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Towards the post-antibiotic era: The role of antibiotic resistant enterococci in different sources

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List of Abbreviations

Ace: Accessory colonisation factor

AMP: Ampicillin

AMR: Antimicrobial Resistance

AR: Antibiotic Resistance

ARB: Antibiotic Resistant Bacteria

AS: Aggregation Substance

BAPS: Bayesian Analysis of Population Structure

BHI: Brain Heart Infusion

BOD_5: Biochemical Oxygen Demand

CA: Community Associated

CATs: Chloramphenicol acetyltransferases

CC: Clonal Complex

CDC: Centers for Disease Control and Prevention

CDS: Coding Sequences

cgMLST: Core Genome Multilocus Sequence Typing

CHL: Chloramphenicol

CLSI: Clinical and Laboratory Standard Institute

COD: Chemical Oxygen Demand

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat

CTns: Conjugative Transposons

CW: Constructed Wetland

Cyl: Cytolysin

ddcP: D-alanyl-D-alanine carboxypeptidase gene

DHF: dihydrofolate reductase

DHPS: dihydropteroate synthetase

EfaA: Endocardis Antige

EFSA: European Food Safety Authority

EPA: Environmental Protection Agency

ERY: Erithromycin

ESKAPE: *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter* spp., *Pseudomonas aeruginosa*, and *Enterobacter* spp.

ESP: Enterococcal Surface Protein

EU: European Union

EUCAST: European Committee of Antimicrobial Susceptibility Testing

FIB: Fecal Indicator Bacteria

GEL E: Gelatinase E

GEN: Gentamycin

GI: Gastro Intestinal

GRAS: Generally Recognized as Safe

HA: Hospital Associated

HLR: High level Resistance

H-SSF: Horizontal Sub-Surface Flow

HYL: hyaluronidase

ICE: Integrative Chromosomal Element

IS: Insertion Sequence

KAA: Kanamicin Aesculin Azide

KAN: Kanamycin

LAB: Lactic Acid Bacteria

Ldtfm: L,D-transpeptidase

lytG: peptidoglycan hydrolase gene

MGEs: Mobile Genetic Elements

MIC: Minimal Inhibitory Concentration

MLST: Multilocus Sequence Typing

MLVA: Multilocus Variable Number Tandem Repeats Analysis

MMH594: Multi-drug-resistant strain of E. faecalis

MRSA: Methicillin Resistant Staphilicoccus aureus

MSCRAMM: Ace microbial surface components recognizing adhesive matrix molecules adhesin of collagen

NAG: N-acetylglucosamine

NAM: N-acetylmuramic acid

NH4: Ammonia

PAI: Pathogenic Island

PBP: Penicillin Binding Protein

PCA: Principal Component Analysis

PDO: Protected Designation of Origin

PEN: Penicillin G

PFGE: Pulsed Field Gel Electrophoresis

PG: peptidoglycan

Pgt: glycosyl transferase group 2 family protein

QPS: Qualified Presuntion of Safety

RIF: Rifampicin

SBA: Slantez Bartley Agar

ST: Sequence Type

STRE: Streptomycin

SUL: Sulphamethoxazole

Te: Teicoplanin

TET: Tetracyclin

TMP: Trimetropin

TN: Total Nitrogen

TP: Total Phosphorus

TSS: Total Suspended Solids

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

VAN: Vancomycin

VNTR: Variable-Number of Tandem Repeats

VRE: Vancomycin Resistant Enterococci

WGS: Whole Genome Sequencing

WHO: World Health Organization

WWTP: Watewater Treatment Plant

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Abstract

The explosive spread of antibiotic resistance (AR) phenomenon has a global dimension. In the past, AR bacteria were predominantly associated with hospitals and care settings, but over the last decade, they were always more frequently found in aquatic environment and in food. Among AR bacteria, enterococci are important nosocomial pathogens and their clinical importance is directly related to AR, which contributes to increase risk of colonization and infection.

The main objective of the present PhD thesis was to investigate the occurrence and the diversity of AR enterococci in two different settings, such as food and aquatic environments. In particular, two traditional cheeses (Chapter 1) and an urban wastewater treatment plant (Chapter 2) were considered. To our knowledge, this is the first evidence of AR enterococci isolated from Ragusano PDO cheese and from an urban wastewater treatment plant located in Sicily.

Results obtained from cheese indicated the presence of a specific population, mostly referred to E. durans*, for which less information on AR is available. High incidence of resistance was detected and numerous isolates exhibited multidrug-resistant phenotypes with strains resistant to vancomycin. Moreover, the multimodal distribution of MIC in enterococcal isolates permits to distinguish two sub-populations, suggesting an acquiring AR mechanism.*

Regarding water associated enterococci, results highlighted the dominance of E. faecium *species in all analysed sites and a high incidence of AR against several antibiotics. Furthermore, numerous pulso-types with a unique PFGE pattern were distinguished and the presence of the same*

clone, in different sites, with different phenotypic resistance patterns was observed.
In conclusion, results here obtained reinforced the need of a constant monitoring and an active surveillance of the presence of antibiotic resistant enteroccci in both cheeses and water, in order to contain the risk to human health.

Sommario

L'esplosione del fenomeno dell'antibiotico resistenza (AR) si configura come un fenomeno globale. Sebbene i batteri AR sia stati per lungo tempo esclusivamente associati ad ambienti ospedalieri e/o sanitari, nell'ultimo decennio sono stati isolati sempre più frequentemente, in numerosi altri ambienti e persino negli alimenti. La diffusione dell'antibiotico resistenza tra gli enterococchi, noti come patogeni nosocomiali, ha contribuito a innalzarne il rischio di colonizzazione e infezione, incrementandone l'importanza clinica.

L'obiettivo principale della presente tesi di dottorato è stato quello di indagare la presenza e la struttura della popolazione enterococcica AR sia in matrici alimentari sia ambientali. In dettaglio, sono stati presi in considerazione due formaggi siciliani (Capitolo 1) e un impianto di depurazione urbano (Capitolo 2). Ad oggi, questa rappresenta la prima evidenza scientifica di AR in enterococchi isolati da formaggio Ragusano DOP e da un impianto di trattamento delle acque reflue urbane siciliano. I risultati ottenuti dal formaggio hanno evidenziato la presenza di una specifica popolazione, per lo più riferita alla specie E. durans*, per la quale meno informazioni sull'AR sono disponibili in letteratura. In particolare, è stata rilevata un'elevata prevalenza di multi-resistenza e alcuni ceppi hanno evidenziato resistenza alla vancomicina. Inoltre, la distribuzione multimodale dei valori di MIC, all'interno della specie, ha consentito la distinzione di due sottopopolazioni, suggerendo meccanismi di acquisizione genica di AR.*

Per quanto riguarda gli enterococchi isolati dal sistema di depurazione di acque urbane, i risultati hanno evidenziato

la dominanza di E. faecium *in tutti i siti analizzati e un'alta prevalenza di AR nei riguardi di numerosi antibiotici. Attraverso l'analisi PFGE numerosi profili unici sono stati ottenuti e la presenza dello stesso clone con differenti profili di resistenza, in differenti siti, è stata osservata. In conclusione, i risultati ottenuti rafforzano la necessità di un monitoraggio costante e di una sorveglianza attiva sulla presenza di enterococchi AR sia nei formaggi che in ambienti acquatici, al fine di contenere il rischio per la salute umana.*

1. Introduction

1.1 A general overview on antibiotic resistance phenomenon

Antibiotic resistance (AR) is a global phenomenon with severe epidemiological ramifications. Since its first warning (McCoy, 1954), the phenomenon has dramatically increased and, currently, represents one of the biggest threats to global health, affecting any one, of any age, in any country. In the past, antibiotic resistant bacteria (ARB) were predominantly associated with hospitals and care settings, but over the last decade, they have been found also in the wider community (O'neill, 2016). Without harmonized and immediate action on a global scale, the world is heading towards a post-antibiotic era in which common infections could once again kill humans (WHO, 2015). Systematic misuse and overuse of antibiotics for veterinary and agricultural purposes determined an explosive spread of multi-resistant microorganisms, and the construction of a complex interactive network ables to exchange resistance genes between organisms, and/or among Mobile Genetic Elements (MGEs). Such a platform (Baquero et al., 2011; Halary et al., 2010; Nogueira et al., 2009; Skippington et al., 2011), promotes the movement of genes in different settings and competence for genetic transformation (Baquero et al., 2011; Couce et al., 2009; Courvalin, 2008). In many countries, antibiotics are administered without professional oversight; important examples of such misuse include the consumption of antibiotics by people with common viral infections or administration of antibiotics to farm-raised fish or livestock as growth promoters (Hay et al., 2018). This escalating evolution of resistance is associated to a reduced

antibiotic pipeline (Richard et al., 2014) (Fig.1). As a consequence the spread of the so-called "superbugs" is acting the reduction of therapeutic options for these microorganisms, the exention of periods of hospital care and, finally, an increasing costs. The term "superbugs" refers to microbes with enhanced morbidity and mortality due to multiple mutations endowing and high levels of resistance to the antibiotic classes specifically recommended for their treatment. In some cases, super-resistant strains have also acquired increased virulence and enhanced transmissibility (Davies, 2010). ARBs and ARGs can circulate through food, water and environment, and transmission is influenced by trade, travel and both human and animal migrations. Tackling AR is a high priority for scientific community and international organizations, as demonstrated by the number of internationally/scientifically relevant initiatives, coordinating a global campaign to raise awareness and encourage best practices among public, policymakers, health and agriculture professionals. Given the global dimensions of the phenomenon with a multi-compartmental nature, there is need to consider all compartments at the same time in order to design therapeutic and preventive interventions aiming to control its emergence and evolution (Baquero et al., 2014). Further, poor infection control, inadequate sanitary conditions, and inappropriate food handling may facilitate the spread of AMR within populations (Fletcher, 2015). These problems are exacerbated in low- and middle income countries, where there is often inadequate surveillance, minimal laboratory capacity, and limited access to essential antimicrobials (Hay et al., 2018). As a critical input to these actions, a high-quality evidence base is needed to support surveillance and data collection efforts, as well as to inform global policy

priorities, set clear international standards and guidelines, establish intervention priorities, and support investment decisions. Despite growing awareness and concern, inertia appears to persist in improving stewardship of existing antimicrobials to prevent a future with more resistant bacteria (Dowling et al., 2013). For instance, in comparison with climate change, while there appears to be increasing scientific consensus about the urgency of countering the impact of global warming, this is perhaps less clear (or at least the emerging consensus is less coherent) for AR. We are now, however, in the midst of a post-antibiotic era. Resistance mechanisms are pandemic and create an enormous clinical and financial burden on health care systems worldwide. There are no simple solutions to the problem. In the United States, the Centers for Disease Control and Prevention (CDC) has conservatively estimated that 2 million patients a year suffer from infections due to drug-resistant bacteria, and 23,000 die annually as a result. AR has a negative impact on morbidity, mortality, and economics, with estimated annual direct and indirect costs of $55 billion in the United States. If resistance continues on its present trajectory, 10 million deaths worldwide are predicted by 2050 (surpassing deaths from cancer) and could cost up to $100 trillion (Luepke et al., 2017). In the end, the treatment of patients infected with drugresistant pathogens is much more expensive for longer hospitalization times and for requiring more expensive last-resort drugs (WHO, 2018). The annual economic burden associated with the treatment of antibiotic-resistant infections has been estimated to be between $21,000 and $34,000 million in the United States alone, and around $1500 million in Europe, which includes the economic impact associated with the number of days of lost

productivity, estimated to be approximately $450 million each year in Europe (ECDC, 2009).

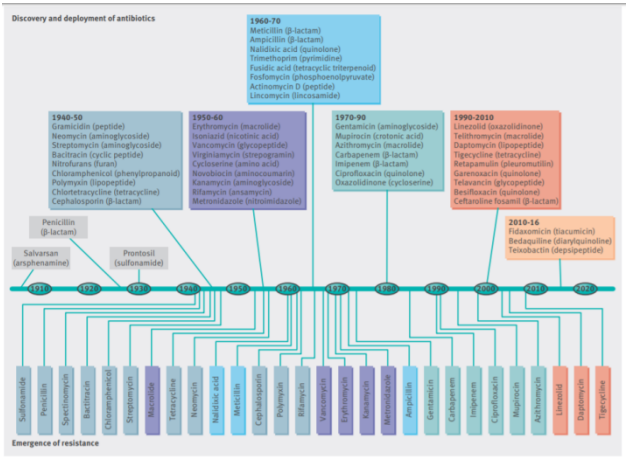

Figure 1. Introduction of antibiotics in clinical practice and emergence of antimicrobial resistance. The timeline shows that most new antibiotics (scaffolds) were discovered up to the 1970s. In subsequent years, these scaffolds were expanded chemically by introducing new functional groups. Few clinically used antibiotics with new scaffolds (Das et al., 2017).

Investment in newer anti-infective platforms is essential and urgent, and requires collaboration among industry, academia and government and a revolution in our understanding of bacterial resistance. However, the era where acute or chronic bacterial infections used to be treated with ‘‘antibiotics-only’’ appears to have come to an abrupt end (Alanis, 2005). As reported by Roca and

coworkers (2015), the following measures can be taken to prevent the emergence and spread of antibiotic resistance worldwide: (a) rational use of antibiotics in all settings; (b) implementation of infection control measures in healthcare settings; (c) development of strategies to mitigate the risks of environmental exposure; (d) development of rapid diagnostic tests; (e) promotion of research on antibacterial resistance prevention and surveillance; (f) promotion of research and development of novel antimicrobial strategies and antibacterial agents and; (g) improving of general awareness of antibiotic use.

2. The *Enterococcus* genus: historical prospective

The history of taxonomy of enterococci started in 1899 when Thiercelin described a saprophytic microrganism that pullulates in human gastrointestinal (GI) tract along with *Bacterium coli* (Thiercelin & Jouhaud, 1899). It was described as a Gram-positive diplococcus for which it was proposed the name "*Enterocoque*" to emphasize its morphology and its intestinal origin. Afterwards, Andrewes and Harder renamed the strain as *Streptococcus faecalis* for its ability to form short or long chains. The carachteristics of the strain were the pathogenicity towards mice and the ability to become pathogenic for humans (Thiercelin & Jouhaud, 1899). Shortly after, numerous strains were isolated from sick patients and the typical features of the genus were defined, recognizing these microorganisms as "*very hardy and tenacious of life*" (MacCallum & Hastings, 1899). Subsequently, Orla-Jensen reported the first description of *Streptococcus faecium*, which differed from

the fermentation patterns described for *Streptococcus faecalis* (Orla-Jensen, 1919). In 1930, the identification and classification were based on the serological groups introduced by Lancefield, regrouping all enterococci strains into the antigen D group together with *Streptococcus bovis* and *Streptococcus equinus*, that showed the presence of traits which allow to group them separately from the enterococci. Sherman (1937) divided the streptococci into four groups: (1) the enterococci; (2) the lactic-; (3) the viridans-; and (4) the pyogenic streptococci. The term enterococci have been used to designate microorganisms able to growth at 10 and 45°C, in 6.5% NaCl, and at pH 9.6 and which survived 60°C for 30 min; the ability to split esculin was also noted (Murray et al., 1990). Many of these characteristics became widely used to distinguish between enterococci and non-enterococcal streptococci. As early as 1970, Kalina (1970) proposed the creation of the taxon *Enterococcus*, based on cellular arrangement and phenotypic characteristics of species in the enterococcal group. However, this genus was not formally accepted until 1984, when Schleifer and Kilpper-Balz (1984) provided genetic evidence that *S. faecalis* and *S. faecium* were sufficiently distant from other members of the *Streptococcus* genus.

2.1 Criteria for identification

Nowadays, the *Enterococcus* genus is classified within the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Enterococcaceae*. This genus is composed of more than 50 species http://www.bacterio.net/enterococcus.html, of which *E. faecium* and *E. faecalis* are the species most

commonly recovered from humans. However, several other species (*E. avium, E. durans, E. hirae, E. casseliflavus, E. gallinarum*) are also able to colonize the GI tract of humans and may sporadically cause human infections (Agudelo et al., 2014; Lebreton et al., 2014). Enterococci are Gram-positive cocci (spherical cells) frequently arranged in pairs (diplococci), non-spore-forming, facultative anaerobes and obligatory fermentative chemoorganotrophs. They typically growth at the optimal temperature of 35°C, being able to growth also in the range from 10 to 45°C (Sherman, 1937). They typically are able to growth in medium containing 6.5% NaCl and to hydrolyze aesculin in the presence of 40% bile salts (Facklam, 1973). They are catalase negative, and do not express complete cytochromes, although some species produce a catalase and appear catalase positive with weak effervescence. This behaviour is due to the lack of cytochrome enzymes which also influences the homofermentative metabolism, lactic acid being the end product of glucose fermentation. They are usually homofermentative, producing lactic acid as the end product of glucose fermentation, without production of gas (Klein, 2003; Murray et al., 1990). Indeed, enterococci are often simply described as lactic-acid–producing bacteria, a designation that understates their vast metabolic potential. The ability to utilize hexose and pentose carbohydrates is a key component of metabolism for enterococci: carbohydrate fermentation allows this genus to thrive in diverse environments. At least 13 sugars are metabolized by *Enterococcus* species and over 30 more are utilized by at least two members of the genus (Gilmore et al., 2014). Some species are motile, such as *E. gallinarum* and *E. casseliflavus* (Graudal, 1957; Mundt, 1986) and others present pigmentation that represents a variable trait: *E.*

sulfureus, *E. casseliflavus*, and *E. mundtii* are yellow-pigmented species, commonly found among plants (Aarestrup et al, 2002; Graudal, 1957; Martinez-Murcia & Collins, 1991; Mundt, 1986). Enterococci are capable of hydrolyzing pyrrolidonyl β-naphthylamide and producing leucine aminopeptidase (Facklam et al., 2002; Naser, et al., 2005; Rahkila, et al., 2011; Švec, et al., 2005; Svec, et al., 2006). In addition, their ability to metabolize a broad range of energy sources (complex carbohydrates, glycerol, lactate, citrate, malate, amino acids such as arginine, and some alpha-keto acids), their capacity to tolerate oxidative stress, as well as a wide variety of compounds (heavy metals, azide, detergents, biocides) and prolonged desiccation allows this bacterial genus the survival in diverse habitats (Faklam et al., 2002; Lebreton et al., 2014; Murray, 1990). Enterococci are also known for being somewhat fastidious bacterial needing a certain number of amino acids and vitamins for maximum growth (Faklam et al., 2002; Lebreton et al., 2014). *Enterococcus faecalis* has been known to require a number of amino acids (including Val, Leu, Ile, Ser, Met, Glu, Arg, His and Trp) and vitamins (including biotin, nicotinic acid, pantothenate, pyridoxine, riboflavin, and sometimes folic acid) for maximal growth, with other species being similar in their fastidiousness (Facklam et al., 2002). Facklam and collaborators (2002) divided enterococcal species into 5 groups based on acid formation in mannitol and sorbose broth, as well as hydrolysis of arginine (Tab. 1). It is important to note that this phenotypic classification should be distinguished from the 16S rRNA sequence relationship, so although these tests are potentially useful for diagnostics, they do not appear to reflect the evolutionary relationships between *Enterococcus* species. **Group I** consists of enterococcal species that form

acid in both carbohydrate broths, but do not hydrolyze arginine. **Group II** includes *E. faecalis* and *E. faecium*, and consists of species that form acid in mannitol broth and hydrolyze arginine, but fail to form acid in sorbose broth. **Group III** includes species that are not able to form acid in either mannitol or sorbitol broth, but that hydrolyze arginine. In **Group IV** the species are negative for acid formation in mannitol and sorbose broth and do not hydrolyze arginine (Table 1). At last, **Group V** consists of the species that form acid on mannitol, but not in sorbose broth, and fail to hydrolyse arginine (Facklam et al., 2002; Gilmore et al., 2014). Recently, Zhong and coworkers (2017) showed that the genes for carbohydrate metabolism were the most common genes found in the genome of *Enterococcus*. On average, 15.4% of genes in each genome were associated with carbohydrate metabolism. However, the number of genes involved in carbohydrate metabolism varied significantly among strains.

Table 1. Facklam et al., Enterococci classification

Group	Species	MAN	SOR	ARG
I	*E. avium, E. malodoratus, E. raffinosus, E. pseudoavium, E. saccharolyticus, E. pallens, E. gilvus, E. phoeniculicoa, E. devriesei, E. canis*	+	+	-
II	*E. faecalis, E. faecium, E. casseliflavus, E. gallinarum, E. canintestini, E. lactis, E. thailandicus, E. sanguinicola*	+	-	+
III	*E. villorum, E. durans, E. dispar, E. hirae, E. silesiacus, E. rotai**	-	-	+
IV	*E. asini, E. sulfureus, E. cecorum, E. aquamarinus, E. plantarum, E. caccae, E. termitis*	-	-	-
V	*E. columbae, E. rivorum, E. hermaniensis, E. camelliae, E. viikiensis*	+	-	-
VI	*E. ureilyticus*	-	+	-

*some variants of *E. faecium* and *E. faecalis* that do not hydrolyse mannitol can also be found in this group. **some variants of *E. casseliflavus, E. gallinarum* and *E. faecalis* that do not hydrolyse arginine can also be included in this group. Abbreviations: MAN, mannitol; SOR, sorbose; ARG, Arginine.

There are 82 genes associated with carbohydrate metabolism in the core-genome of *Enterococcus*, accounting for 13.6% of the core genes. These core genes are mainly involved in two microbial carbohydrate metabolism pathways, the pentose phosphate pathway (Fig. 2) and the glycolysis/gluconeogenesis pathway (Fig. 3).

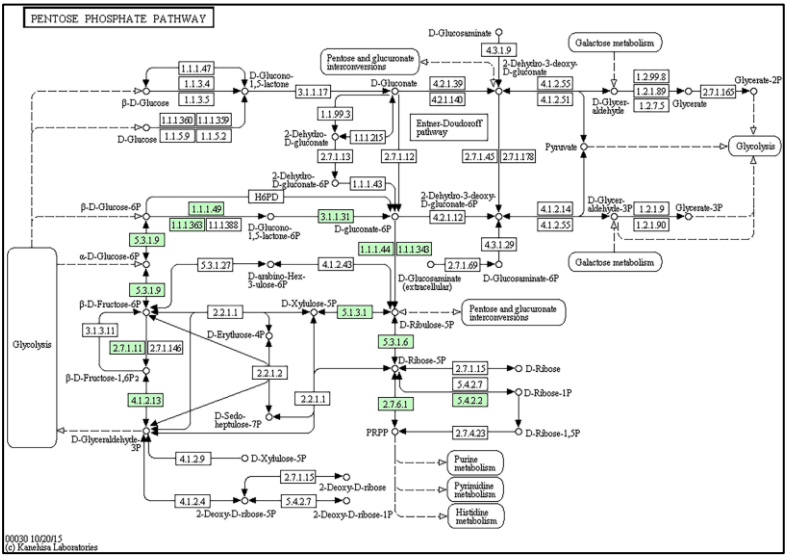

Figure 2. The core genes involved in the pentose phosphate pathway. The pathway map was obtained from KEGG database (Kanehisa et al., 2016). The core genes are labelled with gray in the pathway.

A previous research has shown that the Entner-Doudoroff carbohydrate metabolism pathway is also present in *Enterococcus* species (Gilmore et al., 2014), but only genes associated with the pentose phosphate pathway and the

glycolysis/gluconeogenesis were found in the core-genome. This could indicate that the pentose phosphate and the glycolysis/gluconeogenesis are the main carbohydrate metabolism pathways in *Enterococcus.* Holzapfel and Wood (2014) studing phenotypic traits demonstrated that the transport and utilization of some carbohydrates were species-specific within the *Enterococcus* genus.

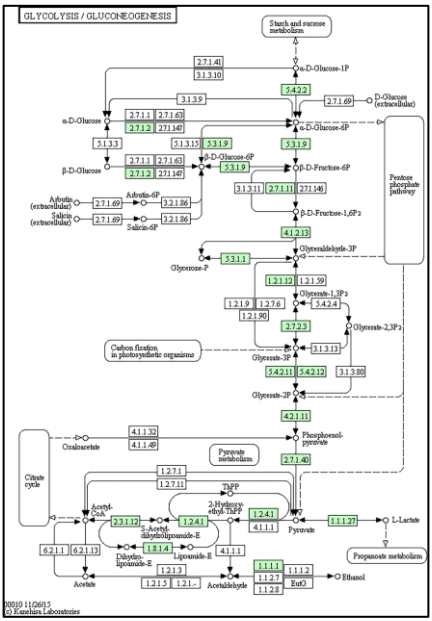

Figure 3. The core genes involved in the glycolysis/gluconeogenesis pathway. The pathway map was obtained from KEGG database (Kanehisa et al., 2016). The core genes are labelled with gray in the pathway.

2.2 Phylogeny

The genus *Enterococcu*s has undergone a considerable taxonomic revision, which has led to a more accurate taxonomic grouping and increased the number of distinguishable species assigned to genus. Phylogenetic analysis of catalase-negative Gram-positive cocci, based on the comparison of ~1400 bases of the 16s rRNA gene, showed that the *Enterococcus* are more closely related to *Vagococcus*, *Tetragenococcus*, and *Carnobacterium* than they are to the *Streptococcus* and *Lactococcus* (Facklam et al., 2002; Gilmore et al., 2014). The G + C content of DNA ranges from 37 to 45 mol%. As a reflection of their highly evolved role as members of a consortium in an extremely competitive environment, enterococci have reduced their genomes, that range from 2.7 Mb to 3.6 Mb across species sequenced so far (Gaechter et al., 2012; Qin et al., 2012; Palmer et al., 2012; Van Schaik & Willems, 2010). Enterococcal genome sizes span a surprisingly wide range from 2.3 Mb (*E. sulfureus*) to 5.4 Mb (*E. pallens*) (Lebreton et al., 2017). These differences in genome size may indicate that the evolution of *Enterococcus* is coupled with different levels of horizontal gene transfer, including gene insertion and deletion (Zhong et al., 2017). Species belonging to *Enterococcus* genus were identified by DNA-DNA reassociation, 16S rRNA gene sequencing, and/or whole-cell protein analyses. According to the topology of robust phylogenies obtained by Zhong and Lebreton (Fig. 3) and previous research (Holzapfel and Wood, 2014), the *Enterococcus* strains can be distinguished into six branches. The *E. faecium* branch contained *E. faecium, E. mundtii, E. durans, E. hirae, E. ratti, E. villorum, E. thailandicus and E. phoeniculicola*, which were mainly isolated from

bloodstream and intestinal tract. The *E. faecalis* branch contained *E. faecalis, E. termitis, E. quebecensis, E. moraviensis, E. caccae, E. haemoperoxidus* and *E. silesiacus*, which were mainly isolated from water and intestinal tract. The *E. dispar* branch contained *E. dispar, E. canintestini* and *E. asini*, which were mainly isolated from the intestinal tracts of humans and mammals. The *E. casseliflavus* branch contained *E. casseliflavus, E. gallinarum, E. aquimarinus, E. saccharolyticus, E. italicus, E. sulfureus, E. cecorum* and *E. columbae*, which were mainly isolated from plant materials and intestinal tracts of birds. The *E. pallens* branch contained *E. pallens, E. hermanniensis, E. devriesei, E. gilvus, E. malodoratus, E. avium* and *E. raffinosus*, which were mainly isolated from humans and mammals. The *E. canis* branch contained only one strain, which was isolated from chronic otitis externa in dogs. This strain was closest to the outgroup strains. When relating the source of *Enterococcus* to the lineages, it has been found that most strains from humans and mammals were dispersed in the branches of *E. faecium*, *E. dispar* and *E. pallens*. The strains isolated from plant material and intestinal tracts of birds were mainly distributed in the *E. casseliflavus* branch. The most of strains isolated from water were dispersed in the *E. faecalis* branch. These results suggest that habitat is very important in the evolution of *Enterococcus*. Genetic relationships were closer in strains that had similar habitats. This potential relationship between source distribution and genealogy provides us with a clue to the evolution of the *Enterococcus* genus (Zhong et al., 2017). So, one possible evolutionary scenario is that humans and mammals may be the original hosts of *Enterococcus*, and then species from humans and mammals made a host-shift to plants, birds, food and other

environments. It is therefore surprising that these two species are at opposite ends of the phylogenetic tree (Fig. 4). *E. faecalis* occurs in one of the oldest branches of the genus, whereas *E. faecium* arose more recently. However, their common occurrence in the human intestine and in hospital infection shows that they possess common features allowing them to inhabit similar ecologies (Van Tyne and Gilmore, 2015).

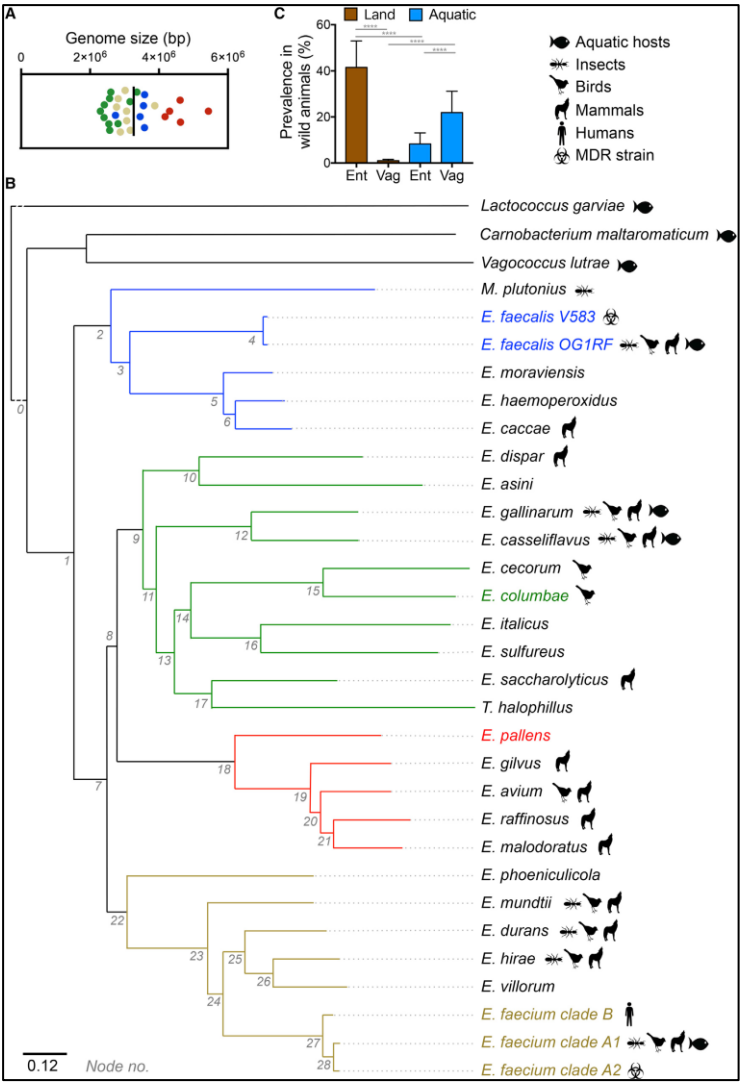

Figure. 4. Genome-Based Phylogeny of the *Enterococcus* genus.

(A) Distribution of *Enterococcus* species genome sizes (color-coded by phylogenetic group).

(B) Phylogenomic tree of *Enterococcus* and outgroup species (gray) based on alignment of 526 single copy core genes. Species groups: *E. faecalis* group (blue), *E. columbae* group (green), *E. pallens* group (red), and *E. faecium* group (yellow). Each node is assigned a unique numeric identifier. Icons represent host associations inferred from both qualitative and quantitative analyses of the literature.
(C) Prevalence of *Enterococcus* and *Vagococcus* in the GI tract of land (n = 492) or aquatic hosts (n = 1,300) isolated from the wild. Presence or absence was determined by 16S rDNA analysis of microbiome samples available in public databases. Mean and SEM are indicated. Statistical significance was evaluated by Fisher's exact test (Lebreton et al., 2017).

2.3 Ubiquity of the genus

The enterococci belong to an ancient genus of microorganisms that are highly adapted to living in complex environments and surviving harsh conditions. Probably, they inhabited the GI tracts of our ancient common ancestors, placing them among the earliest members of a symbiotic consortium, a microbiota that we now appreciate serves roles ranging from facilitating digestion to providing the host with development cues and protection from colonization by more antagonistic microorganisms (Lebreton et al., 2014). However, studies on their native ecology, demonstrate their principal habitat in the GI tracts of a wide variety of animals, from insects to man, foreboding that most of the evolutionary refinement of these organisms occurred prior to the advent of humans. Although enterococci constitute a large proportion of the autochthonous bacteria associated with the mammalian GI tract, they are able to colonise diverse niches because of their exceptional aptitude to resist or grow in hostile environments. Therefore, enterococci are not only

associated with warm-blooded animals, but they also occur in soil, surface waters and on plant and vegetables. Indeed, research spanning more than 3 decades has shown that these bacteria are widely distributed in a variety of environmental habitats, even when there is little or no input from human and/or animal fecal sources. These extra-enteric habitats include soil and sediments, beach sand, aquatic and terrestrial vegetation, and ambient waters (rivers, streams, and creeks) (Fig. 5), heterothermic habitats, in which temperatures are variable, in contrast to the GI tract of warm-blooded animals, where the temperature is relatively constant (Byappanahalli et al., 2012). The transition from the animal GI tract, an environment rich in many nutrients with key nutrients restricted, to oligotrophic environments that are generally nutrient-poor and a variety of environmental stressors such as UV sunlight, salinity, starvation, and predation (Byappanahalli et al., 2012) expose enterococci to nutrient starvation. However, the remarkable ability of enterococci to share new carbon utilization mechanisms between strains and species, often on mobile elements and the metabolism of a broad array of carbohydrates, included naturally abundant carbohydrate polymers, provides them with a robust advantage in colonizing competitive environments, especially the GI tracts of nearly every phylum within the animal kingdom (Ramsey et al., 2014). Their auxotrophic nature (requiring up to nine amino acids and several vitamins) suggests that enterococci thrive in the context of highly co-evolved relationships with other organisms, acquiring nutrients from their host and/or from cross-feeding relationships with other microorganisms.

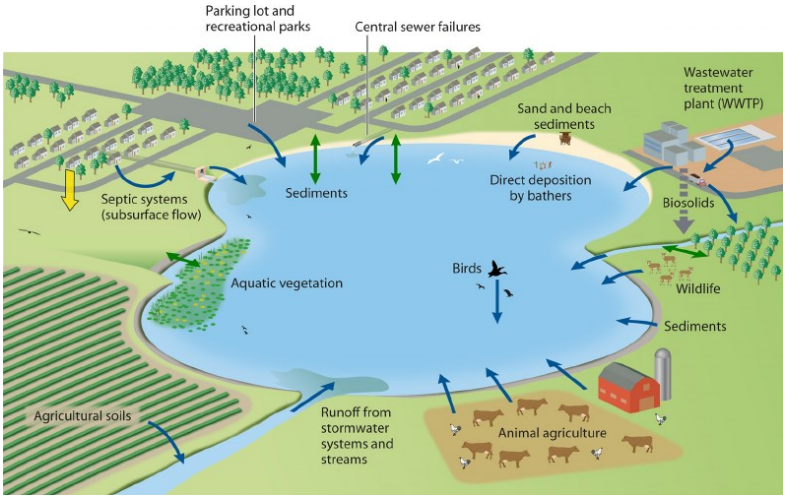

Figure 5. Sources of enterococci in water bodies (blue arrows) as well as sinks where enterococci are immobilized (yellow arrow) and areas of flux, in which enterococci can transition from a reservoir to the water column and vice versa (green arrows). Fluxes act as secondary sources or sinks depending upon the conditions. (Byappanahalli et al., 2012).

2.3.1 Enterococci in human

Enterococci are commonly found in the GI tract of healthy humans and animals (Vankerckhoven et al., 2004). The numerous studies carried out on the human GI tract and faeces showed that the GI tract is a primary habitat for the genus *Enterococcus* and many species have been reported colonizing the GI tracts of humans. In the human gut, the genus can constitute up to 1% of the total bacterial microbiota in healthy individuals, with *Enterococcus faecium* and *Enterococcus faecalis* being the most common

species (Sghir et al., 2000). In detail, enterococci are primarily localized into the human small and large intestine, where they are prominent members of jejunal, ileal, cecal, and recto-sigmoidal consortia (Hayashi et al., 2005), for this reason they are largely present in human faeces (Eckburg, et al., 2005; Sghir et al., 2000). Enterococci are common also in the oral cavity (Smyth et al., 1987), but occur more rarely in the stomach (Bik, et al., 2005; Monstein et al., 2000). So while *E. faecalis* and *E. faecium* are most common in human feces, *E. durans* and *E. avium* are occasionally detected (Finegold et al., 1983; Tannock & Cook, 2002). *E. caccae* was isolated from human faeces, but its prevalence is low (Carvalho, et al., 2006). *E. avium*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. hirae* are species found in human faecal specimens while *E. casseliflavus* and *E. saccharolyticus* were not commonly observed (Layton et al., 2010). Other members of the genus *Enterococcus,* identified in clinical samples, are rarely part of the normal human intestinal microbiota, but are present in the gut of other animals or on plants. Some members of the microbiota, including indigenous commensal enterococci, can act as opportunistic pathogens and translocate across the mucosal barrier causing systemic infection in immune-compromised hosts (Berg, 1996; Donskey, 2004). More commonly, however, infection results from colonization, overgrowth, and translocation of hospital-adapted antibiotic-resistant strains with enhanced pathogenicity, even if studies using animal models have shed light on the mechanisms of microflora-mediated colonization resistance to enterococci (Brandl, et al., 2008; Kinnebrew et al., 2010). *E. faecalis* and *E. faecium* can cause urinary tract infections, wound infections, bacteraemia, and infective endocarditis.

2.3.2 Enterococci in animals

Also animal GI tracts likely represent the greatest reservoir for enterococci (Gilmore, et al., 2013). The most commonly encountered enterococcal species in the gut of mammals are *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* (Devriese & De Pelsmaecker, 1987). Other species are found only occasionally, or in particular age groups (such as *E. cecorum* in older poultry) (Aarestrup et al., 2002). In fact, an age-dependent succession of enterococcal species colonization appears to occur in chickens and in cattle (Devriese et al., 1991, 1994; Kaukas et al., 1987). Chickens are initially colonized by *E. faecalis*, but this population is then displaced, mainly by *E. faecium,* and it has been proposed that the use of tylosin (to which *E. faecium* is commonly resistant) as a growth promoter is the reason of this replacement (Kaukas et al., 1987). These species then appear to be replaced by *E. cecorum* in the mature chicken. It is interesting to highlight that *E. avium* and *E. gallinarum*, originally described from chickens, were rarely found, suggesting that these species may not belong to the normal intestinal microbiota of poultry (Devriese et al., 1991). *E. avium* was originally described from human faeces (Guthof, 1955), but is is common in chicken faeces (Nowlan & Deibel, 1967). The extent to which the microbiota of chickens reflects that native to poultry, or reflects the consequences of intensive production and modern poultry husbandry practices (namely, the frequent use of antibiotics including aminoglycosides), is controversial, but has undoubtedly impacted the representation of various species of enterococci (Aarestrup et al., 2002; Devriese et al., 1994). In preruminant calves, the enterococcal microbiota mainly consists of *E. faecalis*, *E. faecium,* and *E. avium*,

gradually replaced by *E. cecorum* (Devriese et al., 1992). The most prevalent enterococcal species isolated from intestines of swine are *E. faecalis* and *E. faecium*, which occurs in low numbers (Devriese & De Pelsmaecker, 1987; Devriese, et al., 1994). Other enterococcal species found among swine microbiota include *E. hirae* and *E. cecorum* (Devriese et al., 1987; Devriese & Haesebrouck, 1991; Devriese, et al., 1994). *E. faecalis* was also the most frequently encountered enterococcal species in the cats and dogs gut, as well as on the tonsils of these animals, with *E. faecium* and *E. hirae* occurring infrequently (Devriese et al., 1992). Other species, including *E. avium*, *E. raffinosus*, *E. durans*, *E. cecorum*, and *E. gallinarum* were also occasionally isolated (Devriese et al., 1992). *E. canis* and *E. canintestini* have more recently been isolated from healthy dogs (De Graef, et al., 2003; Naser, et al., 2005). *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum*, E. *casseliflavus,* and *E. mundtii* have also been found in horses (Devriese & De Pelsmaecker, 1987; Thal, et al., 1995). Finally, a large variety of insects, including beetles, flies, bees, termites, and worms have been found to harbor enterococci, mostly *E. faecalis* and *E. faecium*, even if other species occur but at lower prevalence (Lebreton et al., 2014).

