As expected, the highest percentage of Ery^R *E. faecium* came from SBA + erythromycin (100 % of isolates from this medium). Quite high percentages of resistant isolates were collected also from the other media (43 and 57% from SBA + tetracycline and SBA + kanamycin, respectively), except from SBA (8 %) (**Fig. 36**).

Analogous behaviour was observed for *E. faecalis*. Only a few Ery^R isolates came from SBA (9 %), while 100 % of isolates from SBA + erythromycin and SBA + kanamycin were resistant to erythromycin. Finally, from SBA + tetracycline was isolated 30 % of Ery^R *E. faecalis* (**Fig. 37**).

Putting aside isolates coming from SBA + erythromycin, that clearly underwent a strong selective pressure, the modal erythromycin MIC value was 2 for *E. faecium* and ≤ 0.5 for *E. faecalis* Fiore Sardo isolates (**Fig. 46a** and **b**).

Lopes and co-workers (2005) stated that Macrolide resistance seems to be related to clinical enterococcal isolates. This is not completely in agreement with the results of this study, as if a selective medium (SBA + erythromycin) is used, isolating Ery^R enterococci, mainly *E. faecium* and *E. faecalis*, was quite frequent. Of course, they are not the largest contingent enterococcal population colonizing Fiore Sardo cheese, since they have a very low incidence among SBA isolates, but they are present.

As regards mainly food isolates, in agreement with the results of this study, a higher incidence of Ery^R *E. faecium* than *E. faecalis* is generally reported in literature.

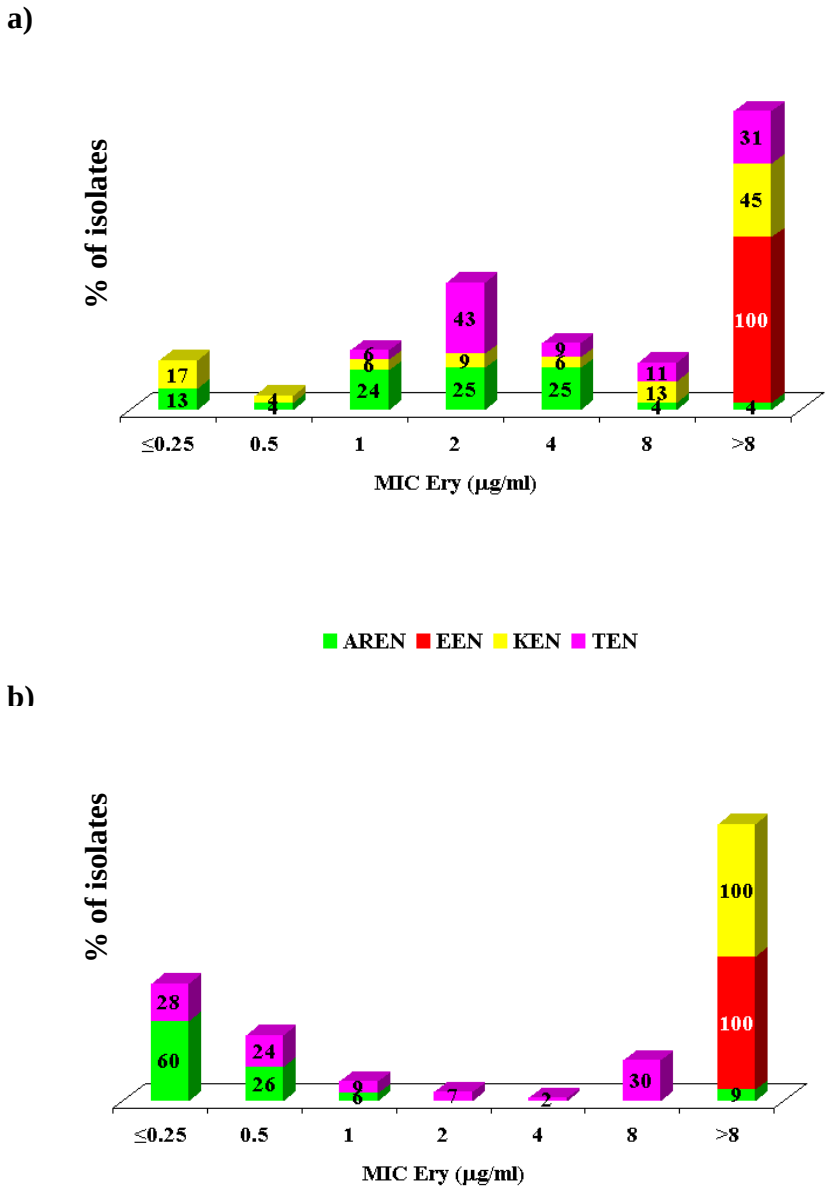


Fig. 46. Macrolides (erythromycin) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Abriouel *et al.* (2008) found similar percentages (> 60%) of Ery^R *E. faecium* in food and clinical isolates, while *E. faecalis* from fruits and vegetable foods showed a lower incidence of resistance against erythromycin compared to clinical isolates (29.41 vs 69.23%). Incidences of 43-55 and 83-97% of Ery^R *E. faecalis* and *E. faecium*, respectively, isolated from different Spanish and Italian foods (Ben Omar *et al.*, 2004; Busani *et al*., 2004), are exceptionally high values. Indeed, percentages of Ery^R enterococcal isolates ranging between 7 and 45 % (highest percentages are usually linked to *E. faecium*) were generally reported by different authors among animal food enterococcal isolates (Peters *et al*., 2003; Bulajić and Mijačević, 2004; Messi *et al*., 2006; Barbosa *et al.*, 2008; Gomes *et al*., 2008; Ogier and Serror, 2008). These percentages could seem low in comparison with the Ery^R incidence found in this study for the two dominant species (see above). However, it should be taken into account that data reported in literature are often related to the whole enterococcal population colonizing the different foods studied. The species to which an isolate is ascribed may influence its antibiotic resistance profile (Lopes *et al*., 2005), and generally *E. faecium* and *E. faecalis* are the most resistant enterococcal species. In fact, when these species were considered separately, higher percentages of Ery^R isolates were reported (Valenzuela *et al*., 2008 and 2009).

Last but not least, in the studies mentioned above, all the isolates came from media without antibiotic adjunct. In Fiore Sardo cheese, only 8 % of *E. faecium* and 9 % *E. faecalis* isolated from SBA without antibiotic, were resistant against erythromycin.

Resistance to erythromycin was detected in very variable percentages (between 9 and 95%) among enterococci isolated from environmental specimens, and, in some cases,

it was the commonest antibiotic resistance among environmental enterococci (Arvanitidou *et al.* 2001; da Costa *et al.*, 2006; Messi *et al.*, 2006; Shaghaghi *et al.*, 2007; Pangallo *et al.*, 2008).

An incidence ranging between 62.5 and 100 % was found among clinical enterococci (Dupré *et al.*, 2003; Abriouel *et al.*, 2008; Mannu *et al.*, 2003; Helmi *et al.*, 2008).

Erythromycin resistance, even though occurring at lower level than tetracycline, could often be found in combination with it (Busani *et al.*, 2004; Templer *et al.*, 2008).

Tetracyclines *(tetracycline)*

E. faecalis turned out to be more resistant against tetracycline than *E. faecium* (82 and 43 % of isolates, respectively) (**Fig. 34a** and **b**; **Fig. 38**). Among non-dominant species only *E. durans* was resistant to this drug (40% of isolates) (**Fig. 35a**, **b** and **c**).

As already seen for erythromycin, 100 % of isolates, both *E. faecium* and *E. faecalis*, coming from SBA + tetracycline were resistant to this drug (**Fig. 36** and **37**).

As regards *E. faecalis* also the isolates coming from SBA + erythromycin and SBA + kanamycin were 100 % Tet^R, and 34 % of those from SBA.

Different behaviour was observed among *E. faecium* isolates. A high percentage of resistant isolates (86%) from SBA + erythromycin, 38 % from SBA + kanamycin, and only 4 % from SBA were isolated (**Fig. 36**).

Thus, it seems that adding erythromycin (apart from tetracycline) or, in case of *E. faecalis* also kanamycin, to the isolation medium could favour the selection of Tet^R isolates. Considering the similar results obtained for erythromycin resistant isolates

(see above), this could mean that tetracycline and erythromycin resistances can be associated (Templer *et al*., 2008).

Both for *E. faecium* and *E. faecalis*, modal MIC value of the isolates coming from the medium without antibiotic addition was ≤ 2, indicating that this two species are not naturally resistant to tetracycline. When a selective pressure (the addition of tetracycline or erythromycin for *E. faecium*, and also of kanamycin for *E. faecalis*) is applied, the modal MIC value increases to > 16 (**Fig. 47a** and **b**).

Resistance to tetracycline is commonly dectected around or over 50% of clinical isolates (Nelson *et al*., 2000; Dupré *et al*., 2003; LeBlanc, 2006; Abriouel *et al*., 2008; Helmi *et al*., 2008; Templer *et al*., 2008), and in recent years, multiresistance to tetracycline, erythromycin, and chloramphenicol has been often described (Templer *et al*., 2008).