2.3.3 Enterococci in the environment

Among the extra-enteric niches where enterococci are routinely isolated, there are soil and sediments, aquatic and terrestrial plants, and ambient waters. It is unclear whether these microganisms are contaminants from faecal consortia that are simply surviving or whether these environments actually constitute a habitat. Despite the auxotrophies of

enterococci, some studies indicate that they are able to replicate in the environment, possibly as the result of collaboration within a polymicrobic consortium. Survival ability results from their tolerance to UV irradiation, salt, starvation, and possibly predation by protozoa and bacteriophages (Van Tyne & Gilmore, 2015; Byappanahalli et al., 2012). This inherent ruggedness has no doubt contributed to their emergence in the harsh environment in modern hospital. Anyhow, the occurrence of enterococci has been investigated in association with various vegetation, including algae (Whitman et al., 2003), beach wrack (Anderson et al., 1997; Grant, et al., 2001; Inamura et al., 2011), submerged vegetation (Badgley et al., 2010), flowering plants (Mundt, 1963), and forage crops (Mülleret al., 2001; Byappanahall et al., 2012). Several recent studies showed that *Enterococcus* species identified on plants, such as *E. plantarum* that was isolated from plant samples obtained from a meadow in the Czech Republic, are not influenced by farm animals and exploited only for hay harvesting (Švec, et al., 2011). Their persistence into environment is also linked to large quantities of human and animal wastes rilesead into the environment through sewage or non-sewage systems. Human wastes are discarged into waterways, usually through a sewage treatment plant, while animal wastes are often used composted (but otherwise untreated) as fertilizers on fields. In these ecosystems *E. faecalis* is the predominant species, but different species, including *E. pseudoavium*, *E. casseliflavus*, *E. faecium*, *E. mundtii*, *E. gallinarum*, *E. dispar*, *E. hirae*, *E. durans*, *E. flavescens*, *E. haemoperoxidus*, and *E. moraviensis* can also be recovered (Kühn, et al., 2003). This may have implications for the identification of faecal pollution based on the presence of specific bacterial types associated with

domestic wastewater (Graves & Weaver, 2010). In water, the species considered as faecal contaminants are mainly *E. faecium* and *E. faecalis*, and the origins of other species are less clear (Aarestrup et al., 2002). Enterococci are found in both fresh (Byappanahalli et al., 2012; Fujioka et al., 1998) and marine water (Boehm, et al., 2002; Korajkic et al., 2011), but it is unclear if the growth in the absence of essential nutrients occur. Enterococci occur in temperate and tropical soils (Byappanahalli et al. 2012; Mundt, 1961), fresh or marine water sediments (Ferguson et al., 2005; Obiri-Danso & Jones, 2000), and can be recovered from sand (Halliday & Gast, 2011; Yamahara et al., 2007). A prolonged survival of enterococci has been observed in freshwater and estuarine sediments (Anderson et al., 2005). Interestingly, many of the early investigations on survival and persistence of enterococci in soil environments focused on watersheds affected by anthropogenic activities, particularly in cattle grazing and field lot operations (Byappanahalli et al., 2012). All samples taken from urban sewage, and from farmland using pig manure, as well as crops grown on this land, readily yielded *Enterococcus* species (Kühn, et al., 2003). In crops, to which animal fertilizer had not been applied, the incidence of *Enterococcus* species is reduced to 33% (Kühn, et al., 2003). *Enterococcus* species can be recovered from sand from freshwater and marine beaches (*E. faecium*, *E. casseliflavus*, *E. durans*), and from marine sediments (*E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, and *E. mundtii*) (Ferguson et al., 2005). As for other environments, whether enterococci grow as free-living organisms in sediments, sands, or soils remains highly speculative.

2.3.3.1 Enterococci as faecal indicators

For over a century, faecal indicator bacteria (FIB) have been used to assess water quality and protect humans from the myriad of enteric pathogens that are transmitted by the water route. However, there is no universal agreement on which indicator organism(s) is most useful, nor are there federal regulations mandating a single standard for bacterial indicators. Thus, different indicators and different indicator levels, identified as standards, are used by water quality programs in different states, countries, and regions. Today, the most commonly measured bacterial indicators are total coliforms, faecal coliforms, and enterococci (Noblea et al., 2003). They are generally commensal inhabitants of the GI tracts of many warm blooded animals and are shed in faeces at high densities; thus, they are easily detected in contaminated waters. Although many researchers believe that enterococci might be more appropriate FIB than *E. coli*, their use as FIB has been criticized almost since their adoption as a regulatory tool (Byappanahalli et al. 2012; Colford et al., 2012), because epidemiology studies on which the standards were based were focused solely on waters contaminated by point source (particularly human sewage) pollution. Characteristics associated with "ideal" FIB include: lack of virulence; existence of a simple, rapid methodology for enumeration; survival characteristics similar to those of pathogens in extra-enteric environments; and a strong association with the presence of pathogens (Cabelli et al., 1979). In contrast, several studies have shown that enterococci may be endogenous in sediments and soils and not exclusively of faecal origin, which may confound accurate water quality assessments (Byappanahalli et al. 2004; Desmarais et al., 2002).

Furthermore, many domestic and wild animals can contribute to release enterococci into water bodies (Table 2), which complicates the FIB pathogen relationship, since the suite of pathogens associated with various animal GI tracts and the risk associated with faecal contamination are highly variable. Other important sources are agricultural contributions, which may come directly from animals, e.g., cattle or swine defecating in and near water bodies, or indirectly from activities such as the spreading of manure or poultry litter on fields (USEPA, 2005.). Wildlife (e.g., birds, deer, feral hogs, and raccoons) can be sources of enterococci in urban and rural environments, either via direct deposition or in runoff. Due to the potential for introduction by sources other than human faeces and the persistence of enterococci in the environment, high levels may not indicate continuous addition of human faecal wastes into an area and therefore may present confounding information for regulators charged with assessing water quality (Byappanahalli et al., 2012). In 2012, the EPA recommends to use as faecal indicators *Escherichia coli* and enterococci, for fresh water and marine water, respectively, based on epidemiological evidences (Cabelli et al., 1982; Cabelli, 1983; Dufour, 1984). Both indicators continue to be used in epidemiological studies conducted throughout the world, including in the European Union (E.U.) and Canada (EP/CEU, 2006; MNHW, 1992). In this scenario it is interesting to highlith that the World Health Organization (WHO) recommends the use of enterococci as water-quality indicators for recreational waters (WHO, 2003).

2.3.4 Enterococci in food

It is noteworthy that the enterococci belong to the lactic acid bacteria (LAB) group, maybe the main microbial group involved in food microbiology. These bacteria may play an important beneficial role in the production of various traditional fermented food products in Europe and have benn successfully used as probiotics. The presence and growth of enterococci in fermented foods, such as cheeses and sausages, results in sensorial unique traits of final products which contribute to the excellence of local cuisine and heritage of many Mediterranean regions, distributed in worlwilde markets (Franz et al., 2003). Endeed, enterococci contribute to the aroma development of these products due to their proteolytic and esterolytic activities, as well as the production of diacetyl. Although, the presence of enterococci in dairy products has long been considered as an indication of insufficient sanitary conditions during the production and process, actually, many authors suggest that the enterococci have a desirable role in some cheeses because they occur in large numbers (up to 10^7 to 10^8 CFU per g) as natural consortia of many cheeses. Bacteriocins production together with desirable technological and metabolic traits make enterococci a part of starter cultures defined for several European cheeses. Clearly, the presence of enterococci is ineluctable in many dairy products so that the Commission Regulation (EC) No 1441/2007 of 5 December 2007 allows derogation from Regulation (EC) No 2073/2005 of 15 November 2005 ‘on microbiological criteria for foodstuffs’, declaring that enterococci in food are not always due to faecal contamination and sets no limit for their presence in foods (Commission Regulation, 2007). The isolation of enterococci from natural milk starter

cultures, which are still widely used for many Italian soft cheeses made with raw or pasteurised milk, can be explained by their thermal resistance. Moreover their persistence during ripening can be attributed to their wide range of growth temperatures and their tolerance to pH and salt. *E. faecium* and *E. faecalis* are the most prevalent species recovered (Franz et al., 1999). However, they are not considered as GRAS (Generally Recognized As Safe) organisms (Giraffa et al., 1997) and have been not recommended in the QPS (Qualified Presumption of Safety) list by the European Food Safety Authority (EFSA) because of their potential role in human clinical infections associated with endocarditis, bacteraemia, urinary tract infections and antimicrobial resistance (EFSA, 2013b). The overall data on AR within food-associated enterococci open the question of their entering the food chain. The extremely high level of AR observed in enterococci and their widespread finding in raw foods are two key elements contributing to the frequent recovery of AR enterococci, in both unfermented and fermented foods. AR enterococci have been found in meat products, dairy products, ready-to-eat foods and even within enterococcal strains proposed as probiotics (Giraffa, 2002). Several studies have provided strong evidences that enterococci originating from foods of animal origin had a remarkable degree of similarity in virulence characteristics with human isolates, implicating animal meat as an important source of virulent enterococcal strains for human colonization. Furthermore, exists a strong epidemiological evidence of link between the use of antibiotics in human medicine and animal husbandry and the emergence, spreading and persistence of resistant strains in animal products (Witte, 2000). Food-associated enterococci could therefore be a reservoir for AR. Once ingested, AR

enterococci can survive gastric passage and multiply, thus leading to sustained intestinal carriage (Sørensen et al., 2001). High level of vancomycin resistant enterococci (VRE) strains, especially belonging to *E. faecium*, was found at high frequencies in beef, poultry, pork and other meat products and also in both industrial and artisan cheeses (Giraffa, 2002). The *van*A type glycopeptide resistance, which confers coupled resistance to both vancomycin and teicoplanin, seems the most frequent vancomycin-resistant phenotype among food-associated VRE. The chronic use of antibiotics as growth promoters in livestock is recognised as factor acting a selective agent in promoting resistant enterococci. Therefore, although a connection between the occurrence of VRE in meat and nosocomial infections has not yet been clearly proved, epidemiological data would suggest that both clonal dissemination through food chain and horizontal gene transfer among a variety of different strains are involved in the VRE spreading outside the hospital (Giraffa et al., 2002). The presence of enterococci in GI tract of animals leads to a high potential for contamination of meat at the time of slaughter. Enterococci were consistently isolated from beef, poultry or pig carcasses or fresh meat and they have been also associated with processed meats. Heating processed meats may confer a selective advantage on enterococci being among the most thermotolerant non-sporulating bacteria (Sanz Perez et al., 1982; Magnus et al., 1988). After surviving the heat-processing step, both *E. faecalis* and *E. faecium* can be involved in spoilage of cured meat products, such as canned hams and chub-packed luncheon meats. Although for many years belived important in the food fermentation, for contributing to sensorial traits of several foodstuffs, these microorganisms are undoubtedly at the crossroads of food

safety and acceptance for use in food. Evidences indicate dissemination of pathogenic strains through food chain (Soares-Santos et al., 2015), making the safety of food products harboring enterococci a matter of concern and highlighting the importance of discriminating between food grade and pathogenic variants.

3. Enterococci population structure

Studies on the population structure of enterococci have been performed to discern whether structure can be identified among enterococcal populations linked to certain ecological niches (Willems et al., 2011). Specifically, considerable research has been carried out to discern whether clinical isolates of *E. faecalis* and *E. faecium* are genetically distinct from strains that inhabit the GI tract of animals or healthy humans (Werner et al., 2013).

Alongside the common methods used to investigate the diversity among enterococcal isolates, the molecular techniques has substantially improved the ability to discriminate enterococcal isolates and has provided critical insights into the epidemiology of enterococci. Several molecular typing techniques now used to trace the dissemination of enterococci in different environments and hosts, to set phylogenetic relationship, and to ouline the evolution of multidrug-resistant strains, are greatly expanding our understanding of enterococcal epidemiology, population structure, antimicrobial resistance, and virulence. Thanks to these tecniques, emergence and global dispersion of certain epidemic enterococcal clonal complexes have been identified. Different methods have been used to discriminate strains with a high epidemicity potential. These

tecniques include PFGE (Pulsed Field Gel Electrophoresis), MLVA (Multilocus Variable number tandem repeats Analysis), MLEE (Multilocus Enzyme Electrophoresis), MLST (Multilocus Sequence Typing) and more recently, cgMLST and whole genome sequencing (WGS) (Coque et al., 1998; Sadowy et al., 2011). Different schemes of MLST analysed by eBURST and goeBURST constitute, nowday, the most used method to analyse the population structure of *Enterococcus* spp. (Chowdhury et al., 2009; Nallapareddy et al., 2002). By PCR amplifying and sequencing seven conserved loci (*aroE, gdh, gki, gyd, pstS, xpt* and *yqiL*), isolates can be further divided into more than 500 unique *E. faecalis* MLST types and 800 types of *E. faecium.* However, these methods showed limitations, such as poor reproducibility and/or high technical complexity. MLST is based on identifying alleles after sequencing of internal fragments of a number of selected housekeeping genes, resulting in a numeric allelic profile. Each profile is assigned to a sequence type (ST). Internet sites with the possibility for data exchange have been developed (www.mlst.net and www.pubMLST.org), which contain MLST protocols for *E. faecium* (http://efaecium.mlst.net/misc/info.asp) and *E. faecalis* (http://efaecalis.mlst.net/misc/info.asp). MLVA is based on differences in variable-number of tandem repeats (VNTR) in multiple loci dispersed over the enterococcal genome. Overall, there is no single definitive method to type enterococci, so a strong match among the results of different typing techniques, particularly those based on different genomic polymorphisms, should be used as indicative of high relatedness (Palmer et al., 2014). Despite the overt lack of reproducibility, PFGE long remained the “gold standard” for molecular typing of *E. faecium* until the introduction of

whole genome sequence (WGS)-based epidemiology (Prieto et al., 2016).

3.1 *E. faecium* population structure

The first analyses of *E. faecium* MLST data performed with the algorithm eBurst (Feil et al., 2004), confirmed a distinct clustering of strains derived from hospital environment. This analysis revealed that clinical and outbreak hospital strains of *E. faecium* grouped in one Clonal Complex (CC), designated CC17 as all STs appeared to descend from the founder ST17, and that these strains were different from those isolated from healthy community humans and animals (Homan et al., 2002; Feil et al., 2004). Strains within CC17 were labelled as hospital-associated *E. faecium* and have been isolated from hospitals worldwide. Most CC17 strains are MDR (resistant to vancomycin, ampicillin, aminoglycosides and fluoroquinolones) and their genomes appear to be enriched in genes coding for putative virulence genes, megaplasmids, and insertion sequences (ISs) (Tedim, 2017). More advanced studies using BAPS (a statistical model based on both clonal ancestry and recombination patterns using concatenated sequences of the 7 MLST genes) revealed the existence of two BAPS groups (2-1 and 3-3), associated with antibiotic resistance (ampicillin and vancomycin) and the hospital setting. When applied to *E. faecium,* MLST data BAPS allowed the partitioning of 519 STs of 1720 *E. faecium* isolates into 13 non-overlapping groups. Of these groups, BAPS 3–3 was significantly associated with isolates from hospitalized patients, while BAPS2–1 and 2–4 were significantly associated with farm animals. These observations again confirmed that existence

of a structure in the *E. faecium* population, with a distinct subpopulation of isolates that are almost exclusively found in hospitalized patients (Willems et al., 2012; Prieto et al., 2013). Later it was demonstrated that clinical isolates of these groups are related to ST17 and ST18 lineages (BAPS 3-3), and ST78 lineage (BAPS 2-1). BAPS analysis also showed that strains normally colonizing healthy (community) humans belong to BAPS 1 and that these isolates can be genetic and evolutionary different from hospital associated strains, which were more closely related to animal strains, indicating a possible role of animals in the emergence of current hospital associated isolates (Palmer et al., 2014). The recent description of a cgMLST allowed to further look in these lineages and revealed epidemiological links (or lack thereof) in groups of strains where this links were not apparent (de Been et al., 2015) making it a valuable tool for future epidemiological analysis. In 2012, the first *E. faecium* closed genome was published (Lam et al., 2012) and up to date only three more closed *E. faecium* genomes have been released (Lam et al., 2013; Qin et al., 2012). Three of these strains are representatives of major lineages of hospital-associated *E. faecium* (ST16, ST17 and ST203) (Palmer et al., 2014; Lam et al., 2013; Qin et al., 2012). The other *E. faecium* genome correspond to ST860, a clonal lineage associated with healthy humans, used for many years as a probiotic (Kopit et al., 2014). Comparative genomics of these and other sequenced *E. faecium* strains from clinical and non-clinical environments allows to firstly suggest a core (±1600 genes) and an accessory genome for this species (with 2272-3318 coding sequences, CDS that represent from 29% to 59% of the *E. faecium* genome) (Palmer et al., 2014; Lam et al., 2012). Recent comparative genomic studies using WGS consistently split the *E.*

faecium populations in two major groups: Community associated (CA) or Clade B and Hospital associated (HA) or Clade A (that also includes animal isolates) (Galloway-Peña et al., 2012; Lebreton et al., 2013). Clade A was further divided in A1, including most clinical isolates, and A2, including most animal strains. Clade B corresponded to BAPS 1 and clade A1 with BAPS subgroups 2-1 and 3-3 (Galloway-Peña et al., 2012). These classifications should be considered as dynamic since recombination can occur between strains from the different clades, generating new hybrid genomes (Galloway-Peña et al., 2012; Palmer et al., 2012). The genome size of strains in clade A1 is larger than that of clades A2 and B, consistently with the suggested recent emergence of the former clade. Conversely, clade A2 had a larger pan-genome, which would reflect the diverse origins of the strains within the clade. Clade A1 is also enriched in MGE as plasmids, ISs, phages and showed more genomic islands compared to clades A2 and B (Galloway-Peña et al., 2012). The high level of diversity between these two clades indicates that the clade– B split is ancient and precedes the modern antibiotic era (Prieto et al., 2016). These findings indicates that *E. faecium* has an open pan-genome capable of effectively acquire and incorporate novel DNA into the collective gene pool as most ubiquitous bacteria and opportunistic pathogens (Palmer et al., 2014; Lam et al., 2012; Galloway-Peña et al., 2012). The enrichment in genetic determinants seems to be a cumulative process, called “genetic capitalism” where the acquisition and integration of adaptive elements facilitates the acquisition of additional adaptive elements and afterwards, the transition of *E. faecium* (particularly of clade A1) from commensal to nosocomial pathogen (Willems et al., 2009; Baquero, 2004).

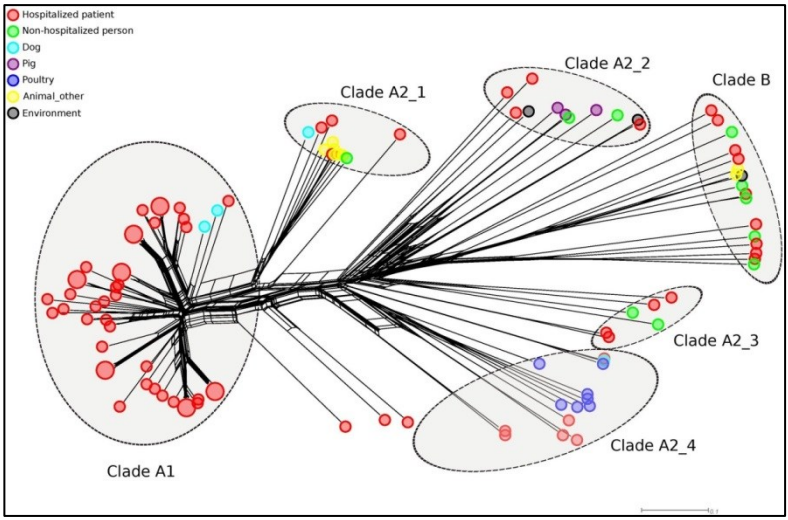

Figure 6. NeighborNet phylogenetic network to visualize the relationships between 196 *E. faecium* isolates (Lebreton et al., 2017; de Been et al., 2015).

3.2 *E. faecalis* population structure

The population structure of *E. faecalis* was also initially establish using MLST and eBURST analyses. Using that more finely tuned approach, MLST results have been able to consistently show that certain enterococcal lineages are associated with invasive diseases, antibiotic resistances and hospital outbreaks (Heimer et al., 2015). Based on sampling across the globe, *E. faecalis* clonal complex 2 (CC2), CC8 and CC9 are known for their AR; whereas in Europe, this is associated with CC2, CC16 and CC87 lineages (Mc Bride et al., 2007; Kuck et al., 2012). Both CC2 and CC87 are almost exclusively found among healthcare-associated infections and are considered to be high-risk lineages (Kuck

et al., 2012; Heimer et al., 2015). It has been proposed that some clonal types are more amenable to genetic exchange than others, especially CC2. These recombination events lead to epidemic populations that rapidly expand and disseminate (Mc Bride et al., 2007). As the backbone of the first VRE healthcare-associated isolates, CC2 representatives are among the largest known *E. faecalis* genomes, enriched in certain colonization traits and PAI modules (Teixeira et al., 1997). Even though CC2 is mostly found among hospital strains, it may also be identified in farm animals reflecting the epidemic *E. faecalis* population structure (Freitas et al., 2011; Kuck et al., 2012; Novais et al., 2013). CC21, CC16 and CC40 have been widely isolated from hospitalized and non-hospitalized humans, meat and farm animals. Similarly to that reported in other continents, a decrease in the occurrence of CC9 and an increase in CC2 occurrence has been observed in the European Union (Ruiz-Garbajosa et al., 2006; Kuck et al., 2012). Analysis of the MLST alleles revealed the *E. faecalis* has a high recombination: mutation rate, gene trees for three individual MLST loci were incongruent and individual MLST alleles are widely distributed among concatenated MLST phylogeny. All these observations indicate that *E. faecalis* has an epidemic population structure that frequently recombines (Willems, 2010; Palmer et al., 2014; Ruiz-Garbajosa et al., 2006). However, an epidemiologic study using an historic *E. faecalis* strain collection (1900s-2006) revealed that CC2 was not found prior to the 1980s, suggesting that this CC might have a recent origin (Willems, 2010; Mc Bride et al., 2007; Kawalec et al., 2007; Solheim et al., 2011). Another feature that is absent of CC2 strains, but present in other less successful CCs in the hospital environment, was the CRISPR (Clustered

Regularly Interspaced Short Palindromic Repeat) loci. This is a defence mechanism against foreign DNA (phages, transposons and plasmids) so the lack of this system would facilitate the acquisition of new genes (Palmer et al., 2014; Kuck et al., 2012; Palmer & Gilmore, 2010). The CC87 isolates have a common phenotype that may contribute to colonization and virulence, CYLlA+, ASA1+, ESP+ and GELE-. The absence of gelE gene seems to improve the adherence of bacteria to the host tissue due to the increased production of MSCRAMM Ace (microbial surface components recognizing adhesive matrix molecules adhesin of collagen) (Pinkiston et al., 2011). The lack of clade structure, or even substantial genetic drift among populations of *E. faecalis*, might be explained by the observation that it is widely disseminated along the food chain (Van Tyne & Gilmore, 2015).

4. Clinical relevance and antibiotic resistance in enterococci

Actually, enterococci are important nosocomial pathogens and enterococcal infections are one of the 21st century medical challenges due to the increasing prevalence of bactericidal activity against these microorganisms (Tedim, 2017). The clinical importance of the genus *Enterococcus* is directly related to its AR, which contributes to the risk of colonization and infection. The increase of enterococcal infections, together with AR led to the inclusion of *E. faecium* species by the IDSA (Infectious Disease Society of America), in the list of ESKAPE pathogens (*Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter* spp., *Pseudomonas aeruginosa*, and

Enterobacter spp.), for all of which new therapies are urgently needed. Furthermore, the CDC has classified VRE as a serious threat to human health, being VRE responsible for about the 30% of the 66,000 *Enterococcus* healthcare-associated infections registered every year in the USA (Arias & Murray, 2012; CDC, 2013). Enterococci are particularly well positioned to both acquire and serve as a depot for antibiotic-resistance factors for two main reasons. Firstly, enterococci exist in complex microbial ecosystems, in intimate contact with a large variety of potential sources of genetic material. Secondly, because of their high level of intrinsic antibiotic resistance, enterococci occur in environments that are substantially enriched for antibiotic-resistance elements (Van Tyne & Gilmore, 2014). The species of the greatest clinical importance are *E. faecalis* and *E. faecium*. Although the resistance characteristics of these two species differ in important ways, they can generally be categorized as intrinsic resistance, acquired resistance, and tolerance. The genus *Enterococcus* is intrinsically resistant to β-lactams, aminoglycosides, and trimethoprim-sulfamethoxazole. The species *E. faecalis* is specifically resistant to lincosamides and streptogramins. Intrinsic resistance is defined as an innate property of a species chromosomally encoded and present in all strains belonging to the species. Enterococci can acquire an extensive variety of resistance mechanisms by mutation or by acquisition of exogenous genes (Arias & Murray, 2012; Gilmore, 2014). In this way, enterococci have been extraordinarily successful at rapidly acquiring resistance to virtually any antimicrobial agent clinically used. Introduction of chloramphenicol, erythromycin and tetracyclines has been quickly followed by the emergence of resistance, in some cases reaching a prevalence that

precluded their empirical use. In response to the growing problem of vancomycin resistance in enterococci, the pharmaceutical industry has developed a number of newer agents that have activity against VRE. However, none of these newly licensed agents (quinupristin-dalfopristin, linezolid, daptomycin, tigecycline) has been entirely free of resistance. Thus, the widespread resistance of enterococci has had a substantial impact on our use of both empirical and definitive antibiotics for the treatment of enterococcal infections, a situation that is likely to persist for the foreseeable future. Enterococci resistance to antimicrobials can be also exibithed as phenotypic tolerance (Aria & Murray, 1990; Murray, 1990). Tolerance can be defined as the ability of a microorganism to resist killing by any antimicrobial well above normal minimal inhibitory concentrations (MIC), and it is caused by a proportion of microbial cells that exist in a transient non-dividing state their bacterial target (Tedim, 2017). Enterococci can exhibit tolerance as to antibiotics that target bacteria cell-wall as β-lactams and vancomycin. It can be overcome by using β-lactams besides high concentrations of aminoglycosides. These therapeutic regimens that combine different antibiotic classes (often including a cell wall active agent) are frequently used to treat severe infections as synergy between different classes of drugs provides a bactericidal effect that is not possible to achieve using monotherapy regimens (Kritish et al., 2014). Acquired resistance may result from one or several mutations in existing gene(s) or the acquisition of exogenous DNA (Kritish et al., 2014; Murray, 1990). Enterococci have acquired resistance to all families of antibiotics (phenicols, tetracyclines, macrolides, aminoglyosides, β-lactams, glycopeptides, quinolones,

streptogramins, oxazolidinones, lipopeptides and glycylcyclines) (Kritish et al., 2014; Murray, 1990).

4.1 Resistance to β-lactams

The species of genus *Enterococcus* usually show a low intrinsic resistance to β-lactam antibiotics like penicillin, ampicillin, piperacilin and imipenem, which exert on them a bacteriostatic effect (Garrido et al., 2014). The potential intrinsical resistance to β-lactams is usually due to the presence of penicillin-binding proteins (PBPs) with low affinity for these antibiotics, particularly PBP5 in *E. faecium*. β-lactams are able to inhibit cell-wall synthesis by serving as subtracts for PBPs, (D,D-transpeptidases) that catalyze the union of the peptidoglycan pentapeptide side chains during the synthesis of mature peptidoglycan. However, the level of tolerance to different classes of β-lactams varies, penicillins exhibiting the highest activity against enterococci (particularly ampicillin, an aminopenicillin), followed by carbapenems and cephalosporins, which barely exhibit activity against enterococci. The level of activity reflects the usefulness of these antibiotics to treat enterococcal infection. While ampicillin is the β-lactam more commonly used to treat enterococcal infections, administration of cephalosporins for the treatment of other bacterial infections constitutes a risk factor for acquiring enterococcal infections (Kritish et al., 2014; Murray, 1990; Shepard & Gilmore, 2002). Although the production of β-lactamases in enterococci is constitutive, up to date two acquired mechanisms of resistance to β-lactams have been described, namely production of β-lactamase and mutation of PBP genes. The

most common mechanism of acquired resistance to β-lactams occurs through mutations and/or hyperproduction of *pbp* genes. This mechanism of resistance was firstly described in 1970s and 1980s in American hospitals associated with the *pbp*5 gene of *E. faecium* and less frequently, of *E. raffinosus* (al-Obeid et al., 1990; Fontana et al., 1994). Recently, other genes that might be responsible for ampicillin resistance were also identified in *E. faecium*, such as, *ddcP* (encoding for D-alanyl-D-alanine carboxypeptidase), *ldtfm* (encoding for a L,D-transpeptidase), *pgt* (predicted to encode a glycosyl transferase group 2 family protein) and *lytG* (predicted to encode an exo-glucosaminidase that might be acting as a peptidoglycan hydrolase) (Zhang et al., 2012). In *E. faecalis*, either the hyperproduction of PBP5 together with deficient binding of penicillin to PBP1 and PBP6 or point mutations in the PBP4, confer resistance to β-lactams (Cercenado et al., 1996; Ono et al., 2005).

4.2 Resistance to aminoglycosides

Enterococci are intrinsically resistant to low levels of aminoglycosides (streptomycine, gentamicin, and kanamycin) due to decreased cellular permeability of these agents (O'Drsicoll & Crank, 2015). In details, they may demonstrate moderate-level (MIC, 62 to 500 g/ml) or high-level (MIC, 2.000 g/ml) resistance (HLR) to aminoglycosides. HLR to aminoglycosides is mediated through modification of the ribosomal attachment sites or the production of aminoglycoside-modifying enzymes (Centinkaya et al., 2000). Both mechanisms are encoded by specific genes and are typically plasmid borne. The

prevalence of HLR to aminoglycosides can vary from 40 to 68% among *Enterococcus* species isolates, depending on geographical location, and differs between *E. faecalis* and *E. faecium* (Dadfarma et al., 2013; Parameswarappa et al., 2013). Intrinsic resistance may be overcome using a combination treatment with a β-lactam and an aminoglycoside, also referred to as a synergy therapy approach. Synergy therapy is based on the principle that lactams will disrupt the cell wall, thereby facilitating increased cellular penetration by the aminoglycoside to reach a sufficient concentration for the inhibition of protein synthesis. This approach is effective in treating infections caused by organisms with moderate-level resistance to aminoglycosides; however, synergy is lost in strains with HLR to aminoglycosides, because the increase in intracellular aminoglycoside concentration cannot overcome the presence of specific aminoglycoside-modifying enzymes (Faron et al., 2016).

4.3 Resistance to glycopeptides

The glycopeptide antibiotics are a class of antimicrobial agents that share a similar, macromolecular structure and basic mechanism of action. The lipoglycopeptides are largely synthetic derivatives of vancomycin modified to have greater potency or better pharmacokinetics and tolerance (Guskei & Tsuji, 2010). Their function is expressed by binding to the terminal D-Ala-D-Ala in the pentapeptide portion of the N-acetylglucosamine (NAG)–N-acetylmuramic acid (NAM) peptidoglycan (PG) cell wall precursor (Fig.7) (Faron et al., 2016). The link between blocks transpeptide of cell wall components results in

reduced integrity and, ultimately, cell death. Resistance to glycopeptides in *Enterococcus* spp. is mediated by the vancomycin resistance (Van) operon. This operon may be carried chromosomally or extrachromosomally on a plasmid.

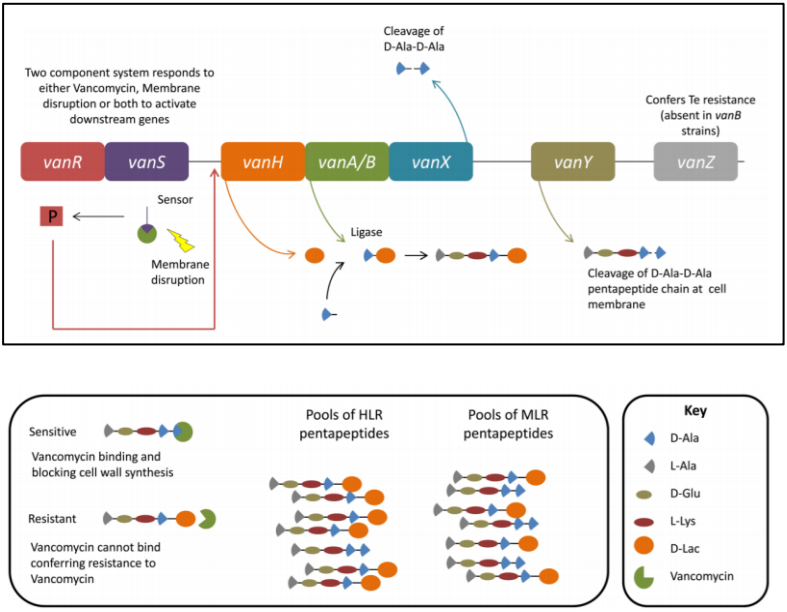

Figure 7. Mechanism of vancomycin resistance (Faron et al., 2016).

The Van operon consists of vanS-vanR, a response regulator; vanH, a D-lactate dehydrogenase gene, vanX, a D-Ala-D-Ala dipeptidase gene; and a variable ligase in which 9 variant genes have been identified (*vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM,* and *vanN*). Expression is inducible by the two-component system

VanS/R, which senses disruptions in the cellular membrane caused by glycopeptides, as well as cell wall damage caused by bacitracin or polymyxin B (Faron et al., 2016). The variable ligase gene is central in determining the level of vancomycin resistance (low, medium, or high), with the most commonly identified genes being *vanA, vanB,* and *vanC*. *VanA* is plasmid borne that confers high-level resistance (MIC, 256 g/ml) to vancomycin, and is most commonly associated with *E. faecium* and *E. faecalis*, while chromosomally encoded *VanC* confers low-level resistance (MIC, 8 to 32 g/ml) to vancomycin and is almost exclusively found in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* (O'Drsiscoll & Crank, 2015). *Enterococcus* spp. carrying *vanA* are highly resistant to glycopeptides and are the dominant VRE variants of *E. faecium* and *E. faecalis* globally. Resistance is mediated by substituting the high-affinity terminal D-Ala-D-Ala peptide on NAM subunits with D-Ala-D-Lac. This amino acid substitution causes a 1.000-fold decrease in the affinity of the pentapeptide for vancomycin (Fig. 7) (Faron et al., 2016). Incorporation of NAM subunits containing the substituted D-Ala-D-Lac peptide into the peptidoglycan layer requires PBPs other than PBP4 and PBP5. In the presence of vancomycin, these alternative PBPs become the dominant proteins for cell wall synthesis. The alternative PBPs used during the presence of vancomycin have enhanced binding to β-lactams, which when used together can allow synergistic treatment (al-Obeid et al., 1992; Arthur et al., 1995). VanA-expressing strains are also resistant to the glycopeptide teicoplanin (Te) due to the presence of an additional gene present on the vanA operon, vanZ, which confers resistance by an unknown mechanism (Arthur et al., 1995). Isolates carrying *vanB* are less prevalent than vanA-carrying strains, but can

be found throughout the world (Coombs et al., 2014; Schouten et al., 2000). Resistance in vanB positive strains is mediated by converting D-Ala-D-Ala to D-Ala-DLac. However, vanB confers varied resistance to vancomycin, ranging from moderate- to high-level resistance (MIC range, 4 to 256 g/ml) (O'Drsiscoll & Crank, 2015). The lower-level resistance compared to that of vanA is likely the result of a lower proportion of D-Ala-D-Lac substitution in the cell wall of strains carrying vanB. Resistance to vancomycin is proportional to the percent composition of D-Ala-D-Lac to D-Ala-D-Ala (Arthur et al., 1996). Smaller amounts of D-Ala-D-Lac incorporation might result from reduced expression of the vanB operon, a reduction in VanX or VanB enzymatic activity, or a combination of minor mechanistic changes. The resistance is not observed in vanB-carrying isolates, as vanZ is not encoded in this operon. Vancomycin is one of the few antibiotics that can be used to treat infections resulting from Gram-positive multidrug-resistant organisms, such as MRSA; therefore, transmission of vancomycin resistance from enterococci to MRSA is of major concern. Horizontal gene transfer has been shown to be a mechanism of transmission between enterococci, and *in vitro* studies have demonstrated that transfer between *Enterococcus* and *S. aureus* can occur (de Niederhäusern et al., 2011).

4.4 Resistance to macrolides

Macrolide antibiotics are used in the treatment of infections in humans, being erythromycin the antibiotic of first choice in patients allergic to penicillins. Tylosin is an antibiotic pertaining to this group that was widely used in pigs

(Garrido et al., 2014). Several studies have demonstrated a dissemination of macrolide resistance in enterococci through three different mechanisms such as target modification by precise mutations, hydrolysis of the lactone ring of the antibiotic molecule, and efflux pumps, that remove antibiotic molecules from inside of the bacterial cell. The more frequent macrolide resistance determinants are *erm* genes. These encode for one methyltransferase that acts on specific residues of the 23S rRNA subunit, causing a N6-dimethylation of an adenine residue in the 23S rRNA subunit and inhibiting that way erythromycin binding (Pechère, 2001). Resistance to macrolides can be transferred from animals to humans, either by dissemination of the resistant bacteria or by horizontal transfer through movable genetic elements. Finally, *Enterococcus* spp. may also contain export mechanisms for macrolide antibiotics. The genes responsible for this trait (*mef)* show a high mobility between diverse Gram-positive species (Garrido et al., 2014).

4.5 Resistance to tetracycline

Resistance to tetracycline is frequent in clinical and animal isolates of enterococci. The presence of strains resistant to tetracycline has also been described in diverse foods of animal origin (Butayeet al., 200; Klein et al., 1998; Pavia et al. 2000). In enterococci, tetracycline resistance is generally associated to the presence of the gene *tet*(M) which confers ribosomal protection, but other related genes affording ribosomal protection have also been described, like *tet*(O) and *tet*(S) (Garido et al., 2014). In clinical isolates, *tet*(M) is frequently associated to Tn916-type transposable elements,

but it can also be found in conjugative plasmids and on the chromosome. The genes *tet*(K) and *tet*(L) encode tetracycline export pumps, the latter one being more frequent (Leclercq, 1997). Other resistance genes encode proteins that bind to the ribosome and modify its conformation, preventing the union of tetracycline.

4.6 Resistance to rifampicin

Enterococci are frequently resistant to rifampicin, even though this antibiotic is not commonly used in enterococcal infections (Andrews et al., 2000). However, the resistance is due to the exposure of commensal microbiota to this antibiotic during treatment of other bacterial infections. Rifampin specifically inhibits bacterial RNA polymerase, the enzyme responsible for DNA transcription, by forming a stable drug-enzyme complex preventing RNA synthesis by physically blocking elongation, and thus preventing synthesis of host bacterial proteins (Wehrli, 1983). Mutations in the RNA polymerase B subunit *rpo*B gene account for most of the observed resistance. One particular mutation (rpoB H486Y) also confers an increased cephalosporin resistance, possibly by increasing transcription of genes involved in intrinsic resistance to this antibiotic (Kristich et al., 2014).

4.7 Resistance to sulphonamides

Sulfonamides represent the first class of antimicrobial agents, discovered in 1932 and put into clinical use in 1935 (Sköld, 2000). Since then, they have been used extensively

in many different clinical indications. The medium long-acting sulfonamides, sulfamethoxazole (Sul) and sulfadiazine, remain the most useful members of this class of antimicrobial agents. Sulfonamides inhibit dihydropteroate synthetase (DHPS), which catalyses the formation of dihydrofolate from para-aminobenzoic acid. In the subsequent step of the pathway, TMP inhibits dihydrofolate reductase (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate. The use of TMP and sulfonamides in combination is thought to have a synergistic effect. Different mechanisms mediate bacterial resistance to TMP and sulfonamides. Because resistance to both drugs can be transferable, these resistance traits are often linked to one other, as is the case in the well-known transposons of the Tn21 family (Huovinen et al., 2001). Bacterial resistance to TMP and to sulfonamides is mediated by the following 5 main mechanisms: (1) the permeability barrier and/or efflux pumps; (2) naturally insensitive target enzymes; (3) regulational changes in the target enzymes; (4) mutational or recombinational changes in the target enzymes, and; (5) acquired resistance by drug-resistant target enzymes.

4.8 Resistance to chloramphenicol

Chloramphenicol has been used as a broad-spectrum antibiotic in human and veterinary medicine since the 1950s. The structure of chloramphenicol is relatively simple, and it was the first chemically synthesized antibiotic on the market (Schwarz et al., 2004). Chloramphenicol inhibits translation in bacteria, by inhibition of the peptidyl transferase reaction of the large subunit of the ribosome.

The inhibition of the peptidyl transferase activity is mediated by binding to several proteins in the 50S ribosomal subunit (Schlunzen et al., 2001). Recent reviews have shown that resistance to phenicols or lincosamides can be based on several different resistance mechanisms specified by a considerable number of genes or mutations. A number of resistance mechanisms to chloramphenicol in bacteria has been described, of which the most common is enzymatic inactivation by acetylation of chloramphenicol via chloramphenicol transferases (CATs). Chloramphenicol acetyltransferases (CATs) have been described in both Gram-positive and Gram-negative bacteria. CATs catalyze the hydroxylation of chloramphenicol, thereby leaving the antibiotic inactive. The inactivation of chloramphenicol can also be performed by xenobiotic acetyltransferases (Murray et al., 1997 Aakra et al., 2010) A third mechanism of chloramphenicol inactivation is performed by chloramphenicol phosphotransferases (Schlunzen et al., 2001).

4.9 Resistance to biocides and copper

Among enterococci, resistance to biocides and disinfectant has been studied to a much less extent compared to antibiotics (Braga et al., 2013; Garrido et al., 2014). In most reports, enterococci have been found to be sensitive or moderately resistant to biocides, such as benzalkonium chloride, triclosan or chlorhexidine. Most results are based on higher than wild type tolerance levels, since there are no breakpoints yet established for biocide resistance. Bacterial resistance to heavy metals is also a matter of concern, since resistance genes are often located on the same mobile

elements as those conferring resistance to antibiotics, raising opportunities for cross-selection of antibiotic resistance.

4.10 Biological cost of antibiotic resistance

Besides conferring resistance to antibiotics, mutations can have other effects in the cell. Since most resistance mutations are located in housekeeping genes, they can interfere with essential cell processes, such as DNA replication and transcription, protein translation and bacterial wall synthesis (Andersson & Levin, 1999; Dahlberg & Chao, 2003; Higgins et al., 2003). Hence, resistant cells physiology is deviated from an optimal state and these cells may grow slower than their drug-susceptible ancestrals. Thus, in the absence of the antibiotic selective pressure, resistance mutations are often deleterious and confer a biological fitness cost to the cell (Andersson & Levin, 1999; Andersson, 2006; Lenski, 1998; Nilsson et al., 2003). This fitness cost can vary widely with the mutation and the strain genetic background (Gagneux et al., 2006). Despite the generally accepted principle that resistance mutations are deleterious, recent studies have suggested that, in specific environments and/or strains, the metabolic modifications produced by resistance mutations can lead to greater fitness (Andersson, 2006; Yu et al., 2005). Several study showed low fitness cost and high stability for vancomycin resistance and for vancomycin resistant plasmid (Freitas et al., 2013; Johnsen et al., 2009; Johnsen et al., 2011). In the absence of the antibiotic selective pressure, it is logical to expect that, when a resistance mutation imposes a fitness cost, the resistant strains will be

outcompeted by the susceptible ones. This assumption suggests that a possible procedure to eliminate antibiotic resistance is to ban the use of the respective antibiotic. It is also natural to assume that the higher the fitness cost, the more effective the ban would be.

5. Mobile genetic elements

Extrachromosomal elements are ubiquitous in the prokaryotic world and play important roles in the adaptation and survival of cell populations, especially in changing environments (Gilmore et al., 2014). MGEs are common in enterococci, with conjugative transposons (CTns), PAIs and plasmids all contributing to resistance, virulence and host adaptation. The mobilome, defined as all MGEs able to move around within or between genomes, contributes to genome plasticity as well as dissemination of antibiotic resistance genes and pathogenicity (Santagati et al., 2012). Conjugative elements, such as plasmids, integrative chromosomal elements (ICEs) and CTns, enable intercellular mobility of traits that provide rapid adaptation to environmental changes, either by catching genes that they then may transfer to recipients or by integrating themselves into other recipients' elements. They also mediate mobilization of chromosomal fragments after integrating into the host genome and transfer variable chromosomal fragments in addition to parts of the conjugative element into a recipient cell or by rearrangements among them. Plasmids are readily found in enterococci, and it is not unusual for clinical and commensal strains (e.g. *E. faecalis* and *E. faecium*) to harbor a number of such elements. Indeed, plasmid-free isolates are only infrequently

identified. Preliminary data from recent studies on horizontally acquired gene clusters, genomic islands and (resistance) plasmids in *Enterococcus* demonstrated that acquisition of additional genetic material may have different influences on the host's fitness and may be dependent on the corresponding context, genetic element, species and even strain background (Foucaultet al., 2009, 2010; Laverde Gomez et al., 2011a;b; Tedim et al., 2011; Werner et al., 2013). The most common way of exchanging MGE is believed to be conjugation, either by horizontal transfer of plasmids or by conjugative transposons (Osborn et al., 2000). Several plasmids have been identified that harbour transposons capable of co-transfer and integration in the chromosome, either by site specific recombination or by homologous recombination (Jensen et al., 2010). Phylogenomic analyses have revealed the influence of HGT in the evolutionary trajectories of some ST within major clonal complexes of *E. faecium* and *E. faecalis*, and also in determining strain-specific properties (de Regt, et al., 2012; van Schaik, et al., 2010). HGT between enterococci and other species occurs readily, with plasmids being the dominant MGE.

5.1 Plasmids

The ever increasing numbers of reports of enterococcal strains that have acquired new adaptive traits that enable them to survive in different conditions (such as resistance to antibiotics, biocides, heavy metals, and different metabolic capabilities, or the ability to colonize different epithelial tissue or cause infection) illustrates the role of HGT in the combinatorial evolution of these microorganisms (Miller et

al., 2015). Plasmids are abundant in enterococci and they comprise a substantial part of the auxiliary genome: they are responsible for much of the horizontal gene transfer that has allowed antibiotic and virulence traits to converge in hospital adapted lineages (Santagati et al., 2012). Plasmids are semi-autonomously replicating extrachromosomal genetic elements. Differences in replication strategies and modular structures profoundly affect plasmid properties, such as size, copy number, host dependence and host range (Thomas, 2004). The essential pillars for successful plasmids include genetic modules encoding self-replication, stable inheritance and the ability to transfer between bacteria. Accessory plasmid content is integrated in between functional plasmid backbone modules and represents a huge reservoir of genetic variability, often with unknown functions, that is shared among different bacterial genera through horizontal gene transfer (Hegstag et al., 2010). There are several criteria to classify plasmids and the basis of the mode of replication has been used to distinguish rolling circle replication plasmids and theta-replicating plasmids. Moreover, based on the presence of conserved domains in their replication initiators it was possible distinguish in: Rep_3, Inc18 and RepA_N families of theta-replicating plasmids and Rep_trans, Rep_1 and Rep_2 classes of RCR plasmids. This classification scheme was initially developed by Jensen and co-workers (2010). Whereas plasmids with different mechanisms of replication, conjugation abilities, and host ranges have been described in enterococci, their occurrence varies among both species and strain origin. Enterococcal plasmids commonly encode: 1) resistance to one or more antibiotics; 2) elevated resistance to ultraviolet light; 3) virulence factors, such as cytolysin and aggregation substance, and; 4) bacteriocin

production. In addition, intercellular transmissibility is frequently a plasmid-determined trait. As in many bacterial species, plasmids generally range in size from 3–4 kb to well over 100 kb and may be present at relatively low copy number (1–2 copies) or up to 20 or more copies per cell. For example, while Inc18 plasmids appear to be widely distributed among different species, other plasmid types seem to be related to either *E. faecium* (e.g. RCR pRI1-like, small theta pCIZ2-like, and pRUM-like plasmids, as well as a variety of megaplasmids) or *E. faecalis* (e.g. pheromone-responsive plasmids) (Freitas, et al., 2010; 2013; Leon-Sampedro, et al., 2012). Antibiotic resistance is frequently associated with Inc18, pRUM-like, and pheromone-responsive plasmids. Nowadays, plasmid categorization is relevant from the public and environmental health perspective to follow the movement of genes coding for resistance to antimicrobials (antibiotics, heavy metals, biocides), colonization and virulence factors for humans and animals, and/or other adaptive traits that drive ecological success (bacteriocins, metabolic traits) and consequently increase the population size of bacteria harboring MGEs. Recent studies revealed that most strains of *E. faecium* and *E. faecalis* carry a number of plasmids of different families that include species specific plasmids (e.g., narrow host range RCRs and RepA_N plasmids such as megaplasmids in *E. faecium* and pheromone-responsive plasmids in *E. faecalis*) and broad host range plasmids (e.g., Inc18), plasmid chimeras being abundant and still difficult to classify. Megaplasmids of *E. faecium* or pheromone-responsive *E. faecalis* plasmids enhance the ability to colonize, invade, and form biofilms. Conjugative plasmids may influence the mobilization of non conjugative elements and chromosomal regions and facilitate the acquisition of

different adaptive traits and genome evolvability (Tedim, 2017). Most enterococcal plasmids are able to acquire and disseminate AR genes by different mechanisms of genetic exchange. However, the role of plasmids in the population structure and evolvability of enterococci has been poorly addressed due to the overrepresentation of recent clinical and animal isolates of specific lineages commonly associated with AR (Tedim, 2017) and due to the lack of available plasmid sequences. Plasmids can be classified according to their modes of replication or their ability to transfer. Even though some plasmids of conserved size can be isolated, plasmids are plastic structures that can recombine, thereby obtaining new genetic traits and changing in size. Therefore composite plasmid structures ("mosaicism") are often reported in enterococci challenging a simplified typing scheme only based on single determinants (Sletvold et al., 2010; Freitas et al., 2013b). Furthermore plasmids are a major part of the bacterial mobilome and play an important role in adaptation to changing environments (Palmeret al., 2010; Santagati et al., 2012). The prevalence and importance of specific plasmids in enterococci and their role in the dissemination of antimicrobial resistance among wild-type enterococci in animals, food and humans is still unknown. Furthermore, studies are hampered by the lack of standardised classification systems to be used in surveys and epidemiological studies (Jensen et al., 2010).

Nowadays, with the many sequences available in public databases, it has become obvious that plasmids are plastic and dynamic structures (Andrup et al., 2003; Thomas, 2000; Tousssaint and Merlin, 2002). They could be described as distinct modules with specific functions such as maintenance (replication, stability and copy control),

transfer (mobilization and transfer), pathogenicity (pathogenicity islands and virulence genes), degradation pathways (biochemical) and resistance functions (antimicrobial, heavy metals and disinfectants), (Jensen et al., 2010).

5.2 Trasposons

Enterococcal transposons can be classified into: (i) composite transposons; (ii) Tn3 family transposons, and; (iii) ICEs, including the classical conjugative transposons (Weaver et al., 2002).

Composite transposons are composed of two IS elements of the same family that move together within the cell and carry the DNA in between them. IS elements typically encode only the enzyme necessary for their own transposition. Tn5281, associated with high-level gentamicin resistance, is widely distributed in enterococci (Feizabadiet al., 2008; Hallgren et al., 2003; Leelaporn et al., 2008; Simjee et al., 1999; Straut et al., 1996; 1997), whereas the occurrence of vanB1-subtype vancomycin resistance has been described as an integral part of a composite transposon for a single isolate (Quintiliani and Courvalin, 1996). Since IS elements seem to represent the major proportion of MGE in clinical enterococcal isolates (Lam et al.,2012; Paulsen et al., 2003) and IS elements of the same family frequently are found flanking resistance genes such as the *cfr* (chloramphenicol florfenicol resistance) gene mediating transferable linezolid resistance (Diaz et al., 2012; Liu et al., 2012; 2013).

The **Tn3 family** of transposons transpose within or between different replicons within the same cell through a replicative mechanism promoted by a transposase (TnpA) and a resolvase (TnpR) (Chandler and Mahillon, 2002). Two well-known and widely distributed Tn3-like transposons in enterococci mediateVanA-type glycopeptide resistance (Tn1546) (Arthur et al., 1993) and macrolide–lincosamide–streptogramin B-resistance (Tn917) (Shaw and Clewell, 1985).

ICEs are conjugative self-transmissible elements that can excise from integrate and replicate along with the host chromosome. An ICE typically contains three modules for: (1) integrationand excision; (2) regulation, and; (3) a full complement of machinery for conjugation into a new host (Burrus et al., 2002; Burrusand Waldor, 2004). Many ICEs additionally carry putative virulence determinants, antibiotic resistance factors and/or genes coding for other beneficial traits and they may also spread such traits through 'hitchhiking' (Burrus and Waldor, 2004). Enterococcal ICEs have been associated with resistance to tetracycline, kanamycin, macrolide/lincosamide/streptogramin B (MLSB)-antibiotics, as well as to glycopeptide. The classical widely distributed conjugative transposon family ICEs Tn916/Tn1545 have a broad host range carrying clinically relevant resistance determinants (*tet*M, *erm*B, *aph*A-3) between a large diversity of bacterial genera (Flannagan et al., 1994; Roberts and Mullany, 2009). Importantly, these enterococcal ICEs are able to co-transfer other plasmids and transposons as well as facilitate transfer of large chromosomal fragments (Rice et al., 2005b; Weaver et al., 2002). Other prevalent ICEs are Tn1549/Tn5382 carrying the vanB2 subtype vancomycin

resistance cluster which have been found both in enterococci (Carias et al., 1998; Dahl et al., 2000; Garnier et al., 2000; McGregor & Young, 2000; Umeda et al., 2002) and in other bacterial genera belonging to the intestinal microbiota (Ballardet al., 2005; Dahl & Sundsfjord, 2003; Domingo et al., 2005; Marvaud et al., 2011).

Bacteriophages may incorporate host-cell DNA into their capsids during their replicative cycle. The bacterial DNA can then be carried and integrated into the new host-DNA upon successful infection. Transfer of both virulence and antibiotic resistance genes by transduction has recently been demonstrated between enterococci by lytic bacteriophages of the very common bacteriophage family Siphoviridae (Mazaheri Nezhad Fard et al., 2011; Yasmin et al., 2010). The importance of bacteriophage transfer compared to conjugation in gene mobilization may be underestimated for small genetic elements that can easily fit into the phage particles since little research on transduction in enterococci has been conducted.

Integrons are genetic units that are able to capture small mobile elements, called gene cassettes, and ensure their expression. An integron can contain from zero to dozens of cassettes which are transcribed from a common strong promoter located in the 5' conserved segment. Integrons are not transferable themselves, but are often carried by mobile genetic elements, which promote their spread within bacterial communities (Cambray et al., 2010; Hall, 2012).

5.3 Pathogenic Island (PAI)

PAIs are large, horizontally transmitted elements found in many gram-positive and gram-negative pathogens (Gal-Mor et al., 2006). They are believed to contribute to the rapid evolution of non pathogenic organisms into pathogenic forms (McBride et al., 2009). It is now well known that virulence of an organism is regulated with virulence coding genes present on the genome in special regions which are termed pathogenicity islands (Hacker & Kaper, 2000). The PAI of *Enterococcus* was first identified in the genome of multi-drug-resistant strain of *E. faecalis* [MMH594] that had caused an outbreak of nosocomial infection in the 1980's (Huycke et al., 1991). The PAI of *E. faecalis* is approximately 150 kb and encodes multiple factors that contribute to its virulence, including the cytolysin toxin, the enterococcal surface protein Esp, and Gls-24-like proteins, as well as traits suspected of contributing to pathogenicity or altering its relationship with the host, including a bile acid hydrolase, carbohydrate utilization pathways, and many additional genes of unknown function (Mc Bride et al., 2003). The PAI, or parts thereof, has been identified in hundreds of *E. faecalis* isolates, and variation in genetic content has been noted (McBride et al., 2007; McBride et al., 2009; Nallapareddy et al., 2005). It is enriched among infection-derived isolates and highly clonal lineages containing multiple antibiotic resistance elements (Shankar et al., 2002). Variation has been found in the occurrence of genes within the PAI, even within a genetic (clonal) lineage, suggesting that segments of the island can vary independently of the whole. Indeed, movement of genes derived from an internal portion of the PAI has been detected (Coburn et al., 2007). Little is known about the

genesis of the enterococcal PAI, how it varies, or how it entered the species. The prototype PAI of strain MMH594 includes known and putative insertion sequence (IS) elements, transposases, conjugal transfer components, and other plasmid derived sequences and at the sequence level appears to have been assembled by a process of accretion. The variability of the PAI among numerous diverse set of clinical isolates has been investigated and compared to variation in the host chromosome (McBride et al., 2009).

6. Virulence factors

Several studies (Hancock & Gilmore, 2000; Huycke et al., 2000) have identified different virulence factors, most important among them being haemolysin, gelatinase, enterococcal surface protein (Esp), aggregation substance (AS), MSCRAMM Ace, serine protease, capsule, cell wall polysaccharide and superoxide. These factors can be grouped into two main groups: i) surface factors that affect colonisation of host cells, and; ii) agents secreted by enterococci, which damage the tissues (Sava et al., 2010) (Table 2).

Table 2. Enterococcal virulence factors

Enterococcal virulence factors	Gene	Function/biological effect
Virulence factors that promote colonisation:		
- aggregation substance (AS),	*agg*	- binding to host cells, enables cell-to-cell contact between donor and recipient strains for conjugation
- collagen-binding protein (Ace),	*ace*	- colonisation by binding to proteins of the extracellular matrix (ECM); it also participates in binding type I and IV collagen
- cell wall adhesin (Efa A),	*efa*A	- virulence factors associated with infective endocarditis
- enterococcal surface protein (Esp),	*esp*	- associated with biofilm production
Virulence factors with affect tissues:		
- cytolysin (Cyl),	*ace*	- bactericidal properties towards Gram-negative bacteria and toxic properties (β-haemolysis) towards erythrocytes, leukocytes, macrophages
- gelatinase (GelE)	$efaA_{fs}$, $efaA_{fm}$	- hydrolyses gelatine, elastin, collagen, haemoglobin, as well as other bioactive peptides, e.g. proteins bound to pheromones
- hyaluronidase (Hyl)	*esp*	- plays a role in destroying mucopolysaccharides of the connective tissue and cartilage

Enterococci are capable to adher to the host's tissues (Tomita & Ike, 2004). This and their resistance to low pH and high concentrations of bile salts contribute to enterococci to be among the most common bacteria colonising the colon (Moreno et al., 2006). Their adhesins enable them to bind to receptors on the mucous membrane or to proteins of the extracellular matrix, which favour colonisation of the epithelium (Stiles at al., 2003). If they could not bind, they would be removed by peristalsis of the intestines. Obviously, colonisation itself is not proof of pathogenicity, but combined with other factors of virulence and with the presence of a number of resistance genes, potentially harmful. Virulence factors that promote colonisation include: AS, collagen-binding protein (Ace), cell wall adhesin (Efa A), Esp (Hollenbeck & Rice, 2012; Strzelecki et al., 2011).

The **AS** has been the first enterococcal surface protein to be described. This protein has a molecular weight of 137 kDa and a hairpin-like structure. The strongly conservative motive motif of LPXTG is an important part of its molecule and its distinctive sequence is regarded as site of recognition and cleavage by sortases which bind them by a covalent bond to the cell wall (Dramsi et al., 2005). The aggregation substance includes a range of highly homologous adhesins, encoded on large conjugative plasmids transferred in a so-called facilitated conjugation system, mediated by sex pheromones (Strzelecki et al., 2011). The process is of special importance in the conjugative transfer of genes between cells. In the presence of pheromones secreted by the recipient's cells, the donor's cells synthesise AS which binds to a related EBS ligand on the recipient cell surface (Dunny et al., 1995). The process

results in the formation of large conjugative aggregates consisting of bacterial cells, which facilitates their exchange of genetic material between cells. Moreover, it plays a role in propagation within a species of plasmids, on which other factors of enterococci virulence are encoded, such as cytolysin and determinants of antibiotic resistance. Finally, the aggregation substance and cytolysin can act synergistically, thereby increasing the strain's virulence by switching on cytolysin regulation in the quorum-sensing system, making it possible to damage deeper tissues (Gilmore et al., 2002; Moreno et al., 2006).

Ace (Accessory colonisation factor) is another surface protein with adhesive properties, with a molecular weight of about 74 kDa, encoded by the ace gene (Rich et al., 1999). The protein was isolated from *E. faecalis* strains both from healthy carriers and from people with enterococcal infections, which suggested that this feature can be used to identify the species (Duh et al., 2001). Like the AS protein, Ace also plays an important role in colonisation by binding to proteins of the extracellular matrix (ECM); it also participates in binding type I and IV collagen (Nallapareddy et al., 2000). Ace is a member of the family of surface proteins described by the acronym MSCRAMM, with LPXTG (L - leucine, P - proline, X e any amino acids, T e threonine, G e glycine), with high affinity and specificity of binding a ligand which is among the components of ECM (Hendrickx et al., 2009). Considering the functionality and structure of the Ace protein, it has been noted that it is similar to one of the first MSCRAMM's discovered in Gram-positive bacteria: the Cna protein which occurs in *S. aureus*. A protein whose structure is similar to Ace in *E. faecalis* has been found in *E. faecium* and Acm. The Acm

protein is encoded by the *acm* gene, which is homologous to ace. Like Ace, it is responsible for binding collagen (Nallapareddy et al., 2008).

EfaA (endocardis antigen) is a protein with a molecular weight of about 34 kDa encoded by the efAfs gene in *E. faecalis* strains, and by efArm in *E. faecium* (Eaton & Gasson, 2001; Sava et al., 2010). The efaA gene is part of the afaCBA operon which encodes the ABC transporter (permease), regulated by magnesium ions (Abrantes et al., 2013). The EfaA protein is homologous to the adhesins present in cell walls of streptococci, e.g. to the FimA protein, produced by *Streptococcus parasanguis*, ScaA in *S. gorgonii*, PsaA in *S. pneumoniae* and SsaB in *S. sanguis* (Archimbaud et al., 2002). It has been shown that homologous genes to efaA are present in strains of *E. avium*, *E. asini*, *E. durans* and *E. solitarius* (Jimenez et al., 2013; Semedo et al., 2003b).

Entercococcal surface protein (Esp) with a molecular weight of about 200 kDa is the largest identified enterococcal protein. The esp gene encoding this protein is located on the PAI, which also contains proteins responsible for the active outflow of antibiotics (Leavis et al., 2004). This location is probably a result of horizontal gene transfer between *E. faecalis* and *E. faecium*. The Esp protein shows some structural similarity to other proteins present in Gram-positive bacteria. Studies of the Esp protein have confirmed its participation in the formation of biofilm which can play an important role in the exchange of genetic material between cells and increase their resistance to antibiotics (Donlan & Costerton, 2002; Foulquie Moreno et al., 2006; Latasa, Solano, Penade, & Lasa, 2006). It has been shown

that the occurrence of the esp gene in *E. faecium* is correlated with resistance to ampicillin, ciprofloxacin and imipenem (Billström et al., 2008). The latest reports suggest a correlation beteween the presence of a surface protein and resistance to vancomycin. It has also been shown that the esp gene can be transferred between strains of *E. faecium* by way of plasmid conjugation, and also between strains of *E. faecalis* by the chromosome-chromosome transposition (Oancea et al., 2004).
After the colonisation process, pathogenic strains of *Enterococcus* spp. secrete toxic substances which have a destructive effect on the host's tissues. Virulence factors secreted by enterococci include: cytolysin (Cyl), gelatinase (GelE) and hyaluronidase (Hyl).

Cytolysin is one of the best characterised enterococcal virulence factors. It is a bacteriocin-type exotoxin, which shows bacteriocidal properties towards Gram-negative bacteria and toxic properties (beta-haemolysis) towards erythrocytes, leukocytes and macrophages (De Vuyst et al., 2003). Production of cytosine is related to operon containing eight genes: *cylR1, cylR2, cylLl, cylLs, cylM, cylB, cylA* and *cylI*. The operon can be located on strongly conservated pheromone-dependent conjugative plasmids (e.g. pAD1) or within the island of pathogenicity in the bacterial chromosome near other determinants of virulence: surface protein Esp and the aggregation substance AS (Eaton & Gasson, 2001; Shankar et al., 2004). Cytolysin produced in a cell is secreted outside with the help of a protein encoded by the cylM gene. Outside the cell, cytolysin is activated by serine protease encoded by the cylA gene, and the cell itself is protected by a surface protein encoded by the cylI gene, which affects its

resistance to cytolysin. The regulatory system is activated by the quorum-sensing mechanism, when the concentration of the smaller sub-unit of cytolysin CylLs outside the cell is sufficiently high (Hallgren et al., 2008; Semedo et al., 2003a). Cytolysin encoding genes have been found both in *Enterococcus* strains isolated from infections and from those belonging to commensal microbiota. There have been a number of reports which indicate their frequent occurrence in strains isolated from animals and food products, both of animal and plant origin (Eaton & Gasson, 2001; Ben Omar et al., 2004; Franz et al., 2001; Trivedi et al., 2011). Cytolysin-encoding genes have been found in the following species: *E. faecalis, E. faecium, E. avium, E. casseliflavus, E. cecorum, E. durans, E. gallinarum, E. malodoratus* and *E. raffinosus* (Semedo et al., 2003a).

Gelatinase is an extracellular, zinc-dependent metalloendopeptidase, with a molecular weight of about 30 kDa. Gelatinase is encoded by the *gelE* gene situated on the chromosome. The gene is controlled by the transmembrane protein FsrB, which is regulated by locus fsr consisting of three genes: *fsrA, fsrB, fsrC* (Hancock & Perego, 2004; Pillai et al., 2004). The *gelE* gene is regulated in the quorum-sensing system and depends on the proper level of the gelatinase biosynthesis activation pheromone (GBAB) (Pinkston et al., 2011; Teixeira et al., 2012). Locus *fsr* in enterococci is functionally similar to locus *agr* in *S. aureus* (Dunman et al., 2001). The presence of the *gelE* gene is one of those determinants of virulence assayed in enterococci, which is found both in clinical and in food-associated strains. It usually occurs in *E. faecalis* and in individual strains of *E. faecium* (Eaton & Gasson, 2001).

Hyaluronidase, an *E. faecium* genome protein with molecular weight close to 45 kDa is encoded by the *hyl* gene. This enzyme plays a role in destroying mucopolysaccharides of the connective tissue and cartilage and, consequently, in spreading bacteria. The *hyl* gene in clinical strains has been usually found in *E. faecium* and it occurs extremely rarely in *E. faecalis* (Vankerckhoven et al., 2004). It has also been found in other strains of species isolated from food including: *E. casseliflavus, E. mundtii* and *E. durans* (Trivedi et al., 2011). Strains of a clinical origin usually contain more factors of virulence than those isolated from other sources, including food, whereas the latter, although not an immediate source of infection themselves, can promote the spreading of genes of virulence (Comerlato et al., 2013; Eaton & Gasson, 2001; Jimenez et al., 2013). A number of enterococcal factors of virulence, such as haemolysin-cytolysin, adhesive substances can be transferred by the mechanism of gene exchange. It is often the case that one plasmid contains genes that encode pheromones, antibiotic resistance and factors of virulence (Franz et al., 2001; Giraffa, 2002). Therefore, considering consumer safety, it seems reasonable to monitor the presence of virulence factors in strains of genus *Enterococcus* isolated from food.

6.1 Biofilm formation

Bacteria survive in nature by forming biofilms on surfaces and probably most, if not all, bacteria (and fungi) are capable of forming biofilms. A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and

extracellular DNA. Bacterial biofilms are resistant to antibiotics, disinfectant chemicals and to phagocytosis and other components of the innate and adaptive inflammatory defense system (Frieri et al., 2017). Gradients of nutrients and oxygen exist from the top to the bottom of biofilms and the bacterial cells located in nutrient poor areas show a decreased metabolic activity and an increased doubling times. These more or less dormant cells are therefore responsible for some of the tolerance to antibiotics. Biofilm growth is associated with an increased level of mutations. Bacteria in biofilms communicate by means of molecules, which activates certain genes responsible for production of virulence factors and, to some extent, biofilm structure (Hoiby et al., 2011). This phenomenon is called quorum sensing and depends upon the concentration of the quorum-sensing molecules in a certain niche, which depends on the number of the bacteria. Biofilms can be prevented by antibiotic prophylaxis or early aggressive antibiotic therapy and can be treated by chronic suppressive antibiotic therapy. Promising strategies may include the use of compounds that can dissolve the biofilm matrix and quorum-sensing inhibitors, which increases biofilm susceptibility to antibiotics and phagocytosis (Hoiby et al., 2011).

References

Aarestrup, F.M., Butaye, P., & Witte, W. (2002). Non-human reservoirs of enterococci. In M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, & L. B. Rice (Eds.), The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance (pp. 55-100). Washington, District of Columbia: ASM Press.

Agudelo, N.I.H. and Huycke, M.M. (2014). Enterococcal Disease, Epidemiology, and Implications for Treatment. In M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, & L. B. Rice (Eds.), The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance (pp. 65-100). Washington, District of Columbia: ASM Press.

Anderson, K.L., Whitlock, J.E., Harwood, V.J. (2005). Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. Applied and Environmental Microbiology; 71(6):3041–3048. PubMed PMID: 15933000.

Anderson, S.A., Turner, S.J., Lewis, G.D. (1997). Enterococci in the New Zealand environment: implications for water quality monitoring. Water Science and Technology; 35(11-12):325–331.

Badgley, B. D., Thomas, F. I., & Harwood, V. J. (2010). The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. (1271-1281, Ed.) Environmental Microbiology, 12(5).

Baquero, F., Coque, T. M., Canton, R. (2014). Counteracting antibiotic resistance: breaking barriers among antibacterial strategies. Expert Opin. Ther. Targets, 18 (8).

Baquero, F., Coque, M. T., de la Cruz, F. (2011). Ecology and Evolution as Targets: the Need for Novel Eco-Evo Drugs and Strategies To Fight Antibiotic Resistance. *Antimicrobial agents and chemotherapy*, p. 3649–3660 Vol. 55, No. 8.

Baquero, F. (2004). From pieces to patterns: evolutionary engineering in bacterial pathogens. Nature reviews | Microbiology Volume 2.

Berg, R.D. (1996). The indigenous gastrointestinal microflora. Trends in Microbiology; 4(11):430–435. PubMed PMID: 8950812.

Bik, E.M., Eckburg, P.B., Gill, S.R., Nelson, K.E., Purdom, E.A., Francois, F., et al. (2005). Molecular analysis of the bacterial microbiota in the human stomach. Proceedings of the National Academy of Sciences of the United States of America, 103(3):732–737.

Boehm, A.B., Grant, S.B., Kim, J.H., Mowbray, S.L., McGee, C.D., Clark, C.D., et al. (2002). Decadal and shorter period variability of surf zone water quality at Huntington Beach, California. Environmental Science & Technology; 18:3885–3892.

Brandl, K., Plitas, G., Mihu, C.N., Ubeda, C., Jia T., Fleisher, M., et al. (2008). Vancomycin-resistant

enterococci exploit antibiotic-induced innate immune deficits. Nature, 455(7214):804–807.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J. (2012). Enterococci in the environment. Microbiology and Molecular Biology Reviews, 76(4):685–706. PubMed PMID: 23204362.

Cabelli, V.J. (1983). Health effects criteria for marine recreational waters. Research Triangle Park: U.S. Environmental Protection Agency.

Cabelli, V.J., Dufour, A.P., McCabe, L.J., Levin, M.A. (1982). Swimming-associated gastroenteritis and water quality. American Journal of Epidemiology, Volume 115, Issue 4, 1, Pages 606–616.

Cabelli, V.J. (1979). Evaluation of recreational water quality, the EPA Approach. In: Biological Indicators of Water Quality. Eds. S. James and L. Evison, Wiley, London, p. 14-1.

Carvalho, M.G., Shewmaker, P.L., Steigerwalt, A.G., Morey, R.E., Sampson, A. J., Joyce K., et al. (2006). *Enterococcus caccae* sp. nov. isolated from human stools. International Journal of Systematic and Evolutionary Microbiology; 56:1505–1508.

Chowdhury, R., Islam, K.M.S., Khan, M.J.,. Karim, M.R, Haque, M.N., Khatun, M., Pesti, G.M. (2009). Effect of citric acid, avilamycin, and their combination on the performance, tibia ash, and immune status of broilers. Poultry Science 88:1616–1622.

Chowdhury, S.A., Arias, C.A., Nallapareddy, S.R., Reyes, J., Willems, R.J.L., Murray, B.E. (2009). A trilocus sequence typing scheme for hospital epidemiology and subspecies differentiation of an important 151 nosocomial pathogen, *Enterococcus faecalis*. J. Clin. Microbiol, 47:2713–2719.

Colford, J.M. Jr, Schiff, K.C., Griffith, J.F., Yau, V., Arnold, B.F., Wright, C.C., et al. (2012). Using rapid indicators for Enterococcus to assess the risk of illness after exposure to urban runoff contaminated marine water. Water Res., 46:2176–2186.

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

Coque, T. M., P. Seetulsingh, K. V. Singh, and B. E. Murray. (1998). Application of molecular techniques to the study of nosocomial infections caused by enterococci. Methods Mol. Med. 15:469–493.

Couce, A., and Blazquez, J. (2009). Side effects of antibiotics on genetic variability. FEMS Microbiol. Rev. 33:531–538.

Courvalin P. (2008). Predictable and unpredictable evolution of antibiotic resistance. J. Intern. Med. 264:4–16.

de Been, M., Pinholt, M., Top, J., Bletz, S., Mellmann, A., van Schaik, W. Brouwer, E. et al. (2015). Core Genome Multilocus Sequence Typing Scheme for High

Resolution Typing of *Enterococcus faecium*. Journal of Clinical Microbiology Volume 53 Number 12.

De Graef, E.M., Devriese, L. A., Vancanneyt, M., Baele, M., Collins, M.D., Lefebvre, K., et al. (2003). Description of *Enterococcus canis* sp. nov. from dogs and reclassification of *Enterococcus porcinus*. International Journal of Systematic and Evolutionary Microbiology, 53:1069-1074.

Desmarais, T.R., Solo-Gabriele, H.M. Palmer, CJ. (2002). Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Applied and Environmental Microbiology, p. 1165–1172.

Devriese, L.A., De Pelsmaeck, K. (1987). The anal region as a main carrier site of *Staphylococcus intermedius* and *Streptococcus canis* in dogs. Veterinary Record, 121:302–303.

Devriese, L.A., Haesebrouck, F. (1991). *Enterococcus hirae* in different animal species. Veterinary Record, 129:391–392.

Devriese, L.A., Pot, B., Van Damme, L., Kersters, K., Haesebrouck, F. (1994). Identification of Enterococcus species isolated from foods of animal origin. International Journal of Food Microbiology, 26:187–197.

Donskey, C.J. (2004). The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. Clinical Infectious Diseases, 39:219–226.

Dowling, P.J., Festing, M., Engle, R. (2013). International human resource management. Thompson learning, London.

Dufour, A.P. (1984). Health effects criteria for fresh recreational waters. Research Triangle Park: U.S. Environmental Protection Agency.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. Science, 308(5728):1635–1638.

EFSA. (2013). Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA J. 11 (11), 3449 (108 pp.).

EP/CEU. (2006). Pan-Canadian primary health care indicator update report. Health System Performance. Canadian Instute for Health Information.

European Centre for Disease Prevention and Control (ECDC), **European Medicines Agency** (EMEA). Technical report. (2009). The bacterial challenge: time to react.

Facklam, R.R. (1973). Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci. Journal of Applied Microbiology, 26:138–145.

Facklam, R.R., Carvalho, M.G., Teixeira, L.M. (2002). Enterococcus. In M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, & L. B. Rice, The

Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance (pp. 1-54). Washington, DC: ASM Press.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., Spratt, B.G. (2004). eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. Journal of Bacteriology, p. 1518–1530.

Ferguson, D.M., Moore, D.F., Getrich, M.A., Zhowandai, M.H. (2005). Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. Journal of Applied Microbiology, 99:598–608.

Finegold, S. M., Sutter, V. L., & Mathisen, G. E. (1983). Normal indigenous intestinal flora. In D. J. Hentges, Human intestinal microflora in health and disease (pp. 3-29). Waltham: Academic Press.

Fletcher, S. (2015). Understanding the contribution of environmental factors in the spread of antimicrobial resistance. Environ Health Prev Med, 20:243–52.

Franz, C.M., Holzapfel, W.H., Stiles, M.E. (1999). Enterococci at the crossroads of food safety? International Journal of Food Microbiology, 47(1-2):1–24. PubMed PMID: 10357269.

Franz, C.M., Stiles, M.E., Schleifer, K.L., Holzapfel, W.H. (2003). Enterococci in foods--a conundrum for food

safety. International Journal of Food Microbiology; 88(2-3):105–122.

Freitas, A.R., Coque, T.M., Novais, C., Hammerum, A.M., Lester, C.H., Zervos, M.J., Don-abedian, S., Jensen, L.B., Francia, M.V., Baquero, F., Peixe, L. (2011). Human andswine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishableplasmids. J. Clin. Microbiol. 49, 925–931.

Fujioka, R.S., Tenno, K., Kansako, S. (1988). Naturally occurring fecal coliforms and fecal streptococci in Hawaii's freshwater streams. Toxicity Assessment, 3, 613-630.

Gaechter, T., Wunderlin, C., Schmidheini, T., Solioz, M. (2012). Genome sequence of *Enterococcus hirae* (*Streptococcus faecalis*) ATCC 9790, a model organism for the study of ion transport, bioenergetics, and copper homeostasis. Journal of Bacteriology, 194:5126–5127.

Galloway-Pena, J., Roh, J.H., Latorre, M., Qin, X., Murray, B.E. (2012). Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of *Enterococcus faecium*. PLoS One, 7(1):30187.

Gilmore, M.S., Lebreton, F., van Schaik, W. (2013). Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. Current Opinion in Microbiology. 16:10–6.

Gilmore, M.S., Clewell, D.B, Ike Y., Shankar N. (2014). The Enterococci: from commensals to leadings causes of drug resistant infection. Massachusetts Eye and Ear Infirmary Boston. ASM Press.

Giraffa, G. (2002). Enterococci from foods. FEMS Microbiology Reviews; 26(2):163–171.

Giraffa, G., Carminati, D. (1997). Control of *Listeria monocytogenes* in the rind of Taleggio, a surface-smear cheese, by a bacteriocin from *Enterococcus faecium* 7C5. Sciences des Aliments,17:383–391.

Grant, S.B., Sanders, B.F., Boehm, A.B., Redman, J.A., Kim, J.H., Mrse, R.D., et al. (2001). Generation of enterococci bacteria in a costal saltwater marsh and its impact on surf zone water quality. Environmental Science & Technology, 15:2407–2416.

Graudal, H. (1957). The classification of motile streptococci within the enterococcus group. Acta Pathologica Microbiota Scandinavica, 41:403–410.

Graves, A.K., Weaver, R.W. (2010). Characterization of enterococci populations collected from a subsurface flow constructed wetland. Journal of Applied Microbiology, 108:1226–1234.

Guthof, O. (1955). A new serologic group of alphahemolytic Streptococci (serologic group Q). Zentralbl Bakteriol Orig, 164:60–3.

Halary, S., Leigh, J. W., Cheaib, B., Lopez P., Bapteste, E. (2010). Network analyses structure genetic diversity in independent genetic worlds. Proc. Natl. Acad. Sci. U. S. A. 107:127–132.

Halliday, E., Gast, R.J. (2011). Bacteria in beach sands: an emerging challenge in protecting coastal water quality and bather health. Environmental Science & Technology, 45:370–379.

Hay, S.I., Rao, P.C., Dolecek, C., Day, N.P.J., Stergachis A., Lopez A.D., Murray, C.J.L. (2018). Measuring and mapping the global burden of antimicrobial resistance. BMC Medicine 16:78.

Hayashi, H., Takahashi, R., Nishi, T., Sakamoto, M., Benno, Y. (2005). Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. Journal of Medical Microbiology, 54:1093–1101.

Heimer, S.R., Morrison, D., Gilmore, M.S. (2015). The Enterococci. Molecular Medical Microbiology. Elsevier Ltd.

Holzapfel, W.H.N, Wood, Brian J.B. (2014). The Genera of Lactic Acid Bacteria. Food Science & Nutrition.

Homan, W.L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., et al. (2002). Multilocus sequence typing scheme for *Enterococcus faecium*. Journal of Clinical Microbiology; 40:1963–1971.

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. International Journal of Food Microbiology, 88(2-3):123–131.

Lam, M.M., Seemann, T., Tobias, N.J., Chen, H., Haring, V., Moore, R.J., Ballard, S., Grayson, L.M., Johnson, P.D. et al. (2013). Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant *Enterococcus faecium*. BMC Genomics, 14, 595.

Lam, M.M., Seemann, T., Bulach, D.M., Gladman, S.L., Chen, H., Haring, V., Moore, R.J., Ballard, S., Grayson, M.L., Johnson, P.D., Howden, B.P., Stinear, T.P. (2012). Comparative analysis of the first complete *Enterococcus faecium* genome. J. Bacteriol.194, 2334–2341.

Lebreton, F., van Schaik, W., McGuire, A.M., Godfrey, P., Griggs, A., Mazumdar, V., et al. (2013). Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. MBio, 4(4):e00534–00513.

Lebreton, F., Manson, A.L., Saavedra, J.T., Straub, T.J., Earl, A.M., Gilmore, M.S. (2017). Tracing the Enterococci from Paleozoic Origins to the Hospital. Cell 169, 849–861, Elsevier Inc.

Luepke, K.H., Suda, K.J., Boucher, H., Russo, R.L., Bonney, M.W., Hunt, T.D., Mohr, G.F. (2017). Past, Present, and Future of Antibacterial Economics: Increasing

Bacterial Resistance, Limited Antibiotic Pipeline, and Societal Implications. Pharmacotherapy, 37:71-84.

Inamura, G.J., Thompson, R.S., Boehm, A.B., Jay, J. A. (2011). Wrack promotes the persistence of fecal indicator bacteria in marine sands and seawater. FEMS Microbiology Ecology.;77:40–9.

Kalina, A.P. (1970). The position of enterococci in the system of microorganisms. Zhurnal Mikrobiologii, Epidemiologii, I Immunobiologii, 47:20–1.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M. & Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res., 44, D457–462.

Kaukas, A., Hinton, M., Linton, A.H. (1987). The effect of ampicillin and tylosin on the faecal enterococci of healthy young chickens. Journal of Applied Bacteriology, 62:441–447.