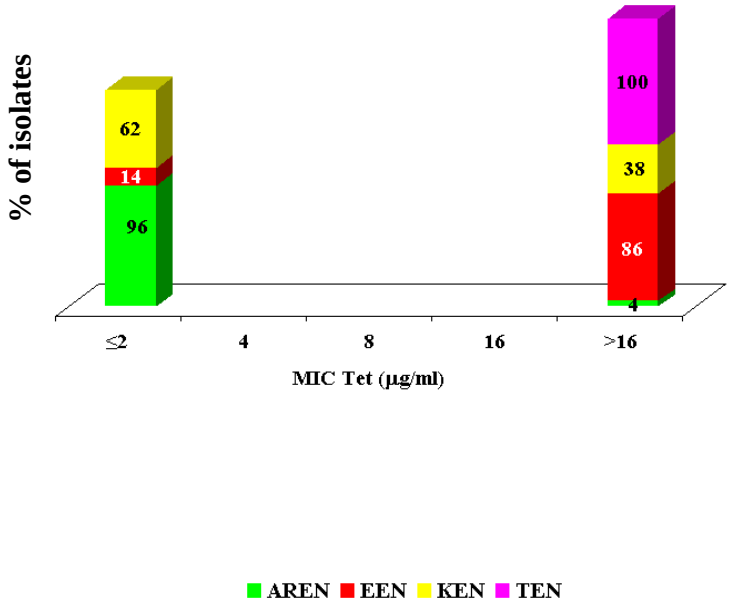
Different incidences of tetracycline resistance were reported for enterococci from food. Among *E. faecium* isolated from meat, dairy and vegetables foods, only a very low percentage (between 4 and 7%) was resistant to tetracycline, while a higher incidence (from 31 to 87 %) of Tet^R *E. faecalis* was detected (Peters *et al*., 2003; Drahovská *et al*., 2004; Huys *et al*., 2004; Gomes *et al*., 2008; Ogier and Serror, 2008; Valenzuela *et al*., 2008). These percentages are quite in agreement with those obtained for *E. faecium* (4 %) and *E. faecalis* (34 %) from Fiore Sardo cheese without any antibiotic addition to the isolation medium. As already highlighted for erythromycin, in order to calculate the antibiotic resistance incidence it is important to take into account both the enterococcal species and the culture medium used for isolation. At a first glance, Barbosa *et al.* (2008) isolated a lower percentage (22.5%)

of Tet^R enterococci in Portuguese meat products compared to the total percentages of Tet^R *E. faecium* (40%) and *E. faecalis* (50%) from Fiore Sardo cheese and other foods.

However, they considered different species altogether and did not add antibiotics to the culture medium. Usually, the majority of resistant enterococci belong to *E. faecium* and *E. faecalis* species, and, if no antibiotic is added to the cultivation medium, very low percentages of resistant isolates can be detected, as they are not dominant among food enterococcal microflora. If exclusively SBA isolates from Fiore Sardo are considered, without distinction of species, only 12.3 % (15/122) of the them are resistant to tetracycline.

Among environmental specimens, Tet^R enterococci are commonly found in percentages ranging between 20 and 34.6 % (da Costa *et al*., 2006; Shaghaghi *et al*., 2007; Pangallo *et al*., 2008).

a)



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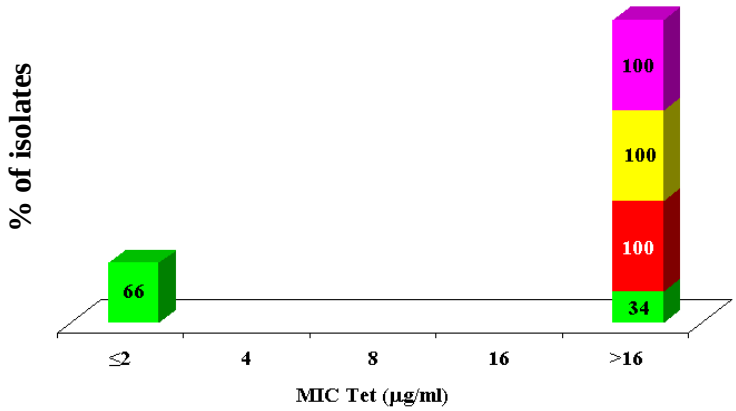


Fig. 47. Tetracyclines (tetracycline) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin.

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

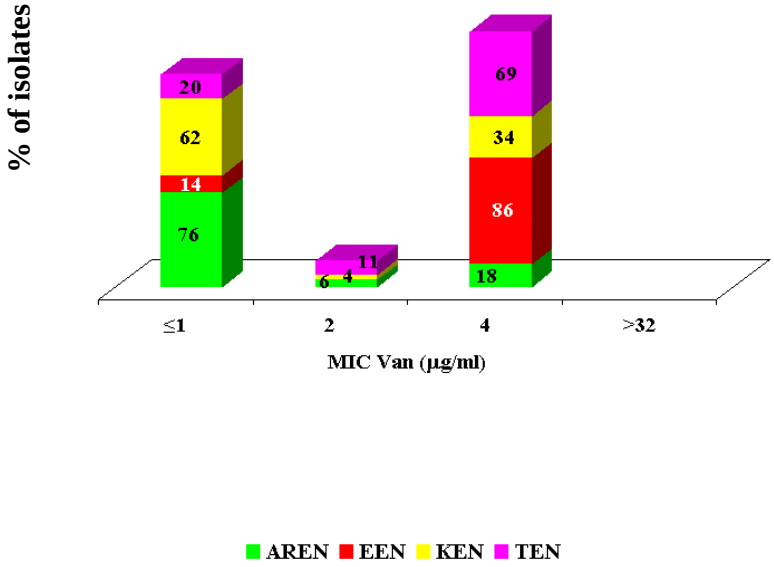
Glycopeptides *(vancomycin)*

Among the 322 enterococci isolated from Fiore Sardo cheese, only one isolate belonging to the species *E. faecalis*, isolated from SBA + erythromycin was resistant to vancomycin (MIC > 32 µg/ml) (**Figs 34a** and **b**; **35a**, **b** and **c**; **36**; **37**). *E. faecium* vancomycin MIC values ranged between 1 and 4, *E. faecalis* between 2 and 4, with the exception of the sole Van^R isolate (**Fig. 48**).

Since vancomycin is an important alternative against multiple-resistant enterococci (and other Gram-positive bacteria), VRE (Vancomycin Resistant Enterococci) constitute a serious risk group among bacterial nosocomial pathogens and their presence in hospitals is met with great concern (Franz *et al.*, 1999). They had been first described in France and UK, but were subsequently found in many parts of the world, especially in US hospitals (Klare *et al.*, 2003).

Our results are similar to those reported by other authors, confirming that vancomycin resistant enterococci are very rarely isolated from dairy products, while slightly more frequently from other animal foods, environmental and clinical specimens (Giraffa and Sisto, 1997; Giraffa *et al.*, 2000; Arvanitidou *et al.*, 2001; Franz *et al.*, 2001; Hällgren *et al.*, 2001; Dupré *et al.*, 2003; Kayser, 2003; Mannu *et al.*, 2003; Peters *et al.*, 2003; Ben Omar *et al.*, 2004; Cosentino *et al.*, 2004; Drahovská *et al.*, 2004; Čanžek Majhenič *et al.*, 2005; Kaçmaz and Aksoy, 2005; da Costa *et al.*, 2006; Messi *et al.*, 2006; Fracalanzza *et al.*, 2007; Abriouel *et al.*, 2008; Gomes *et al.*, 2008; Helmi *et al.* 2008; McGowan-Spicer *et al.*, 2008; Templer *et al.*, 2008; Barbosa *et al.*, 2009).

a)



b)

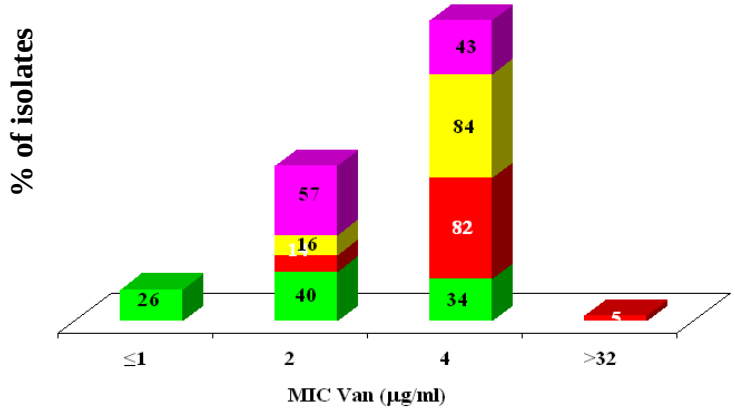


Fig. 48. Glycopeptides (vancomycin) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin.
AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

The very different percentages (from 0 to 100%) of VRE isolated from various sources would lead the conclusion that antibiotic resistance against vancomycin, and also other drugs, is not just linked to the origin of the isolates (food, environment, healthy or not humans and animals), but that it has also a geographic and above all a strain dependent characteristic (Čanžek Majhenič *et al*., 2005).

However, it is important to highlight that, in many research works, VRE recovery could have been reduced because no vancomycin was added to the isolation medium. In fact, scientific literature indicates that an enrichment step in medium supplemented with vancomycin enhanced the detection rate of VRE by approximatively three times (Wegener *et al*., 1997). In the present study, even though SBA + vancomycin was used as well as SBA without antibiotic supplementation, only one VRE isolate was detected and that was from SBA + erythromycin.