Kawalec, M., Pietras, Z., Daniłowicz, E., Jakubczak, A., Gniadkowski, M., Hryniewicz, W., et al. (2007). Clonal structure of *Enterococcus faecalis* isolated from Polish hospitals: characterization of epidemic clones. Journal of Clinical Microbiology, 45:147–153.

Kinnebrew, M.A., Ubeda, C., Zenewicz, L.A., Smith, N., Flavell, R.A., Pamer, E.G. (2010). Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. The Journal of Infectious Diseases, 201:534–543.

Kopit, L.M., Kim, E.B., Siezen, R.J., Harris, L.J., Marco, M.L. (2014). Safety of the surrogate *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. Appl. Environ. Microbiol. 80: 1899-1909.

Korajkic, A., Brownell, M.J., Harwood, V.J. (2011). Investigation of human sewage pollution and pathogen analysis at Florida Gulf Coast beaches. Journal of Applied Microbiology, 110:174–183.

Kristich, C., Rice, L., Arias, C. (2014). Enterococcal Infection—Treatment and Antibiotic Resistance. In Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Massachusetts Eye and Ear Infirmary, Boston.

Kühn, I., Iversen, A., Burman, L.G., Olsson-Liljequist, B., Franklin, A., Finn, M., et al. (2003). Comparison of enterococcal populations in animals, humans, and the environment - a European study. International Journal of Food Microbiology, 88(2-3):133–145.

Layton, B.A., Walters, S.P., Lam, L.H., Boehm, A.B. (2010). *Enterococcus* species distribution among human and animal hosts using multiplex PCR. Journal of Applied Microbiology, 109:539–547.

MacCallum, W.G., Hastings, T.W. (1899). A case of acute endocarditis caused by Micrococcus zymogenes (Nov. Spec.), with a description of the microorganism. The Journal of Experimental Medicine, 4(5-6):521–534.

Magnus, C.A., McCurdy, A.R., Ingledew, W.M. (1988). Further studies on the thermal resistance of *Streptococcus faecium* and *Streptococcus faecalis* in pasteurized ham. Canadian Institute of Food Science and Technology Journal, 21:209–212.

Martinez-Murcia, A.J., Collins, M.D. (1991). *Enterococcus sulfureus*, a new yellow-pigmented *Enterococcus* species. FEMS Microbiology Letters, 80(1):69–74.

McBride, S.M., Fischetti, V.A., Leblanc, D.J., Moellering Jr., R.C., Gilmore, M.S. (2007). Genetic diversity among *Enterococcus faecalis*. PLoS One 2, e582.

McCoy E. (1954). Changes in the host flora induced by chemotherapeutic agents. Annu Rev Microbiol, 8: 257–272.

Miller, W.R, Munita, J.M., Arias, C.A. (2015). Mechanisms of antibiotic resistance in enterococci. Expert Rev. Anti. Infect. Ther. 12: 1221–1236.

MNHW, (1992). Canadian Recreational Water Guidelines. Canadian Government Publishing Centre, Ottawa, Cat. H49-70/1991E, 101pp.

Monstein, H.J., Tiveljung, A., Kraft, C.H., Borch, K., Jonasson J. (2000). Profiling of bacterial flora in gastric biopsies from patients with *Helicobacter pylori*-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. Journal of Medical Microbiology, 49:817–822.

Müller, T., Ulrich, A., Ott, E.M., Müller, M. (2001). Identification of plant-associated enterococci. Journal of Applied Microbiology, 91:268–278.

Mundt, J.O. (1963). Occurrence of enterococci on plants in a wild environment. Journal of Applied Microbiology,11(2):141–144.

Mundt, J.O. (1986). Enterococci. In J. G. Holt, P. H. Sneath, & N. R. Krieg, Bergey's Manual of Systematic Bacteriology, vol. 2. (pp. 1063-1065). Baltimore: Williams & Wilkins.

Murray, B.E. (1990). The life and times of the *Enterococcus*. Clinical Microbiology Reviews, 3(1):46–65.

Nallapareddy, S.R., Duh, R.W., Singh, K.V., Murray, B.E. (2002). Molecular typing of selected *Enterococcus faecali*s isolates: pilot study using multilocus sequence typing and pulsed- field gel electrophoresis. Journal of Clinical Microbiology, 40(3):868–876.

Nallapareddy, S.R., Duh, R.W., Singh, K.V., Murray, B.E. (2002). Molecular typing of selected *Enterococcus faecalis* isolates: pilot study using multilocus sequence typing and pulsed-field gel electrophoresis. J. Clin. Microbiol, 40:868–76.

Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., et al. (2005a). Application of multilocus sequence analysis (MLSA) for rapid

identification of *Enterococcus* species based on rpoA and pheS genes. Microbiology, 151(Pt 7):2141–2150.

Naser, S.M., Vancanneyt, M., De Graef, E., Devriese, L.A., Snauwaert C., Lefebrve K., et al. (2005b). *Enterococcus canintestini* sp. nov., from faecal samples of healthy dogs. International Journal of Systematic and Evolutionary Microbiology, 55(Pt 5):2177–2182.

Noblea, R.T., Mooreb, D.F., Leecasterc, M.K., McGeed, C.D., Weisberge, S.B. (2003). Comparison of total coliform, fecal coliform, and *Enterococcus* bacterial indicator response for ocean recreational water quality testing. Water Research, 37, 1637–1643.

Nogueira T., Rankin, D.J., Touchon, M., Taddei, F., Brown, S.P., Rocha, E.P. (2009). Horizontal gene transfer of the secretome drives the evolution of bacterial cooperation and virulence. Curr. Biol. 19:1683–1691.

Novais, C., Leon, R., Tedim, A., Freitas, A., Peixe, L., Baquero, F., et al. (2013). Spread of CTn5801 among enterococcal species from different origins. 23rd European Congress of Clincal Microbiology and Infections Diseases. Berlin.

Nowlan, S.S., Deibel, R.H. (1967). Group Q streptococci. I. Ecology, serology, physiology, and relationship to established enterococci. Journal of Bacteriology; 94:291–296.

O'neill, J. (2016). Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance.

Obiri-Danso, K., Jones, K. (2000). Intertidal sediments as reservoirs for hippurate negative *Campylobacters*, *Salmonellae* and faecal indicators in three EU recognised bathing waters in north west England. Water Research, 34:519–527.

Orla-Jensen, S. (1919). The lactic acid bacteria. Memoirs of the Academy of the Royal Society of Denmark. Section of Sciences Series, 85:81–197.

Palmer, K., Godfrey, P., Griggs, A., Kos, V. N., Zucker, J., Desjardins, C., et al. (2012). Comparative genomics of enterococci: variation in *Enterococcus faecalis*, clade structure in *E. faecium*, and defining characteristics of *E. gallinarum* and *E. casseliflavus*. mBio, 3(1):e00318–00311.

Pinkston, K.L., Gao, P., Diaz-Garcia, D., Sillanpää, J., Nallapareddy, S.R., Murray, B.E., et al. The Fsr quorum-sensing system of *Enterococcus faecalis* modulates surface display of the collagen-binding MSCRAMM Ace through regulation of gelE. J Bacteriol, 193: 4317–4325.

Prieto, A.M.G., van Schaik, W., Rogers, M.R.C., Coque, T.M., Baquero, F., Corander, J., 5 Willems, R.J.L. (2016). Global emergence and dissemination of enterococci as nosocomial pathogens: Attack of the Clones? Frontiers in Microbiology Volume 7 Article 788.

Qin, X., Galloway-Peña, J.R., Sillanpaa, J., Roh, J.H., Nallapareddy, S.R., Chowdhury, S., et al. (2012). Complete genome sequence of *Enterococcus faecium* strain TX16 and comparative genomic analysis of *Enterococcus faecium* genomes. BMC Microbiology, 12:135.

Qin, X., Galloway-Peña, J.R., Sillanpaa, J., Roh, J.H., Nallapareddy, S.R., Chowdhury, S., et al. (2012). Complete genome sequence of *Enterococcus faecium* strain TX16 and comparative genomic analysis of *Enterococcus faecium* genomes. BMC Microbiology, 12:135.

Rahkila, R., Johansson, P., Sade, E., Björkroth J. (2011). Identification of Enterococci from broiler products and a broiler processing plant and description of *Enterococcus viikkiensis* sp. nov. Applied and Environmental Microbiology, 1196–1203.

Richard, J.F. and Yitzhak, T. (2014). Antibiotics and Bacterial Resistance in the 21st Century. Perspectives in Medicinal Chemistry, 25–64.

Sanz Pérez, B., Lòpez Lorenzo, P., Garcia, M.L., Hernandez, P.E. and Ordonez, J.A. (1982). Heat resistance of enterococci. Milchwissenschaft, 7, 724–726.

Schleifer, K.H., Kilpper-Balz, R. (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. International Journal of Systematic and Evolutionary Microbiology, 34:31–34.

Sghir, A., Gramet, G., Suau, A., Violaine, R., Pochart, P., Dore, J. (2000). Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Applied and Environmental Microbiology, 66:2263–2266.

Sherman, J.M. (1937). The streptococci. Bacteriological Reviews, 1:3–97.

Skippington, E., Ragan, M.A. (2011). Lateral genetic transfer and the construction of genetic exchange communities. FEMS Microbiol. Rev. 35, 707–735.

Smyth, C.J., Matthews, H., Halpenny, M. K., Brandis, H., Colman, G. (1987). Biotyping, serotyping and phage typing of *Streptococcus faecalis* isolated from dental plaque in the human mouth. Journal of Medical Microbiology, 23:45–54.

Soares-Santos, V., Barreto, A.S., Semedo-Lemsaddek, T. (2015). Characterization of Enterococci from Food and Food-Related Settings. Journal of Food Protection, 78: 1320–1326.

Solheim, M., Brekke, M.C., Snipen, L.G., Willems, R.J., Nes, I.F., Brede, D.A. (2011). Comparative genomic analysis reveals significant enrichment of mobile genetic elements and genes encoding surface structure-proteins in hospital-associated clonal complex 2 *Enterococcus faecalis*. BMC Microbiol.,11, 3.

Sørensen, T.M, Marianne Blom, M.D., Monnet, D.M., Frimodt-Møller, N., Poulsen, R.L., Espersen, F. (2001).

Transient intestinal carriage after ingestion of antibiotic-resistant *Enterococcus faecium* from chicken and pork. N. Engl. J. Med., Vol. 345, No. 16.

Švec, P., Vancanneyt, M., Koort, J., Naser, S.M., Hoste, B., Vihavainen, E., et al. (2005). *Enterococcus devriesei* sp. nov. associated with animal sources. International Journal of Systematic and Evolutionary Microbiology, 55:2479–2484.

Švec, P., Vancanneyt, M., Sedlácek, I., Naser, S.M., Snauwaert, C., Lefebvre, K., et al. (2006). *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. International Journal of Systematic and Evolutionary Microbiology, 56(Pt 3):577–581.

Švec, P., Vandamme, P., Bryndová, H., Holochova, P., Kosina, M., Maslanova, I., et al. (2011). *Enterococcus plantarum* sp. nov. isolated from plants. International Journal of Systematic and Evolutionary Microbiology, 62(Pt 7):1499–1505.

Strzelecki, J., Hryniewicz, W., Sadowy, E. (2011). Gelatinase-associated phenotypes and genotypes among clinical isolates of *Enterococcus faecalis* in Poland. Pol J Microbiol, 60:287–92.

Tannock, G. W., Cook, G. (2002). In M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, & L. B. Rice, The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. Washington, D. C.: ASM Press.

Tedim A.S. (2017). Análisis de elementos genéticos móviles en *Enterococcus faecium*: coste biológico e impacto en la diversificación clonal. PhD Thesis. Universidad Complutense De Madrid, Facultad De Farmacia, Departamento de Microbiología II.

Teixeira, L.M., Carvalho, M.G., Merquior, V.L., Steigerwalt, A.G., Teixeira, M.G., Brenner, D.J., et al. (1997). Recent approaches on the taxonomy of the enterococci and some related microorganisms. Advances in Experimental Medicine and Biology, 418:397–400. PubMed PMID: 9331679.

Thal, L. A., Chow, J.W., Mahayni, R., Bonilla, H., Perri, M.B., Donabedian, S.A., et al. (1995). Characterization of antimicrobial resistance in enterococci of animal origin. Antimicrobial Agents and Chemotherapy, 39(9):2112–2115.

Thiercelin, M.E., Jouhaud, L. (1899). Sur un diplococque saprophyte de l'intestin susceptible de devenir pathogene. CR Soc. Biol.5:269–271.

USEPA, United States Environmental Protection Agency (2012). Recreational Water Quality Criteria. Office of Water 820-F-12-058.

USEPA, United States Environmental Protection Agency (2003). Guidelines for water reuse. U.S. Environmental Protection Agency, Washington, DC, EPA/625/R-04/108 (NTIS PB2005 106542).

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle,1 S., Rossi, R., Jabes, D., Goossens, H. (2004). Development of a Multiplex PCR for the Detection of asa1, gelE, cylA, esp, and hyl Genes in Enterococci and Survey for Virulence Determinants among European Hospital Isolates of *Enterococcus faecium*. J Clin Microbiol, 4473–4479.

van Schaik, W., Willems, R.J. (2010). Genome-based insights into the evolution of enterococci. Clinical Microbiology and Infection, 16:527–532.

Yamahara, K.M., Layton, B.A., Santoro, A.E., Boehm, A.B. (2007). Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. Environmental Science & Technology, 41:4515–4521.

Whitman, R.L., Shively, D.A., Pawlik, H., Nevers, M.B., Byappanahalli, M.N. (2003). Occurrence of *Escherichia coli* and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. Applied and Environmental Microbiology, 69:4717–4719.

Werner, G., Coque, T.M, Franzc, C.M.A.P., Grohmannd, E., Hegstade, K., Jenseng, L., van Schaikh, W., Weaveria, K. (2013). Antibiotic resistant enterococci—Tales of a drug resistance gene trafficker. International Journal of Medical Microbiology.

WHO, World Health Organisation (2018). Antimicrobial resistance. http://www.who.int/news-room/facts-in-pictures/detail/antimicrobial-resistance.

WHO, World Health Organisation (2015). Antibiotic Resistance: Multi-country public awareness survey. WHO Library Cataloguing. ISBN 978 92 4 150981 7.

WHO, World Health Organisation (2003). Guidelines for safe recreational water environments. VOLUME 1: Coastal and Fresh Waters.

Willems, R.J.L., Hanage, W.P., Bessen, D.E., Feil, E.J. (2011). Population biologyof Gram-positive pathogens: high-riskclones for dissemination of antibiotic resistance. FEMS Microbiol Rev, 35, 872–900.

Witte, W. (2000). Ecological impact of antibiotic use in animals on different complex microflora: environment. International Journal of Antimicrobial Agents, 14: 321-325.

Zhi Zhong, Z., Zhang, W., Song, Y., Liu, W., Xu, H., Xi, X., Menghe, B., Zhang, H., Sun, Z. (2017). Comparative genomic analysis of the genus *Enterococcus*. Microbiological Research, 95–105.

Hypothesis and objectives

Over the past two decades infections due to AR bacteria have escalated world-wide, affecting patient morbidity, mortality, and health care costs. Among these bacteria, enterococci represent those that have been distinguished for the ability to acquire and disseminate antibiotic resistant determinants, causing difficult to treat infections. A plethora of studies published treated this argument in hospital setting, whereas food sector has been highlighted in recent researches. For this purpose, this PhD project focused on the two main sources of transmission route of enterococci to humans: reclaimed water for agriculture reuse and dairy products.

The **HYPOTHESIS** was the presence of specific species of enterococci, strongly related to matrix studied and the occurrence of resistant enterococcal genomics groups to specific geographic niches that might confirm the general differences in enterococci lineages–clinical and non-clinical clades and to confirm niche-adapted genetic resources.

The **MAIN OBJECTIVE** of this dissertation was to get insights on the ecological and epidemiological role of antibiotic resistant enterococci in different setting, comparing diversity of antibiotic resistance pattern and assess the prevalence of resistance of water-and food-associated enterococci.

The more specific **OBJECTIVES** were the following:

1. Evaluation of the AR prevalence of enterococcal isolates from Sicilian cheeses, in order to assess their

role in the risk of dissemination of antibiotic resistance in animal origin food, by testing the biodiversity and the AR profile of enterococci from Ragusano PDO and Pecorino Siciliano, at different ripening stages.

1.1. Genotyping and sequencing of the enterococci isolates to compare their characteristics and clonality.

2. Assessment of the removal efficiency of a full-scale horizontal subsurface flow CW combined with UV for reusing treated urban wastewater in agriculture. For this purpose, removal of microbial indicators of faecal contamination (*Escherichia coli*, Total coliforms and enterococci), ARGs and antibiotic resistant enterococci were monitored before and after the applied treatment.

3. Evaluation of the seasonal efficiency of lagooning and UV disinfection systems, as tertiary treatment processes, in the removal of *E. coli*, total coliform, *Enterococcus* spp., somatic coliphages and *C. perfringens* and by monitoring both physico-chemical and microbiological indicators for a one year period.

4. Comprehensive assessment of system efficiency in removing *Enterococcus* population in order to evaluate the effects of the system in the selection of resistant strains.

4.1 Study of clonal relatedness of the enterococcal population to obtain information about the

ecological distribution in a determined geographical area.

The application of such a polyphasic approach, including conventional and molecular methods, was belived to provide additional insights into antibiotic resistance prevalence and dissemination within the *Enterococcus* genus contributing to the detection of specific genetic targets for antibiotic resistances.

CHAPTER 1

ENTEROCOCCI IN CHEESE

Backround

Enterococci are ubiquitous microorganisms related to the LAB group. Although their presence in dairy products is usually associated with inadequate hygiene practices as a consequence of faecal contamination (Franciosi et al., 2009; Suzzi et al., 2000), nowadays, it is widely accepted and signed by the European Regulation that enterococci in food are not always due to faecal contamination and sets no limit for their presence in foods (Commission Regulation, 2007). Indeed, enterococci are still considered an important component of the natural microbiota responsible for ripening and aroma development of certain traditional cheeses and sausages, especially those produced in the Mediterranean area. For their ability to survive and compete in the human GI tract, they have been used as active strains in commercial probiotic preparations (Foulquié-Moreno et al., 2006; Franz et al., 1999; Holzapfel et al., 1998), and also as starter cultures (Foulquié-Moreno et al., 2006), and bio-preservatives for the production of enterocin (Stiles and Holzapfel, 1997). Enterococci are the most thermotolerant non-sporulating bacteria and can survive to pasteurization temperatures. Tolerance to harsh environmental conditions explains their survival during processing of cooked and uncooked cheeses and cured meats and their ability to multiply during fermentations (Hugas et al., 2003). This high survival capacity in changing environments such as food might be explained in part by their capacity to form

biofilms (Creti et al., 2004; Jahan and Holley, 2014; Tendolkar et al., 2006). However, over the past decade, enterococci have emerged as important nosocomial pathogens (Vankerckhoven et al., 2004), for their enhanced AR that can be found also in large numbers in foods of animal origin (Aslam et al., 2012; Giraffa, 2002; Jamet et al., 2012) and can be transmitted to humans through consumption and handling of contaminated animal-derived food (Sørensen et al., 2001). Therefore, it is also supposed that enterococci from foods of animal origin may play a significant role in the dissemination of AR genes, particularly since these genes are located on transferable genetic elements (Clewell et al., 1995; Vignaroli et al., 2011). The food chain is potentially one of the main routes for transmission of AR bacteria between animal and human populations (Witte, 1997). These considerations reinforce the concern expressed elsewhere about the safety of enterococcal strains present in foods, particularly in commercially food of animal origins where viable antibiotic resistant enterococci may be present.

References

Aslam, M., Diarra, M.S., Checkley, S.L., Bohaychuk, V., Masson, L. (2012). Characterization of antimicrobial resistance and virulence genes in *Enterococcus* spp. isolated from retail meats in Alberta, Canada. International journal of food microbiology, 156(3):222-30.

Clewell, D.B., Flannagan, S.E., Jaworski, D.B. (1995). Unconstrained bacterial promiscuity: the Tn916–Tn1545 family of conjugative transposons. Trends Microbiology 3, 229–236.

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

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R., Baldassarri, L. (2004). Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. J. Med. Microbiol., 53(Pt 1):13-20.

Foulquié Moreno MR1, Sarantinopoulos P, Tsakalidou E, De Vuyst L. (2006). The role and application of enterococci in food and health. Int. J. Food Microbiol.106, (1):1-24.

Franciosi, E., Settanni, L., Cavazza, A., Poznanski, E. (2009). Presence of enterococci in raw cow's milk and "Puzzone di Moena" cheese. J. Food Process. Preserv. 33, 204–217.

Franz, C.M.A.P., Worobo, R. W., Quadri, L.E.N., Schillinger, U., Holzapfel, W.H., Vederas, J.C. & Stiles, M.E. (1999). Atypical genetic locus associated with constitutive production of enterocin B by *Enterococcus faecium* BFE 900. Appl. Environ. Microbiol 65, 2170–2178.

Giraffa, G. (2002). Enterococci from foods. FEMS Microbiology Reviews; 26(2):163–171. PubMed PMID: 12069881.

Holzapfel, W.H, Haberer P., Snel, J., Schillinger, U., Huis in't Veld, J.H.J. (1998). Overview of Gut Flora and Probiotics'. International Journal of Food Microbiology, 41(2):85-101.

Hugas, M., Garriga, M., Aymerich, M.T. (2003). Functionality of enterococci in meat products. International Journal of Food Microbiology, 18: 223-233.

Jahan, M., Holley, R.A. (2014). Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25°C and 37°C. Int. J. Food Microbiol., 17;170:65-9.

Jamet, E., Akary, E., Poisson, M. A., Chamba, J. F., Bertrand, X., and Serror, P. (2012) Prevalence and characterization of antibiotic resistant Enterococcus faecalis in French cheeses. Food Microbiol. 31, 191-198.

Sørensen, T.M, Marianne Blom, M.D., Monnet, D.M., Frimodt-Møller, N., Poulsen, R.L., Espersen, F. (2001).

Transient intestinal carriage after ingestion of antibiotic-resistant enterococcus faecium from chicken and pork. N. Engl. J. Med., Vol. 345, No. 16.

Stiles, M.E., Holzapfel, W.H. (1997). Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol., 36(1):1-29.

Suzzi, G., Caruso, M., Gardini, F., Lombardi, A., Vannini, L., Guerzoni, M., Andrighetto, C., Lanorte, M.T., 2000. A survey of the enterococci isolated from an artisanal Italian goat's cheese (semicotto caprino). J. Appl. Microbiol. 89, 267–274.

Tendolkar, P.M., Baghdayan, A.S., Shankar, N. (2006). Putative surface proteins encoded within a novel transferable locus confer a high-biofilm phenotype to *Enterococcus faecalis*. J. Bacteriol., 188: 2063–2072.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H. (2004). Development of a Multiplex PCR for the detection of *asa1, gelE, cylA, esp,* and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. J Clin Microbiol. 42: 4473–4479.

Vignaroli, C., Zandri, G., Aquilanti, L., Pasquaroli, S., Biavasco, F. (2011). Multidrug-resistant enterococci in animal meat and faeces and co-transfer of resistance from an Enterococcus durans to a human *Enterococcus faecium*. Curr. Microbiol. 62:1438-47.

Witte, W. (1997). Impact of antibiotic use in animal feeding on resistance of bacterial pathogens in humans. Ciba Foundantion Symposium, 207, 61-71.

Enterococcus spp. in Ragusano PDO and Pecorino Siciliano cheese types: a snapshot of their antibiotic resistance distribution

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Abstract

In the present study, 110 enterococci were isolated from two Sicilian cheese types. Isolates, firstly identified by MALDI-TOF/MS and a multiplex PCR assay, were tested for susceptibility to eleven main antimicrobials. Clonal relationship among isolates were evaluated by pulsed-field-gel electrophoresis (PFGE) analysis and the presence of vanA and vanB genes, in vancomycin resistant enterococci (VRE), was investigated. Overall, *E. faecalis*, *E. durans* (35% for each species) and *E.faecium* (28%) were the major identified species. Different occurrence between cheese types was revealed. Most isolates from Ragusano PDO cheese were identified as *E. durans* (46%) and/or *E.faecalis* (43%), while *E. faecium* (605) was mainly detected in Pecorino Siciliano cheese. High incidence of resistance (97% of total strains) was detected for rifampicin, erythromycin and ampicillin. Moreover, 83 isolates (75%) exhibited multidrug-resistant phenotypes and the one VRE (vanB) isolate was identified as *E. durans*. PFGE analysis clustered isolates into 22 genotypes and the presence of the same PFGE types, for both *E. durans* and *E. faecalis*, in the two cheese types, suggest the link between enterococci and geographical area of production. Results of present study raise concerns about possible role of dairy enterococci as reservoirs of antibiotic resistance.

Keywords: enterococci, antibiotic resistance, PFGE, MIC, cheese

1. Introduction

In recent years, there is a growing concern for the antibiotic resistance of food-related enterococci, no more regarded as GRAS (Generally Recognized as Safe) organisms. Enterococci are highly widespread microorganisms, thanks to their strong adaptability and resistance to adverse environmental conditions. They are present in soil, water, and food, such as meat, milk and cheese, but the gastrointestinal tract of humans and animals remains the larger reservoir of enterococci (Aarestrup et al., 2002a; Gilmore et al., 2013; Hugas et al., 2003; Ogier and Serror, 2008). Enterococci have extensively been studied for their ability to carry out beneficial technological effects, in different fermented food, especially in traditional Mediterranean cheeses (Bonacina et al., 2017). *Enterococcus* species are also known to produce bacteriocins active against many human pathogens (Izquierdo et al., 2009; Ness et al., 2014). Nevertheless, they have been revealed as food spoiler (Ogier and Serror, 2008) and, contrarily to other lactic acid bacteria, as opportunistic pathogens, being a major cause of nosocomial infections (Vergis et al., 2001;Guzman Pietro et al., 2016). Although these bacteria were considered to have low virulence, the high documented mortality rates have been related to the increasing of acquired antibiotic resistance (Na et al., 2012). Indeed, for most enterococci, the resistance to several antimicrobial agents is a remarkable species-characteristic and the emergence and spread of Vancomycin Resistant Enterococci (VRE) has focused greater attention on this genus (Franz et al., 1999). Enterococci are intrinsically resistant to several antibiotics and can readily accumulate mutations and exogenous genes

conferring additional resistances (Guzman Prieto et al., 2016; Bocanegra-Ibarias et al., 2016; Rossi et al., 2014). The acquisition of resistance genes often occurs via conjugation with plasmids or conjugative transposons that can potentially carry multiple antibiotic resistance genes (Arias et al., 2012; Clewell et al 2014; Coque et al., 1998).
Recently, antimicrobial resistance and virulence factors have been detected in retail foods including cheeses (Hammad et al., 2015; Koluman et al., 2009), being food chain one of the main routes of antibiotic resistance dissemination. In most cases, studies focused on *E. faecalis* and *E. faecium* species (Aarestrup et al., 2002b), which represent, about 75% and 20% of enterococcal-related infections in the world (Sánchez Valenzuela et al., 2008; Peel et al., 2011). Moreover *Enterococcus* spp. were found able to transfer antibiotic resistance genes to *Staphylococcus aureus* and *Listeria* spp. in human or animal intestinal tract, in environment, and in food (Walsh et al., 2001; Pesavento et al., 2010; Sparo et al., 2011). In addition, *E. faecium* has been classified as one of the key problem bacteria (named as ESKAPE), by the Infectious Diseases Society of America, (Bonten et al., 2001) and, most recently have not been included in the qualified presumption of safety (QPS) list, by European Food Safety Authority (EFSA, 2017), according to the putative virulence profile (Freitas et al., 2018). Based on the above considerations the aim of the present study was to evaluate the prevalence of antibiotic resistance of enterococcal isolates from Sicilian cheeses in order to assess their role in the risk of dissemination of antibiotic resistance in animal origin food. For this purpose, the biodiversity and the antibiotic resistance profile of enterococcal population from

Ragusano PDO and Pecorino Siciliano, at different ripening stages, were tested.

2. Materials and methods

2.1 Sample collection and enterococci isolation

Samples of Ragusano PDO and Pecorino Siciliano cheese, at different ripening stages (90 and 180 days) were collected from two dairy factories located in Western Sicily region (South Italy). Samples were transported to the Laboratory of Food Microbiology of University of Catania. For the isolation of enterococci, cheese samples (25 g) were taken with sterile borer (1 cm in diameter) from four individual cheese samples of each manufacturer. Cheeses were homogenized in sterile physiological solution (0.9 % w/v NaCl) in a Stomacher Lab-Blender 400 (Seward Medical, London, United Kingdom) for approximately 5 min and diluted tenfold in physiological solution. Aliquots of the highest dilutions were inoculated onto Slanetz Bartley agar medium (Liofilchem, Milan, Italy) and plates incubated at 37 °C for 48 h, under aerobic conditions. Ten to fifteen colonies were randomly selected from the highest dilution plates and purified twice. Overall, 140 isolates were collected and analyzed for Gram staining, catalase reaction, growth at 45°C and 10°C, growth in presence of 6.5% (w/v) NaCl and at pH 9.6, and growth on esculin hydrolysis on bile-esculin agar (Liofilchem). Isolates were maintained in Brain Heart Infusion medium (BHI), (Liofilchem).

2.2 Enterococci identification

2.2.1 Identification by MALDI-TOF/MS

The identification of the 140 isolates was performed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry MALDI-TOF/MS (Bruker Daltonics, Germany). Sample preparation, data processing and analyses were carried out as previously described by Freiwald and Sauer (2009). Mass spectra were acquired using a Microflex™ mass spectrometer (Bruker Daltonik) and identified with manufacturer's software MALDI BioTyper™ 3.0. Standard Bruker interpretative criteria were applied; scores ≥ 2.0 were accepted for species assignment and scores ≥ 1.7 but ≤ 2.0 for genus identification.

2.2.2 Identification by multiplex PCR

Genomic DNA from isolates was extracted from a loopful of overnight BHI cultures and transferred to lysis buffer (0.25% SDS and 0.05M NaOH). The suspension was incubated at 95–100 °C for 5 min and centrifuged for 5 min at 13000 rpm. Then, 180 µL of Tris-HCl 10 mM (pH= 8.5) were added and the suspension was centrifuged for 7 min at 13000 rpm. The supernatant was transferred in clean eppendorf and storage at -20 °C until use. Genus- and species-specific multiplex PCR were applied in order to distinguish isolates into most common species of enterococci, such as *E. faecalis, E. faecium, E. durans, E. casseliflavus*, and *E. gallinarum.* For species-specific identification, the manganese-dependent superoxide

dismutase gene (sodA) was chosen (Poyart et al., 2000). Six sets of PCR primers (Table 1) were used, as previously reported (Deasy et al., 2000; Jackson et al., 2004). Two PCR reactions, consisting of different primer sets, were prepared: The first one included *E. durans* ATCC19432, *E. faecalis* ATCC19433, and *E. faecium* ATCC19434; and the second one consisted of *E. casseliflavus* ATCC25788 and *E. gallinarum* ATCC49673. The master mix EconoTAQ plus 2X was used and 0.5 µL of each genus primer was added, with the exception of *E. faecalis* (FL1, FL2 primers), and *E. gallinarum* (GA1, GA2 primers), for which 1 µL was added to the base mix. The PCR mixtures were performed in a final volume of 25 µL consisting of 24 µL of master mix and 1.0 µL of DNA template previously obtained. After 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 for 1 min, and elongation at 72 °C for 1 min. Amplification was followed by a final extension at 72 °C for 7 min. Three microliters of product were electrophoresed on a 2% 1X Tris-acetate-EDTA agarose gel containing Gel Red Nucleic Acid stain (Biotium, California). DNA molecular weight marker 100 bp (Invitrogen- Life Technologies) was used as the standard.

Table 1. PCR primers, products and reference strains for *Enterococcus* spp.

Reference strains	Primer	Sequence (5'-3')	Product size (bp)	Multiplex group	Reference
Enterococcus spp.	E1	TCAACCGGGGAGGGT	733	1 and 2	Deasy et al., 2000
	E2	ATTACTAGCGATTCCGG			
E. casseliflavus ATCC 25788	CA1	TCCTGAATTAGGTGAAAAAAC	288	2	Jackson et al., 2004
	CA2	GCTAGTTTACCGTCTTTAACG			
E. durans ATCC 19432	DU1	CCTACTGATATTAAGACAGCG	295	1	Jackson et al., 2004
	DU2	TAATCCTAAGATAGGTGTTTG			
E. faecalis ATCC 19433	FL1	ACTTATGTGACTAACTTAACC	360	1	Jackson et al., 2004
	FL2	TAATGGTGAATCTTGGTTTGG			
E. faecium ATCC 19434	FM1	GAAAAAACAATAGAAGAATTAT	215	1	Jackson et al., 2004
	FM2	TGCTTTTTTGAATTCTTCTTTA			
E. gallinarum ATCC 49673	GA1	TTACTTGCTGATTTTGATTCG	173	2	Jackson et al., 2004
	GA2	TGAATTCTTCTTTGAAATCAG			

2.3 Antimicrobial susceptibility testing

Enterococcal isolates were characterized for their susceptibility to the most relevant clinical antibiotics (Sigma-Aldrich). In details, the MIC values were detected between different range for each antibiotics, such as: tetracycline (0.5-16-µg/mL), erythromycin (1-32 µg/mL), streptomycin (32-1024 µg/mL), gentamycin (8-256 µg/mL), ampicillin (32-1 µg/mL), rifampicin (64-2 µg/mL), penicillin G (0.5-16 µg/mL), sulphametoxazole (128-4096 µg/mL), chloramphenicol (2-64 µg/mL), vancomycin (1-32 µg/mL) and kanamycin (128-4096 µg/mL). The MICs were determined by micro-dilution method using Mueller-Hinton broth. MIC microtiter tests were performed in 384-well plates, filled with an automatic liquid handling system

(EpMotion, Eppendorf, Italy) to a final volume of 80 µL. The bacteria were incubated in absence (positive control) and in presence of each antibiotic at six different concentrations. Each isolates were exposed in duplicate to each antimicrobial concentration tested at a final inoculum density of 10^8 bacteria/mL, from overnight cultures. The bacterial cell concentration was determined by flow cytometry (BD Accuri™ C6 Plus Flow Cytometer, BD Biosciences, Milan, Italy). The 384-well plates were then incubated at 37 °C for 24-48 h, and the cell density was evaluated measuring the OD600 (MicroWave RS2, Biotek, USA) and the Gene5 software (Biotek, USA). The MIC was determined as the lowest antimicrobial concentration that resulted in no visible growth. Interpretations were made according to CLSI and EUCAST-approved clinical breakpoint and epidemiological cut-off values, with exception of sulfamethoxazole and kanamycin, for which no breakpoint and/or epidemiological cut-off are available (CLSI, 2016; EUCAST, 2017).

2.4 PCR detection of antibiotic resistance genes

The VREs were subjected to detection of both vanA and vanB genes, according to Depardieu et al. (2004). PCR was performed using 1 µL of DNA template, extracted as previously described, and 24 µL of mixture, containing the master mix EconoTAQ plus 2X (Lucigen) and 0.5 µL of each primer: EA1 GGGAAAACGACAATTGC and EA2 GTACAATGCGGCCGTTA for vanA and EB3 ACGGAATGGGAAGCCGA and EB4 TGCACCCGATTTCGTTC for vanB genus. Amplification was carried out with the following thermal cycling profile: 3

min at 94 °C and 30 cycles of amplification consisting of 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C, with 7 min at 72°C for the final extension. DNA fragments were analysed by electrophoresis in 0.5 Tris-borate-EDTA on a 1% agarose gel containing Gel Red Nucleic Acid stain (Biotium, California). DNA molecular weight marker 100 bp (Invitrogen-Life Technologies) was used as standard.

2.5 Clonal relatedness

The clonal relationship among isolates was revealed by comparison of *SmaI* digested DNA profiles using PFGE. High-molecular-weight DNA was isolated from 1 mL of overnight culture as previously reported (Novais et al., 2004; Tenover et al. 1995). The digested plugs were electrophoresis using the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA) in a 1.2% agarose gels at 6 V/cm, with linear switching interval ramps from 35 s to 25 s for 25 h at 14 °C for the first block. Lambda ladder (New England BioLabs, Beverly, MA, UK) was run as molecular weight markers. After staining with gel red (Biotium), DNA bands were visualized by UV. The images acquisition was performed by using ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc. Hercules, California, USA). The obtained PFGE profiles were converted to TIFF files and subjected to cluster analysis, using BioNumerics v. 7.5 software (Applied Maths, Sint-Martens-Latem, Belgium). PFGE profiles were interpreted according to criteria of Tenover et al. (1995). PFGE band patterns were generated by BioNumerics v. 7.5 software (Applied Maths, Sint-Martens-Latem, Belgium) with tolerance position of 1%. Clustering was based on the

unweighted pair group method with arithmetic averages (UPGMA). The Dice correlation coefficient was used to analyse the similarities of banding patterns.

2.6 Data analysis

In order to evaluate the distribution of antibiotic resistance among isolates species and cheese type, data obtained at different ripening time were subjected to principal component analysis (PCA) using MATLAB, achieving high data compression efficiency of the original data.

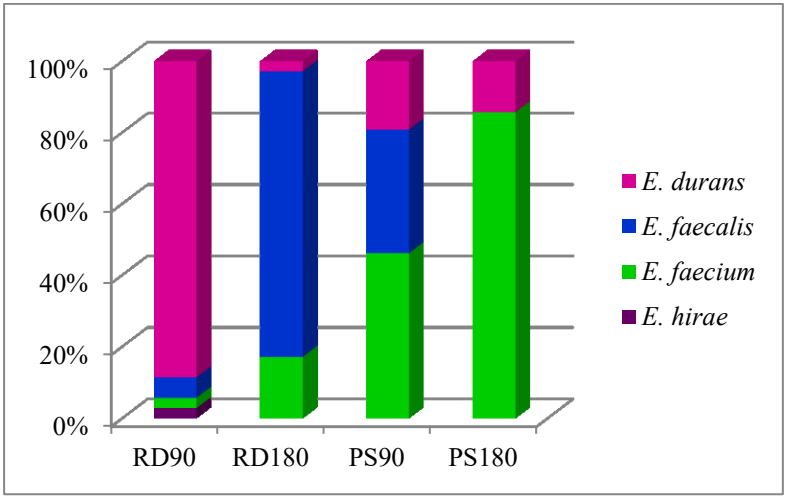
3. Results

3.1 Identification of enterococcal isolates

In this study, 140 isolates, collected from two Sicilian cheese types, both at early and late stage of ripening, were selected, identified by MALDI-TOF MS and 110 of them subjected to multiplex PCR analyses (Fig. 1S). Overall, of 110 isolates, 39 (35%) were ascribed to *E. durans*, 39 isolates (35%) to *E. faecalis*, 31 isolates (28%) to *E. faecium*, and only one isolate to *E. hirae*. In detail, the 70 isolates obtained from Ragusano PDO cheese type, 32 (46%) were *E. durans,* 30 (43%) *E. faecalis*, 7 (10%) *E. faecium* and the only *E. hirae*. Looking at the occurrence trough ripening time, in Ragusano PDO cheese type, the dominance of *E. durans* (88%) and *E. faecalis* (80%), at early and late stage of ripening, respectively, was observed (Fig. 1). The Pecorino Siciliano cheese type appeared more heterogeneous, indeed, out of 40 isolates, 24 (60%) were identified as *E. faecium*, 9 (22%) as

E. faecalis, and 7 (17%) as *E. durans*, with a clear prevalence of *E. faecium* (86%) and *E. faecalis* (34%), at late and early stages of ripening, respectively (Fig. 1).

Figure 1. Enterococcal species distribution in cheese types at different ripening stages.



RD$_{90}$: Ragusano PDO at 90 ripening days
RD$_{180}$: Ragusano PDO at 180 ripening days
PS$_{90}$: Pecorino Siciliano at 90 ripening days
PS$_{180}$: Pecorino Siciliano at 180 ripening days

3.2 Antimicrobial susceptibility testing

In the present study, the susceptibility of isolates to the most clinical antibiotics was examined by microdilution method, according to Eucast (2017) and CLSI (2016) guidelines. The minimum inhibitory concentration (MIC) values, for

each antimicrobial agent, are reported in Table 2. In particular, 107 (97%) isolates, out of 110, exhibited a multidrug-resistant phenotype (resistance to at least three antimicrobials) with 14 strains, 13 *E. faecalis* ($RD_{90}2.2$, $RD_{180}1.2$, $RD_{180}1.5$, $RD_{180}1.6$, $RD_{180}1.7$, $RD_{180}1.8$, $RD_{180}1.10$, $RD_{180}1.13$, $RD_{180}1.14$, $RD_{180}1.15$, $RD_{180}1.16$, $RD_{180}1.17$, $RD_{180}1.22$) and one *E. faecium* (RD1801.12), resistant to 7 out of 9 antimicrobials tested with known break-point and/or cut-off. Furthermore, 19 strains (15 *E. durans*, 2 *E. faecalis* and 2 *E. faecium*) showed resistance to 5 antimicrobials. In particular, out of 15 *E. durans*, all were resistant to erythromycin, 14 to rifampicin and ampicillin and 7 to penicillin. Only three (2.7 %) strains (*E. durans* $PS_{90}2.14$, *E. faecalis* $PS_{90}3.18$, and *E. faecium* PS_{90} 2.4) were sensible to all tested antibiotics.