Oxazolidinones *(Linezolid)*

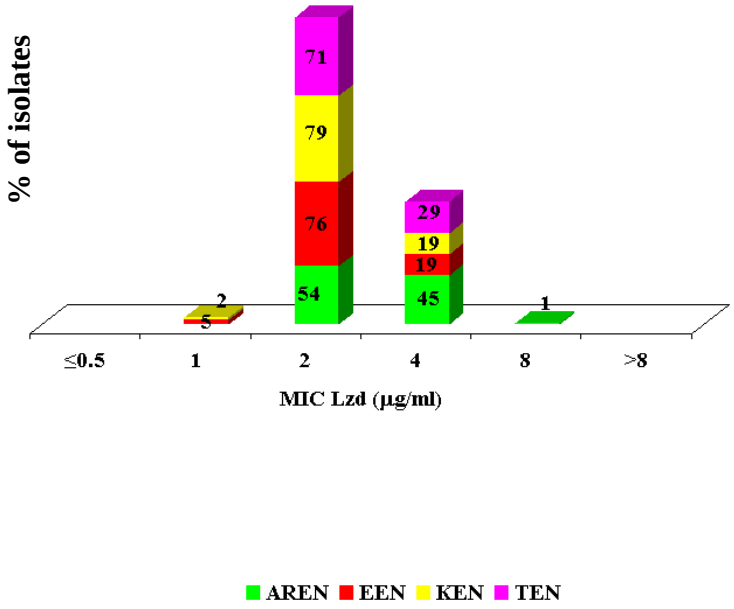
About 1% of *E. faecium* and *E. faecalis* isolates was resistant to linezolid, while all the isolates belonging to non-dominant species were susceptible to this antibiotic (**Figs 34a** and **b; 35a**, **b** and **c**). All Lzd^R *E. faecium* isolates came from SBA (1% of isolates from this medium), while Lzd^R *E. faecalis* isolates came from SBA + erythromycin (5 %) (**Figs 36** and **37**). On the whole, a slightly higher percentage of sensitive Lzd^R *E. faecalis* than Lzd^R *E. faecium* was isolated (**Fig. 38**).

Modal MIC values were between 2 and 4 for both species (**Fig. 49**).

The occurrence of a few isolates presenting resistance to this drug is important since linezolid is a useful agent in treatment of infections with Glycopeptide- or multiple-

resistant enterococci, even though, in recent years, the increased consumption of this drug in USA caused the onset of outbreaks of *E. faecalis* and *E. faecium* isolates (Klare *et al*., 2003; Leclercq, 2009). However, as reported by various authors, Lzd^R enterococci are only sporadically detected among isolates from human sources worldwide, while they are generally not found among isolates from food such as fruit, vegetables and meat products (Bersos *et al*., 2004; Fracalanzza *et al*., 2007; McGowan-Spicer *et a*l., 2008; Casal *et al*., 2009).

a)



b)

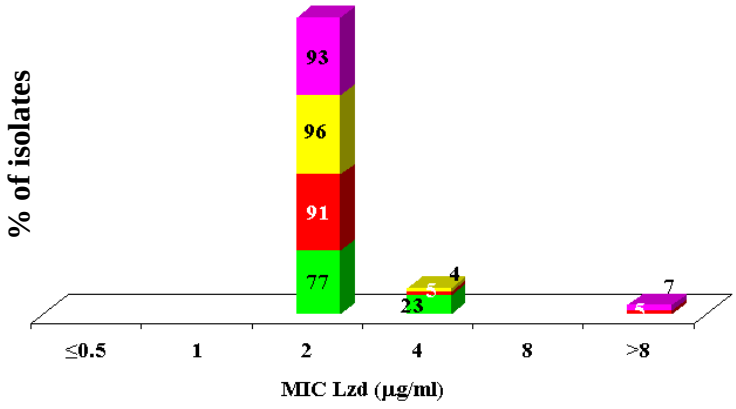


Fig. 49. Oxazolidinones (linezolid) MIC value distribution of (**a**) *E. faecium* (174) and (**b**) *E. faecalis* (128) isolates depending on their medium of origin.

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

On the whole, resistance to ciprofloxacin was most frequently spread in the *E. faecium* isolates with 71% of occurrence, followed by the resistance to clindamicin and HL streptomycin (60%), tetracycline (43%), erythromycin (40%), rifampin (37%), levofloxacin (18%), gatifloxacin (17%), ampicillin (13%), penicillin (12%), quinupristin/dalfopristin (10%), HL gentamicin (3%), linezolid (1%). No *E. faecium* was resistant to vancomycin.

Among *E. faecalis*, resistance to clindamicin was the most spread (98%), followed by resistance to quinupristin/dalfopristin (95%), HL streptomicin (83%), tetracycline (82%), rifampin (53%), erythromycin (50%), ciprofloxacin (20%), gatifloxacin (15%), levofloxacin (9%), linezolid, penicillin and vancomycin (around 1%). No *E. faecalis* was resistant to ampicillin and gentamicin.

Summarizing briefly, all enterococci isolated from Fiore Sardo, except one, were sensitive to vancomycin. The highest frequency of resistant strains were detected in *E. faecalis* (100 % of isolates were resistant to at least two of the tested antibiotics), while around 2 % of *E. faecium* were susceptible to all antibiotics and 11 % were resistant against only one.

Multidrug resistant isolates were quite frequently detected. Phenotypic determination of resistance to antimicrobial agents of the 174 *E. faecium* resulted in seventy resistance patterns, plus one consisting of isolates susceptible to all drugs tested. Resistant isolates showed from one up to eight resistances. Sixty-five isolates (37.4%), mainly coming from cultivation media containing antibiotic, displayed resistance to between five and eight out of the fourteen antibiotics tested (**Table 10**).

Resistance patterns (17) constituted of three antibiotics, were the most frequently detected (20.7 % of isolates).

Table 10. Resistance profiles of *E. faecium* isolates.

Antibiotics		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of resistances	
N.	Name	N.	%		N.	%
0	None	3	1.7	AREN	3	1.7
1	Cip	9	5.2	AREN	19	10.9
	Cli	2	1.1	KEN		
	Str	8	4.6	n. 7 AREN[a] n. 1 KEN		
2	Cip, Cli	7	4.0	KEN	28	16.1
	Cip, Rif	3	1.7	AREN		
	Cip, Str	7	4.0	AREN		
	Cip, Tet	5	2.9	n. 1 AREN n. 4 TEN		
	Cli, Ery	1	0.6	AREN		
	Cli, Rif	1	0.6	AREN		
	Cli, Str	3	1.7	n. 2 AREN n. 1 KEN		
	Gat, Str	1	0.6	AREN		
3	Cip, Cli, Gat	1	0.6	AREN	36	20.7
	Cip, Cli, Levo	1	0.6	KEN		
	Cip, Cli, Rif	2	1.1	AREN		
	Cip, Cli, Syn	2	1.1	AREN		
	Cip, Cli, Tet	1	0.6	KEN		
	Cip, Gat, Str	1	0.6	AREN		
	Cip, Gat, Tet	1	0.6	TEN		
	Cip, Levo, Rif	3	1.7	n. 2 AREN n. 1 KEN		
	Cip, Levo, Tet	7	4.0	n. 1 AREN n. 6 TEN		
	Cip, Rif, Str	5	2.9	n. 3 AREN n. 2 KEN		
	Cli, Ery, Rif	1	0.6	AREN		
	Cli, Ery, Str	5	2.9	KEN		
	Cli, Gat, Str	1	0.6	AREN		
	Cli, Rif, Str	1	0.6	KEN		
	Cli, Str, Tet	1	0.6	KEN		
	Ery, Rif, Str	2	1.1	AREN		
	Gat, Rif, Str	1	0.6	AREN		

[a]: n. 2 isolates are the same PFGE-type
[b]: all isolates are the same PFGE-type
Unless otherwise specified, all isolates belonged to different PFGE-types

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Continued on following page

Table 10. *Continued.*

Antibiotics		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of resistances	
N.	Name	N.	%		N.	%
	Cip, Cli, Ery, Tet	2	1.1	n. 1 KEN n. 1 TEN		
	Cip, Cli, Gat, Str	1	0.6	AREN		
	Cip, Cli, Rif, Str	1	0.6	KEN		
	Cip, Cli, Rif, Tet	1	0.6	TEN		
	Cip, Cli, Str, Tet	1	0.6	TEN		
	Cip, Cli, Syn, Rif	1	0.6	AREN		
	Cip, Ery, Levo, Rif	1	0.6	KEN		
4	Cip, Ery, Rif, Tet	5	2.9	n. 1 AREN n. 4 TEN[a]	23	13.2
	Cip, Gat, Levo, Rif	1	0.6	AREN		
	Cip, Levo, Rif, Str	2	1.1	AREN		
	Cli, Ery, Rif, Str	1	0.6	KEN		
	Cli, Ery, Rif, Tet	1	0.6	EEN		
	Cli, Ery, Str, Tet	1	0.6	KEN		
	Cli, Gat, Rif, Str	2	1.1	AREN		
	Cli, Syn, Rif, Str	1	0.6	AREN		
	Ery, Gat, Str, Tet	1	0.6	TEN		
	Amp, Cli, Ery, Str, Tet	3	1.7	n. 2 EEN n. 1 KEN		
	Cip, Cli, Ery, Rif, Str	3	1.7	KEN		
5	Cip, Cli, Ery, Str, Tet	9	5.2	n. 2 EEN[b] n. 4 KEN[b] n. 3 TEN	25	14.4
	Cip, Cli, Gat, Levo, Lzd	1	0.6	AREN		
	Cip, Cli, Syn, Rif, Str	7	4.0	AREN		
	Cip, Gat, Levo, Rif, Str	2	1.1	AREN		
	Amp, Cli, Ery, Pen, Str, Tet	8	4.6	n. 1 EEN n. 3 KEN[b] n. 4 TEN[a]		
	Cip, Cli, Ery, Gat, Str, Tet	2	1.1	EEN		
	Cip, Cli, Ery, Levo, Str, Tet	1	0.6	EEN		
6	Cip, Cli, Ery, Levo, Syn, Tet	1	0.6	KEN	21	12.1
	Cip, Cli, Ery, Rif, Str, Tet	1	0.6	TEN		
	Cip, Cli, Ery, Syn, Rif, Str	5	2.9	n. 1 AREN n. 1 KEN n. 3 EEN[b]		
	Cip, Cli, Levo, Pen, Str, Tet	1	0.6	TEN		
	Cip, Cli, Levo, Rif, Str, Tet	2	1.1	TEN		
	Amp, Cip, Cli, Ery, Pen, Str, Tet	1	0.6	KEN		
	Amp, Cip, Ery, Gat, Pen, Str, Tet	1	0.6	EEN		
	Amp, Cli, Ery, Gat, Pen, Str, Tet	2	1.1	EEN		
7	Cip, Cli, Ery, Gat, Levo, Str, Tet	1	0.6	EEN	10	5.7
	Cip, Cli, Ery, Gat, Rif, Str, Tet	1	0.6	EEN		
	Cip, Cli, Ery, Pen, Rif, Str, Tet	1	0.6	TEN		
	Cip, Cli, Gat, Levo, Rif, Str, Tet	3	1.7	TEN		
	Amp, Cip, Cli, Ery, Gat, Gen, Pen, Tet	3	1.7	EEN		
	Amp, Cip, Cli, Ery, Gen, Levo, Pen, Tet	3	1.7	KEN		
8	Amp, Cip, Cli, Ery, Pen, Rif, Str, Tet	1	0.6	KEN	9	5.2
	Cip, Cli, Ery, Gat, Levo, Rif, Str, Tet	1	0.6	EEN		
	Cip, Cli, Gat, Levo, Syn, Rif, Str, Tet	1	0.6	TEN		
		174				

[a]: n. 2 isolates are the same PFGE-type

[b]: all isolates are the same PFGE-type

Unless otherwise specified, all isolates belonged to different RAPD M13-types

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Eighty-eight *E. faecalis* isolates (68.8%) showed resistance to between five and nine (only one isolate) out of the fourteen drugs tested. Resistance patterns (10) constituted of five antibiotics, were the most frequently detected (33.6% of isolates) among *E. faecalis* isolates (**Table 11**).

Table 11. Resistance profiles of *E. faecalis* isolates.

Antibiotics		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of resistances	
N.	Name	N.	%		N.	%
2	Cli, Syn	4	3.1	AREN[a]	6	4.7
	Rif, Tet	1	0.8	TEN		
	Syn, Tet	1	0.8	TEN		
3	Cli, Syn, Str	4	3.1	AREN[a]	7	5.5
	Cli, Syn, Tet	2	1.6	TEN		
	Syn, Str, Tet	1	0.8	TEN		
4	Cip, Cli, Syn, Str	1	0.8	AREN	27	21.1
	Cip, Cli, Syn, Tet	1	0.8	AREN		
	Cli, Ery, Syn, Tet	1	0.8	TEN		
	Cli, Gat, Rif, Tet	1	0.8	TEN		
	Cli, Rif, Str, Tet	1	0.8	TEN		
	Cli, Syn, Rif, Str	6	4.7	AREN		
	Cli, Syn, Rif, Tet	8	6.3	TEN[b]		
	Cli, Syn, Str, Tet	8	6.3	n. 2 AREN n. 6 TEN[c]		
5	Cip, Cli, Ery, Str, Tet	1	0.8	KEN	43	33.6
	Cip, Cli, Gat, Syn, Str	1	0.8	AREN		
	Cip, Cli, Levo, Rif, Tet	1	0.8	AREN		
	Cip, Cli, Syn, Rif, Str	2	1.6	AREN		
	Cip, Cli, Syn, Rif, Tet	1	0.8	AREN		
	Cli, Ery, Rif, Str, Tet	3	2.3	KEN		
	Cli, Ery, Syn, Str, Tet	27	21.1	n.2 AREN n. 7 EEN[d] n. 13 KEN[a] n. 5 TEN[d]		
	Cli, Gat, Syn, Str, Tet	1	0.8	TEN		
	Cli, Levo, Syn, Rif, Str	1	0.8	AREN		
	Cli, Syn, Rif, Str, Tet	5	3.9	TEN		
6	Cip, Cli, Ery, Syn, Str, Tet	2	1.6	TEN	31	24.2
	Cip, Cli, Gat, Levo, Syn, Rif	1	0.8	AREN		
	Cip, Cli, Gat, Syn, Rif, Str	3	2.3	AREN[e]		
	Cip, Cli, Syn, Rif, Str, Tet	3	2.3	AREN[e]		
	Cli, Ery, Gat, Syn, Str, Tet	4	3.1	EEN[a]		
	Cli, Ery,Levo, Syn, Str, Tet	1	0.8	TEN		
	Cli, Ery, Syn, Rif, Str, Tet	15	11.7	n. 5 EEN[a] n. 7 KEN[c] n. 3 TEN		
	Cli, Gat, Syn, Rif, Str, Tet	1	0.8	TEN		
	Cli, Levo, Syn, Rif, Str, tet	1	0.8	AREN		
7	Cip, Cli, Ery, Gat, Syn, Str, Tet	1	0.8	EEN	11	8.6
	Cip, Cli, Ery, Syn, Rif, Str, Tet	4	3.1	n. 1 EEN n. 1 AREN n. 2 TEN		
	Cli, Ery, Gat, Syn, Rif, Str, Tet	1	0.8	EEN		
	Cli, Ery, Levo, Syn, Rif, Str, Tet	1	0.8	EEN		
	Cli, Gat, Levo, Syn, Rif., Str, Tet	4	3.1	TEN[a]		
8	Cip, Cli, Ery, Gat, Syn, Rif, Str, Tet	1	0.8	EEN	2	1.6
	Cip, Cli, Ery, Levo, Syn, Rif, Str, Tet	1	0.8	TEN		
9	Cip, Cli, Ery, Lzd, Pen, Syn, Rif, Str, Van	1	0.8	EEN	1	0.8
		128				

[a]: n. 3 RAPD M13-types **[b]: n. 7 RAPD M13-types** **[e]: n. 2 RAPD M13-types**

[c]: n. 5 RAPD M13-types **[d]: n. 4 RAPD M13-types**

Unless otherwise specified, all isolates belonged to different RAPD M13-types

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Seven out of ten *E. durans* isolates showed resistance against only one antibiotic, and three a double resistance (**Table 12**).

Table 12. Resistance profiles of *E. durans* isolates.

Antibiotics		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of resistances	
N.	Name	N.	%		N.	%
1	Str	6	60.0	AREN[a]	7	70.0
	Tet	1	10.0	TEN		
2	Cli, Tet	2	20.0	TEN	3	30.0
	Str, Tet	1	10.0	TEN		
		10				

[a]: n. 5 RAPD M13-types
Unless otherwise specified, all isolates belonged to different RAPD M13-types
AREN and TEN codes indicate isolates from SBA and SBA+Tet, respectively

Six out of nine *E. hirae* isolates did not display any resistance, one isolate was resistant against streptomycin, and two isolates showed a triple resistance profile (**Table 13**).

Table 13. Resistance profiles of *E. hirae* isolates.

Antibiotics		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of resistances	
N.	Name	N.	%		N.	%
0	None	6	66.7	AREN[a]	6	66.7
1	Str	1	11.1	AREN	1	11.1
3	Cli, Gat, Str	1	11.1	AREN	2	22.2
	Cli, Syn, Str	1	11.1	AREN		
		9				

[a]: n. 5 RAPD M13-types
Unless otherwise specified, all isolates belonged to different RAPD M13-types
AREN code indicates isolates from SBA

The sole *E. gallinarum* isolated displayed a triple resistance against ciprofloxacin, clindamicin and streptomycin (**Table 14**).

Table 14. Resistance profile of *E. gallinarum* isolate.

Antibiotics		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of resistances	
N.	Name	N.	%		N.	%
3	Cip, Cli, Str	1	100.0	AREN	1	100

AREN code indicates isolates from SBA

According to the results of the present and previous studies, *Enterococcus* genus is still represented by strains intrinsically susceptible to the majority of antibiotics used in human medicine, but care is advisable since some dairy isolates already started to show resistances usually associated with clinical isolates (Lopes *et al*., 2005). Teuber *et al*. (1999) ascertained that VRE incidence among enterococci from European cheeses was as low as 4%, while resistance to other drugs (as chloramphenicol, streptomycin, tetracycline, erythromycin, ciprofloxacin, gentamicin, penicillin and ampicillin) varied between 7 and 80 %.

Antibiotic resistant enterococci were isolated also from Fiore Sardo cheese, and they are commonly isolated from other different, non dairy, animal foods. However, only a low proportion of them are resistant to the clinically important antibiotics such as ampicillin, penicillin, gentamicin and, especially to the last resort antibiotic vancomicin (Franz *et al*., 2001; Lopes *et al*., 2003; Mannu *et al*., 2003; Peters *et al*., 2003; Cosentino *et al*., 2004; Čanžek Majhenič *et al*., 2005; Ogier *et al*., 2008).

Concerning the other antimicrobials, antibiotic resistance among food isolates varies among different studies and is often species-, strain- and region-dependent, and may differ according to the isolation method used (Klein, 2003; Čanžek Majhenič *et al*., 2005; Ogier and Serror, 2008).