Table 2. MIC values detected for enterococcal isolates to the eleven tested antibiotics.

Strains	Species	STRE (>512)*	ERY (≥8)*	VAN (>4)*	RIF (≥4)*	GEN (>128)*	TET (≥16)*	SUL	AMP (>8)*	KAN	CHL (≥32)*	PEN (≥16)*	Total resistance
		Range µg/mL											
		(32-1024)	(1-32/ 8-256)	(1-32)	(2-64)	(8-256)	(0.5-16)	(128-4096)	(1-32)	(128-4096)	(2-64)	(0.5-16)	
RD_{90} 1.2	*E. durans*	64	$>32^R$	<1	8^R	<8	<0.5	>4096	8	<128	32^R	8	3
RD_{90} 1.4	*E. durans*	$>1024^R$	$>32^R$	4	8^R	16	<0.5	>4096	8	<128	16	8	3
RD_{90} 1.6	*E. durans*	$>1024^R$	$>32^R$	<1	16^R	16	1	>4096	16^R	<128	32^R	8	5
RD_{90} 1.7	*E. durans*	128	$>32^R$	4	16^R	16	1	>4096	8	<128	32^R	8	3
RD_{90} 1.10	*E. durans*	128	$>32^R$	<1	16^R	16	1	>4096	16^R	<128	32^R	16^R	5
RD_{90} 1.12	*E. durans*	$>1024^R$	$>32^R$	4	16^R	<8	1	>4096	16^R	<128	32^R	8	5
RD_{90} 1.13	*E. durans*	512	$>32^R$	2	16^R	16	1	>4096	8	<128	32^R	8	3
RD_{90} 1.16	*E. durans*	128	$>32^R$	4	16^R	16	<0.5	>4096	16^R	<128	32^R	8	4

RD_{90} 1.17	*E. durans*	256	>32^{R}	2	<2	32	1	>4096	16^{R}	<128	16	8	2
RD_{90} 1.19	*E. durans*	512	>32^{R}	2	16^{R}	64	>16^{R}	>4096	16^{R}	256	32^{R}	8	5
RD_{90} 1.20	*E. durans*	128	>32^{R}	2	32^{R}	16	8	>4096	32^{R}	<128	32^{R}	16^{R}	5
RD_{90} 1.24	*E. durans*	128	>32^{R}	2	16^{R}	<8	8	>4096	16^{R}	<128	32^{R}	16^{R}	5
RD_{90} 1.25	*E. durans*	256	>32^{R}	4	16^{R}	16	>16^{R}	>4096	16^{R}	<128	32^{R}	8	5
RD_{90} 1.28	*E. durans*	128	>32^{R}	<1	16^{R}	16	1	>4096	16^{R}	<128	32^{R}	8	4
RD_{90} 1.29	*E. durans*	256	>32^{R}	2	32^{R}	32	2	>4096	32^{R}	512	32^{R}	16^{R}	5
RD_{90} 1.30	*E. durans*	256	>32^{R}	2	32^{R}	32	8	>4096	16^{R}	<128	32^{R}	8	4
RD_{90} 1.31	*E. durans*	128	>32^{R}	4	16^{R}	16	1	>4096	32^{R}	256	32^{R}	16^{R}	5
RD_{90} 1.35	*E. durans*	>1024^{R}	>32^{R}	<1	16^{R}	16	1	>4096	32^{R}	<128	32^{R}	8	5
RD_{90} 1.36	*E. durans*	128	>32^{R}	4	16^{R}	16	1	>4096	16^{R}	<128	32^{R}	8	4
RD_{90} 1.37	*E. durans*	256	>32^{R}	4	16^{R}	<8	1	>4096	16^{R}	<128	32^{R}	8	4
RD_{90} 1.40	*E. durans*	128	>32^{R}	2	16^{R}	>256^{R}	2	>4096	>32^{R}	>4096	64^{R}	>16^{R}	6
RD_{90} 1.41	*E. durans*	128	>32^{R}	2	16^{R}	32	8	>4096	16^{R}	<128	64^{R}	16^{R}	5
RD_{90} 1.42	*E. durans*	>1024^{R}	>32^{R}	2	16^{R}	<8	1	2048	16^{R}	<128	32^{R}	16^{R}	6
RD_{90} 2.3	*E. durans*	>1024^{R}	>32^{R}	2	64^{R}	16	1	>4096	16^{R}	<128	32^{R}	8	5
RD_{90} 2.8	*E. durans*	>1024^{R}	>32^{R}	2	16^{R}	16	>16^{R}	>4096	16^{R}	4096	32^{R}	8	6
RD_{90} 2.10	*E. durans*	128	>32^{R}	4	8^{R}	16	1	>4096	16^{R}	<128	32^{R}	8	4
RD_{90} 2.11	*E. durans*	128	>32^{R}	4	8^{R}	16	1	>4096	16^{R}	<128	32^{R}	8	4
RD_{90} 2.12	*E. durans*	>1024^{R}	>32^{R}	<1	16^{R}	32	>16^{R}	>4096	8	<128	32^{R}	8	5
RD_{90} 2.13	*E. durans*	128	>32^{R}	4	8^{R}	16	1	>4096	8	<128	32^{R}	8	3
RD_{90} 2.15	*E. durans*	>1024^{R}	>32^{R}	4	16^{R}	<8	1	>4096	8	<128	32^{R}	8	4
RD_{90} 3.2	*E. durans*	512	>32^{R}	4	16^{R}	64	>16^{R}	>4096	16^{R}	256	32^{R}	8	5
RD_{180} 1.1	*E. durans*	1024^{R}	<8	4	32^{R}	<8	>16^{R}	4096	8	512	8	4	3
PS_{90} 2.14	*E. durans*	<32	<8	<1	<2	<8	1	2048	<1	<128	4	<0.5	0
PS_{90} 3.8	*E. durans*	64	<8	>32^{R}	32^{R}	<8	16^{R}	2048	4	<128	8	2	3
PS_{90} 4.1	*E. durans*	128	>32^{R}	>32^{R}	8^{R}	16	4	>4096	32^{R}	512	16	16^{R}	5
PS_{90} 4.5	*E. durans*	128	>32^{R}	>32^{R}	16^{R}	16	16^{R}	>4096	32^{R}	256	16	16^{R}	6
PS_{90} 4.20	*E. durans*	64	>32^{R}	>32^{R}	16^{R}	<8	>16^{R}	>4096	16^{R}	<128	32^{R}	8	6
PS_{180} 1.6	*E. durans*	64	>32^{R}	4	<2	<8	<0.5	>4096	16^{R}	<128	16	8	2
PS_{180} 3.4	*E. durans*	64	<8	2	8^{R}	<8	>16^{R}	4096	4	<128	8	2	2
RD_{90} 2.1	*E. faecalis*	>1024^{R}	>32^{R}	4	8^{R}	32	<0.5	>4096	32^{R}	512	16	16^{R}	5
RD_{90} 2.2	*E. faecalis*	>1024^{R}	>32^{R}	2	16^{R}	64	>16^{R}	>4096	16^{R}	256	32^{R}	16^{R}	7
RD_{180} 1.2	*E. faecalis*	1024^{R}	>32^{R}	2	16^{R}	64	>16^{R}	>4096	>32^{R}	256	64^{R}	16^{R}	7
RD_{180} 1.4	*E. faecalis*	512	>32^{R}	2	8^{R}	64	>16^{R}	>4096	32^{R}	256	64^{R}	16^{R}	6
RD_{180} 1.5	*E. faecalis*	>1024^{R}	>32^{R}	2	32^{R}	64	>16^{R}	>4096	32^{R}	256	32^{R}	16^{R}	7
RD_{180} 1.6	*E. faecalis*	1024^{R}	>32^{R}	2	16^{R}	64	>16^{R}	>4096	16^{R}	256	32^{R}	16^{R}	7

RD_{180} 1.7	*E. faecalis*	1024^{R}	$>32^{R}$	2	32^{R}	64	$>16^{R}$	>4096	16^{R}	256	32^{R}	16^{R}	7
RD_{180} 1.8	*E. faecalis*	1024^{R}	$>32^{R}$	2	16^{R}	64	$>16^{R}$	>4096	32^{R}	512	32^{R}	16^{R}	7
RD_{180} 1.10	*E. faecalis*	1024^{R}	$>32^{R}$	2	16^{R}	64	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	7
RD_{180} 1.13	*E. faecalis*	1024^{R}	$>32^{R}$	2	32^{R}	128	$>16^{R}$	>4096	32^{R}	512	64^{R}	16^{R}	7
$RD1_{180}$ 1.14	*E. faecalis*	$>1024^{R}$	$>256^{R}$	2	$>64^{R}$	256^{R}	$>16^{R}$	>4096	4	512	32^{R}	16^{R}	7
RD_{180} 1.15	*E. faecalis*	$>1024^{R}$	$>256^{R}$	2	16^{R}	128	$>16^{R}$	>4096	$>32^{R}$	256	32^{R}	16^{R}	7
RD_{180} 1.16	*E. faecalis*	$>1024^{R}$	$>256^{R}$	4	$>64^{R}$	64	$>16^{R}$	>4096	$>32^{R}$	256	32^{R}	16^{R}	7
RD_{180} 1.17	*E. faecalis*	1024^{R}	$>256^{R}$	4	16^{R}	64	$>16^{R}$	>4096	$>32^{R}$	256	32^{R}	16^{R}	7
RD_{180} 1.20	*E. faecalis*	512	$>256^{R}$	2	8^{R}	64	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	6
RD_{180} 1.21	*E. faecalis*	512	$>256^{R}$	2	16^{R}	64	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	6
RD_{180} 1.22	*E. faecalis*	1024^{R}	$>256^{R}$	2	8^{R}	64	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	7
RD_{180} 1.23	*E. faecalis*	512	$>256^{R}$	2	8^{R}	128	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	6
RD_{180} 1.25	*E. faecalis*	512	$>256^{R}$	2	16^{R}	64	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	6
RD_{180}1.26	*E. faecalis*	512	$>256^{R}$	2	8^{R}	64	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	6
RD_{180} 1.28	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	16^{R}	512	16	8	3
RD_{180} 1.29	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	512	16	8	2
RD_{180} 1.30	*E. faecalis*	512	<8	2	$>64^{R}$	<8	$>16^{R}$	4096	8	512	16	8	2
RD_{180} 1.31	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	512	16	8	2
RD_{180} 1.32	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	512	16	8	2
RD_{180} 1.33	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	512	16	4	2
RD_{180} 2.1	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	1024	8	8	2
RD_{180} 2.2	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	512	16	8	2
RD_{180} 2.3	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	512	16	4	2
RD_{180} 2.4	*E. faecalis*	512	<8	2	32^{R}	<8	$>16^{R}$	4096	8	256	16	8	2
PS_{90} 2.1	*E. faecalis*	512	$>32^{R}$	2	16^{R}	64	$>16^{R}$	>4096	32^{R}	256	32^{R}	16^{R}	6
PS_{90} 2.13	*E. faecalis*	<32	<8	2	32^{R}	<8	$>16^{R}$	<128	8	<128	16	8	2
PS_{90} 3.2	*E. faecalis*	$>1024^{R}$	64^{R}	$>32^{R}$	32^{R}	64	4	512	16^{R}	512	4	$>16^{R}$	6
PS_{90} 3.9	*E. faecalis*	1024^{R}	<8	2	32^{R}	<8	$>16^{R}$	4096	8	1024	8	4	3
PS_{90} 3.11	*E. faecalis*	512	<8	2	16^{R}	<8	$>16^{R}$	4096	4	256	8	2	2
PS_{90} 3.18	*E. faecalis*	<32	<8	2	<2	<8	8	4096	4	<128	8	2	0
PS_{90} 4.3	*E. faecalis*	512	128^{R}	2	16^{R}	<8	$>16^{R}$	4096	8	512	8	4	3
PS_{90} 4.6	*E. faecalis*	256	128^{R}	2	32^{R}	<8	$>16^{R}$	4096	8	512	8	4	3
PS_{90} 4.17	*E. faecalis*	128	128^{R}	2	64^{R}	<8	$>16^{R}$	4096	16^{R}	1024	16	16^{R}	5
RD_{90} 2.14	*E. faecium*	128	$>32^{R}$	2	8^{R}	16	1	>4096	16^{R}	<128	32^{R}	8	4
RD_{180} 1.3	*E. faecium*	256	$>32^{R}$	2	<2	32	$>16^{R}$	>4096	16^{R}	512	32^{R}	16^{R}	5
RD_{180} 1.9	*E. faecium*	256	$>32^{R}$	2	4^{R}	64	$>16^{R}$	>4096	16^{R}	512	32^{R}	16^{R}	6
RD_{180} 1.11	*E. faecium*	512	16^{R}	2	4^{R}	<8	$>16^{R}$	4096	4	512	4	4	3

RD_{180} 1.12	*E. faecium*	1024^{R}	$>32^{R}$	2	8^{R}	64	$>16^{R}$	>4096	16^{R}	512	32^{R}	16^{R}	7
RD_{180} 1.18	*E. faecium*	512	$>256^{R}$	2	8^{R}	64	$>16^{R}$	>4096	16^{R}	256	32^{R}	16^{R}	6
RD_{180} 1.24	*E. faecium*	128	$>256^{R}$	<1	<2	16	<0.5	>4096	8	256	16	8	1
PS_{90} 1.1	*E. faecium*	64	$>32^{R}$	<1	<2	<8	<0.5	>4096	16^{R}	512	32^{R}	16^{R}	4
PS_{90} 1.3	*E. faecium*	64	$>32^{R}$	<1	8^{R}	<8	<0.5	>4096	16^{R}	<128	32^{R}	8	4
PS_{90} 2.2	*E. faecium*	64	$>32^{R}$	2	8^{R}	16	<0.5	>4096	16^{R}	<128	32^{R}	16^{R}	5
PS_{90} 2.4	*E. faecium*	256	<8	<1	<2	<8	1	2048	<1	512	4	1	0
PS_{90} 2.5	*E. faecium*	128	$>32^{R}$	2	16^{R}	16	16^{R}	>4096	$>32^{R}$	512	32^{R}	$>16^{R}$	6
PS_{90} 2.6	*E. faecium*	256	$>32^{R}$	2	$>64^{R}$	<8	<0.5	>4096	8	256	32^{R}	4	3
PS_{90} 2.7	*E. faecium*	64	$>32^{R}$	2	4^{R}	<8	2	>4096	16^{R}	<128	16	8	3
PS_{90} 2.9	*E. faecium*	64	$>32^{R}$	2	16^{R}	16	8	4096	4	512	32^{R}	16^{R}	4
PS_{90} 3.17	*E. faecium*	128	<8	2	32^{R}	<8	16^{R}	4096	16^{R}	1024	8	8	3

*CLSI and EUCAST-approved clinical breakpoint and epidemiological cut-off values.
STRE: streptomycin; ERY: erythromycin; VAN: vancomycin; RIF: rifampicin; GEN: gentamicin; TET: tetracycline; SUL: sulfamethoxazole; AMP: ampicillin; KAN: kanamycin; CHL: chloramphenicol; PEN: penicillin G. Total resistance (number of antibiotics to which a strain showed resistance).
R: resistant.

Considering total resistance (as number of isolates showing resistance to antibiotics), results highlighted the highest occurrence of resistance for rifampicin, erythromycin and ampicillin (84%, 81% and 64%, respectively), and the lowest for high level of gentamicin (2%). PCA plot of distribution of antibiotic resistance of isolates from Pecorino Siciliano and Ragusano PDO, at early at late stage of ripening, is reported in Figure 3. Based on the loadings (data not shown), component 1 represents the 33% of the variability, while the second principal component represents a variance of 20%. Score plot is effective in showing the difference among samples and in separating them in the graph. Overall, three main groups (A, B, and C) were detected. In detail, a positive contribution by component 1

was determined by penicillin G, ampicillin and chloramphenicol antibiotics, for which a high level of resistance was observed among several strains from Ragusano PDO cheese (group A). The highest level of resistance was achieved by the *E. durans* RD_{90} 1.40 strain. On the contrary, several strains from Pecorino Siciliano cheese did not show resistance to the aforementioned antibiotics (group B). Strains clustered into the group C exhibited a high level of resistance to rifampicin, tetracycline, and streptomycin.

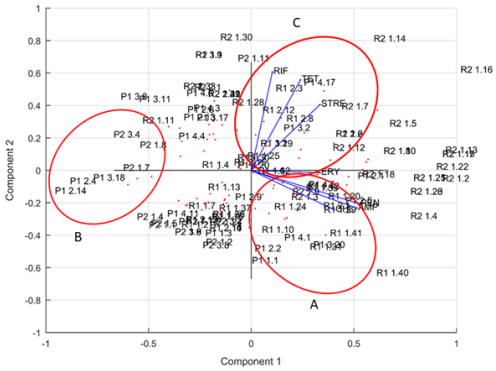


Figure 3. Biplot of PCA analysis showing the antibiotic resistance distribution among enterococcal strains at different ripening days.

The *E. faecalis* RD_{180} 1.14 and *E. faecalis* RD_{180} 1.16 strains, both isolated from Ragusano PDO cheese, at late stage of ripening, showed the highest level of resistance the same antibiotics. Finally five isolates, four *E. durans* strains (*E. durans* PS_{90}3.8, 4.1, 4.5, and 4.20) and one *E. faecalis* (PS_{90}3.2), exhibited resistance to vancomycin (Table 2).

Focusing on antibiotic resistance among species, *E. faecalis* was the major resistant species, followed by *E. durans* (Fig. 2). Overall, the 97% and 92% of *E. faecalis* strains showed resistance to rifampicin and tetracycline, respectively, while the majority of *E. durans* isolates were resistant to rifampicin and tetracycline (92% and 90%, respectively). Finally, *E. faecium* strains showed the highest incidence of resistance to erythromycin (90%), and ampicillin (61%). The unique *E. hirae* isolate was found resistant to penicillin, ampicillin, tetracycline, rifampicin, erythromycin and chloramphenicol (Table 2).

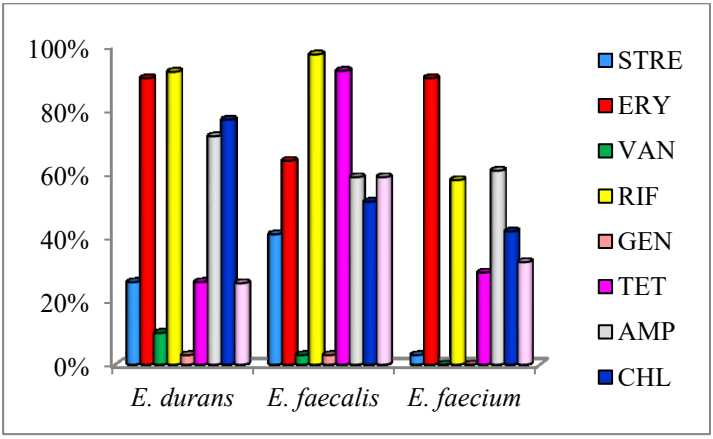
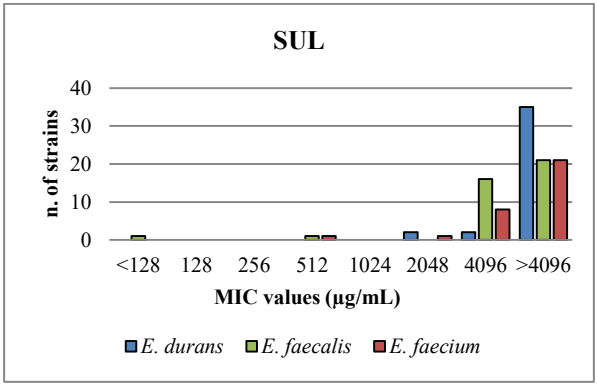


Figure 2. Incidence of antibiotic resistance among *E. durans*, *E. faecalis* and *E. faecium* species to 9 antibiotics.

Moreover, zooming on the MIC distribution within the three species, multimodal distributions for different antibiotics were observed (data not shown). Within *E. durans* species, multimodal distribution for tetracycline, streptomycin, vancomycin and gentamicin was observed, indicating the

coexistence of different subpopulations, with a different level of susceptibility. Among *E. faecalis*, multimodal distribution was observed for streptomycin, rifampicin, gentamicin and ampicillin, and among *E. faecium* for rifampicin, gentamicin and tetracycline, (data not shown). In the present study, the susceptibility of enterococcal strains to sulfamethoxazole and kanamycin, for which no breakpoint and/or epidemiological cut-off are available, were also tested. In detail, for sulphametoxazole, 90% of *E. durans*, 68% of *E. faecium* and 54% of *E. faecalis* highlighted MIC values above the highest tested concentration (>4096 µg/mL).
Regarding kanamycin, lower MIC values were registered for *E. durans* and *E. faecalis*, while a bimodal distribution of MIC values was observed for *E. faecium* isolates (Fig. 4).



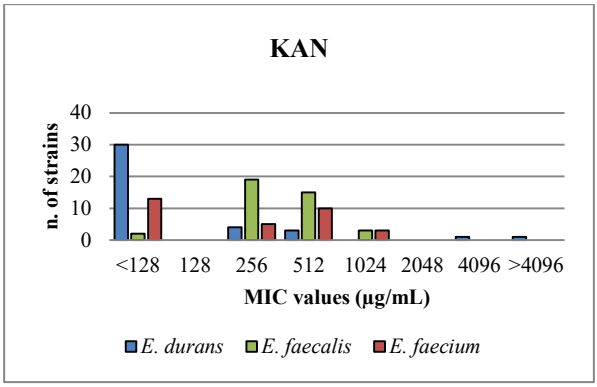


Figure 4. MIC distribution for sulphametoxazole and kanamycin antimicrobials among the enterococcal species.

3.3 Clonal relatedness

Isolates, belonging to the three major enterococcal species, were typed by PFGE anlalysis and the obtained profiles were subjected to BioNumerics v.7.5 (Applied Maths, Sint-Martens- Latem, Belgium). Isolates with a similarity higher than 81% were considered as related and assigned to the same clone. The dendrogram showed significant clustering by origin of isolation (Fig. 5).The results allowed to allocate the enterococcal isolates into 22 genotypes, here indicated as Edu for *E. durans*, Efl for *E. faecalis*, and Efm *E. faecium* (Figure 9), some of which included sub-genotypes, here indicated with a tip number. The 35% (39/110) of *E. durans* isolates were distributed in eight clones. In details, Edu1 (9/39), Edu11 (23/39), Edu12 (1/39), Edu13 (1/39), Edu2 (1/39), Edu3 (1/39), Edu4 (1/39) and Edu5 (1/39) clones clustered isolates from both cheese types, although a prevalence of isolates from Ragusano PDO cheese, at early stage of ripening, was observed (Table 3). Similarly, the

35% (39/110) of *E. faecalis* strains were clustered in five clones, among which the most numerous Efl2 (35/39) clone included strains mainly isolated from Ragusano cheese at late stage of ripening. Finally, 28% (31/110) of the *E. faecium* strains were distributed in nine clones. Clones Efm1 (1/31), Efm2 (6/31), Efm3 (1/31), Efm4 (4/31), Efm41 (12/31), Efm5 (1/31), Efm6 (1/31), Efm7 (1/31) and Efm8 (4/31) clustered strains mainly originating from late stage of ripening of both cheese types (Table 3 and Fig. 5).

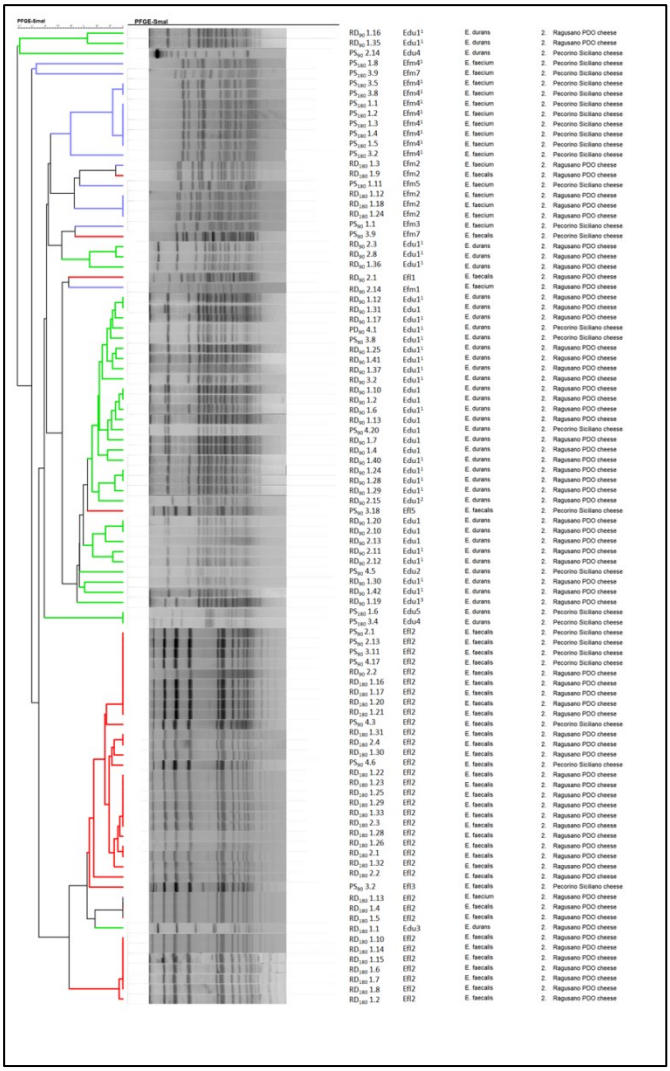


Figure 5. Dendogram and PFGE profiles of enterococcal strains.

Table 3. PGFE clusters and AR of tested strains.

	Total of isolates	n° of isolates at different ripening days		Species (n° of strains)	PFGE types	Antimicrobial resistance (n° of resistant strains)
Ragusano PDO	70	90	35	*E. durans* (31)	Edu1(8), Edu1[1](21), Edu1[2](1), Edu1[3](1)	STRE (9), ERY (31), RIF (30), GEN (1), TET (5), AMP (24), CHL (29), PEN (8)
				E. faecium (1)	Efm1(1)	ERY, RIF, AMP, CHL
				E. faecalis (2)	Efl1(1), Efl2(1)	STRE (2), ERY (2), RIF (2), TET (1), AMP (2), CHL (1), PEN (2)
				E. hirae (1)		ERY, RIF, TET, AMP, CHL, PEN
		180	35	*E. durans* (1)	Edu3(1)	STRE, RIF, TET
				E. faecium (6)	Efm2(6)	STRE (1), ERY (6), RIF (4), TET (5), AMP (4), CHL (4), PEN (4)
				E. faecalis (28)	Efl2(28)	STRE (12), ERY (20), RIF (28), GEN (1), TET (30), AMP (20), CHL (20), PEN (20)
Pecorino Siciliano	40	90	26	*E. durans* (5)	Edu1(1), Edu1[1](2), Edu2(1), Edu4(1)	ERY (3), VAN (4), RIF (4), TET (3), AMP (3), CHL (1), PEN (2)
				E. faecium (12)	Efm3(1), Efm4(4), Efm4[1](3), Efm8(4)	ERY (10), RIF (9), TET (2) AMP (8), CHL (6), PEN (5)
				E. faecalis (9)	Efl2(6), Efl3(1), Efl4(1), Efl5(1)	STRE (2), ERY (5), VAN (1), RIF (8), TET (7), AMP (3), CHL (1), PEN (3)
		180	14	*E. durans* (2)	Edu4(1), Edu5(1)	ERY (1), RIF (1), TET (1), AMP (1)
				E. faecium (12)	Efm4[1](9), Efm5(1), Efm6(1), Efm7(1)	ERY (11), RIF (4), TET (2), AMP (6), KAN (1), CHL (2), PEN (1)

3.4 *VanA* and *VanB* detection

The five VRE strains (*E. durans* PS_{90}3.8, 4.1, 4.5, 4.20 and *E. faecalis* PS_{90}3.2), exhibiting resistance to vancomycin were screened for the presence of resistant genes (*vanA* and *vanB*). None, out of 5 phenotypic vancomycin resistant isolates, exibited the vanA gene, whilst only the *E. durans* PS_{90}4.20 strain showed the presence of the vanB gene.

4. Discussion

The ubiquity of enterococci in food is mainly a result of their widespread in different environmental sites and their high adaptability to the different parameters applied during food processing and storage conditions. Food chain is considered the main route for the introduction of animal and environment-associated antibiotic resistant bacteria into the human gastro-intestinal tract, where the genes may be transferred to pathogenic and opportunistic bacteria (Hasman et al., 2005; Rizzotti et al., 2009; Rossi et al., 2014). In recent years, concern has been raised about commensal bacteria supplied by food. Cheese, as other fermented food, harbors numerous living microorganisms of different genera, among which enterococci are considered a relevant part of dominant microbiota (Giraffa, 2003; Jamet et al., 2012).

In the present study, 110 enterococci, collected from Ragusano PDO and Pecorino Siciliano cheese types, at early and late stages of ripening, were characterized for their antibiotic phenotypic resistance patterns. Since mechanisms of antibiotic resistance are species-specific, the isolates were subjected to multiplex PCR and ascertained as belonging to *E. faecium, E. durans, E. faecalis* and *E. hirae*, confirming MALDI-TOF/MS analysis as effective in identification of *Enterococcus* spp. (Fang et al., 2012). Overall, the prevalence of *E. faecium/E. durans*, followed by *E. faecalis* was revealed, according to previous studies carried out on traditional European and Italian cheeses (Andrighetto et al., 2001; Jamet et al., 2012; Ortigosa et al., 2008; Templer and Baumgartner, 2007). The occurrence of *E. faecalis* and *E. faecium* has been associated to geographical location and to hygiene measures during

cheese processing, while little is known about *E. durans* distribution (Boehm and Sassoubre, 2014). In the present study *E. durans* was detected mainly in Ragusano PDO, in accordance with results reported in Fiore Sardo cheese, Bryndza, and Turkish white cheese (Cosentino et al., 2004; Jurkovic et al., 2006; Ispirli et al., 2017), suggesting that this species has been often underestimated and, probably, misidentified (Ogier and Serror 2008). Several studies have been carried out in order to understand the source and the way of contamination of dairy enterococci, such as raw milk, environment, wooden equipment, animal rennet (Cruciata et al., 2014; Gaglio et al., 2016; Gelsomino et al., 2002; Scatassa et al., 2015). In this study, we evaluated the antibiotic resistance phenotype of *E. faecium, E. faecalis, E. durans* and *E. hirae* isolates. All the isolates displayed the highest incidence of resistance against rifampicin and erythromycin and the lowest to high level of gentamicin, as previously reported (Jamet et al., 2012; Kurekci et al., 2016; Teuber et al., 1999). The high incidence of resistance to chloramphenicol and tetracycline is in agreement with the observed increased presence of tetracycline and erythromycin resistance genes in environment, animal facilities and, consequently, in food of animal origin (Fuka et al., 2017; Kürekci et al., 2016; Ogier and Serror, 2008). Tetracycline resistance is attributed to the overexploitation of these antibiotics in veterinary practices and, of serious concern, is its dissemination from enterococci to a large diversity of bacterial genera, through conjugative transposon (Hammad et al., 2015). Although our results are in accordance with previous reports on high prevalence of tetracycline, erythromycin, and chloramphenicol resistance among food-associated enterococci, the observed resistance to penicillins (penicillin and ampicillin) is in contrast to

numerous previous studies (Cariolato et al., 2008; Franz et al., 2001; Sánchez Valenzuela et al., 2008; Jamet et al., 2012). It is remarkable to underline that in our investigations low resistance to vancomycin was detected. Although vancomycin resistance is mostly reported in *E. faecium*, in the present work, one *E. faecalis* was found resistant, in agreement to a recent higher incidence of VR phenotype among food related *E. faecalis* (Porto et al., 2016). The emergence of enterococci resistance to glycopeptides (including vancomycin and teicoplanin), has been rapidly spreading and, in the past, it has been associated to use of glycopeptide antibiotics in clinical medicine and of avoparcin as a feed additive (Bager et al., 1997; Koluman et al., 2009). Different types of acquired vancomycin resistance have been detected (Courvalin, 2006; Werner et al., 2008a; 2008b) and several genotypes of vancomycin resistance, *vanA–G*, have been described. The different van gene clusters consist of up to nine genes encoding proteins with different functions. Variants of vanA and vanB types are found worldwide, with vanA predominating. In the present study, no *vanA* gene was detected in any vancomycin resistant strains, whilst the *vanB* gene was observed only in one *E. durans* strain, even if *VanB*, together with *VanA*, is considered usually associated with *E. faecium*. Zooming on antibiotic resistance within the three enterococcal species, relevant differences were found, with *E. faecalis* confirmed as the most resistant species, in agreement with Pesavento et al. (2014). Furthermore the multimodal distributions of MIC values observed for some antibiotics indicates that the continous exposure to sub-lethal concentrations of antibiotics can contribute to select resistant subpopulations (Rodríguez-Rojas et al., 2013). This would imply that co-

selection or cross-selection of antibiotic resistance should not be a rare occurrence in the same populations. Marked heterogenity among strains isolated from each cheese type, mostly for *E. durans* and *E. faecium,* were revealed through PFGE typing. In partcular, high genetic variability was found among *E. faecium* isolates, whereas the persistance of the most abundant clone of *E. faecalis*, in stretched Ragusano cheese, confirms that conditions applied during cheese manufacturing have strong selective effect on the indigenous microbiota (Bonomo and Salzano, 2013). Moreover, it is rilevant to point out that within the same PFGE type, strains exhibited a different phenotipic resistance patterns, confirming the high-level of plasticity of the enterococcal genome (Vignaroli et al., 2011). Finally, the presence of same PFGE types, both for *E. durans* and *E. faecalis,* in the two different cheese types, suggest a link between enterococci and geographical area of production. Although *E. durans* has been less frequently envolved in enterococcal infection (Na et al., 2010), the prominent resistance phenotypes against rifampicin, erythromycin and, to a lesser extent, chloramphenicol, detected in the present study, highlighs the importance to better understand their implication in the risk of transfer antitimicrobial resistance to human strains.

5. Conclusions

This study highlighted the presence of enterococci into two Sicilian cheese types at different ripening stages, with *E. durans*, *E. faecalis*, and *E. faecium* being the predominant species. Although no correlation has yet been demonstrated between ingestion of food containing enterococci and

infection, the high rate of multi-resistant enterococci detected and the rapid acquisition of antimicrobials resistance among enterococci should be taken into account in order to detain the high frequency of antibiotic resistance transmission in human gastro intestinal tract. Hence, the role of enterococci in cheese ripening remains questionable mostly for *E. durans*, for which less information is available. To our knowledge, this is the first evidence of antimicrobial resistant enterococci in Ragusano PDO cheese at different ripening stages. Furthermore, the multimodal distribution of MIC observed within the main three enterococcal species reinforces the need of a constant monitoring and an active surveillance in cheeses.

Conflict of interest

The Authors declare that there are no conflict of interest.

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References

Aarestrup, F.M., Butaye, P., Witte, W. (2002a). Nonhuman reservoirs of enterococci. In: Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E., Rice, L.B. (Eds.). The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. ASM, Washington, D. C, pp. 55-99.

Aarestrup, F.M., Hasman, H., Jensen, L.B., Moreno M., Herrero, I.A., Dominguez. L., et al. (2002b). Antimicrobial resistance among enterococci from pigs in three European countries. Appl. Environ. Microbiol. 68, 4127–4129.

Andrighetto, C., Knijff, E., Lombardi, A., Torriani, S., Vancanneyt, M., Kersters, K., Swings, J., Dellaglio, F. (2001). Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. J. Dairy Res. 68, 303–316.

Arias, C.A., Murray, B.E. (2012). The rise of the *Enterococcus*: beyond vancomycin resistance. Nat. Rev. Microbiol. 10, 266-78.

Bager, F., Madsen, M., Christensen, J., Aarestrup F.M. (1997). Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Prev. Vet. Med. 31, 95-112.

Bocanegra-Ibarias, P., Flores-Treviño, S., Camacho-Ortiz, A., Morfin-Otero, R., Villarreal- Treviño, L., Llaca-Díaz, J., Martínez-Landeros, E.A., Rodríguez-

Noriega, E., Calzada- Güereca, A., Maldonado-Garza, H.J., Garza-González, E. (2016). Phenotypic and genotypic characterization of vancomycin-resistant *Enterococcus faecium* clinical isolates from two hospitals in Mexico: First detection of VanB phenotype-vanA genotype. Enferm. Infecc. Microbiol. Clin. 34, 415-421.

Boehm, A.B., Sassoubre, L.M. (2014). Enterococci as indicators of environmental fecal contamination. In M. S. Gilmore, D. B. Clewell, Y. Ike, & N. Shankar (Eds.), Enterococci: from commensals to leading causes of drug resistant infection (pp. 1-17). Boston: Massachusetts Eye and Ear Infirmary.

Bonacina, J., Suárez, N., Hormigo, R., Fadda, S., Lechner, M., Saavedra, L. (2017). A genomic view of food-related and probiotic *Enterococcus* strains. DNA Resv. 24(1); FebPMC5381348.

Bonomo, M.G, Salzano, G. (2013). Genotypic and technological diversity of *Leuconostoc mesenteroides* and *Lactobacillus paracasei* subsp. *paracasei* strains for use as adjunct starter cultures in Pecorino di Filiano cheese. Int. J. Dairy Technol. 66, 4012- 409.

Bonten, M.J., Willems, R., Weinstein, R.A. (2001). Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis.1, 314–25.

Cariolato, D., Andrighetto, C., Lombardi, A. (2008). Occurrence of virulence factors and antibiotic resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected

from dairy and human samples in North Italy. Food Control, 19, 886-892.

Clewell, D.B., Weaver, K.E., Dunny, G.M., Coque, T.M., Francia, M.V., Hayes, F. (2014). Extrachromosomal and mobile elements in Enterococci: Transmission, maintenance, and Epidemiology. In: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N. Ed. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Boston: Massachusetts Eye and Ear Infirmary.

Clinical and Laboratory Standards Institute (CLSI) (2016). Performance Standards for Antimicrobial Susceptibility Testing, 26th Edition CLSI supplement M100S, CLSI, Wayne, PA, USA.

Cosentino, S., Pisano, M. B., Corda, A., Fadda, M. E., Piras, C. (2004). Genotypic and technological characterization of enterococci isolated from artisanal Fiore Sardo cheese. J. Dairy Res., 71, 444–450.

Coque, T.M., Seetulsingh, P., Singh, K.V., Murray, B.E. (1998). Application of molecular techniques to the study of nosocomial infections caused by enterococci. Methods Mol. Med. 15, 469-493.

Courvalin, P. (2006). Vancomycin resistance in gram-positive cocci. Clin. Infect. Dis. 42, S25–S34.

Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., De Filippis, F., Mancuso, I., La Storia, A., Moschetti, G., Settanni, L. (2014). Animal rennets as sources of dairy

lactic acid bacteria. Appl. Environ. Microbiol. 80, 2050–2061.

Deasy, B.M., Rea, M.C., Fitzgerald, G.F., Cogan, T.M., Beresford, T.P. (2000). A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus*. Syst. Appl. Microbiol. 23, 510–522.

Depardieu, F., Perichon, B., Courvalin, P. (2004). Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. J Clin Microbiol. 42, 5857–5860.

EFSA, (2017). Scientific opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (EFSA Journal, 2018 15, Issue 3, March 2017).

EUCAST, European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017. http://www.eucast.org.

Fang, H., Ohlsson, A.-K., Ullberg, M., Özenci, V. (2012). Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK 2 system for the identification of clinical *Enterococcus* isolates. Eur. J. Clin. Microbiol. Infect. Dis. 31, 073–3077.

Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E. (1999). Enterococci at the crossroads of food safety? Int. J. Food Microbiol. 47, 1-24.

Franz, C.M.A.P., Muscholl-Silberhorn, A.B., Yousif, N.M.K., Vancanneyt, M., Swings, J., Holzapfel, W.H. (2001). Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Appl. Environ. Microbiol. 67, 4385–4389.

Freitas, A.R., Tedim, A.P., Novais, C., Coque, T.M., Peixe, L. (2018). Distribution of putative virulence markers in *Enterococcus faecium*: towards a safety profile review. J Antimicrob Chemother. 73, 306-319.

Freiwald, A., Sauer, S. (2009). Phylogenetic classification and identification of bacteria by mass spectrometry. Nat. Protoc. 4, 732–742.

Fuka, M., Maksimovic, A., Tanuwidjaja, I., Hulak, N., Schloter, M. (2017). Characterization of enterococcal community isolated from an artisan Istrian raw milk cheese: Biotechnological and safety aspects. Food Technol. Biotechnol. 55, 368-380.

Gaglio, R., Couto, N., Marques, C., de Fatima Silva Lopes, M., Moschetti, G., Pomba, C., Settanni, L. (2016). Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. Int. J. Food Microbiol. 236 107–114.

Gelsomino, R., Vancanneyt, M., Cogan, T. M., Condon, S., Swings, J. (2002). Source of enterococci in a farmhouse raw-milk cheese. Appl. Environ. Microbiol. 68, 3560–3565.