The high frequency of resistance to tetracycline and erythromycin among isolates of animal origin is likely related to the wide use of these drugs in husbandry activities. Due to the frequent association of these two genetic determinants of resistance, the use of either antibiotic selects at the same time for both resistances (Busani *et al*., 2004). Finally, although the origin of a strain may not always influence its antibiotic resistance profile, the species to which it belongs to may do so. In fact, many differences between dairy isolates belonging to *E. faecium* and *E. faecalis* in comparison to other less frequently isolated enterococcal species (in this study *E. durans, E. hirae,* and *E. gallinarum*), often occur in the antibiotic resistance pattern (Lopes *et al*., 2005).

3.3.2 Presence of antibiotic resistance genes

Enterococci's acquired resistance based on acquisition of plasmids and trasposons, has relevance for Chloramphenicol, Lincosamides and Aminoglycosides, β-Lactams, Fluoroquinolones, Glycopeptides, Macrolides and Tetracyclines (Franz *et al*., 1999). Among the antibiotics belonging to the two last classes, resistance to erythromycin and tetracycline is quite widespread in many Gram-negative and Gram-positive bacteria, and it occurred also among enterococci isolated from Fiore Sardo cheese (Cosentino *et al*., 2004, present study). There are various resistance mechanisms to

these antibiotics encoded from different genes (see Paragraph **1.4.3 Antibiotic resistance in Enterococci**).

In this study the presence of *erm*A, *erm*B and *erm*C genes among phenotypically Ery^R isolates, and of *tet*L, *tet*M, *tet*S and *tet*W genes among phenotypically Tet^R isolates was investigated. In some cases, both antibiotic resistances were present, so all genes were searched. Most of Ery^R and/or Tet^R isolates belonged to *E. faecium* and *E. faecalis* species, while among non dominant species only four *E. durans* isolates (three different RAPD M13-types) came out to be resistant to tetracycline, all harbouring both *tet*M and *tet*L resistance genes (data not shown).

The unique *E. faecalis* vancomycin resistant strain (MIC > 32) harboured neither *van*A nor *van*B genes. It is well-known that nearly all strains of *E. gallinarum*, as other motile enterococci (*E. casseliflavus* and *E. flavescens*), are resistant to low levels of vancomycin (Kak and Chow, 2002). The sole *E. gallinarum* (MIC = 8) isolated during this study harboured the *van*C-1 gene. This gene is confined to strains of *E. gallinarum* species and confers on them an intrinsic vancomycin resistance (Clark *et al*., 1998). A positive PCR result for this gene can be used to identify the *E. gallinarum* species within the genus *Enterococcus* (Leclercq *et al.*, 1992, Vincent *et al*., 1992; Dutka-Malen *et al*., 1995).

In **Table 15**, the incidence of erythromycin and tetracycline resistance genes among the phenotypically Ery^R and/or Tet^R *E. faecium* isolates is reported.

Table 15. Presence of erythromycin and tetracycline resistance genes in phenotypically Ery^R and Tet^R *E. faecium* isolates.

E. faecium isolates		Ery^R isolates		with resistance genes						Tet^R isolates		with resistance genes							
				erm A		*erm* B		*erm* C				*tet* L		*tet* M		*tet* S		*tet* W	
Medium of origin ID code	N.	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%
AREN	71	6	8.5	-	-	-	-	-	-	3	4.2	2	66.7	3	100	-	-	-	-
EEN	21	21	100	-	-	15	71.4	-	-	18	85.7	18	100	18	100	-	-	-	-
KEN	47	27	57.4	-	-	9	33.3	-	-	18	38.3	12	66.7	18	100	-	-	-	-
TEN	35	15	42.9	-	-	5	33.3	-	-	35	100	29	82.9	35	100	-	-	-	-
TOTAL	**174**	**69**	**39.7**	-	-	**29**	**42.0**	-	-	**74**	**42.5**	**61**	**82.4**	**74**	**100**	-	-	-	-

-: not detected

Twenty-nine (42%) out of the 69 Ery^R isolates, representing 42% of total *E. faecium* isolates, harboured the *erm*B resistance gene. Most of them (15) were isolated from SBA + erythromycin, none from SBA without antibiotic addition. None isolate harboured *erm*A or *erm*C resistance genes.

All the 74 Tet^R isolates (42.5% of all *E. faecium* isolates) harboured the *tet*M gene, 61 (82.4% of Tet^R *E. faecium*) the *tet*L, while *tet*S and *tet*W genes were not detected in any isolate. On the whole, 50 *E. faecium* were both Ery^R and Tet^R, 19 were only Ery^R, and 24 only Tet^R.

Most of Ery^R *E. faecium* harbouring *erm* genes came from SBA + erythromycin, while most of Tet^R isolates harbouring *tet* resistance genes came from SBA + tetracycline, so, on the whole, around 90% of Ery^R and/or Tet^R *E. faecium* harbouring resistance genes were isolated from culture media containing an antibiotic as selective agent.

Arranging the 93 Ery^R and/or Tet^R *E. faecium* isolates according to the presence of resistance genes resulted in five resistance patterns besides one pattern constituted of 16 (17.2%) isolates without resistance genes (**Table 16**). Almost half (41) of resistant isolates harboured two genes, against both the antibiotics or only against tetracycline (*erm*B/*tet*M or *tet*L/*tet*M), while around 25 % showed a triple genotypic profile (*erm*B/*tet*L/*tet*M).

Table 16. Erythromycin and tetracycline resistance genes profiles detected among phenotypically Ery^R and/or Tet^R *E. faecium* isolates.

Resistance genes profiles		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of AR genes	
N.	Name	N.	%		N.	%
0	None	16	17.2	n. 5 AREN n. 11 KEN[a]	16	17.2
1	*erm* B	3	3.2	EEN[b]	13	14.0
	tet M	10	10.8	n. 1 AREN n. 3 KEN[b] n. 6 TEN[c]		
2	*erm* B *tet* M	3	3.2	KEN[b]	41	44.1
	tet L *tet* M	38	40.9	n. 2 AREN n. 6 EEN[d] n. 6 KEN[e] n. 24 TEN[f]		
3	*erm* B *tet* L *tet* M	23	24.7	n. 12 EEN[a] n. 6 KEN[d] n. 5 TEN[g]	23	24.7
Total N. of resistant isolates		93			93	

[a]: n. 6 PFGE-types [c]: n. 5 PFGE-types [e]: n. 2 PFGE-types [g]: n. 4 PFGE-types

[b]: all the isolates are the same PFGE-type [d]: n. 3 PFGE-types [f]: n. 21 PFGE-types

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

In **Table 17**, the incidence of erythromycin and tetracycline resistance genes among the Ery^R and/or Tet^R *E. faecalis* isolates is reported.

All the 64 phenotypically Ery^R isolates, coming from all the isolation media and representing 50 % of all *E. faecalis* isolates, harboured the *erm*B resistance gene. Most of them (73.4%) were isolated from SBA + erythromycin or SBA + kanamycin (34.4 and 39.1 %, respectively), 21.9 % from SBA + tetracycline and 4.7% from SBA without antibiotic addition. No *E. faecalis* harboured *erm*A or *erm*C resistance genes.

All the 105 phenotipically Tet^R isolates, representing 82% of all *E. faecalis* isolated, harboured the *tet*M gene, 39 (37.1% of Tet^R *E. faecalis*) the *tet*L, and 30 (29%) the *tet*S, while *tet*W gene was not detected in any isolate.

On the whole, all the 64 Ery^R *E. faecalis* were also Tet^R, while 41 Tet^R isolates were not Ery^R.

Table 17. Presence of erythromycin and tetracycline resistance genes in phenotypically Ery^R and Tet^R *E. faecalis* isolates.

E. faecalis isolates		Ery^R isolates		with resistance genes						Tet^R isolates		with resistance genes							
				erm A		*erm* B		*erm* C				*tet* L		*tet* M		*tet* S		*tet* W	
Medium of origin ID code	N.	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%
AREN	35	3	8.6	-	-	3	100	-	-	12	34.3	2	16.7	12	100	-	-	-	-
EEN	22	22	100	-	-	22	100	-	-	22	100	12	54.5	22	100	13	59	-	-
KEN	25	25	100	-	-	25	100	-	-	25	100	11	44.0	25	100	13	52	-	-
TEN	46	14	30.4	-	-	14	100	-	-	46	100	14	30.4	46	100	4	9	-	-
TOTAL	128	64	50.0	-	-	64	100.0	-	-	105	82.0	39	37.1	105	100	30	29	-	-

-: not detected

Arranging the 105 Tet^R and/or Ery^R *E. faecalis* isolates according to the presence of resistance genes resulted in seven resistance patterns (**Table 18**). Fifty-six (53. 3%) of resistant isolates harboured three genes, against both erythromycin and tetracycline (*erm*B/*tet*L/*tet*M or *erm*B/*tet*M/*tet*S), while 33.3% showed unique genotypic profile *tet*M. Four isolates from SBA + erythromycin, constituted of three RAPD M13-types, harboured four resistance genes (*erm*B/*tet*L/*tet*M/*tet*S). Mostly (85.7%) of Ery^R and/or Tet^R *E. faecalis* were isolated from culture media containing an antibiotic as selective agent.

Table 18. Tetracycline and erythromycin resistance genes profiles detected among phenotypically Ery^R and/or Tet^R *E. faecalis* isolates.

Resistance genes profiles		Isolates *per* resistance profile		Medium of origin ID Code	Isolates *per* N. of AR genes	
N.	Name	N.	%		N.	%
1	***tet*M**	35	33.3	n. 9 AREN[a] n. 26 TEN[b]	35	33.3
2	***erm*B *tet*M**	4	3.8	n. 1 AREN n. 1 KEN n. 2 TEN	10	9.5
	***tet*L *tet*M**	5	4.8	TEN		
	tetM tetS	1	0.9	TEN		
3	***erm*B *tet*L *tet*M**	30	28.6	n. 2 AREN N. 8 EEN[c] n. 11 KEN[d] n. 9 TEN[e]	56	53.3
	erm*B *tet*M *tetS	26	24.8	n. 10 EEN[d] n. 13 KEN[b] n. 3 TEN		
4	***erm*B *tet*L *tet*M tetS**	4	3.8	EEN[d]	4	3.8
Total N. of resistant isolates		**105**			**105**	

[a]: n. 8 RAPD M13-types [c]: n. 4 RAPD M13-types [e]: n. 5 RAPD M13-types
[b]: n. 20 RAPD M13-types [d]: n. 3 RAPD M13-types

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

As already noticed, the medium used for isolation has a great importance for the recovery of resistant strains and the higher number of resistant isolates found in the present study in comparison with previous studies on Fiore Sardo cheese (Mannu *et al*, 2003; Cosentino *et al*., 2004) and on other animal food products (see Paragraph **3.3.1 MICs determination**) is due to the use of more effective selection media, not to an increased incidence of resistant isolates in this product.

The widespread use of antiobics for various purposes (from the treatment of disease to the practice of feeding them to food-production animals for growth promotion) contributed to the dissemination of antibiotic resistance genes, selecting for antibiotic resistance-harbouring bacteria. Moreover, the acquisition of the antibiotic resistance genotype may actually increase the fitness of certain bacteria also in the absence of antibiotic selective pressure, thus allowing their rapid emergence and dissemination on a worldwide scale (Enne *et al*., 2004; Luo *et al*., 2005). In this case, the dissemination of antibiotic resistance becomes a self-perpetuating process replacing the antibiotic susceptible genotype in the absence of any antibiotic selective pressure. Therefore, it becomes of huge importance to control the release of antibiotic resistance genes into the environment. In fact, in the areas with historically low level of antibiotic usage in agriculture the frequency of antibiotic resistance genes carriers is also very low. The presence of antibiotic resistance genes in bacteria isolated from apparently antibiotic free environments could be due to different reasons. For example, a low level of pre-existing natural gene pool in soil or in acquatic microbiota. Another explanation could be a previous strong antibiotic driven selection and 'integration' of antibiotic resistance genes into the bacterial genotype

(for instance in clinical isolates), with compensatory mutations reducing the fitness cost. Commensal bacteria as enterococci may subsequently become widespread in different environments and compete with susceptible genotypes in the absence of selection by antibiotics. Then, antibiotic resistant bacteria could be selected by other factors than antibiotics, such as heavy metals. Many antibiotic resistance genes reside on large selftransmissible genetic elements such as conjugative plasmids and transposons, which have sufficient capacity to carry multiple genes, including those encoding antibiotic, heavy metal, and biocide resistances. The physical linkage between the R plasmids and resistances to heavy metals was noticed almost 40 years ago (Smith, 1967). In the environment, bacteria in metal-contaminated areas appeared to be more tolerant to metals and antibiotics than in control sites (Stepanauskas *et al*., 2005 and 2006; Baker-Austin *et al*., 2006; Wright *et al*., 2006). Finally, microbial ecosystems are not isolated, there is extensive gene exchange between different compartments, and antibiotics can become widespread in the environment in different ways: veterinary antimicrobials can be found even in groundwater as deep as > 10 m and in surface water such as a river, in areas impacted by agricultural activities (Batt *et al*., 2006; Yang and Carlson, 2003).

Thus, a carefully designed analysis of a particular ecosystem for antibiotic resistance genes is of fundamental importance to exclude the possibility of contamination by antibiotics, even in the areas with light agricultural activity and with low human and animal population density (Aminov and Mackie, 2007).

From the clinical point of view, the most important and worrying event is the entry of antibiotic resistance genes into pathogenic bacteria.

Genetic mechanisms involved in acquisition of antibiotic resistance genes from the environmental antibiotic 'resistome' both by commensal and pathogenic bacteria may include transformation by naked DNA, conjugative transfer and transduction, but the ecological aspects of this process are still poorly understood (Aminov and Mackie, 2007). In any case, both to exert a beneficial (probiotic) or a negative effect (not only pathogenic, but e.g. the exchange of antibiotic resistance or virulence genes), a strain, after ingestion, must be able to survive and to colonize the gastrointestinal tract (GIT) by competing with the local natural flora. For probiotics, for instance, a daily intake of at least 10^8–10^9 viable cells has been suggested as the minimum intake to provide a therapeutic effect (Roy, 2005). Therefore, at least 10^6 CFU/g of a strain should be present in a food (Codex Alimentarius, 2003). In this case an amount of 10^8 cells will be ingested when 100 g of food are eaten. If lower amounts of bacterial cells are ingested it is unlikely that they could manage to survive in the GIT and carry out their positive or negative function (Talwalkar and Kailasapathy, 2004).

Several authors have highlighted the importance of a high number (10^7–10^8 CFU g^{-1}) of donors on the efficacy of gene transference *in vivo*, demonstrating that a low number (10^4 CFU g^{-1}) of cell donors containing antibiotic resistance genes was insufficient to transfer these genes to the intestinal microflora (Licht and Wilcks 2006; Jacobsen *et al.*, 2007; Zonenschain *et al.*, 2009).

All these things considered, from this point of view Fiore Sardo cheese can be deemed safe, as enterococci harbouring antibiotic resistance genes were detected at low concentration ($\leq$ 3 CFU g^{-1}).

3.4 Presence of virulence determinants

A total of 202 isolates (93/174 *E. faecium*, 105/128 *E. faecalis*, and 4/10 *E. durans*), all Ery^R and/or Tet^R, were analysed for the presence of several virulence determinants by PCR technique using primers previously designed at this laboratory or present in literature.

Among the two dominant species *E. faecium* and *E. faecalis*, the last turned out to have the highest number of virulent determinants.

None of the *E. durans* isolates harboured any investigated virulence trait (**Tables 19** and **20**).

The ***cyl*A** was the only gene not detected in any of the investigated isolates. Therefore, considering that the cheeses analysed in this study were produced in almost half of the total dairy farms producing Fiore Sardo, no haemolytic activity should be expected by the enterococcal population colonising this cheese. As seen in other studies (Ben Omar *et al*., 2004; Drahovská *et al*., 2004; Vankerckhoven *et al*., 2004; Abriouel *et al*., 2008; Billström *et al*., 2008; Gomes *et al*., 2008; Hällgren *et al*., 2009), generally, *cyl*A gene does not appear to be characteristic, either, of clinical *E. faecium* isolates.

On the other hand, among *E. faecalis*, its incidence can vary a lot (from 0 to 61.53%) depending on the origin of the isolates.

Table 19. Presence of virulence determinants genes in Ery^{+} and Tet^{+} *E. faecium* isolates.

Medium of origin ID code	Total isolates	Ery^{R}/Tet^{R} isolates		Isolates with virulence determinants genes													
				agg		*ace*		*gel* E		*esp*		*efa* A		*efa* A*fm*		*cpd*		*cyl* A	
	N.	N.	%	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]
AREN	71	8	11.3	-	-	-	-	-	-	1	12.5	-	-	8	100	-	-	-	-
EEN	21	21	100	-	-	-	-	-	-	8	38.1	1	4.8	21	100	1	4.8	-	-
KEN	47	29	61.7	-	-	-	-	-	-	-	-	-	-	28	97	-	-	-	-
TEN	35	35	100	-	-	1	2.9	-	-	22	62.9	8	22.9	35	100	8	22.9	-	-
TOTAL	**174**	**93**	**53.4**	-	-	**1**	**1.1**	-	-	**31**	**33.3**	**9**	**9.7**	**92**	**99**	**9**	**9.7**	-	-

-: not detected

[a]: calculated on Ery^{R}/Tet^{R} isolates NOT on the total amount

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Table 20. Presence of virulence determinants genes in Ery^{+} and Tet^{+} *E. faecalis* isolates.

Medium of origin ID code	Total isolates	Ery^{R}/Tet^{R} isolates		Isolates with virulence determinants genes													
				agg		*ace*		*gel* E		*esp*		*efa* A		*efa* A*fm*		*cpd*		*cyl* A	
	N.	N.	%	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]	N.	%[a]
AREN	35	12	34.3	6	50.0	9	75.0	6	50.0	10	83.3	12	100	1	8.3	12	100	-	-
EEN	22	22	100	12	54.5	18	81.8	9	40.9	22	100	22	100	-	-	22	100	-	-
KEN	25	25	100	11	44.0	23	92.0	12	48.0	25	100	24	96.0	-	-	25	100	-	-
TEN	46	46	100	22	47.8	23	50.0	34	73.9	35	76.1	46	100	1	2.2	46	100	-	-
TOTAL	**128**	**105**	**82.0**	**51**	**48.6**	**73**	**69.5**	**61**	**58.1**	**92**	**87.6**	**104**	**99.0**	**2**	**1.9**	**105**	**100**	-	-

-: not detected

[a]: calculated on Ery^{R}/Tet^{R} isolates NOT on the total amount

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

The highest percentages are detected among clinical strains, but it is generally not detected among food strains (Archimbaud *et al*., 2002; Ben Omar *et al*., 2004; Creti *et al*., 2004; Abriouel *et al*., 2008; Gomes *et al*., 2008; McGowan-Spicer *et al*., 2008; Pangallo *et al*., 2008; Hällgren *et al*., 2009).