Giraffa, G., (2003). Functionality of enterococci in dairy products. Int. J. Food Microbiol. 88, 215-222.

Gilmore, M.S., Lebreton, F., van Schaik, W. (2013). Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. Curr Opin Microbiol. 16, 10-16.

Guzman Prieto, A.M., van Schaik, W., Rogers, M.R.C., Coque, T.M., Baquero, F., Corander, J., Willems, R.J.L. (2016). Global emergence and dissemination of Enterococci as nosocomial pathogens: Attack of the clones? Front. Microbiol.7, 788-803.

Hammad, A.M., Hassan, H.A., Shimamoto, T. (2015). Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. in Egyptian fresh raw milk cheese. Food Control 50, 815- 820.

Hasman, H., Villadsen, A.G., Aarestrup, F.M. (2005). Diversity and stability of plasmids from glycopeptide-resistant *Enterococcus faecium* (GRE) isolated from pigs in Denmark. Microb. Drug Resist. 11, 178-184.

Hugas, M., Garriga, M., Aymerich, M.T. (2003). Functionality of enterococci in meat products. Int. J. Food Microbiol. 8, 223–233.

Ispirli, H., Demirbas, F., Dertli, E. (2017). Characterization of functional properties of Enterococcus spp. isolated from Turkish white cheese. LWT-Food Sci. Technol. 75, 358- 365.

Izquierdo, E., Cai, Y., Marchioni, E., Ennahar, S. (2009). Genetic identification of the bacteriocins produced by *Enterococcus faecium* IT62 and evidence that bacteriocin 32 is identical to enterocin IT. Antimicrob. Agents Chemother. 53, 1907–1911.

Jackson, C.R., Fedorka-Cray, P.J., Barrett, J.B. (2004). Use of a genus- and species-specific multiplex PCR for identification of enterococci. J. Clin. Microbiol. 42, 3558-3565.

Jamet, E., Akary, E., Poisson, M.A., Chamba, J.F., Bertrand, X., Serror, P. (2012). Prevalence and characterization of antibiotic resistant *Enterococcus faecalis* in French cheeses. Food Microbiol. 31, 191–198.

Jurkovic, D., Križková, L., Sojka, M., Belicová, A., Dusinsky, R., Krajcovic, J., Snauwaert, C., Naser, S., Vandamme, P., Vancanneyt, M. (2006). Molecular identification and diversity of enterococci isolated from Slovak Bryndza cheese. J. Gen. Appl. Microbiol., 52, 329–337.

Koluman, A., Akan, L.S., Çakiroglu, F.P. (2009). Occurrence and antimicrobial resistance of enterococci in retail foods. Food Control 20, 281-283.

Kürekci, C., Önen, S.P., Yipel, M., Aslantaş, Ö., Gündoğdu, A. (2016). Characterization of phenotypic and genotypic antibiotic resistance profile of enterococci from cheeses in Turkey. Korean J Food Sci Anim Resour. 36, 352-358.

Na, S., Park H.J., Park, K.H., Cho, O.H., Chong, Y.P., Kim, S.H., Lee, S.O., Sung, H., Kim, M.N., Jeong, J.Y., Kim, Y.S., Woo, J.H., Choi, S.H. (2012). *Enterococcus avium* bacteremia: a 12-year clinical experience with 53 patients. Eur. J. Clin. Microbio.l Infect. Dis. 31, 303–310.

Ness, I.F., Diep, D.B., Ike, Y. (2014). Enterococcal bacteriocins and antimicrobial proteins that contribute to niche control. In: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N, editors. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Boston: Massachusetts Eye and Ear Infirmary.

Nilsson, O., Greko, C., Bengtsson, B. (2009). Environmental contamination by vancomycin resistant enterococci (VRE) in Swedish broiler production. Acta Vet. Scand. 51:49.

Novais, C., Coque, T.M., Sousa, J.C., Baquero, F., Peixe, et al. (2004). Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. Antimicrob. Agents Chemother. 48, 3613-3617.

Ogier, J.C., Serror, P. (2008). Safety assessment of dairy microorganisms: The *Enterococcus* genus. Int. J. Food Microbiol. 126, 291–301.

Ortigosa, M., Irigoyen, A., Urdin, M., García, S., Ibañez, F.C., Torre, P. (2008). Identification of enterococci and isolation of vancomycin-resistant strains in Spanish cheeses. Milchwissenschaft, 63, 164–167.

Peel, T., Cheng, A.C., Spelman, T., Huysmans, M., Spelman, D. (2011). Differing risk factors for vancomycin-resistant and vancomycin-sensitive enterococcal bacteraemia. Clin. Microbiol. Infect. 18, 388-394.

Pesavento, G., Ducci, B., Nieri, D., Comodo, N., Lo Nostro, A. (2010). Prevalence and antibiotic susceptibility of *Listeria* spp. isolated from raw meat and retail foods. Food Control. 21, 708–713.

Pesavento, G., Calonico, C., Ducci, B., Magnanini, A., Lo Nostro, A. (2014). Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. Food Microbiol. 41, 1–7.

Porto, B.C., Fujimoto, G., Borges, M.D.F., Bruno, L.M., Gasparin Carvalho, J.D. (2016). Determinants of virulence in *Enterococcus* endogenous to artisanal cheese. Rev. Cienc. Agron. 47, 69-76.

Poyart, C., Quesnes, G., Trieu-Cuot, P. (2000). Sequencing the gene encoding manganese dependent superoxide dismutase for rapid species identification of enterococci. J. Clin. Microbiol. 38, 415-418.

Rizzotti, L., La Gioia, F., Dellaglio, F., Torriani, S. (2009). Molecular diversity and transferability of the tetracycline resistance gene *tet*(M), carried on Tn916-1545 family transposons, in enterococci from a total food chain. Antonie Van Leeuwenhoek 96, 43– 52.

Rodríguez-Rojas, A., Rodríguez-Beltrán, J. Couce, A., Blázquez, J. (2013). Antibiotics and antibiotic resistance: A

bitter fight against evolution. Int. J. Med. Microbiol. 303, 293- 297.

Rossi, F., Rizzotti, L. Felis, G.E., Torriani, S. (2014). Horizontal gene transfer among microorganims in food: Current knowledge and future perspectives. Food Microbiol. 42, 232–243.

Sánchez Valenzuela, A., Omar, N.B., Abriouel, H., López, R.L., Ortega, E., Martínez Cañamero, M., Gálvez, A. (2008). Risk factors in enterococci isolated from foods in Morocco: Determination of antimicrobial resistance and incidence of virulence traits. Food Chem Toxicol. 46, 2648-2652.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G. (2015). Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41.

Sparo, M., Urbizu, L., Solana, M.V., Pourcel, G., Delpech, G., Confalonieri, A., Ceci, M., Sanchez Bruni, S.F. (2011). High-level resistance to gentamicin: genetic transfer between *Enterococcus faecalis* isolated from food of animal origin and human microbiota. Lett. Appl. Microbiol. 54, 119-125.

Tan, C.K., Lai, C.C., Wang, J.Y., Lin, S.H., Liao, C.H., Huang, Y.T., Wang, C.Y., Lin, H.I., Hsueh, P.R. (2010). Bacteremia caused by *non-faecalis* and *non-faecium*

Enterococcus species at a Medical center in Taiwan, 2000 to 2008. J. Infect. 61,34–43.

Templer, S.P., Baumgartner, A. (2007). Enterococci from Appenzeller and Schabziger raw milk cheese: antibiotic resistance, virulence factors, and persistence of particular strains in the products. J. Food Prot. 70, 450-455.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33, 2233-2239.

Teuber, M., Meile, L., Schwarz, F. (1999). Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Van Leeuwenhoek 76, 115–137.

Vergis, E.N., Hayden, M.K., Chow, J.W., Snydman, D.R., Zervos, M.J., Linden, P.K., Wagener, M.M., Schmitt, B., Muder, R.R. (2001). Determinants of vancomycin resistance and mortality rates in enterococcal bacteremia. A prospective multicenter study. Ann. Intern. Med. 135, 484–492.

Vignaroli, C., Zandri, G., Aquilanti, L., Pasquaroli, S., Biavasco F. (2011). Multidrug-resistant enterococci in animal meat and faeces and co-transfer of resistance from an *Enterococcus durans* to a human *Enterococcus faecium*. Curr Microbiol. 62, 1438-1447.

Walsh, D., Duffy, G., Sheridan, J.J., Blair, I.S., McDowell, D.A. (2001). Antibiotic resistance among

Listeria, including *Listeria monocytogenes*, in retail foods. J. Appl Microbiol. 90, 517-522.

Werner, G., Strommenger, B., Witte, W. (2008a). Acquired vancomycin resistance in clinically relevant pathogens. Future Med. 3, 547-562.

Werner, G., Coque, T.M., Hammerum, A.M., Hope, R., Hryniewicz, W., Johnson, A., Ingo, K., Kristinsson, K.G., Leclercq, R., Lester, C.H., Lillie, M., Novais, C., Olsson-Liljequist, B., Peixe, L.V., Sadowy, E., Simonsen, G.S., Top, J., Vuopio-Varkila, J., Willems, R.J., Witte, W., Woodford N. (2008b). Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill. 13, 1-11.

Supplementary data

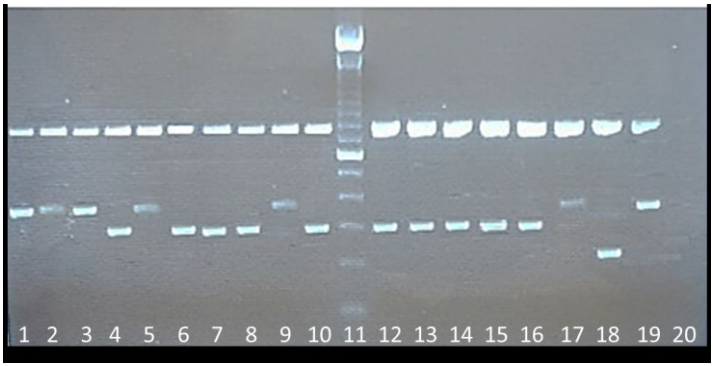
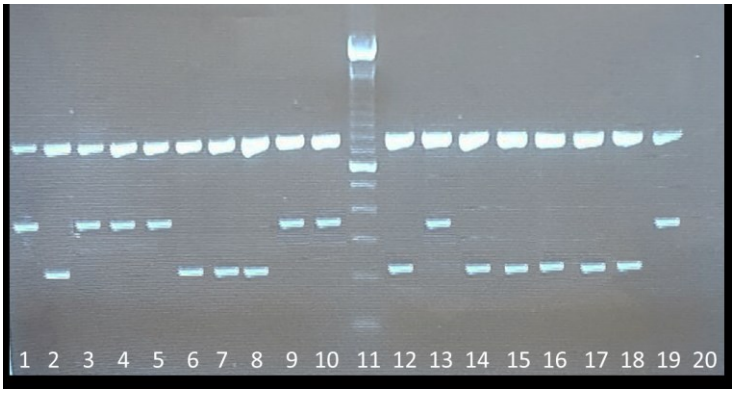


Figure 1S. Genus and species multiplex PCR of enterococci: **(A)** line 1: *E. faecalis* ATCC 19433; line 2: RD_{180} 1.10; line 3: RD_{180} 1.13; line 4: RD_{90} 1.2; line 5: RD_{180} 1.14; line 6: RD_{90} 1.4; line 7: RD_{90} 1.6; line 8: RD_{90} 1.7; line 9: PS_{90} 2.1; line 10: *E. durans* ATCC 19432; line 11: DNA ladder; line 12: PS_{90} 4.5; line 13: PS_{90} 4.20; line 14: PS_{90} 3.8; line 15: PS_{180} 1.6; line 16: PS_{180} 3.4; line 17: PS_{90} 2.13; line 18: PS_{90} 3.20; line 19: PS_{90} 3.11; line 20: negative control.



(B) line 1: *E. faecalis* ATCC 19433; line 2: *E. faecium* ATCC 19434; line 3: RD_{90} 2.1; line 4: RD_{90} 2.2; line 5: RD_{180} 1.2; line 6: RD_{180} 1.9; line 7: RD_{180} 1.11; line 8: RD_{180} 1.12; line 9: RD_{180} 1.5; line 10: RD_{180} 1.6; line 11: DNA ladder; line 12: RD_{180} 1.18; line 13: RD_{180} 1.7; line 14: PS_{90} 1.3; line 15: PS_{90} 2.2; line 16: PS_{90} 2.4; line 17: PS_{90} 2.5; line 18: PS_{90} 2.6; line 19: RD_{180} 1.8; line 20: negative control.

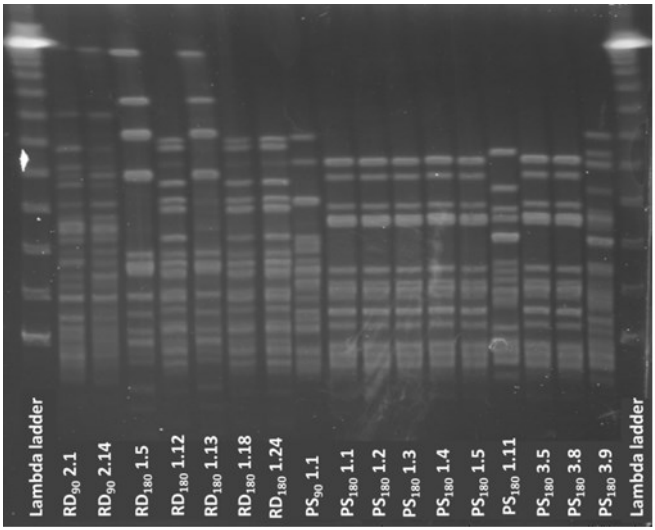


Figure 2S. *SmaI*-PFGE pattern of enterococcal strains. Line 1: Lambda ladder; line 2: *E. faecalis* $RD_{90}2.1$; line 3: *E. faecium* RD_{90} 2.14; line 4: *E. faecalis* RD_{180} 1.5; line 5: *E. faecium* RD_{180} 1.12; line 6: *E. faecalis* RD_{180} 1.13; line 7: *E. faecium* RD_{180} 1.18; line 8: *E. faecium* RD_{180} 1.24; line 9: *E. faecium* $PS_{90}1.1$; line 10: *E. faecium* $PS_{180}1.1$; line 11: *E. faecium* $PS_{180}1.2$; line 12: *E. faecium* $PS_{180}1.3$; line 13: *E. faecium* $PS_{180}1.4$; line 14: *E. faecium* $PS_{180}1.5$; line 15: *E. faecium* $PS_{180}1.11$; line 16: *E. faecium* $PS_{180}3.5$; line 17: *E. faecium* $PS_{180}3.8$; line 18: *E. faecium* $PS_{180}3.9$; line 19: Lambda ladder.

MLST analysis

Currently, MLST have become the standard for epidemiological studies on enterococci (Willems and van Schaik, 2009). In MLST schemes, fragments of housekeeping genes are sequenced and each sequence is assigned an allele, resulting in a 'barcode' consisting of seven allele numbers, which can be assigned a ST. MLST data can be deposited in a freely accessible database and because of the large number of sequences that are available in these databases, the data allow a truly global view of the population structure of these bacteria (Maiden, 2006). Through this analysis it was demonstrated that a relatively limited number of clonal complexes (a group of closely related STs) are responsible for the majority of clinical infections (Freitas et al., 2009; Kawalec et al., 2007; Kuch et al., 2012). These high-risk enterococcal clonal complexes (or HiRECCs), such as CC2, CC9, and CC87, are not exclusively limited to clinical settings, but also in pigs (Freitas et al., 2011) and healthy babies (Solheim et al., 2009). However, a recent study into the gene content of CC2 *E. faecalis* showed that mobile genetic elements and genes encoding cell surface structure are overrepresented in this HiRECC (Solheim et al., 2011). Studies of the population structure of *E. faecium* were initially fueled by its rapid acquisition of antibiotic resistance determinants (notably to vancomycin) and have continued since its emergence as a nosocomial pathogen of major importance. An MLST-scheme for *E. faecium* was described in 2002 (Homan et al., 2002) and analyses based on MLST have consistently shown that the majority of *E. faecium* strains causing clinical infections were distinct from strains that had been isolated from other sources, like animals or

healthy individuals (Willems and van Schaik, 2009). This sub-population of clinical isolates is also characterized by their resistance to ampicillin and their enrichment in several genes with a proposed role in pathogenesis, like esp and "hylEfm", carbohydrate metabolism or in other cellular functions, most notably Insertion Sequence elements (Laverde Gomez et al., 2011; Leavis et al., 2007; Rice et al., 2003; Willems et al., 2001). Vancomycin resistance is not a specific marker of clinical *E. faecium* strains, at least not in Europe where the use of the vancomycin-analog avoparcin as a growth promoter until 1996 has led to widespread dissemination of vancomycin resistance genes among animals that are raised for food production (Werner et al., 2008; Willems et al., 2005).

In our study, 5 *E. faecium* strains, isolated from Ragusano PDO and Pecorino Siciliano cheeses, were subjected to MLST analyses. Seven housekeeping loci were selected for the characterization. Internal 400- to 600-bp fragments of the following genes were amplified by PCR: *adk* (adenylate kinase), *atpA* (ATP synthase, alpha subunit), *ddl* (D-alanine: D-alanine ligase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosylaminoimidazol carboxylase ATPase subunit), and *pstS* (phosphate ATP-binding cassette transporter). Fragments were amplified from bacterial lysates by PCR with the sets of primers showed in the table 1.

Table 1. Sets of primers for housekeeping genes amplification.

Genes	Sequences
adk1	5'-TATGAACCTCATTTTAATGGG-3'
adk2	5'-GTTGACTGCCAAACGATTTT-3'
atpA1	5'-CGGTTCATACGGAATGGCACA-3'
atpA2	5'-AAGTTCACGATAAGCCACGG-3'
ddl1	5'-GAGACATTGAATATGCCTTATG-3'
ddl2	5'-AAAAAGAAATCGCACCG-3'
gdh1	5'-GGCGCACTAAAAGATATGGT-3'
gdh2	5'-CCAAGATTGGGCAACTTCGTCCCA-3'
gyd-1	5'-CAAACTGCTTAGCTCCAATGGC-3'
gyd2	5'-CATTTCGTTGTCATACCAAGC-3'
purK1	5'-GCAGATTGGCACATTGAAAGT-3'
purK2,	5'-TACATAAATCCCGCCTGTTTC/T-3'
pstS1	5'-TTGAGCCAAGTCGAAGCTGGAG-3'
pstS2	5'-CGTGATCACGTTCTACTTCC-3'

PCR conditions for all amplification reactions were as follows: initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; and extension at 72 °C for 5 min. Reactions were performed in 50 µl volumes with buffers and Taq polymerase from AmpliTaq (Thermo Fisher Scientific). PCR products were purified with a PCR purification kit from Qiagen Inc. (Hilden Germany) and send to sequence with PCR forward

and reverse primers, to Macrogen, Spain. The sequences obtained have been deposited in MLST database and the results are showed in the table 2.

Table 2. MLST results.

***E. faecium* strains**	**atpA**	**ddl**	**gdh**	**purk**	**gyd**	**pstS**	**adk**	**ST**
PS_{90} 1.1	NEW-83	NEW-8	8	NEW-8	NEW-2	27	6	NEW ST
PS_{180} 1.11	65	2	1	11	1	10	15	675
PS_{180} 3.9	NEW-5	2	1	11	2	14	5	NEW ST
RD_{90} 2.14	14	40	12	3	1	27	1	NEW ST
RD_{180} 1.24	NEW-83	NEW-8	8	NEW-22	2	1	6	NEW ST

The results were subsequently submitted to Ribosomal MLSTlocus/sequence definitions database to obtain the STs.

References

Freitas, A.R., Coque, T.M., Novais, C., Hammerum, A.M., Lester, C.H., Zervos, M.J., Don-abedian, S., Jensen, L.B., Francia, M.V., Baquero, F., Peixe, L. (2011). Human andswine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishableplasmids. J. Clin. Microbiol. 49, 925–931.

Freitas, A.R., Novais, C., Ruiz-Garbajosa, P., Coque, T.M., Peixe, L. (2009). Clonal expansion within clonal complex 2 and spread of vancomycinresistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal. J Antimicrob Chemother 63: 1104-1111.

Homan, W.L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., et al. (2002). Multilocus sequence typing scheme for *Enterococcus faecium*. Journal of Clinical Microbiology; 40:1963–1971.

Kawalec, M., Pietras, Z., Daniłowicz, E., Jakubczak, A., Gniadkowski, M., Hryniewicz, W., Willems, R.J.L. (2007). Clonal Structure of *Enterococcus faecalis* Isolated from Polish Hospitals: Characterization of Epidemic Clones. J Clin Microb, p. 147–153.

Kuch A., Willems R. J., Werner G., Coque T. M., Hammerum A. M., Sundsfjord A., et al. (2012). Insight into antimicrobial susceptibility and population structure of contemporary human *Enterococcus faecalis* isolates from Europe. J of Antim Chemo. 67(3):551–558.

Laverde Gomez, J.A., van Schaik, W., Freitas, A.R., Coque, T.M., Weaver, K.E., Francia, M.V., Witte, W., Werner, G.A. (2011). A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital *Enterococcus faecium* isolates. Int J Med Microbiol.301(2):165-75.

Leavis, H.L., Willems, R.J., van Wamel, W.J., Schuren, F.H., Caspers, M.P., Bonten, M.J. (2007). Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of E. faecium. PLOS Pathogens.3(1)-7.

Maiden, M.C.J. (2006). Multilocus sequence typing of bacteria. Annu Rev Microbiol 60:561–588.

Rice L. B., Carias L., Rudin S., Vael C., Goossens H., Konstabel C., et al. (2003). A potential virulence gene, hylEfm, predominates in *Enterococcus faecium* of clinical origin. The J of Inf Dis. 187(3):508–512.

Solheim, M., Aakra, A., Snipen, L., Nes, I.F. (2009). Comparative genomics of Enterococcus faecalis from healthy Norwegian infants. BMC Genomics 10(1):194.

Solheim, M., Brekke, M.C., Snipen, L.G., Willems, R.J., Nes, I.F., Brede, D.A. (2011). Comparative genomic analysis reveals significant enrichment of mobile genetic elements and genes encoding surface structure-proteins in hospital-associated clonal complex 2 *Enterococcus faecalis*. BMC Microbiol.,11, 3.

Werner, G., Strommenger, B., Witte, W. (2008). Acquired vancomycin resistance in clinically relevant pathogens. Future Med. 3, 547-562.

Willems, R.J., Homan, W. Top, J., van Santen-Verheuvel, M. Tribe, D., Manzioros, X., Gaillard, C., Vandenbroucke-Grauls, C.M., Mascini, E.M., van Kregten, E., van Embden, J.D., Bonten, M.J. (2001). Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. Lancet 357:853–855.

Willems, R.J., van Schaik W. (2009). Transition of Enterococcus faecium from commensal organism to nosocomial pathogen. Future Microbiol 4:1125-1135.

Willems, R.J.,J. Top, van Santen, M., Robinson, D.A., Coque, T.M., Baquero, F., Grundmann, H.,. Bonten. M.J. (2005). Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex. Emerg. Infect. Dis. 11:821–828.

Chapter 2

Enterococci in aquatic environment

Backround

Water reuse is an increasingly common sustainable water management practice motivated by climate change, urbanization, energy efficiency, and environmental protection (USEPA, 2012). Reclaimed or recycled wastewater is treated by municipalities for a variety of purposes, including non-potable urban reuse (Aiello et al., 2012; Grant et al., 2012; USEPA, 2012). Although a lack of public acceptance has sometimes been a barrier to using this option, in recent years, the use of treated wastewater has increased in water-scarce regions, where a high percentage of the national crop production is irrigated with wastewater (Pedrero et al., 2010). The removal of organic matter, chemical pollulants and undesirable microorganisms from sewage, using a combination of physico-chemical and biological treatments, was a major technological achievement of the last century, allowing to the environment of water with good quality. The quality of treated wastewater is guaranteed by their microbiological traits that mainly consiste on the assence of potential human pathogens and parasites, regarded as respect of estabilished standards, such as limit for faecal indicators and nematode eggs. Moreover, the guidelines aim at preventing also potential effects on soil productivity and fertility, due to disturbance of physico-chemical properties of soil, and possible risks for human health, through the presence of toxic compounds (Aquarec, 2006; EPA, 2012; WHO,

2006). However, the emerging risks linked to the spread of AR through the transmission pathways environment–plants–humans or the removal of novel classes of contaminants, represent a relevant omission of current guidelines. Regarding AR bacteria, the abundance of carbon sources and other nutrients, a variety of possible electron acceptors such as oxygen or nitrate, the presence of particles onto which bacteria can adsorb, or a fairly stable pH and temperature are examples of conditions favouring the remarkable diversity of microorganisms in this peculiar habitat. To overcome these limits, the introduction of different technologies based on sedimentation, filtration or disinfection processes, such as chlorine dioxide, UV, ozone or TiO_2 has been proposed (Norton-Brandão et al., 2013). Nevertheless, the final effluent are far from being sterile, hence releasing into the environment high amount of bacteria of animal and human origins (Manaia et al., 2017; Rizzo et al., 2013), many of them harbour AR genes and are potential carriers for the dissemination of these genes in the environmental microbiome (Berendonk et al., 2015; Manaia, 2017; Pruden , 2014). Urban WWTPs, bringing together AR bacteria, antibiotic residues and other potential selectors, favour the selection towards these bacteria, and simultaneously, offer an environment capable of facilitating the horizontal gene transfer of AR determinantes. These features make of the WWTPs environment one of the most exciting niches to unveil the fate of AR bacteria and genes (Manaia et al., 2018). In this contest, great attention is paid to enterococci that, among faecal indicators, have emerged as the most important agents of nosocomial infections in the last period, for their enhanced AR. For this microbial group neither the Italian guidelines nor the new European proposal directive provide limits in reclaimed wastewater. In the light

of what was stated, it is necessary to focus on the presence of AR enterococci to mitigate possible health effects associated with the use of treated wastewater in agriculture and to estimate the risk associated with their environmental dispersal.

References

Aiello, R., Cirelli, G., Consoli, S., Giuffrida, F., Leonardi, C., Licciardello, F. (2012). Treated municipal wastewater reuse in vegetable production. Agricultural Water Management, 104.

AQUAREC. (2006). Reclamation and reuse of municipal wastewater in Europe – current status and future perspectives analysed by the AQUAREC research project.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L. Tackling antibiotic resistance: the environmental framework. Nat Rev Microbiol,13:310-7.
EPA. (2012). Guidelines for Water Reuse. Environmental Protection Agency (EPA), Wasghinton DC (EPA/600/R-12/618): 2012.

Grant, S.B., Saphores, J.D., Feldman, D.L., Hamilton, A.J., Fletcher, T.D., Cook, P.L.M., et al. (2012). Taking the "waste" out of "wastewater" for human water security and ecosystem sustainability. Science 337: 681–686.

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Nunes, O.C. (2018). Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environment International, 115: 312-324.

Norton-Brandão, D., Scherrenberg, S.M., van Lier, JB. (2013). Reclamation of used urban waters for irrigation

purposes--a review of treatment technologies. J. Environ. Manage, 15:85-98.

Pedrero, F., Kalavrouziotis, I., Alarcòn, J.J., Koukoulakis, P., Asano, T. (2010). Use of treated municipal wastewater in irrigated agriculture: Review of some practices in Spain and Greece. Agric. Water Manage. 2010; 97:1233–1241.

Pruden, A., Arabi, M., Storteboom, H.N. (2012). Correlation between upstream human activities and riverine antibiotic resistance genes. Environ. Sci. Technol. 46, 11541–11549.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci Total Environ. 447:345-60.

USEPA, United States Environmental Protection Agency **(2012).** Recreational Water Quality Criteria. Office of Water 820-F-12-058.

WHO. (2006). Guidelines for the safe use of wastewater, excreta and greywater. Volume 2: Wastewater use in agriculture**.**

Constructed wetlands combined with disinfection systems for the removal of urban wastewater contaminants

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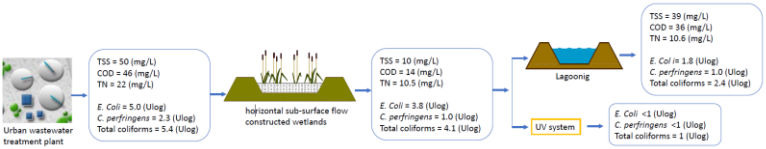
Graphical abstract



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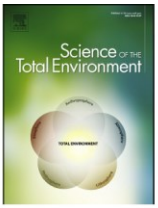

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Abstract

The removal efficiency of an urban wastewater treatment plant (WWTP) to obtain an effluent suitable for agriculture reuse was evaluated in a one-year period, taking into account the Italian wastewater limits and the recent European proposal for the minimum requirements water quality for agricultural irrigation. The secondary effluent of WWTP was treated by three full-scale horizontal sub-surface flow (H-SSF) constructed wetlands (CWs), working in parallel, planted with different macrophytes species, and combined with a UV device and a lagooning system running in series. The H-SSF CW system effectively reduced physico-chemical pollutants and its efficiency was steady over the investigation period, while, Escherichia coli densities always exceed the Italian limits required for wastewater reuse in agriculture. The UV system significantly reduced the microbiological indicators, eliminating E. coli, in compliance with the Italian regulation, and somatic coliphages, although a variable efficacy against total coliforms 27 and enterococci, especially in winter season, was achieved. Although the lagooning unit provides a high removal of the main microbial groups, it did not reduce physico-chemical parameters. Even if the overall performance target, for the whole treatment chain, met the recent log10 reduction (≥5.0), required by the European Commission, the persistence of enterococci, especially in winter season, poses a matter of concern for public health, for the potential risk to serve as a genetic reservoir of transferable antibiotic-resistance.

Keywords: fecal indicators; enterococci; wastewater reuse, lagooning, UV system.

1. Introduction

Reclaimed water provides a valid opportunity to supplement water resources, alleviate environmental loads and address the imbalance between water demand and water supply (Lyu et al., 2016). Agricultural reuse is one of the most worldwide significant use of reclaimed water (EPA, 2012) and Constructed Wetlands (CWs) are widely applied as a low-cost alternatives or supplementary systems for wastewater treatment, especially in small and medium communities where low maintenance and easy operation are required (Marzo et al., 2018; Toscano et al., 2013; Vymazal et al., 2002; 2009). Irrigation with treated wastewater is already implemented in different countries, especially in arid zones and urban areas, such as France, Italy, Spain, Cyprus, Malta, Israel, Jordan, and USA (Pedrero et al., 2010; EPA, 2012; Kalavrouziotis et al., 2015). However, the human and environmental health implications of treated wastewater reuse for agricultural scope poses still some concerns (Phung et al., 2011). In particular, the microbiological parameters represent the biggest threat to municipal wastewater agricultural reuse due to the frequent presence of pathogens in effluents of conventional wastewater treatment plants (WWTPs) (Ghermandi et al., 2007; Calheiros et al., 2017). For each application of treated wastewater reuse, safety criteria must be observed and the potential risks associated with the processes must be defined, according to World Health Organisation guidelines (WHO, 2006). Standard criteria are based on the establishment of threshold values for specific physico-chemical and microbiological parameters. These values must be fulfilled before using treated wastewater for irrigation purpose. In Italy, the use of reclaimed wastewater

is regulated by the Ministerial Degree N. 185, 12/06/2003 (Ministry for Environment, 2003) which establishes standard values for fifty-four parameters, many of which are the same required for drinking water (Cirelli et al., 2008). At European level, new guidelines for minimum requirements water quality for agricultural irrigation have been recently announced (European Commission, 2018). It sets minimum requirements for treated wastewater from urban WWTPs, referring to four parameters (*Escherichia coli*, BOD_5, TSS and Turbidity), and establishing different water quality for different crop categories and irrigation methods to guarantee the safety use of reclaimed water. Conventional indicator organisms, such as *Escherichia coli*, total and fecal coliforms, are mostly used worldwide (Lyu et al., 2016). However, their presence is not always correlated with the presence of certain pathogens (Saxena et al., 2015) and their reliability has been questioned. In addition, to provide insights into the comprehension on efficiency of natural wastewater (WW) treatment systems, alternative indicators, including enterococci, have been suggested by many researchers (Karpiscak et al., 2001; Stott et al., 2003). Currently, European Commission (2018) proposes to assess the performance target of the treatment chain (in $\log_{10}$ reduction) for some selected indicator microorganisms: F+ specific coliphages, somatic coliphages or coliphages as indicator for pathogenic viruses; *Clostridium perfringens* spores or spore-forming sulfate-reducing bacteria for protozoa, and confirms *E. coli* as indicator for pathogenic bacteria. In order to reduce the microbiological risks associated with the use of treated wastewater in food crop irrigation, additional treatments to WWTP have to be considered (Toscano et al., 2013; Licciardello et al., 2018). Among complementary treatment technologies recently

proposed, UV disinfection have attracted an increasing interest (Gomez et al., 2007) to be a fast, safe, and cost-effective process against a wide range of pathogens (Guo et al., 2011; Toscano et al., 2013). In addition, artificial lagoons appear an effective complementary solution (Campos 78 et al., 2002; Oragui et al., 2011) and, thanks to the high microbial inactivation rates, represent an affordable and easy way to produce reclaimed water in small communities, mostly where the norm limits are less restrictive (Mara et al., 1992; Peña et al., 2000). The aims of the present study were to evaluate the horizontal sub-surface flow CWs efficiency, in terms of water quality improvement, and to evaluate the efficiency of lagooning and UV disinfection systems, as tertiary treatment processes, in the removal of *E. coli*, total coliform, *Enterococcus* spp., somatic coliphages and *C. perfrigens* spores. Moreover, in order to evaluate the seasonal efficency of the whole system, both physico-chemical and microbialogical indicators were monitored for one year period.

2. Materials and methods

2.1 Experimental plant

The present study was carried out in three horizontal sub-surface flow CWs, namely CW1, CW2 and CW3, which receive the secondary effluents of the urban WWTP from San Michele di Ganzaria (37°17’0” N and 14°26’0” E). San Michele di Ganzaria is a small community (about 3,200 inhabitants, in 2016, as repoted by the Italian National Institute of Statistics: www.istat.it of Eastern Sicily), located

in Csa Hot-summer mediterranean climate, according to Köppen classification (also known as a mediterranean climate), with a mean annual temperature of 18 °C and mean annual rainfall of 500 mm. Basically two seasons can be distinguished: from April to September (summer, dry and hot, with mean temperatures of 20 °C and mean rainfall of 20 mm/month), and from October to March (winter, wet and cool, with mean temperatures of 11 °C and mean rainfall of 91 mm/month). CW1, CW2 and CW3 are part of the largest natural WWTP of South Italy that includes four CWs operating in parallel, followed by three wastewater storage reservoirs, realized for tertiary treatment of municipal wastewater aimed at agricultural reuse (Aiello et al., 2016; Castorina et al., 2016). CW1 has been operating for twelve years (since 2006). It has a surface area of about 2,000 m^2, is filled with 10-15 mm volcanic gravel, treats a wastewater flow of about 2 L/s and is planted with *Phragmites australis* (about 350 stems per m^2). CW2 has been operating since summer 2012 and presents the same design characteristics as CW1 (area, porous medium, flow rate, vegetation type and density). CW3, also in operation since summer 2012, is the smallest CW (surface area of about 1,200 m^2) and is planted with *Typha latifolia*, at a density of four rhizomes per m^2 Its stem density is about 170 per m^2. The main design and operation characteristics of wetland beds are reported in Table 1.

An UV unit and a lagooning system, running in parallel, were designed and connected in series to the CW2 bed to treat part of CW effluent. The type of UV device was selected considered: the flow rate (1-1.5 l/s) to be treat, the wastewater transmittance (60-70%), and the required *E. coli* log reduction (5 ulog) to achieve the Italian standards for wastewater reuse. UV radiation was applied using LBX 10

(WEDECO), a tubular model with low pressure lamps (80 W, λ = 254 nm) and a reactor volume of 13 l. An automatic wiping system keeped the sleeves clean, minimizing manual cleaning effort. For a maximum flow rate of 6 m^3/h the installation provides a minimum dose of 700 J/m^2 to the water with 70% transmittance. However, UV dose varied according to the flow rate (hence, the detention time in the reactor) and the average light intensity (hence, the transmittance of the wastewater) (Amin et al., 2010). The UV unit tested was equipped with a sensor which continuously monitors the UV intensity and with a LC display where was possible to read the UV intensity. If the minimum UV intensity (set to 48 W/m^2) is reached, a visual alarm appears on the display. During the research activity, no alarm message was registered. This means that the minimum dose of 700 J/m^2 declared by the UV producer was always maintained. The flow rate to the UV unit was not continuous and set at 1-1,5 L/s. The lagooning, a maturation pond, waterproofed by a plastic liner, was designed for pathogen removal. Its storage volume is about 60 m^3 and the maximum depth is 1.0 m. The mean influent flow rate treated by the maturation pond was about 3 m^3/day and the hydraulic retention time (HRT)was about 20 days.

Table 1. Constructed wetland characteristics.

Constructed wetlands	Operation time (year)	Flow rate (m^3 /day)	Width (m)	Length (m)	HRT (day)	Area (m^2)	Gravel				Macrophytes planted
							Type	Size (mm)	Nominal porosity	Depth (m)	
CW1	12	240	28.5	70	2,3	2000	volcanic	8-15	0.47	0.6	*Phragmites australis*
CW2	6	240	28.5	70	2.3	2000					
CW3	6	150	20	60	2.3	1200					*Typha latifolia*

2.2 Sampling points

Influent and treated wastewater samples were collected every 2 or 3 weeks for over one year at: (1) CW influent (i.e., following WWTP); (2) CW1 effluent; (3) CW2 effluent; (4) CW3 effluent; (5) after the lagooning; and (6) after the UV treatment (Figure 1).

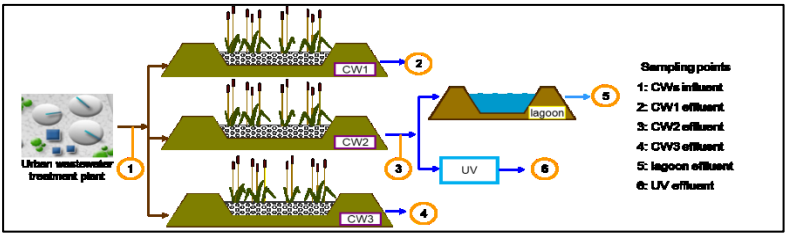

Figure 1. Layout of experimental plant

All samples were collected in sterile bottles and transported, in refrigerated conditions, to the laboratories of the Department of Agricultural, Food and Environment, University of Catania, for further analyses. The quality of the effluents was compared to the Italian legal limits

(Ministerial Decree No. 185/2003) and with European proposal regulation (European Commission, 2018) for wastewater reuse in irrigation (Table 2).

Table 2. Italian standards and European reclaimed water quality for water reuse in agriculture.

Parameters	Italian limits	European guideline	
		Quality requirements	Water quality class
BOD_5 (mg/L)	20	10	A[(1)*]
		25 (According to Council Directive 91/271/EEC)	B[(2)],C[(3)] and D[(4)]
COD (mg/L)	100	-	
TSS (mg/L)	10	10	A[(1)*]
		35 (According to Council Directive 91/271/EEC)	B[(2)],C[(3)] and D[(4)]
NH_4 (mg/L)	2	-	
TN (mg/L)	35	-	
TP (mg/L)	10	-	
Escherichia coli (CFU/100 mL)	10 (80% of samples)	≤10 or below detection limit	A[(1)*]
		≤100	B[(2)]
	100 (max value)	≤1000	C[(3)]
		≤10000	D[(4)]

(1) Crop category irrigable with water quality of Class A: All food crops, including root crops consumed raw and food crops where the edible part is in direct contact with reclaimed water. All irrigation methods allowed
(2)Crop category irrigable with water quality of Class B: Food crops consumed raw where the edible part is produced above ground and is not in direct contact with reclaimed water, processed food crops and non-food crops including crops to feed milk- or meat-producing animals. All irrigation methods allowed
(3) Crop category irrigable with water quality of Class C: the same for the class B but only drip irrigation is admitted.
(4) Crop category irrigable with water quality of Class D: Industrial, energy, and seeded crops. All irrigation methods allowed
*Performance targets for the treatment chain (log10 reduction) only required in class A: *E. coli* ≥ 5.0; Total coliphages/ F-specific coliphages/somatic coliphages/coliphages ≥ 6.0; Clostridium perfringens spores/spore-forming sulfate-reducing bacteria ≥6.

2.3 Microbiological analyses

Influent, after decanting, and effluent samples were subjected to microbiological analyses by membrane filtration method, according to *Standard Methods for the Examination of Water andWastewater* (APHA, 2006). Briefly, samples were opportunely diluted in a sterile saline solution and 100 mL of each dilution were filtered through a 0.45 μm pore-size sterilized membrane filters (Microfil V, Merk Millipore, Italy). The enumeration of conventional fecal indicator bacteria (i.e., *Escherichia coli*, total coliforms and enterococci) were performed according to the ISO procedures (ISO, 2001). The results were expressed as $\log_{10}$ colony forming units (CFU) per unit of volume. Somatic coliphages were quantified according to the ISO 10705-2:2000 method, incubating samples with appropriate host strain. The results were expressed as $\log_{10}$ of Plaque-Forming Units (PFU) per unit of volume. Spores of *C. perfringens* were quantified according to the ISO 7937:2004

procedure. The analyses were performed in triplicate and the results expressed as log_{10} colony forming units (CFU) per unit of volume.