However, exceptions may occur. Indeed Drahovská *et al*. (2004) and Čanžec Majhenič *et al*., (2002) found *cylA* present in 25 and 50% respectively, of *E. faecalis* isolated from cheese, while Valenzuela *et al*. (2008) detected it in 26.7% of *E. faecium* and in 17.4% of *E. faecalis* isolated from foods in Morocco.

No studies were previously carried out on the presence of Cytolisin genes among Fiore Sardo cheese enterococci.

No *E. faecium* isolate was shown to have the ***agg*** gene, while it was present in 48.6% of *E. faecalis* isolates coming from media both with and without antibiotics almost in equal percentages.

Previous studies carried out by Mannu *et al*. in 2003 did not show the presence of genes encoding for an AS among *E. faecium* isolated from Fiore Sardo cheese.

The *agg* gene was rarely detected among *E. faecium* isolates both of food (from 0 to 20%) and clinical (from 0 to 5%) origin (Eaton and Gasson, 2001; Mannu *et al*., 2003; Abriouel *et al*., 2008; Cariolato *et al*., 2008; Valenzuela *et al*., 2008 and 2009; Vankerckhoven *et al*., 2008; Hällgren *et al*., 2009), while it was found at higher incidences among *E. faecalis* isolates, mostly of clinical origin (from 18 to 100%, and from 59 to 100%, respectively among food and clinical isolates) (Eaton and Gasson, 2001; Čanžec Majhenič *et al*., 2002; Semedo *et al*., 2003; Abriouel *et al*., 2008;

McGowan *et al*., 2008; Cariolato *et al*., 2008; Valenzuela *et al*., 2008 and 2009; Hällgren *et al*., 2009). So the results obtained in this study are consistent with those already reported in literature.

The ***ace*** gene was present in only one *E. faecium* isolated from SBA + tetracycline, and in 69.5% of *E. faecalis* coming mostly from SBA + kanamycin.

This gene is generally not detected among both *E. faecium* and *E. faecalis* isolated from food, while its incidence can reach more than 80% among clinical strains, especially those belonging to *E. faecalis* species (Dupré *et al*., 2003; Ben Omar *et al*., 2004; Creti *et al*., 2004; Abriouel *et al*., 2008; Gomes *et al*., 2008). Exceptions are represented by results shown in Cariolato *et al.* (2008), who detected it in around 76% of both clinical and food *E. faecalis* strains, and by those of Valenzuela *et al.* (2008) who found this gene in 60.86% of food *E. faecalis* strains.

The results of this study are in agreement with those of Cariolato *et al.* (2008) and Valenzuela *et al.* (2008). Only in one previous study in which virulence traits of *E. faecium* from Fiore Sardo cheese were investigated, was the *ace* gene not found (Mannu *et al*., 2003). However, it is noteworthy that Mannu *et al*., 2003 carried their study on samples coming from a restricted number of farms (only 3) and without using antibiotics in the primary isolation medium. The unique *E. faecium* harbouring the *ace* gene found in this current study was isolated from SBA + tetracycline, was phenotypically resistant to ciprofloxacin, erythromycin, rifampin and tetracycline (it harboured *tet*L and *tet*M genes) and displayed four other virulence traits (*Efa*A, *Efa*A*fm*, *esp*, and *cpd*) besides *ace*. So it could be supposed that this trait would be

more likely found in particularly virulent *E. faecium* strains, maybe arrived to food from humans. However, in Fiore Sardo cheese this was a very exceptional case.

Similar results were obtained for ***gel*E** gene. It was present only in *E. faecalis* (58.1% of isolates) isolated mostly from SBA + tetracycline (73.9% of investigated isolates coming from this medium). This is one of the most frequently detected virulence genes, mostly among enterococci of food origin. Its incidence varies in a range from 43 to 96% for food *E. faecalis* isolates, and from 58 to 81% for clinical ones. Except for rare cases in which it was not detected (Ben Omar *et al*., 2004), this gene is commonly found also among *E. faecium*, mainly of food origin (up to 85% as opposed to around 20% among clinical strains) (Eaton and Gasson, 2001; Archimbaud *et al*., 2002; Čanžec Majhenič *et al*., 2002; Dupré *et al*., 2003; Mannu *et al*., 2003; Creti *et al*., 2004; Drahovská *et al*., 2004; Abriouel *et al*., 2008; Billström *et al*., 2008; Cariolato *et al.* 2008; McGowan-Spicer *et al*., 2008; Valenzuela *et al*., 2008 and 2009; Vankerckhoven *et al*., 2008).

Gelatinase activity was phenotypically detected in previous studies on enterococci isolated from Fiore Sardo, but only among *E. faecalis* strains (20%) (Cosentino *et al*., 2004), while the *gel*E gene was not found among *E. faecium* isolated from Fiore Sardo tested by Mannu *et al*. in 2003.

The ***esp*** gene was found in 33.3% of the *E. faecium* (mainly isolated from SBA + tetracycline, none from SBA + kanamycin), and in 87.6 % of the *E. faecalis* analysed. This gene is not commonly detected among food *E. faecium* isolates (Eaton and

Gasson, 2001; Mannu *et al*., 2003; Ben Omar *et al*., 2004; Drahovská *et al*., 2004; Gomes *et al*., 2008; Valenzuela *et al*., 2008), but it was found in 27.2 and 43.7% of *E. faecium* isolated from fruit and vegetables by Abriouel *et al*. (2008) and from artisanal food of animal origin by Valenzuela *et al*. (2009), respectively.

It has often a high incidence (65-83%) among *E. faecium* isolates of human origin (Eaton and Gasson, 2001; Dupré *et al*., 2003; Mannu *et al*., 2003; Abriouel *et al*., 2008; McGowan-Spicer *et al*., 2008; Vankerckhoven *et al*., 2008; Hällgren *et al*., 2009).

The occurrence of this gene among food *E. faecalis* isolates can vary a lot (from 0 up to 77%), as well as among clinical ones (from 20 up to 87%) (Eaton and Gasson, 2001; Archimbaud *et al*., 2002; Čanžec Majhenič *et al*., 2002; Dupré *et al*., 2003; Ben Omar *et al*., 2004; Creti *et al*., 2004; Drahovská *et al*., 2004; Abriouel *et al*., 2008; Gomes *et al*., 2008; Valenzuela *et al*., 2008 and 2009; Hällgren *et al*., 2009). Therefore, our results are in the range of those reported by other authors for *E. faecium* and *E. faecalis* species.

The *E. faecalis* ***efa*A** endocarditis gene was detected in all but one *E. faecalis* investigated, while it was present only in 9 (9.7%) of *E. faecium* (1 from SBA + erythromycin, and 8 from SBA + tetracycline). The primers used in this study (Mannu *et al*., 2003) to amplify *efa*A gene, as those used by Creti *et al*. (2004), were designed on the endocarditis specific antigen A gene of *E. faecalis* (GeneBank accession number EFU03756), but their sequence has 100% of homology also with plasmids and transposons DNA sequences found in *E. faecium* (plasmid pHT beta

DNA, GenBank accession number AB183714.1; plasmid pMG1 DNA, GenBank accession number AB206333.1; pVEF3 plasmid, GeneBank accession number AM931300.1; D344R transposon Tn5386, GeneBank accession number DQ321786.1). Therefore, PCR products can be obtained also from *E. faecium* strains. Mannu *et al.* (2003) and Gomes *et al.* (2008) obtained 50 and 67.6% of positive results, respectively, using these primers to investigate the presence of *efa*A gene in *E. faecium* from different origins (milk, cheese and meat products), while Dupré *et al.* (2003) obtained amplification products only from *E. faecalis* DNA.

Both EfaAfs and EfaAfm are cell adhesins, the first generally expressed in serum by *E. faecalis*, the second by *E. faecium*. However, differing degrees of homology among *efa*A genes in the different enterococcal species were demonstrated in the Southern blots by Eaton and Gasson (2001). Therefore, many authors used distinct couples of primers (for *efa*A*fs* and *efa*A*fm*) to investigate the presence of this determinant in the two species (Eaton and Gasson, 2001; Abriouel *et al*., 2008; Cariolato *et al*., 2008; Pangallo *et al*., 2008) obtaining species-specific results.

When *efa*A*fm* primers were used in order to detect the endocarditis antigen A in *E. faecium* isolated in this study, 100% of positive results were obtained, while when they were used to amplify DNA from *E. faecalis* strains only two isolates gave a positive result (1 from SBA and 1 from SBA + tetracycline). *E. faecalis* harbouring e*fa*A*fm* gene are also reported in literature (Čanžek Majhenič *et al*., 2005; McGowan-Spicer *et al*., 2008).

Nevertheless, so far, only the *efa*A gene from *E. faecalis* has been shown to influence pathogenicity in animal models, while the role of the *efa*A*fm* gene has not yet been

demonstrated (Eaton and Gasson, 2001). All *E. faecium* isolated in this study showed to have only *efa*A*fm* gene. Our results are consistent to those reported by the authors cited above.

Finally, the ***cpd*** gene was detected in all *E. faecalis* investigated, while only 9.7% of *E. faecium* (the same showing the presence of *E. faecalis efa*A gene) harboured this gene. Similar incidences were reported in literature for both species (from 85 up to 100 %, and 0%, for *E. faecalis* and *E. faecium*, respectively). Discordant results were reported by some Spanish authors. Indeed, they found an occurrence (20 %) of *cpd* gene higher than usual among *E. faecium* isolated from fruit and vegetables foods (Abriouel *et al*., 2008), and lower (33.3 %) among *E. faecalis* coming from various artisanal foods of animal origin (Valenzuela *et al*., 2009).

As shown above and in the following **Tables 21** and **22**, *E. faecium* and *E. faecalis* strains displayed very different patterns in the incidence of virulence determinants.