2.4 Physico-chemical analyses

Influent and treated wastewater samples were analyzed for physico-chemical parameters including: Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), Total Nitrogen (TN), Ammonia (NH_4), and Total Phosphorus (TP). Analyses were performed following standard methods (APHA, AWWA, AEF, 2005). COD of CW effluent were evaluated on samples filtered by GF/C Whatmann fiberglass.

2.5 Data analyses

The statistical significance between data of water quality before and after each treatment unit was evaluated by t-paired test (Microsoft Office Excel 2010). At UV influent and effluent only the microbiological parameters were compared. The t-test was also performed between seasons at the effluent of each treatment unit. Results were taken to be significant at the 5% level ($P = 0.05$). In addition, the XLSTAT statistical software was used to identify clusters at each sampling point and sampling period, in order to visualize correlations between microbial indicators and physic chemical parameters.

3. Results

3.1 Microbial removal

The microbial removal in CWs (I stage), lagooning system and UV treatment (II stage) was evaluated by monitoring *E. coli*, total coliforms, *Enterococcu*s spp., somatic coliphages and *C. perfringens* spores. Results of microbiological mean values are shown in Figure 2, whereas the reduction throughout the whole monitoring period, in summer and in winter seasons, at the first, and at the second stage, are reported in Table 2. Results indicated that in CW influent the mean values of *E. coli*, total coliforms and enterococci were 5.0, 5.4 and 4.6 log units in 100 mL, respectively, and the mean initial concentration of somatic coliphages and *C. perfringens* spores were 2.9 PFU/mL and 2.3 CFU/mL, respectively (Figure 2). Considering the first stage of the system and the whole monitoring period (Table 2), the mean reduction for all microbiological indicators were in the range of 0.9 (±1.2) - 1.7 (±0.5) Ulog. In details, for *E. coli*, total coliforms and somatic coliphages, a similar mean reduction (1.2 Ulog, 1.3 Ulog, 1.1 Ulog, respectively) was detected along the continuum, while for enterococci and *C. perfringens* spores a slightly higher average reduction (1.9 Ulog and 1.4 Ulog, respectively) was recorded (Table 2). The p-values between samples collected at the inlet and at the outlet of CWs were always lower than 0.05, revealing a significant reduction of the considered microbiological indicators. Although the CWs showed a high efficiency in microbial reduction, *E. coli* densities, in all CWs effluents (Figure 2), were always higher than limit for wastewater reuse in agriculture, fixed by Italian legislation (80 percentile equal to 10 CFU/100 mL with a maximum

admitted value equal to 100 CFU/100 mL) and higher than EU water quality required for class A (10 CFU/100 mL), class B (100 CFU/100 mL) or class C (1000 CFU/100 mL). No statistical differences between the CW units were observed. regarding the seasonality (Figure 2) even if a certain variability was detected.

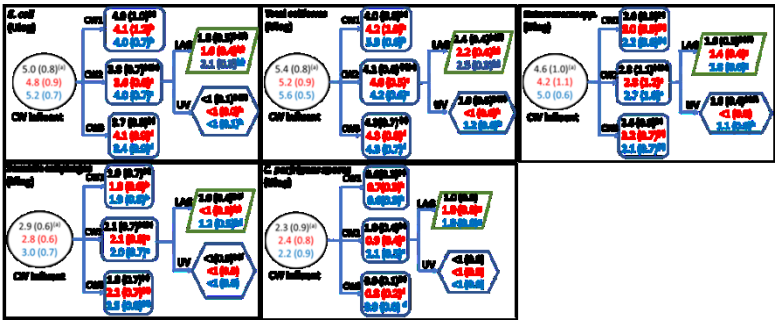

Figure 2. Mean concentration (Ulog) of microbiological indicators at different sampling points in the whole year (black), in summer (red) and in winter (blue).
E. coli, Total coliforms, and *Enterococcus* spp. densities are expressed as log CFU/100 mL; Somatic coliphages as PFU/mL and *C. perfringens* spores as log CFU/mL. T-test was performed to compare mean concentration between: (i) inlet and outlet of each unit for the whole year; (ii) summer and winter at the effluent of each treatment unit; (iii) UV and lagoon effluents of the whole year. Mean values followed by the same letter enclosed in parenthesis are different ($P < 0.05$). Standard deviation in parenthesis. (LAG: lagoon system).

Focusing on the second stage of the system, consisting of lagooning or UV treatments, the microbial mean reduction was considerably higher than those detected in CWs (Table 3). In details, after lagooning system, the highest mean reduction for the whole year (1.9 Ulog) was obtained for *E. coli,* the lowest for *C. perfrigens* spores (0.2 Ulog). Overall data obtained after UV treatment indicated the highest mean reduction, extending to value of 3 Ulog for both *E. coli* and total coliform (Table 3).

Table 3. Mean reduction of microbiological indicators (expressed in Ulog) in the whole year, in summer and in winter. Standard deviations in parentheses.

Parameters	Period	I stage			II stage (after CW2)	
		CW1	CW2	CW3	Lagoon	UV
E. coli	Whole year	1.1 (0.9)	1.3 (0.9)	1.3 (0.9)	1.9 (0.4)	3.0 (0.7)
	Summer	0.9 (0.8)	1.5 (1.0)	0.9 (0.8)	2.0 (0.3)	2.5 (0.9)
	Winter	1.3 (0.9)	1.2 (0.7)	1.7 (0.7)	1.9 (0.5)	2.8 (1.1)
Total coliforms	Whole year	1.4 (0.7)	1.4 (0.7)	1.1 (0.8)	1.8 (0.6)	3.1 (0.9)
	Summer	1.0 (0.7)	1.3 (1.0)	0.9 (0.8)	1.8 (0.6)	2.9 (1.2)
	Winter	1.7 (0.5)	1.4 (0.5)	1.3 (0.7)	1.7 (0.6)	3.0 (0.9)
Enterococcus spp.	Whole year	2.0 (1.2)	1.8 (1.3)	2.0 (1.3)	1.0 (1.3)	1.8 (1.0)
	Summer	1.0 (0.7)	1.2 (1.3)	0.9 (0.6)	0.9 (1.3)	1.9 (1.2)
	Winter	2.8 (0.9)	2.3 (1.1)	3.0 (0.9)	1.1 (1.4)	1.7 (0.8)
Somatic coliphages	Whole year	1.1 (0.9)	0.9 (1.2)	1.2 (0.9)	1.0 (0.9)	1.2 (0.7)
	Summer	1.0 (0.8)	0.7 (1.2)	0.8 (0.9)	1.1 (0.9)	1.2 (0.8)
	Winter	1.2 (1.0)	1.1 (1.2)	1.5 (0.9)	1.0 (0.8)	1.1 (0.7)
C. perfrigens spores	Whole year	1.7 (0.8)	1.3 (1.0)	1.4 (0.8)	0.2 (0.3)	0.1 (0.4)
	Summer	1.7 (0.9)	1.5 (1.0)	1.6 (0.8)	0.1 (0.2)	0.2 (0.2)
	Winter	1.6 (0.7)	1.1 (1.0)	1.3 (0.9)	0.3 (0.3)	0.2 (0.5)

3.2 Physico-chemical parameters removal efficiency

The mean values of physico-chemical parameters recorded at the different sampling points for the whole period and for the two seasons are shown in Figure 3, whereas the mean removal efficiency is reported in Table 4. Since the mean EC value measured in the CW influent, about 1171 (µS/cm), was already suitable for crop irrigation, it was not further considered. As for microbiological parameters, no statistical differences between the CW units were observed for physico-chemical parameters, even if CWs were planted with different plant species (*Phragmites australis* and *Typha latifolia*).

Table 4. Mean removal efficiencies of physico-chemical parameters (expressed as percentage) for the whole year, summer and winter. Standard deviations in parentheses.

Parameters	Period	I stage			II stage (after CW2)
		CW1	CW2	CW3	Lagoon
TSS	Whole Year	77 (10)	80 (9)	81 (11)	-46 (120)
	Summer	76 (11)	78 (9)	77 (11)	-525 (168)
	Winter	78 (9)	82 (9)	83 (10)	-42 (102)
BOD_5	Whole Year	62 (22)	63 (18)	61 (25)	-22 (130)
	Summer	63 (13)	57 (19)	61 (27)	-276 (92)
	Winter	61 (28)	68 (16)	60 (25)	-18 (91)
COD	Whole Year	63 (23)	66 (16)	59 (27)	-21 (120)
	Summer	63 (14)	60 (18)	60 (26)	-255 (110)
	Winter	62 (30)	71 (14)	59 (30)	-19 (132)
TN	Whole Year	48 (19)	44 (25)	44 (24)	-2 (43)
	Summer	37 (24)	33 (29)	31 (26)	-2 (52)
	Winter	53 (18)	54 (16)	56 (16)	-1 (47)
NH_4	Whole Year	42 (25)	40 (27)	39 (30)	-9 (74)
	Summer	32 (19)	25 (27)	23 (23)	-12 (62)
	Winter	51 (28)	49 (53)	53 (30)	-22 (51)
TP	Whole Year	25 (20)	24 (19)	20 (18)	-17 (21)
	Summer	21 (12)	18 (13)	10 (12)	-17 (27)
	Winter	28 (25)	29 (23)	29 (19)	-19 (30)

In detail, TSS values decreased from a mean value of 50 mg/L to 10 mg/L, throughout all of the three CWs. The mean TSS removal efficiency was high and quite constant during the monitoring period (77 ± 10% for CW1, 80 ± 9% for CW2 and 81 ± 11% for CW3, respectively, Table 4), as confirmed by the low standard deviation of values in effluent with standard deviation less of 11. Statistical analyses indicate significant differences in TSS values between influent and effluent of all CWs ($P<0.05$), while no significant differences were revealed considering the seasons (Figure 3).

The treated wastewater was characterized by a BOD_5/COD ratio >0.6, indicating the presence of biodegradable

compounds. Therefore, the mean percentage of organic compound removal was similar for both parameters (about 60%) in all the CW beds (Table 4), with a BOD_5 effluent values ranging from 2 mg/L to 23 mg/L and COD values ranging from 5 to 30 mg/L. The t-paired test indicated that both BOD_5 and COD values were significantly lower in the CW effluent compared to CW influent, and that no differences in the mean reduction between the two seasons were detected (Figure 3).

Mean value of TN decreased from 21.6 mg/L to 10.2 mg/L. Although a lower TN removal was observed, compared to both organic matter and TSS (Table 4), a quite similar removal efficiency was observed for the three beds (48 ± 19% for CW1, and 44 ± 25% for CW2 and CW3). Although, an improvement in both TN and NH_4 removal during summer season was expected, any statistical differences between seasons was observed (Figure 3). Despite the TN values in all the CW effluents met the Italian standard required for irrigation (35 mg/L), the 5% (CW2) and the 10% (CW1 and CW3) of tested the samples exceeded the European discharge limit (10 mg/L). Regarding the TP values, they were low throughout the monitoring period, ranging from 3.8 to 9.7 mg/L, below the Italian law standards for reuse of wastewater in agriculture (fixed as 10 mg/L). The TP concentration was significantly modified through treatment 234 in the CW beds, from a mean value of 4.4 mg/L in CW1 and CW2 effluents, to a mean value of 5.0 mg/L in CW3 effluent (Figure 3).

Different results were detected in the lagooning unit. Even if the HRT of the maturation pond was very high (over the recommended maximum value of 10 days, according to Mara, 2003), the efficiency in reducing the most physico-chemical parameters was quite low. The means values of

TSS, BOD_5 and COD in lagooning system effluent were found significantly higher than those detected in the influent (CW2 effluent). This is also confirmed by the TSS, BOD_5 and COD values in the pond effluent, detected as significantly lower during the winter than in the summer season (Table 4), when algae bloom on the water surface was observed. Lagooning unit was also unable to further lower TN, NH_4, and TP levels, which indeed showed relative increases during the monitoring period.

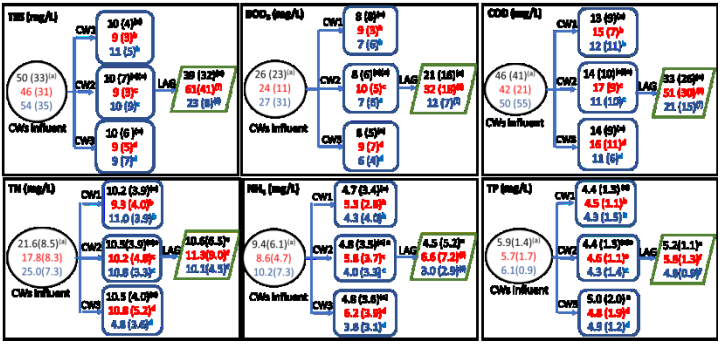

Figure 3. Mean concentration (mg/L) of the physico-chemical parameters at the different sampling points in the whole year (black), in summer (red) and winter (blue).

T-test was performed to compare mean concentration between: (i) inlet and outlet of each unit for the whole year; (ii) summer and winter at the effluent of each treatment unit; (iii) UV and lagoon effluents for the whole year. Mean values followed by the same letter enclosed in parenthesis are different ($P < 0.05$). Standard deviation in parenthesis. (LAG: lagoon system).

3.3 Correlation between physico-chemical and microbiological parameters

In order to visualize correlations between microbial indicators and physico-chemical parameters, XLSTAT statistical software was used. Results are shown in Figure 4. Overall, statistical data revealed a positive correlation among all considered microbial indicators and between microbial indicators and the TN and NH_4 values (Figure 4). In particular, enterococci, together with *C. perfringens* spores, were positively correlated with all considered physico-chemical parameters, whereas a variable correlation was observed considering the other physico-chemical parameters. In addition, a negative correlation was observed between *E. coli* and TP/TSS/BOD_5/COD, while for both total coliforms and somatic coliphages negative correlation was observed towards TSS, BOD_5 and COD (Figure 4).

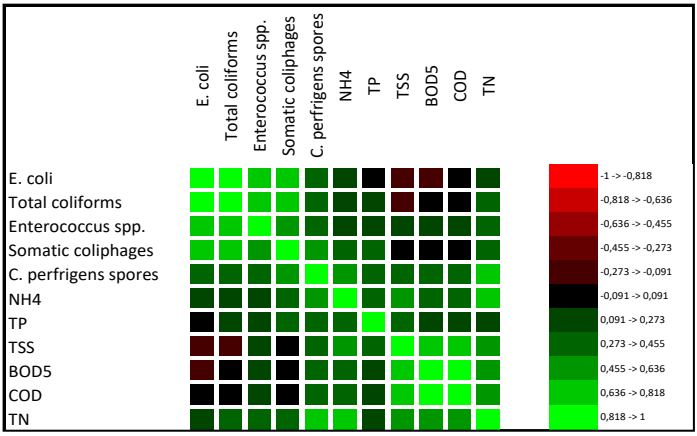

Figure 4. Significant correlations between microbial indicators (log CFU/mL) and physic chemical parameters.

The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (green) and -1 indicating a perfectly negative correlation (red). Only significant correlations (False Discovery Rate <0.05) are shown.

4. Discussion

In this study, the removal efficiency of H-SSF CWs, combined with lagooning systems or UV treatment, in twelve consecutive months was evaluated. The performance of the whole system was assessed by comparing the main parameters of the influent with those of the effluent, at each treatment unit, considering both the Italian regulation for wastewater reuse in irrigation (Ministerial Decree No. 185/2003) and the recent European proposal regulation (European Commission, 2018). Regarding physico-chemical parameters, the mean TSS removal efficiency was high and quite constant during the monitoring period, in accordance to previous studies (Toscano et al., 2015; Vymazal, 2002). No difference in TSS removal related to seasons was observed, as reported by other authors (Llorens et al., 2009; Ouellet-Plamondon et al., 2006; Steer et al., 2002). TSS removal in wetlands is mainly due to physical processes, such as sedimentation and filtration, which are not temperature dependent process (Kadlec and Wallace, 2009). TSS were always detected at levels below the threshold value (35 mg/L) propose by the European for reclaimend water quality in class B, C and D and in 95% of samples also below the Italian law limit for wastewater reuse in agriculture (10 mg/L), the same required for EU water quality in class A. The mean percentage of organic

components removal was quite the same for COD and BOD_5 parameters since municipal wastewater usually contained elevated concetration of easily degradable organic compounds (Vymazal et al., 2009). No differences were revelead in the mean reduction of BOD_5 and COD values between the two seasons highlithing that temperature dependence is not significant for both parameters, as reported by several authors (Akratos et al., 2007; Kadlec and Knight; 1996; Kadlec and Reddy, 2001; Steinmann et al., 2003). In addition, this result could be referred to the buffer effect of both substrate and plant root systems in mantaining, during winter, the wastewater temperature higher than the air temperature, at least by 2–3 °C against climatic fluctuations (Steer et al., 2002; Walaszek et al., 2018). BOD_5 and COD daily concentrations in the CWs effluents were always below the European standard for water quality in class B, C and D (25 mg/L for BOD_5) and below the Italian regulation for reuse of wastewater in irrigation (20 mg/L for BOD_5 and 100 mg/L for COD). The observed lower mean TN removal, compared to those found for organic matter and TSS, has already been reported in HF CW systems (Aiello et al., 2016; Akratos et al., 2007) and a TN removal quite similar for all the beds has been already observed (Katsenovich et al., 2009; O'Luanaigh et al., 2010; Vymazal, 2009). Although the temperature dependence is well documented for TN and NH_4 removal (Akratos and Tsihrintzis, 2007; García et al., 2010; Garfí et al., 2012), no improvement in both TN and NH_4 removal efficiency was detected. This result could be explained by the warm climate of Mediterranean area allows year-around plant growth and a constant microbial activity (Kadlec and Wallace, 2009; Khisa and Mwakio, 2011). The microbial species involved into TN reduction showed an optimal activity at temperature

above 15° C (Saeed and Sun, 2012; Andreo-Martínez et al., 2017). The mean air temperature during winter was 11°C. Since the CW substrate allows maintaining the wastewater temperature higher than that of the air at least by 2–3 °C (Steer et al., 2002), it is possible to asses that the microbial activity was not affected by temperature in winter. Furthermore, this result could be also related by the higher mean rainfall during winter season, that could have a dilution effect in the CWs.

Mean value of TN at CW effluent met the Italian law requirements for wastewater irrigation (35mg/L). No seasonal difference was observed for TP since its removal is mainly due to physical and chemical mechanisms, such as sorption and precipitation, which are not temperature dependent processes (Spieles and Mitsch, 2000; Ding et., 2014; Thongtha et al., 2014). Regarding the lagooning unit, different results were observed. Even if the HRT of the maturation pond was high, the average effluent values of TSS, BOD_5 and COD were significantly higher than the influent, highlighting a negative removal efficiency results. Similar results have been previously observed by other authors (Barbagallo et al., 2011; Dias et al., 2014; Von Sperling et al., 2006), which attributed the increase of TSS and organic compounds values to algae growth and decomposition in the pond, especially during the summer period. Furthermore, the higher mean rainfall during winter season could also contribute to reduce the pollutants concertation in the pond effluent. This is confirmed by the TSS, BOD_5 and COD values found as significantly lower in the pond effluent during the winter than in the summer season. Lagooning unit was also unable to further lower total TN, NH_4, and TP levels and this can be attributed to the low influent values of TN, NH_4, and TP (close to the

background concentrations) and also to the anaerobic decomposition of algal substances. The microbiological results showed that the densities of conventional fecal indicators (*E. coli*, total coliforms and enterococci) in the influents were consistent across the sampling period, and that the WWTP was able to achieve a mean of 1.5-log unit removal along the whole system, revealing a statistical significant improvement of water quality. These removal efficiencies were comparable to those previously reported by other authors, in different geographical locations, mainly in US, Canada, France and Belgium and Spain (Andreo-Martínez et al., 2017; Harwood et al., 2002; Harwood et al., 2005; Saleem et al., 2000; 2003; Wery et al., 2008; Zhang and Farahbakhsh, 2007). In CWs the highest mean removal efficiency was observed for *C. perfrigens* spores and for *Enterococcus* spp., while the lowest for *E. coli* and total coliforms, in agreement with previous reports (Whu et al., 2016). In contrast with many observations, which report a better efficacy in summer season, in the present work, the CW removal efficiency was not influenced by season. The lower efficiencies observed in summer time is in agreement with observations reported by Garcia et al., (2008) and Tunçsiper et al., (2012) that revealed an higher incidence of both *E. coli* and total coliforms in the summer season, related to animal activity and seasonal variation in plant growth (Thurston et al., 2001). In the present work the lower removal efficiency in summer season could be related to proliferation of microganisms in CWs that, as already reported, is highly dependent on many factors such as temperature, water composition and solar intensity (Dixon et al., 2000). Combining CW with UV treatment a considerable improvement in microbiological water quality was obtained, with a complete removal of *E. coli*, somatic

coliphages, and *C. perfringens* spores and a drastic reduction of other microbial indicators. Opposite trend was registered for enterococci, which were significantly removed by the CWs, but persist after both the lagooning system and the UV treatment, especially in winter season. This is probably due to the known genome reparation mechanisms of enterococci and to their cellular structure (with higher peptidoglycan content, presence of theicoic acids and polysaccharides) (Batch 337 et al., 2004; Gao and Williams, 2013; Gravetz and Linden 2005; Oguma et al., 2001). Effectiveness of UV disinfection against different organisms in wastewater systems are reported as variable, and reductions in levels of enterococci has been reported ranging from 2 to 5 orders of magnitude, depending on the treatment processes applied prior to UV exposure and on the type and intensity of the UV source (Hijnen et al., 2006; Koivunen and Heinonen-Tanski, 2005). Overall, the average concentration of *E. coli* in the UV effluent satisfied the Italian reuse standards. Furthermore, the *E. coli* reduction, from raw wastewater effluent entering the same urban wastewater treatment plant to UV effluent, reported as about 7 log CFU/100 mL by Cirelli et al., (2007), was higher than 5 log units, in compliance with the performance targets for the treatment chain reported by the last proposal law (European Commission, 2018). However, in the present study, total coliforms and enterococci, were never completely removed and remain a critical factor with linkage to human health. From a microbiological point of view the persistence of enterococci, above all after lagooing and UV treatment remains a public concern, being WWTPs recognized as a hot spot for antibiotic resistance environmental dissemination (Michael et al., 2013; Novo et al., 2013; Oravcova et al., 2017; Patra et al., 2012). The

results presented here suggest that for combined treatments it will be more appropriate to use at least two microbial indicators in order to validate the performance of the whole treatment, as already proposed by others (Byappanahalli et al., 2012; Lucena et al., 2004). Although lagooning system produced a low mean reduction of physico-chemical parameters, a high removal efficiency, especially in summer season, against somatic coliphages, *E. coli*, total coliforms, enterococci, was observed, whereas no removal effects was observed against *C. perfrigens* spores, considered as conservative surrogates for protozoa such as *Cryptosporidium parvum* and *Giardia lamblia* (oo)cysts. These results are in accordance to previous reports, which highlighted a higher resistance of spores to environmental stress and a longer persistence (several weeks) in water, depending on the temperature, physico-chemical parameters and sunlight (Ahmed et al., 2008; Araki et al., 2001; Fayer et al., 1998; Karim et al., 2004). For this reason the simultaneous use of *E. coli* and *C. perfrigens* spores as indicators of recent and remote fecal contamination, respectively, has been proposed (Byamukama et al., 2005; Mayer et al., 2016). However, the effluents satisfy the WHO guidelines for unrestricted irrigation (WHO, 2006), presenting an *E. coli* mean value lower than 1,000 CFU/100 mL, but fails to meet the strict reuse standard of Italian legislation (50 CFU/100 mL required for 80% of samples in the case of natural treatments).

Finally, the positive correlation between microbial indicators and the TN value is in agreement with previous reports (Wu et al., 2016), highlighting that these microorganisms might survive longer or replicate faster in presence of available nitrogen. In addition, the positive correlation between somatic coliphages and TP, could be

also related to the presence of both organic and inorganic matter which represent the most important factors influencing survival of coliphages (EPA, 2012). The positive correlation between *C. perfringens* spores densities and all the considered physic chemical parameters is in agreement with results reported by Tunçsiper and co-workers (2012), and could also be correlated to higher nutrient concentration. The negative correlation between *E. coli*/total coliforms/somatic coliphages and TSS/BOD_5/COD, suggests that chemical and physical parameters, such as pH, nutrient concentration, dissolved oxygen, turbidity, and conductivity, must all be within a certain range to allow bacteria survival (Wickham et al., 2006).

5. Conclusions

In conclusion, results of the present work highlight that the H-SSF CW can effectively represent a feasible solution for secondary treatment of urban wastewater, producing effluent, which except for microbiological parameters, complies with Italian standards on wastewater reuse in agriculture. The lack of significant correlation between pollutants removal efficiency and temperature underlay that the wetland performance can be considered reasonably constant during the year-around in Mediterranean climate. Furthermore, the different plant species (*Phragmites australis* and *Typha latifolia)* seem not influence the CW performance since no statistical different was detected among the CWs. Although the lagooning system produced a good microbiological removal efficiency, its performance was inefficient for the main physico-chemical parameters.

The combination of CWs with UV treatment produced highly effective results, meeting both the strict Italian legislation and the new European proposal for agricultural reuse of reclaimed water. Although neither Italian nor European legislation provides a limit for enterococci, the risk associated with their environmental dispersal is difficult to estimate and further improvement removal efficiencies are required to obtain a better water quality with minimal detrimental health and environment impacts.

Conflict of interest

The Authors declare that there are no conflict of interest.

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References

Ahmed, W., Sritharan, T., Palmer, A., Sidhu, J.P.S., Toze, S. (2013). Evaluation of bovine feces-associated microbial source tracking markers and their correlations with fecal indicators and zoonotic pathogens in a Brisbane, Australia, reservoir. Appl Environ Microbiol, 79:2682–2691.

Ahn, D.H., Chang, W.S., Yoon, T.I. (1999). Dyestuff wastewater treatment using chemical oxidation, physical adsorption and fixed bed biofilm process. Process Bioch, 34: 429–439.

Aiello, R., Bagarello, V., Barbagallo, S., Iovino, M., Marzo, A., Toscano, A. (2016). Evaluation of clogging in full-scale subsurface flow constructed wetlands. Ecol Eng, 95: 505-513.

Akratos, C.S., Tsihrintzis, V.A. (2007). Effect of temperature, HRT, vegetation and porous media on removal efficiency of pilot-scale horizontal subsurface flow constructed wetlands. Ecol Eng, 29: 173–191.

Amin, M.M., Hashemi, H., Bina, B., Movahhedian, A.H., Farrokhzadeh, H., Ghasemian, M. (2010). Pilot-scale studies of combined clarification, filtration, and ultraviolet radiation systems for disinfection ofsecondary municipal wastewater effluent. Desalination, 260: 70-78.

Andreo-Martínez, P., García-Martínez, N., Quesada-Medina, J., Almela, L. (2017). Domestic wastewaters reuse reclaimed by an improved horizontal subsurface-flow

constructed wetland: A case study in the southeast of Spain. Bioresour Technol, 233: 236-246.

APHA. (2006). Standard Methods for the Examination of Water. American Public Health Association, New York.

Araki, S., Martín-Gómez, S., Bécares, E., De Luis, E., Rojo-Vazquez, F. (2001). Effect of high-rate algal ponds on viability of *Cryptosporidium parvum* oocysts. Appl Environ Microbiol, 67:3322–3324.

Barbagallo, S., Cirelli, G.L., Marzo, A., Milani, M., Toscano, A. (2011). Hydraulic behavior and removal efficiencies of two H-SSF constructed wetlands for wastewater reuse with different operational life. Water Sci Technol, 64:1032-1039.

Batch, L.F., Schulz, C.R., Linden, K.G. (2004). Evaluating water quality effects on UV disinfection of MS2 coliphage. J AW WA, 96: 75-87.

Byamukama, D., Mach, R.L., Kansiime, F., Manafi, M., Farnleitner, A.H. (2005). Discrimination efficacy of fecal pollution detection in different aquatic habitats of a high-altitude tropical country, using presumptive coliforms, *Escherichia coli,* and *Clostridium perfringens* spores. Appl Environ Microbiol, 71:65–71.

Byappanahalli, M.N., Meredith, B., Asja, K., Zachery, R.S., Valerie, J.H. (2012). Enterococci in the environment. Microbiol Mol Biol Rev, 76: 685–706.

Calheiros, C.S.C., Ferreira, V., Magalhães, R., Teixeira, P., Castro, P.M.L. (2017). Presence of microbial pathogensand genetic diversity of Listeria monocytogenes in a constructed wetland system. Ecol Eng, 102: 344-351.

Campos, L.C., Su, M.F.J., Graham, N.J.D., Smith, S.R. (2002). Biomass development in slow sand Filters. Water Res, 36: 4543-51.

Castorina, A., Consoli, S., Barbagallo, S., Branca, F., Farag, A., Licciardello, F., Cirelli, G.L. (2016). Assessing environmental impacts of constructed wetland effluents for vegetable crop irrigation. Int J Phytorem, 18: 626-633.

Cirelli, G.L., Consoli, S., Di Grande, V., Milani, M., Toscano, A. (2007). Subsurface constructed wetlands for wastewater treatment and reuse in agriculture: five years of experiences in Sicily, Italy. Water Sci Technol, 56:183-191.

Cirelli, G.L., Consoli, S., Di Grande, V. (2008). Long term storage of reclaimed water: the case studies in Sicily (Italy) Desalination, 218: 62-73.

Decreto Ministeriale n. 185 del 12 giugno, G.U.R.I. n. 169, 2003, Regolamento recante norme tecniche per il riutilizzo delle acque reflue in attuazione dell'articolo 26, comma 2, del decreto legislativo 11 maggio 1999, n. 152.

Dias, D.F.C., Possmoser-Nascimento, T.E., Rodrigues, V.A.J., von Sperling, M. (2014). Overall performance evaluation of shallow maturation ponds in series treating UASB reactor effluent: Ten years of intensive monitoring of a system in Brazil. Ecol Eng, 71:206–214.

Ding, Y., Wang, W., Song, X.S., Wang, G., Wang, Y.H. (2014). Effect of spray aeration on organics andnitrogen removal in vertical subsurface flow constructed wetland.Chemosphere, 117:502–505.

Dixon, A., Butler, D., Fewkes, A., Robinson, M. (2000). Measurement and modelling of quality changes in stored untreated grey water. Urban Water, 1:293–306.

EN ISO 9308-1:2001 "Water quality-Detection and enumeration of Escherichia coli and coliform bacteria - Part 1: Membrane filtration method".

EPA. Guidelines for Water Reuse. (2012). Environmental Protection Agency (EPA), Wasghinton DC (EPA/600/R-12/618).

European Commission. Proposal for a Regulation of the European Parliament and of the Council on minimum requirements for water reuse. 2018. Brussels, 28.5.2018 COM (2018) 337 final 2018/0169 (COD).

Fayer, R., Trout, J.M., Jenkins, M.C. (1998). Infectivity of *Cryptosporidium parvum* oocysts stored in water at environmental temperatures. J Parasitol 1998, 84:1165–1169.

Gao, W. and Williams, A. (2013). Response of different strains of *Enterococcus faecalis* to UV Inactivation after freezing. Int J Environ Sci Devel, 4.

García, M., Soto, F., González, J.M., Bécares, E. (2008). A comparison of bacterial removal efficiencies in constructed wetlands and algae-based systems. Ecol Eng, 32: 238-243.

García, J., Rousseau, D.P.L., Morató, J., Lesage, E., Matamoros, V., Bayona, J.M. (2010). Contaminant removal processes in subsurface-flow constructed wetlands: a review. Crit Rev Environ Sci Technol, 40(7):561–661.

Garfí, M., Pedescoll, A., Bécares, E., Hijosa-Valsero, M., Sidrach-Cardona, R., García, J. (2012). Effect of climatic conditions, season and wastewater quality on contaminant removal efficiency of two experimental constructed wetlands in different regions of Spain. Sci Total Environ, 437: 61-67.

George, I., Crop, P., Servais, P. (2002). Fecal coliform removal in wastewater treatment plants studied by plate counts and enzymatic methods. Water Research, 36, 2607-2617.

Ghermandi, A., Bixio, D., Thoeye, C. (2007). The role of free water surface constructed wetlands as polishing step in municipal wastewater reclamation and reuse. Sci Total Environ, 380: 247-258.

Gomes, H.T., Figueiredo, J.L., Faria, J.L. (2007). Catalytic wet air oxidation of olive mill wastewater Catal Today, 124: 254-259

Gravetz, H.M. and Linden, KG. (2005). Relationship between physiochemical properties, aggregation and u.v.

inactivation of isolated indigenous spores in water. J Appl Microbiol, 98: 351-363.

Guo, M.T., Huang, J.J., Hu, H.Y., Liu, W.J. (2011). Growth and repair potential of three species of bacteria in reclaimed wastewater after UV disinfection. Biomed Environ Sci, 24:400 - 407.

Harwood, V.J., Whitlock, J., Withington, V. (2002). Classification of Antibiotic Resistance Patterns of Indicator Bacteria by Discriminant Analysis: Use in Predicting the Source of Fecal Contamination in Subtropical Waters. Appl. Environ. Microbiol. 3698–3704

Harwood, V. J., Levine, A. D., Scott, T. M., Chivukula, V., Lukasik, J., Farrah, S. R. & Rose, J. B. (2005). Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Environ. Microbiol. 71(6), 3163–3170

Hijnen, W.A., Beerendonk, E.F., Medema, G.J. (2006). Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. Water Res, 40:3–22.

Kadlec, R.H. and Knight, R.L. (1996). Treatment Wetlands. Lewis Publishers, Boca Raton, p.893.

Kadlec, R.H. and Reddy, K.R. (2001). Temperature effects in treatment wetlands. Water Environ Res, 73: 543-557.

Kadlec RH, Wallace S. (2009). Treatment wetlands. 2nd ed. Boca Raton: CRC Press.

Kalavrouziotis, I.K., Kokkinos, P., Oron, G., Fatone, F., Bolzonella, D., Vatyliotou, M. et al. (2015). Current status in wastewater treatment, reuse and research in some Mediterranean countries. Desalin Water Treat, 53:2015-2013.

Karim, M.R., Manshadi, F.D., Karpiscak, M.M., Gerba, C.P. (2004). The persistence and removal of enteric pathogens in constructed wetlands. Water Res, 38:1831–7.

Karpiscak MM, Whiteake LR, Artiola J, Foster KE. Nutrient and heavy metal uptake and storage in constructed wetland systems in Arizona. Water Sci Technol 2001; 44:455-462.

Katsenovich, Y., Hummel-Batista, A., Ravinet, A.J., Miller, J.F. (2009). Performance evaluation of constructed wetlands in a tropical region. Ecol Eng, 35: 1529–1537.

Khisa, K., Mwakio, T. (2011).The efficacy of a tropical constructed wetland for treating wastewater during the dry season: the Kenyan experience. Water Air Soil Pollut, 215:137–43.

Koivunen, J. and Heinonen-Tanski, H. (2005). Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. Water Res, 39:1519 –1526.

Licciardello, F., Milani, M., Consoli, S., Pappalardo, N., Barbagallo, S., Cirelli, G. (2018). Wastewater tertiary treatment options to match reuse standards in agriculture. Agric Water Manag, 210: 232-242.

Llorens, E., Matamoros, V., Domingo, V., Bayona, J.M., García, J. (2009). Water quality improvement in a full scale tertiary constructed wetland: effects on conventional and specific organic contaminants. Sci Total Environ, 407:2517–24.

Lucena, F., Duran, A.E., Morón, A., Calderón, E., Campos, C., Gantzer, C., Skraber, S., Jofre, J. (2004). Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. J Appl Microbiol, 97:1069-76.

Lyu, S., Chen, W., Zhang, W., Fan, Y., Jiao, W. (2016). Wastewater reclamation and reuse in China: Opportunities and challenges. J Env Sci, 39: 86-96.

Mara, D.D., Mills, S.W., Pearson, H.W., Alabaster, G.P. (1992). Waste stabilization ponds: a viable alternative for small community treatment systems. Water Environ J, 6:72–78.

Mara, D.D. (2003). Domestic Wastewater Treatment in Developing Countries. Earth-scan, London, pp. 293.

Marzo, A., Ventura, D., G.L., Cirelli, G.L., Aiello, R., Vanella, D., Rapisarda, R., Barbagallo, S., Consoli, S. (2018). Hydraulic reliability of a horizontal wetland for

wastewater treatment in Sicily. Sci Total Environ, 636: 94-106.

Mayer, R.E., Bofill-Mas, S., Egle, L., Reischer, G.H., Schade, M., Fernandez-Cassi, X., Fuchs, W., Mach, R.L., Lindner, G, Kirschner, A., Gaisbauer, M., Piringer, H., Blaschke, A.P., Girones, R., Zessner, M., Sommer, R., Farnleitner, A.H. (2016). Occurrence of human-associated Bacteroidetes genetic source tracking markers in raw and treated wastewater of municipal and domestic origin and comparison to standard and alternative indicators of faecal pollution. Water Res, 90: 265–276.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T. et al. (2013). Urban wastewater treatment plants as hot spots for the release of antibiotics in the environment: a review. Water Res, 47: 957–995.

Novo, A., André, S., Viana, P., Nunes, O.C., Manaia, C.M. (2013). Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res, 47:1875-1887.

O'Luanaigh, N.D., Goodhue, R., Gill, L.W. (2010). Nutrient removal from on-site domestic wastewater in horizontal subsurface flow reed beds in Ireland. Ecol Eng, 36:1266–1276.

Oguma, K., Katayama, H., Mitani, H., Morita, S., Hirata, T., Ohgaki, S. (2001). Determination of pyrimidine dimers in Escherichia coli and Cryptosporidium parvum during UV light inactivation, photoreactivation, and dark repair. Appl Microbiol Biotechnol, 67:4630–4637.

Oragui, J.I., Curtis, T.P., Silva, S.A., Mara, D.D. (2011) The removal of excreted bacteria and viruses in deep waste stabilization ponds in Northeast Brazil. IWA Publishing.

Oravcova, V., Mihalcin, M., Zakova, J., Pospisilova, L., Masarikova, M., Literak, I. (2017). Vancomycin-resistant enterococci with vanA gene in treated municipal wastewater and their association with human hospital strains. Sci Total Environ, 609:633-643.

Ouellet-Plamondon, C., Chazarenc, F., Comeau, Y., Jacques Brisson, J. (2006). Artificial aeration to increase pollutant removal efficiency of constructed wetlands in cold climate. Ecol Eng, 27:258–64.

Patra, S., Das, T.K., Avila, C., Cabello, V., Castillio, F., Sarkar, D., et al. (2012). Cadmium tolerance and antibiotic resistance in *Escherichia coli* isolated from waste stabilization ponds. Indian J Exp Biol, 50: 300–307.

Pedrero, F., Kalavrouziotis, I., Alarcòn, J.J., Koukoulakis, P., Asano, T. (2010). Use of treated municipal wastewater in irrigated agriculture: Review of some practices in Spain and Greece. Agric. Water Manage, 97:1233–1241.

Peña, M., Rodriguéz, J., Mara, D.D., Sepulveda, M. (2000). UASBs or anaerobic ponds in warm climates? a preliminary answer from Colombia. Water Sci Technol, 42: 59–65.

Phung, M., Pham, T., Castle, J., Rodgers, J. Jr. (2011). Application of water quality guidelines and water quantity calculations to decisions for beneficial use of treated water. App Water Sci, 1:85–101.

Saeed, T., Sun, G. (2012). A review on nitrogen and organics removal mechanisms in subsurface flowconstructed wetlands: Dependency on environmental parameters, operating conditions and supporting media. J Environ Manage, 112:429–448.

Saleem, M., Bukhari, A.A., and Al-Malack, M.H. (2000). Removal efficiencies of indicator micro-organisms in the Al-Khobar wastewater treatment plant. Environ. Eng. Sci. 17(4): 227-232.

Saleem, M., Bukhari, A.A., and Al-Malack, M.H. (2003). Seasonal variations in the bacterial population in an activated sludge system. J. Environ. Eng. and Sci., 2(2): 155-162.

Saxena, G., Bharagava, R.N., Kaithwas, G., Raj, A. (2015). Microbial indicators, pathogens and methods for their monitoring in water environment. J Water Health, 13:319-339.

Spieles, D.J., Mitsch, W.J. (2000). The effects of season and hydrologic and chemical loading on nitrate retention in constructed wetlands: a comparison of low and high nutrient riverine systems. Ecol Eng, 14:77–91.

Steer, D., Fraser, L., Boddy, J., Seibert, B. (2002). Efficiency of small constructed wetlands for subsurface

treatment of single-family domestic effluent. Ecol Eng, 18:429–440.

Steinmann, C.R., Weinhart, S., Melzer, A. (2003). A combined system of lagoon and constructed wetland for an effective treatment. Water Res, 337:2035–2042.

Stott, P.A., Jones, G.S., Mitchell, J.F.B. (2003). Do models underestimate the solar contribution to recent climate change? J Cli, 4079-4093.