All the *E. faecalis* tested harboured multiple virulence determinants (between 3 and 7), 80.9 % of isolates investigated harboured between four and six virulence genes, while the majority of *E. faecium* (66.7%) harboured just one virulence gene (*efa*A*fm*). Only a few *E. faecium* isolates (9, 9.7%) showing the presence of four or five virulence determinants (*efa*A*fm* was one of those genes) were detected.

The *efa*A*fm* gene was the most frequently detected among *E. faecium*, while *cpd* was among *E. faecalis* (100%).

Table 21. Virulence determinants gene profiles detected among *E. faecium* isolates.

Virulence determinants gene profiles		Isolates *per* virulence profile		Medium of origin ID Code	Isolates *per* N. of virulence determinants genes	
N.	Name	N.	%		N.	%
1	*efa Afm*	62	66.7	n. 7 AREN n.12 EEN[a] n. 29 KEN[b] n. 13 TEN	62	66.7
2	*efa Afm esp*	22	23.7	n.1 AREN n. 7 EEN[a] n. 14 TEN[c]	22	23.7
4	*efaA efa Afm esp cpd*	8	8.6	n. 7 TEN[d] n. 1 EEN	8	8.6
5	*ace esp efa* A *efaAfm cpd*	1	1.1	TEN	1	1.1
Total N. of Ery$^+$/Tet$^+$ isolates		**93**			**93**	

[a]: n. 5 PFGE-types
[b]: n. 13 PFGE-types
[c]: n. 13 PFGE-types
[d]: n. 6 PFGE-types

Unless otherwise specified, all isolates belonged to different PFGE-types

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

Table 22. Virulence determinants gene profiles detected among *E. faecalis* isolates.

Virulence determinants gene profiles		Isolates *per* virulence profile		Medium of origin ID Code	Isolates *per* N. of virulence determinants genes	
N.	Name	N.	%		N.	%
	ace esp cpd	1	1.0	KEN		
3	*esp efa* A *cpd*	13	12.4	n. 4 EEN[a] n. 2 KEN N. 7 TEN[b]	19	18.1
	gelE efa A *cpd*	5	4.8	TEN		
	ace esp efa A *cpd*	24	22.9	N. 5 AREN[c] n. 6 EEN[d] n. 10 KEN[d] n. 3 TEN		
	agg esp efa A *cpd*	2	1.9	n. 1 TEN n. 1 AREN		
4	*agg gelE efaA cpd*	8	7.6	n. 2 AREN n. 6 TEN[b]	38	36.2
	esp efaA efaAfm cpd	1	1.0	TEN		
	gelE esp efa A *cpd*	3	2.9	n. 3 TEN[a]		
5	*ace gelE esp efa* A *cpd*	7	6.7	n. 1 AREN n. 1 KEN n. 5 TEN	10	9.5
	agg ace esp efa A *cpd*	3	2.9	n. 3 EEN		
6	*agg ace gelE esp efaA cpd*	37	35.2	n. 3 AREN n. 9 EEN[c] n. 11 KEN[d] n. 15 TEN[e]	37	35.2
7	*agg ace gelE esp efa* A *efaAfm cpd*	1	1.0	AREN	1	1.0
Total N. of Ery$^+$/Tet$^+$ isolates		**105**			**105**	

[a]: n. 2 RAPD M13-types
[b]: n. 5 RAPD M13-types
[c]: n. 4 RAPD M13-types
[d]: n. 3 RAPD M13-types

Unless otherwise specified, all isolates belonged to different RAPD M13-types

AREN, EEN, KEN, and TEN codes indicate isolates from SBA, SBA+Ery, SBA+Kan, and SBA+Tet, respectively

E. faecium coming from SBA without antibiotic addition and from SBA + kanamycin showed the lowest percentage of virulent factors.

A different situation was found for *E. faecalis*. In fact it was possible to detect high percentages of virulent isolates from all the media used.

Usually, *E. faecalis* harbours more and multiple virulence determinants than other enterococcal species, while in *E. faecium* their incidence is quite low (Bhardwaj *et al*., 2008), but it must be taken into account that the incidence of virulence determinants is strain specific (Franz *et al*., 2001).

Franz *et al.* (2001) reported a percentage of 10.4% and 78.7% of *E. faecium* and E. *faecalis*, respectively, positive for one or more virulence determinants. Eaton and Gasson (2001) found the presence of virulence genes in all *E. faecalis* tested, while, apart from *efa*A*fm* and *esp*, no other virulence genes were found among *E. faecium* tested by these authors.

Virulence factors are mainly detected among clinical isolates, although several traits among food and environmental enterococci were detected as well (Eaton and Gasson, 2001; Semedo *et al*., 2003; Drahovská *et al*, 2004; Čanžek Majhenič *et al*., 2005; Abriouel *et al*., 2008; Cariolato *et al*., 2008; McGowan-Spicer *et al*., 2008; Pangallo *et al*., 2008; Valenzuela *et al*., 2008 and 2009). N.B. some virulence factors may occur in food isolates with a frequency identical to or even higher than that observed within strains of human origin (Cariolato *et al*., 2008).

Major differences on the incidence of genetic determinants for virulence factors involve a remarkably higher average number of traits in *E. faecalis* isolates,

irrespective of their origin, or even the absence of traits in *E. faecium* mainly isolated from foods and environmental sources (Abriouel *et al*., 2008). Moreover, *E. faecium* strains harbouring different virulence traits are found very rarely in cheeses. Particularly in Fiore Sardo they were isolated in so low concentrations (2.43 ± 1.95 CFU/g from SBA + erythromycin, and 2.78 ± 0.96 CFU/g from SBA + tetracycline) that they are unlikely to be ingested in significant amounts by the consumers for efficiently transferring their virulence genes to bacteria colonizing GIT (see Paragraph **3.3.2 Presence of antibiotic resistance genes**).

Taking these observations into consideration, our results indicate that Fiore Sardo's enterococcal microflora could be considered safe in regard to the risk of antibiotic resistance and virulence gene transmission.

In this study the sole *E. faecium* strain harbouring five virulence genes was found in only one sample of cheese, with a low concentration (3 log CFU/g) and in single copy (unique PFGE-type), so its presence should not be of concern.

Our study is quite consistent with the results reported in literature that *E. faecalis* has been found to harbour more virulence genes than *E. faecium* and with incidences similar to those reported for other foods produced in different geographical areas.

Moreover, as the literature has often reported both the presence of silent genes [Gelatinase activity is present only in 36-44% of strains harbouring gelE gene (Creti *et al*., 2004; Drahovská *et al*., 2004; Cariolato *et al*., 2008) or as being totally absent (Biavasco *et al*., 2007)]. Further studies should be carried out to test the virulence of Fiore Sardo's strains from the phenotypic point of view.

4. CONCLUSIONS

In general, the results presented in this study are consistent with those previously reported in literature for food isolates of the same species, and differently from results obtained in a recent study on *Lb. paracasei* isolated from Fiore Sardo (Comunian *et al*., 2009a), the incidences of enterococci resistant to erythromycin and tetracycline found in this work were similar to those found for the same species isolated from other food products.

The incidences of resistance to the other antibiotics tested and the presence of virulence determinants varied a lot among isolates, especially depending on their medium of origin (with or whithout antibiotics), and were higher for *E. faecalis* isolates.

Even though, at present, there is no evidence suggesting enterococci as food borne pathogens, because of their robust nature, their wide distribution and their stability in the environment, a major concern for their presence in foods is their inclination for horizontal transfer of genes for pathogenic factors, as those associated with virulence and antibiotic resistance.

However, isolates harbouring this kind of genes were detected at so low a concentration in Fiore Sardo cheese that their amount is considered insufficient for them to efficiently transfer antibiotic resistance and virulence genes to other bacteria during their transit in the GIT. Therefore, the presence of enterococci in Fiore Sardo cheese should not represent a threat to human health.

Furthermore, since enterococci play an important role in the manufacture of cheeses typical of Mediterranean countries, contributing not only to their own sensory identity, but also, by means of bacteriocins production, to preserve them from spoilage and food borne pathogen bacteria (not forgetting that they might have also probiotic properties), it would be unthinkable to eliminate their presence from such typical dairy products. A future development of the study of Sardinian dairy enterococci could be the assessment of these positive features to show also their useful potential. Moreover, further studies would be needed also to assess the possible contribution of enterococci from Sardinian ewe's milk and cheeses to human clinical illness by means of their ability to transfer virulence and resistance genes to other bacteria.

Other important results obtained in this study were those regarding genotypic biodiversity of the isolates.

Fiore Sardo cheese was chosen to carry out this study on enterococcal microflora of Sardinian ovine cheeses because it was supposed that it is the maximum expression of the microbial biodiversity of dairy products typical of the Sardinia island, and actually a high level of biodiversity was found, particularly at strain level, among enterococci. They were represented by five species, two dominant (*E. faecium* and *E. faecalis*), and three less-abundance species (*E. durans*, *E. hirae*, and *E. gallinarum*).

Using the best molecular technique (PFGE) among those applied for strain typing [rep-$(GTG)_5$ and RAPD M13 PCR, besides PFGE], a Diversity Index of 0.995 was calculated for the most numerous species *E. faecium* (1 is the ideal D.I. of a

population constituted by all different strains). A high level of biodiversity (D.I. 0.963) was estimated also for *E. faecalis* (the second numerically dominant species) even though a less discriminatory technique (RAPD M13 PCR) was applied for typing the isolates. It is likely that a level similar to that found for *E. faecium* would be obtained if PFGE would had been applied.

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