Thongtha, S., Teamkao, P., Boonapatcharoen, S., Tripetchkul, S., Techkarnjararuk, S., Thiravetyan, P. (2014). Phosphorus removal from domestic wastewater by Nelumbo nucifera Gaertn. and Cyperus alte rnifolius L. J. Environ Manag,137:54–60.

Symons, J.M. (1960). A procedure for determination of the biological treatability of industrial wastes J. Water Pollut Control Fed, 32:841–851.

Thurston, J.A., Gerba, C.P., Foster, K.E., Karpiscak, M.M. (2001). Fate of indicator microorganisms, Giardia and *Cryptosporidium* in subsurface flow constructed wetlands. Water Res, 35: 1547-1551.

Toscano, A., Hellio, C., Marzo, A., Milani, M., Lebret, K., Cirelli, G.L., Langergraber, G. (2013). Removal efficiency of a constructed wetland combined with ultrasound and UV devices for wastewater reuse in agriculture. Environl Technol, 34:2327-2336.

Toscano, A., Marzo, A., Milani, M., Cirelli, G.L., Barbagallo, S. (2015). Comparison of removal efficiencies in Mediterranean pilot constructed wetlands vegetated with different plant species. Ecol Eng, 75:155–160.

Tunçsiper, B., Ayaz, S.Ç., Akça, L. (2012). Coliform bacteria removal from septic wastewater in a pilot-scale combined constructed wetland system Bilal. Environ Eng Manag J, 11:1873-1879.

Von Sperling and M., Andrada, J.G.B. (2006). Simple wastewater treatment (UASB reactor, shallow polishing ponds, coarse rock filter) allowing compliance with different reuse criteria. Water Sci Technol, 54:199–205.

Vymazal, J. (2009). The use constructed wetlands with horizontal sub-surface flow for various types of wastewater. Ecol Eng, 35:1–17.

Vymazal, J. (2002). The use of sub-surface constructed wetlands for wastewater treatment in the Czech Republic: 10 years' experience. Ecol Eng, 218: 633–646.

Vymazal, J., Kröpfelová, L. (2009). Removal of organics in constructed wetlands with horizontal sub surface flow: A review of the field experience. Sci. Total Environ, 407: 3911-392.

Walaszek, M., Bois, P., Laurent, J., Lenormand, E., Wanko, A. (2018). Urban stormwater treatment by a constructed wetland: Seasonality impacts on hydraulic efficiency, physico-chemical behaviour and heavy metal occurrence. Sci Total Environ, 637–638: 443-454.

Wery, N., Lhoutellier, C., Ducray, F., Delgenes, J.P., Godon, J.J. (2008). Behaviour of pathogenic and indicator bacteria during urban wastewater treatment and sludge composting, as revealed by quantitative PCR. Water Res. 42 (1e2), 53-62.

Wickham, J.D., Nash, M.S., Wade, T.G., Currey, L. (2006). Statewide empirical modeling of bacterial contamination of surface waters. J Am Water Resour Assoc, 42:583-591.

World Health Organisation. Guidelines for the Safe Use of Wastewater Excreta and Grey Water World Health Organisation (2006). Volume 2: Wastewater Use in Agriculture. World Health Organization Press, Geneva, Sweezerland. 2006.

Wu, S., Carvalho, P.N., Müller, J.A., Manoj, V.R., Dong, R. (2016). Sanitation in constructed wetlands: A review on the removal of human pathogens and fecal indicators. Sci Total Environ, 541: 8-22.

Zhang, K. And Farahbakhsh, K. (2007). Removal of native coliphages and coliform bacteria from municipal wastewater by various wastewater treatment processes: Implications to water reuse. Water Res, 41:2816–2824.

Antibiotic resistance and microbial indicator removals in a constructed wetland treating wastewater for agricultural reuse

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Abstract

Objective of the study was the assessment of hygienization efficiency of a horizontal sub-surface flow constructed wetland (CW), followed by UV disinfection, for treating and reusing wastewater in agriculture. Treatments efficiencies were evaluated in terms of removal of microbial indicators, antibiotic resistance genes (ARGs) and antibiotic resistant enterococci. Good hygienization efficiencies were obtained by the studied wastewater treatment solution: CW achieved average removal of microbial indicators ranging from 2.10±0.94 log CFU/100 ml for *E. coli* to 1.62±0.59 for enterococci; UV disinfection reduced both *E. coli* and enterococci to a un-detectable level. Different effects of the applied treatments were observed on the monitored antibiotic resistance determinants. CW reduced only ARGs (average removals of 0.87-1.71 log unit gene copies/ml) although with variable performance during time. UV treatment showed any significant effect on the ARGs concentrations.

Keywords: constructed wetlands; UV disinfection; water reuse; microbial indicators; antibiotic resistance.

1. Introduction

Treated wastewater reuse in agriculture is increasingly being applied to achieve sustainable water management in arid region. Innovative and sustainable systems needed to be developed and tested as an alternative to conventional systems to reclaim wastewaters for agricultural reuse, especially in small and medium communities where low maintenance and easy operation are essential (Toscano et al. 2013). Constructed wetlands (CWs) are engineered systems designed and constructed to utilize the natural processes to assist in treating wastewater (Vymazal, 2011). One of the main concern for reclaimed water reuse is the microbiological quality of water and thereby the risk of spreading diseases. Although CWs are widely applied as potential alternatives for wastewater treatment, further advanced treatments are needed to achieve targeted applications for specific water qualities (e.g. food crop irrigation), such as ultraviolet (UV) light disinfection. UV disinfection efficiently inactivate viruses and bacteria at low cost and differently from chlorination do not produce chemical by-products (Hijnen et al. 2006). WWTP effluents were shown to be a “hotspots” for the spread of antibiotic resistance genes (ARGs) and antibiotic resistance bacteria (ARB) in the environment (Martinez, 2009). In order to mitigate possible health effects associated to the use of treated wastewater in agriculture, the level of microbiological contamination and the occurence of antibiotic resistance should be carefully monitored. The aims of this study was to evaluate the hygienization efficiency of a full-scale horizontal sub-surface flow CW combined with UV for treating and reusing wastewater in agriculture. For this purpose removal of microbial indicators

of faecal contamination (*Escherichia coli*, total coliforms and enterococci), ARGs and ARBs were monitored before and after the applied treatments.

2. Materials and methods

2.1 System description and sampling campaigns

The experiment was carried out in a horizontal subsurface flow (H-SSF) CW and UV unit functioning in series, located in San Michele di Ganzaria (Eastern Sicily, Italy), 90 km South-West of Catania. The CW is part of the biggest natural wastewater plant of the South Italy, which included four H-SSF reed beds, followed by three wastewater storage reservoirs, used for tertiary treatment for wastewater reuse in agriculture. For this research, the activity was focused on one of the four reed beds, i.e. H-SSF3 (about 2,000 m^2) in operation since 2012, planted with *Phragmites australis* and designed with a flow rate of 2 L/s. In order to improve the microbiological water quality, a part of the CW effluent was pumped through a UV disinfection unit (WEDECO AG, LBX 10) with three UV lamps. During the monitoring campaign wastewater samples were collected at (i) CW influent, (ii) CW effluent and (iii) after UV treatment during 5 consecutive months.

2.2 Microbiological indicators

2.2.1 Sample collection and microbiological analyses

Water samples were processed at the laboratory of Microbiology of the Di3A. Influent samples were analyzed within 2-3 hours. Microbiological analyses were performed using the membrane filtration method according to APHA (2006). Briefly, volumes of 100 ml were diluted and filtered and each membrane filter was placed on the following selective agar media: RAPID' *E. coli* 2 agar (BIORAD, Italy) for direct enumeration of *E. coli* and total coliforms, incubated at 37°C for 24 h, and Slanetz Bartley Agar (BIOLIFE, Italy) for enumeration of *Enterococcus* spp, incubated at 37°C for 4 h and subsequently at 45°C for 48 h. Analyses were performed in duplicate.

2.2.2 Enterococci isolation

Colonies from Slanetz Bartley Agar were randomly isolated and screened for Gram stain, catalase, growth and aesculin degradation on BEA medium (Liofilchem), and for growth at 45°C in Brain Hearth Infusion (BHI, Oxoid) supplemented with 6.5% (w/v) NaCl. Isolates were maintained at –80°C in BHI supplemented with 20% (v/v) glycerol and subsequently investigated for antimicrobial resistance.

2.2.3 Antibiotic resistance of *Enterococcus* spp. through microdilution tests

Minimal Inhibition Concentration (MIC) was determined by broth micro-dilution method according to the S*tandards for Antimicrobial Susceptibility Testing* (CLSI 2002; 2005). The following antimicrobial agents were selected as representatives of different antibiotic classes: erythromicyn (range 1.0-16 µg/ml), tetracycline (0.5-16 µg/ml), sulfonamides (256-4.0 µg/ml). After incubation for 18 h at 37 °C, the strains were classified as susceptible, intermediate or resistant, according to EFSA recommendations (2008). The lowest concentration of antibiotic that prevented visible growth of bacteria represented the endpoint of the test (MIC).

2.3 ARGs quantification assay

For ARGs analysis, samples were filtrated on 0.45 µm pore-size polycarbonate membrane filters (diameter: 47 mm, Whatman, UK). DNA was extracted directly from the filter membranes following a phenol:chloroform:isoamyl alcohol protocol (Miller et al. 1999) with minor modifications. Four antibiotic resistance genes (*ermB*, *sul1*, *sul2*, *tetA*) were quantified by q-PCR. Previously described q-PCR TaqMan assays were utilised for *ermB* (Bockelman et al. 2009), *sul1* (Heuer and Smalla 2007) and *sul2* (Heuer et al., 2008). *tetA* was quantified according to Ng et al. (2001) utilising SYBR Green based q-PCR. NTCs with no template DNA were included in each qPCR assays and 1:10 and 1:50 dilution of the template DNA were also analysed to avoid false negative results due to QPCR inhibitions. Each

reaction was run in triplicate and results are reported as the mean of measurements with standard deviations.

3. Results and discussion

3.1 Microbial indicators removal

The removal level obtained by CW and UV treatment is shown in Figure 1. *Escherichia coli*, total coliforms, and enterococci concentrations in the influent wastewater revealed a mean values of 4.93 ± 1.11 log CFU/100 ml, 5.18 ± 1.09 log CFU/100 ml and 3.88 ± 1.38 log CFU/100 ml, respectively. CW system significantly reduced microbial indicator levels, with a mean logarithmic removal ranging from 2.10 ± 0.94 log CFU/100 ml for *E. coli* to 1.62 ± 0.59 log CFU/100 ml for enterococci. Although higher removal efficiencies were previously reported for the same CW system (Toscano et al. 2013), the observed hygienization performances were comparable to those reported for conventional activated sludge secondary treatment (Lucena et al. 2004, Zhang and Farahbakhsh 2007).

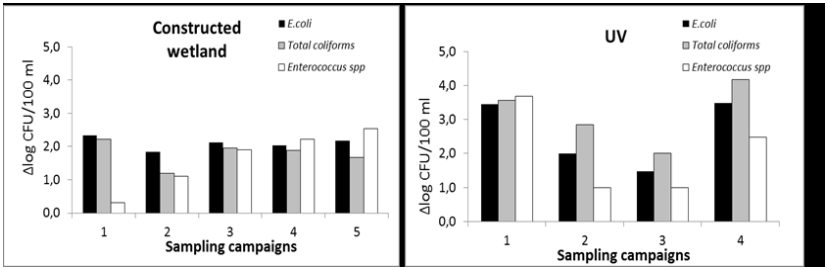

Figure 1. Average reduction efficiency of microbial indicators in constructed wetland and after UV disinfection

UV treatment, as expected, drastically reduced all microbial indicator levels, achieving *E. coli* limits (i.e. Italian limits of *E. coli* <10 cfu/100 ml) required for wastewater reuse in agriculture.

3.2 Antibiotic resistance determinants: ARGs and AR enterococci

Antibiotic resistance genes present in influent wastewater were identified by PCR screening of 9 ARGs (*tetA, tetO, tetW, sul1, sul2, vanA, ermB, ampC* and *mecA*) commonly detected in WWTP. Totally four genes (*ermB, sul1, sul2, tetA*), encoding for the resistance to erythromycin, sulfonamides and tetracycline were detected in influent wastewater samples. The level of the detected ARGs, and the frequencies of resistance of the isolated enterococci strains are reported in Figure 2 and Table 1, respectively.

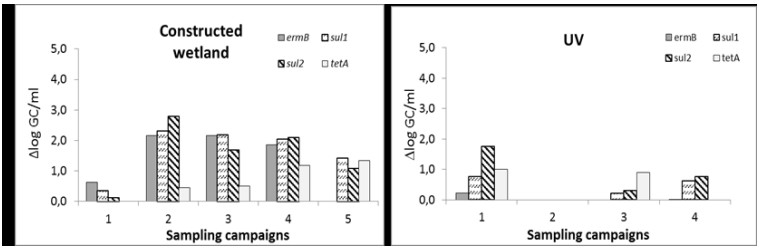

Figure 2. ARGs logarithmic removals achieved by constructed wetland system (CW) and UV disinfection.

Table 1. Frequency (expressed as percentage) of AR enterococci isolated strains.

		Erythromycin	Tetracycline	Sulfonamides
CW	Influent	55±10%	95±10%	57,5±9%
	Effluent	70±26%	100±0%	70±26%

High levels of all ARGs from 2.98 of *ermB* to 8.11 log GC/ml of *sul1* were observed in influent wastewater. As shown in Figure 2, CW reduced ARGs concentrations although a variable efficiency between genes and sampling campaigns was observed. In particular, for *sul1* and *sul2* genes, the removal was comparable or higher than those reported for conventional WWTPs (Yang et al. 2014), achieving 1.66 ± 0.81 and 1.56 ± 1.01 log units removal of GC/ml, respectively, during the 3rd and the 4rd sampling campaigns. On the contrary, no effect or slight increase in ARBs frequency was observed through CW treatment (Tab. 1). Finally, UV treatment reduced to un-detectable level cultivable enterococci, whereas in agreement with what observed by other authors (Munir et al., 2011; McKinney and Pruden, 2012) it did not effect the ARGs concentrations. Therefore in spite of the absence of ARBs, ARGs encoding DNA might be realeased in the final UV tertiary effluent. Nevertheless, in the present study the

ARGs levels in the outgoing tertiary effluents (from 3.12 to 5.61 log ARGs GC/ml) were comparable to those reported in river water (Luo et al., 2010) and reclaimed wastewaters suitable for agricultural irrigation (Fahrenfeld et al., 2013).

4. Conclusions

Microbial indicators and ARGs removals performances, similar than those reported for conventional activated sludge WWTPS, were achieved in the monitored CW. Improved hygienization was obtained by the combined CW+UV system that reduced AR enterococci to un-detectable level and always produce effluent suitable for the water reuse in agriculture according to the Italian *E. coli* limits (<10 cfu/100 ml). Different effects of the applied treatments were observed on the monitored antibiotic resistance determinants showing that the risk of spreading AR in the environment might be underestimated. The results of the present work highlight the need to set the standards for monitoring the AR fate in wastewater treatment processes.

Conflict of interest

The Authors declare that there are no conflict of interest.

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References

APHA, Standard Methods for the Examination of Water, American Public Health Association, New York, 2006.

Böckelmann, U., Dörries, H.H., Ayuso-Gabella, M.N., de Marçay, M.S., Tandoi, V., Levantesi, C., Mascipinto, C., Van Houtte, E., Szewzyk U., Wintgens, T., Grohmann, E. (2009). Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems, Applied and Environmental Microbiology, 75, 154-163.

CLSI, Clinical and Laboratory Standards Institute, (formerly NCCLS) (2005). Performance standards for antimicrobial susceptibility testing; fifteenth informational supplement. CLSI document M100-S15. CLSI, Wayne, PA.

Farhrenfel, N., Ma Y., O'Brien, M., Pruden, A. (2013). Reclaimed water as reservoirs of antibiotic resistance genes: distribution system and irrigation implications. Frontiers in Microbiology, 4; 1-10.

Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., Spiteller, M. (2008). Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil, Soil Biology and Biochemistry, 40 1892-1900.

Heuer, H. and Smalla, K. (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in

soil over at least two months, Environmental Microbiology, 9 657–666.

Hijnen, W.A.M., Beerendonk, E.F., Medema, G.J. (2006) Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review, Water Research, 40 3-22.

Lucena, F., Duran, A.E., Morón, A., Calderón, E., Campos, C., Gantzer, C., Skraber, S., Jofre, J. (2004). Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. J Appl Microbiol, 97(5):1069-76.

Luo, Y., Mao, D., Rysz, M., Zhou, Q., Zhang, H., Xu, L., Alvarez, P.J.J. (2010). Trend in antibiotic resistance genes occurrence in the Haihe river, China, Environmental Science and Technology, 44; 7220-7225.

McKinney, C.W. and Pruden A. (2012). Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. Environ Sci Technol. 46 (24):13393-400.

Miller, D.N., Bryant, J.E., Madsen, E.L., Ghiorse, W.C. (1999). Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol. (11):4715-24.

Munir, M., Wong, K., Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45(2):681-93.

Ng, L.K., Martin, I., Alfa, M., Mulvey, M. (2001). Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes. 15(4):209-15.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci Total Environ, 447:345-60.

Toscano, A., Hellio, C., Marzo, A., Milani, M., Lebret, K., Cirelli, G.L, Langergraber, G. (2013). Removal efficiency of a constructed wetland combined with ultrasound and UV devices for wastewater reuse in agriculture. Environmental Technology, ISSN: 0959-3330.

Vymazal, J. (2011). Constructed wetlands for wastewater treatment: five decades of experience. Environmental Science and Technology, 45; 61-69.

Yang, Y., Li, B., Zou, S., Fang, H.H., Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. Water Res, 1;62:97-106.

Zhang, K., Farahbakhsh, K. (2007). Removal of native coliphages and coliform bacteria from municipal wastewater by various wastewater treatment processes: implications to water reuse. Water Res, 41(12):2816-24.

Occurrence, diversity, and persistence of antibiotic resistant enterococci in full-scale constructed wetlands treating urban wastewater in Sicily

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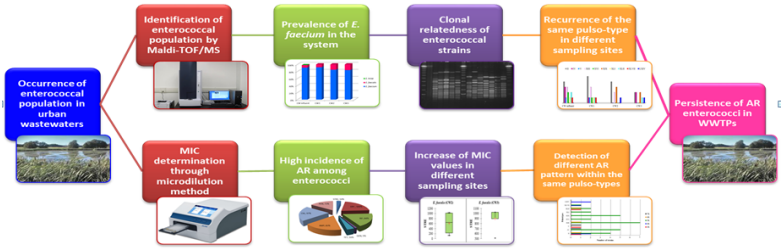
Graphical abstract



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Special issue of Bioresource Technology on Fate, transport and removal of antibiotics in engineered and natural systems: Current status and perspectives

Abstract

Enterococci isolated from different sites of an urban wastewater treatment plant (consisting of three horizontal subsurface flow constructed wetlands) were investigated. One-hundred-thirty isolates were identified at species level and tested for resistance to eleven antibiotics, by microdilution method, and their clonal relatedness was established by *Sma*I-PFGE analysis. Results highlighted the persistence of enterococcal population in all effluents and the dominance of *E. faecium* species. A high incidence of antibiotic resistance against erythromicin, chloramphenicol, rifampicin and ampicillin was observed, with 120 strains (93%) showing a multi-drug-resistance. Numerous pulso-types with a unique pattern were detected indicating a high diversity within enterococcal population. The recurrence of some pulso-types in different effluents was disclosed and, within the same pulso-types, different resistance patterns were observed. Comparing the MIC values of strains from inlet and outlet, different trends were observed, highlighting a certain variability among constructed wetlands in affecting the antibiotic resistance among enterococcal population.

Keywords: Urban Wastewater Treatment Plant; Constructed Wetland; Antibiotic Resistance; *Enterococcus faecium*; *Enterococcus faecalis*; clones.

1. Background

Antimicrobial resistance (AR) is a complex phenomenon and represents a public concern, compromising the ability to cure a wide range of infectious diseases (Martinez and Baquero, 2014; Hay et al., 2018; WHO, 2017). The overuse of antimicrobials may have greatly accelerated the spread of different resistance mechanisms (Hay et al., 2018). Large amounts of antimicrobial compounds together with resistant bacteria can reach wastewater treatment plants (WWTPs). The entrance of antibiotics into biological WWTPs, mainly at sub-therapeutic concentrations can reduce their efficiency and promote the selection of AR among bacteria population (Kim et al., 2007). Enterococci are members of intestinal microbiota of a wide variety of hosts and are able to survive to harsh conditions. Their presence in WWTPs could be detected at high densities (Byappanahalli et al., 2012). Enterococci are intrinsically resistant to a plethora of antibiotics and for the high frequency of multiple antibiotic resistance they have emerged as opportunistic pathogens (Gao et al., 2018). Furthermore their ability to acquire different adaptive traits and to transfer resistance to pathogenic species, makes this group a relevant healthcare problem (Oravcová et al., 2017; Werner et al., 2013). Recently the World Health Organization (WHO) has considered the vancomycin resistant *Enterococcus faecium* a high priority pathogen (WHO, 2017). WWTPs, for their intrinsic parameters, such as pH, temperature, high nutrient concentration, presence of antibiotics (and/or their metabolites) and for supporting a close contact between bacteria, provide an ideal ecosystem to promote selection of resistant strains and transferring of resistance genes (Rodriguez-Mozaz et al., 2015). Among the different types

of WWTPs, constructed wetlands (CWs) represent a natural treatment system widely used thanks to their capability to efficiently treat wastewater from several sources (i.e., small/medium communities, agricultural drainage, agro-industries, road runoff, landfill leachate) with low energy, easier maintenance and low operational costs (Lavrnić et al., 2018). Furthermore, CWs can be used as treatment systems for wastewater reuse in agriculture (Barbagallo et al., 2011), also combined with other technologies. Even if numerous studies have been conducted on hospital wastewater treatment systems, to the best of our knowledge, this is the first study carried out on persistence of antibiotic resistant enterococci in a natural urban wastewater treatment plant (CWs) in Sicily. Therefore, the current study aims to evaluate: (i) the phenotypic antibiotic resistance of enterococcal isolates; (ii) the occurrence and the persistence of antibiotic resistant enterococci both in influent and in effluents of three subsurface flow CW treatment systems; (iii) the clonal relatedness among isolated strains.

2. Materials and methods

2.1 Experimental plant

The present study was carried out in three horizontal subsurface flow CWs, namely CW1, CW2 and CW3, which receive the secondary effluents of the urban WWTP from San Michele di Ganzaria (37°17'0" N and 14°26'0" E). San Michele di Ganzaria is a small community (about 3,200 inhabitants, in 2016 website http://www.comuni-italiani.it/087/011/statistiche/popolazione.html) of Eastern Sicily, located in Csa Hot-summer Mediterranean climate,

according to Köppen classification (also known as a Mediterranean climate), with a mean annual temperature of 18 °C and mean annual rainfall of 500 mm. Basically two seasons can be distinguished: from April to September (summer, dry and hot, with mean temperatures of 20 °C and mean rainfall of 20 mm/month), and from October to March (winter, wet and cool, with mean temperatures of 11 °C and mean rainfall of 91 mm/month). CW1, CW2, and CW3 are part of the largest natural WWTP of South Italy that includes four CWs operating in parallel, followed by three wastewater storage reservoirs, realized for tertiary treatment of municipal wastewater aimed at agricultural reuse (Cirelli et al., 2007). The main design and operation characteristics of wetland beds are reported in Table 1.

Table 1. Constructed wetland characteristics.

Constructed wetlands	Operation time (year)	Flow rate (m^3/day)	Area (m^2)	Gravel				Macrophytes planted
				Type	Size (mm)	Nominal porosity	Depth (m)	
CW1	12	240	2000	volcanic	8-15	0.47	0.6	*Phragmites australis*
CW2	6	240	2000					
CW3	6	125	1200					*Typha latifolia*

2.2 Sampling sites

The study was performed between April and September 2016. Influent and treated wastewater samples were collected in April, May, June, July and September 2016 at: (1) Influent (i.e., following WWTP); (2) CW1 outlet effluent; (3) CW2 outlet effluent; and (4) CW3 outlet effluent. The CWs were used for tertiary treatment for wastewater reuse in agriculture. Samples were collected in sterile bottles and transported in refrigerated conditions to the laboratory of Microbiology at the Department of Agriculture, Food and Environment, University of Catania, and analysed within 24 h from sampling.

2.3 Enumeration of enterococci

Influent, after decanting, and effluent samples were subjected to microbiological analyses by membrane filtration method, according to Standard Methods for the Examination of Water and Wastewater (APHA, 2006). Briefly, samples were diluted in a sterile saline solution and 100 mL of each dilution were filtered through a 0.45 μm pore-size sterilized membrane filters (Microfil V, Merk Millipore, Italy), according to ISO 9001:2008 procedures. Each membrane filter was placed on Slanetz Bartley Agar (SBA, BIOLIFE, Italy) for enumeration of *Enterococcus* spp. The plates were incubated at 37°C for 48 h, under aerobic condition. Colonies grown on filters were counted and the enterococcal abundance was reported as $\log_{10}$ colony-forming units (CFU/100 mL of water). The analysis was performed in duplicate.

2.4 Isolation and phenotypic characterization of enterococci

Colonies from SBA plates were randomly isolated and analyzed for colony characteristics and cell morphology. Overall, 130 isolates were collected and analyzed for Gram staining, catalase reaction, growth at 45°C and 10°C, growth in presence of 6.5% (w/v) of NaCl and at pH 9.6, and growth on on Kanamycin Aesculin Azide agar (Liofilchem). Presumptive enterococci were streaked three times and the pure cultures were stored at −80°C in Brain Heart Infusion (BHI, Oxoid) broth supplemented with 20% (v/v) glycerol for the further analyses. Overall, 130 Gram-positive, catalase negative cocci in pairs or as short chains were considered as belonging to *Enterococcus* genus, based on their ability to grow in Kanamicyne Aesculine Azide agar, and in BHI at 45 °C and 10 °C, at pH 9.6 and in the presence of 6.5% of NaCl, after 48 h.

2.5 Maldi-TOF/MS analysis

The 130 presumptive enterococci were subjected to species identification by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry MALDI-TOF/MS (Bruker Daltonics, Germany). Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonik, Germany) using FlexControl software (version 3.0). The spectra were imported into the integrated MALDI Biotyper software (version 3.0) and analyzed by standard pattern matching with default settings. The strains were treated as previously reported (Doan et al., 2012). The spectrum of each isolate was compared with those present in the

database and identification was provided with a score of reliability. Standard Bruker interpretative criteria were applied; scores ≥ 2.0 were accepted for species assignment and scores ≥ 1.7 but ≤ 2.0 for genus identification.

2.6 Antimicrobial susceptibility testing

Enterococcal strains were characterized for their susceptibility to a panel of eleven antibiotics, according to Russo and co-workers (2018). In details, the Minimum Inhibitory Concentration (MIC) values were evaluated for each antibiotic within different range, as follow: tetracycline (from 0.5 to 16-µg/mL), erythromycin (from 1 to 32 µg/mL), streptomycin (from 32 to 1024 µg/mL), gentamycin (from 8 to 256 µg/mL), ampicillin (from 32 to 1 µg/mL), rifampicin (from 2 to 64 µg/mL), penicillin G (from 0.5 to 16 µg/mL), sulphametoxazole (from 128 to 4096 µg/mL), chloramphenicol (from 2 to 64 µg/mL), vancomycin (from 1 to 32 µg/mL) and kanamycin (from 128 to 4096 µg/mL). All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MICs were determined by micro-dilution method, using Mueller-Hinton broth (Liofilchem) and MIC tests were performed in a 384-well plates, filled with an automatic liquid handling system (EpMotion, Eppendorf, Italy) to a final volume of 80 µL. The bacteria were incubated in absence (control) and in presence of each antibiotic at six different concentrations. *E. faecalis* ATCC 29212 was used as reference strain. Each strain was exposed, in duplicate, to each antimicrobial concentration, at a final inoculum density of 0.5 McFarland, starting from overnight cultures. The bacterial cell concentration was determined by flow cytometry (BD

Accuri™ C6 Plus Flow Cytometer, BD Biosciences, Milan, Italy). The 384-well plates were incubated at 37 °C for 24 h and the cell density evaluated by OD measuring, using a spectrophotometer (MicroWave RS2, Biotek, USA) and the Gene5 software (Biotek, USA). The MIC was determined as the lowest antimicrobial concentration that inhibited visible bacterial growth and the results were interpreted according to CLSI (2016) and EUCAST (2018)-approved clinical breakpoint and epidemiological cut-off values, with exception of sulfamethoxazole and kanamycin, for which no breakpoint and/or epidemiological cut-off are available (CLSI, 2016; EUCAST, 2018). Enterococci resistant to at least one agent of three or more antimicrobial classes were considered as multidrug resistant (MDR).

2.7 Clonal relatedness

The clonal relationship among the 130 isolates was revealed by comparison of *Sma*I digested DNA profiles, using PFGE analysis. High-molecular-weight DNA was isolated from 1 mL of an overnight culture, as previously reported (Novais et al., 2004; Tenover et al., 1995). The digested plugs were subjected to electrophoresis, using the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA), in a 1.2% agarose gels at 6 V/cm, with linear switching interval ramps from 35 s to 25 s for 25 h at 14 °C for the first block. Lambda ladder (New England BioLabs, Beverly, MA, UK) was run as molecular weight markers. After staining with gel red (Biotium), DNA bands were visualized by UV. The images acquisition was performed by using ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc. Hercules, California, USA). The PFGE profiles were converted to

TIFF files and subjected to cluster analysis, using BioNumerics v. 7.5 software (Applied Maths, Sint-Martens-Latem, Belgium). PFGE profiles were interpreted according to criteria of Tenover et al. (1995). PFGE band patterns were generated by BioNumerics v. 7.5 software (Applied Maths, Sint-Martens-Latem, Belgium) with tolerance position of 1%. Clustering was based on the unweighted pair group method with arithmetic averages (UPGMA). The Dice correlation coefficient was used to detect the similarities of banding patterns.

2.8 Data processing

The multiple antibiotic resistance (MAR) index was computed for both each strain and each sampling site, according to Krumperman (1983). The MAR index referred to a single strain, was calculated as = *a/b*, where *a* represents the number of antibiotics to which isolate resulted resistant, and *b* represents the number of antibiotics to which isolate was exposed. MAR index referred to sampling sites was calculated as = c /(n · b), where c represents the total number of resistance scores; n = number of isolates from the site; b number of tested antibiotics.

2.9 Statistical analysis

All experiments were performed in duplicate and results were reported as average values, provided with Standard Deviation. All microbiological statistical analyses were performed using XLSTAT statistical software. The statistical significance between enterococcal density in

samples before and after each CW unit was evaluated by one-way analyses of variance (ANOVA). Results were considered significant at the 5% level (P=0.05). In addition, the XLSTAT statistical software was used to correlate antibiotic resistance patterns within enterococcal population and also to correlate detected MIC values with enterococcal species.

3. Results

3.1 Microbiological analysis

Results of microbiological mean values, at the different sampling points and sampling times are shown in Table 2. Considering the different sampling times, the mean $\log_{10}$ values of enterococci in inlet samples (influent) was 4.04, with the highest enterococcal density in September (about 5.4 log CFU/100 mL) and the lowest in July (2.5 log CFU/100 mL). In CW1, CW2, and CW3 effluents the mean enterococcal load was 3.03, 2.85 and 3.22, respectively. An increase of enterococci counts was observed, both in CW2 and CW3 effluents sampled in May. Different persistence rates (calculated as percentage ratio between inlet and outlet loads) were detected in the three effluents, with values ranging from 86% to 67% in the CW1; from 86% to 50% in CW2 and; from 79% to 72% in CW3 effluents (data not shown). However, no significant differences were detected for enterococcal density, between inlet and outlet samples, except for CW2, where an increase of density was detected, and for CW3, sampled in September, where a decrease was observed.

Table 2. Enterococcal cell density in WWTP. Data are reported as mean values (expressed as log10 CFU/100mL) and Standard Deviation of two independent samples.

	Influent	Effluents		
Sampling month		**CW1**	**CW2**	**CW3**
April	$4.50^{a} \pm 0.41$	$3.88^{a} \pm 0.07$	$3.89^{a} \pm 0.17$	$3.47^{a} \pm 0.20$
May	$3.37^{a} \pm 0.55$	$2.77^{a} \pm 0.11$	$4.12^{b} \pm 0.18$	$3.60^{a} \pm 0.29$
June	$4.46^{b} \pm 0.37$	$3.23^{a} \pm 0.31$	$2.44^{a} \pm 0.36$	$3.17^{a} \pm 0.14$
July	$2.51^{b} \pm 0.32$	$1.70^{a} \pm 0.06$	$1.08^{a} \pm 0.06$	$1.98^{a} \pm 0.02$
September	$5.38^{b} \pm 0.01$	$3.60^{a} \pm 0.48$	$2.70^{a} \pm 0.36$	$3.87^{c} \pm 0.12$
Mean value	4.04 ± 1.12	3.03 ± 0.85	2.85 ± 1.23	3.22 ± 0.73

^{abc} for each sampling point, in the same row followed by different lower case letters are statistically different ($P \leq 0.05$).

3.2 Identification of enterococcal isolates

Results of the MIC value, expressed as μg/mL, of enterococcal strains are reported in Table 3. It is relevant to point out that all of the 130 strains showed antibiotic resistance to erytromycin and susceptibility to vancomycin. Considering total resistance, results highlighted the highest occurrence of resistance for choramphenicol (97%), followed by rifampicin (84%), ampicillin (83%), and penicillin (73%), and the lowest for gentamicin (5.4%). Comparing to *E. faecium*, *E. faecalis* strains showed higher prevalence of resistance against streptomycin (57% vs 8%), tetracycline (36% vs 14%) and gentamycin (21% vs 3%). The unique *E. hirae* strain, isolated from inlet, showed

resistance to erythromycin, streptomycin, rifampicin, ampicillin and chloramphenicol. In addition, all enterococci were analyzed for the sulphametoxazole and kanamycin resistance, for which no breakpoints are available. For both the aforementioned antibiotics 27% of strains showed a MIC value of 4096 µg/mL, which was the highest among the tested range. Evaluating the multidrug resistance of strains, 70(54%) were resistant to 5 antibiotics, 16(12%) to 6 antibiotics, and 7(4%) to 7 antibiotics. Furthermore, high prevelence of MDR strains was detected in each sampling site. Considering the inlet, where the value of MDR isolates was 91%, a decrease was observed only in CW1 site (Table 4). The highest MAR index was 0.89, for one *E. faecalis* strain isolated from CW3 effluent, whereas the lowest (0.11) was detected for one *E. faecium* strain, isolated from CW2 effluent, that showed resistance exsclusively to erythromycin (Table 3). The MAR index considered for each sampling site was 0.52 for CWs influent and 0.50, 0.53 and 0.51 for CW1, CW2 and CW3 effluents, respectively. With the aim to find out an association between strain resistance patterns and tested antibiotics, a correlation analysis was performed. The results revealed the clustering of enterococcal population into two large groups, highlihting a correlation among susceptibility to streptomycin, gentamicin and tetracycline. On the same time, the resistance to ampicillin was correlated to resistance to penicillin, rifampicin and chloramphenicol (data not shown).

3.3 Phetotypic antibiotic resistance of enterococci

Results of the MIC value, expressed as µg/mL, of enterococcal strains are reported in Table 3. It is relevant to

point out that all of the 130 strains showed antibiotic resistance to erytromycin and susceptibility to vancomycin. Considering total resistance, results highlighted the highest occurrence of resistance for choramphenicol (97%), followed by rifampicin (84%), ampicillin (83%), and penicillin (73%), and the lowest for gentamicin (5.4%). Comparing to *E. faecium*, *E. faecalis* strains showed higher prevalence of resistance against streptomycin (57% vs 8%), tetracycline (36% vs 14%) and gentamycin (21% vs 3%). The unique *E. hirae* strain, isolated from inlet, showed resistance to erythromycin, streptomycin, rifampicin, ampicillin and chloramphenicol. In addition, all enterococci were analyzed for the sulphametoxazole and kanamycin resistance, for which no breakpoints are available. For both the aforementioned antibiotics 27% of strains showed a MIC value of 4096 µg/mL, which was the highest among the tested range.

Table 3. MIC values to the eleven tested antibiotics, resistance score and MAR index detected for enterococcal strains.

Strains	Species	STRE	ERY	VAN	RIF	GEN	TET	SUL	AMP	CHL	PEN	Resistence score	MAR Index
		Range µg/mL											
		(32-1024)	(1-32)	(1-32)	(2-64)	(8-256)	(0.5-16)	(128-4096)	(1-32)	(2-64)	(0.5-16)		
IN 2 A	*E. faecium*	256	>32^{R}	4	64^{R}	64	8	4096	32^{R}	64^{R}	16^{R}	5	0.56
IN 12 A	*E. faecium*	256	>32^{R}	4	1	64	2	2048	32^{R}	64^{R}	16^{R}	3	0.33
IN 19 A	*E. faecium*	128	>32^{R}	4	1	128	8	4096	8	64^{R}	4	2	0.22
H_2 1 A	*E. faecium*	256	>32^{R}	4	1	32	8	4096	4	32^{R}	4	2	0.22

H_2 3 A	*E. faecium*	128	>32^R	4	1	32	8	4096	4	64^R	4	2	0.22
H_2 5 A	*E. faecium*	256	>32^R	4	1	64	2	4096	4	64^R	8	2	0.22
H_2 6 A	*E. faecium*	256	>32^R	4	4^R	32	2	4096	32^R	64^R	16^R	5	0.56
H_2 8 A	*E. faecium*	256	>32^R	4	1	64	4	1024	4	32^R	8	2	0.22
H_3 1 A	*E. faecium*	256	>32^R	4	>64^R	>256^R	4	4096	>32^R	64^R	>16^R	6	0.67
H_3 3 A	*E. faecium*	256	>32^R	4	1	128	8	4096	16^R	64^R	8	3	0.33
H_3 4 A	*E. faecium*	256	>32^R	2	>64^R	128	2	2048	16^R	64^R	8	4	0.44
H_3 9 A	*E. faecium*	128	>32^R	4	>64^R	32	8	4097	>32^R	64^R	>16^R	5	0.56
H_4 3 A	*E. faecium*	256	>32^R	4	64^R	32	2	2048	16^R	64^R	16^R	5	0.56
H_4 6 A	*E. faecium*	256	>32^R	4	32^R	64	2	2048	16^R	64^R	>16^R	5	0.56
IN 19 A'	*E. faecium*	128	>32^R	2	1	32	2	2048	4	64^R	4	2	0.22
IN 22 A'	*E. faecium*	128	>32^R	2	1	32	1	2048	4	64^R	4	2	0.22
IN 25 A'	*E. faecium*	256	>32^R	4	64^R	32	2	4096	32^R	64^R	16^R	5	0.56
H_2 3 A'	*E. faecium*	128	>32^R	4	64^R	32	1	2048	32^R	64^R	16^R	5	0.56
H_2 5 A'	*E. faecium*	128	>32^R	4	64^R	32	8	4096	8	64^R	8	3	0.33
H_2 7 A'	*E. faecium*	256	>32^R	4	64^R	64	2	2048	16^R	64^R	16^R	5	0.56
H_2 15 A'	*E. faecium*	256	>32^R	2	64^R	32	2	2048	32^R	64^R	>16^R	5	0.56
H_2 16 A'	*E. faecium*	512	>32^R	4	64^R	64	2	4096	32^R	64^R	16^R	5	0.56
H_2 19 A'	*E. faecium*	256	>32^R	2	64^R	64	2	2048	32^R	64^R	>16^R	5	0.56
H_3 3 A'	*E. faecium*	256	>32^R	2	64^R	64	2	4096	32^R	64^R	16^R	5	0.56
H_3 9 A'	*E. faecium*	256	>32^R	4	1	32	16^R	4096	16^R	64^R	16^R	5	0.56
H_3 14 A'	*E. faecium*	256	>32^R	4	>64^R	64	8	4096	32^R	64^R	16^R	5	0.56
H_3 21 A'	*E. faecium*	256	>32^R	2	1	16	2	4096	32^R	64^R	16^R	4	0.44
H_4 2 A'	*E. faecium*	128	>32^R	4	>64^R	32	2	4096	32^R	64^R	16^R	5	0.56

IN 4 B	*E. faecium*	256	32^R	4	$>64^R$	64	16^R	4096	16^R	64^R	8	5	0.56
IN 5 B	*E. faecium*	256	32^R	2	64^R	64	1	2048	$>32^R$	32^R	$>16^R$	5	0.56
IN 6 B	*E. faecium*	256	32^R	4	64^R	64	4	2048	32^R	64^R	16^R	5	0.56
IN 8 B	*E. faecium*	256	32^R	4	64^R	32	1	2048	8	$>64^R$	16^R	4	0.44
IN 11 B	*E. faecium*	$>1024^R$	32^R	4	1	16	$>16^R$	2048	16^R	64^R	16^R	6	0.67
IN 12 B	*E. faecium*	256	32^R	2	64^R	64	1	2048	32^R	32^R	16^R	5	0.56
IN 14 B	*E. faecium*	128	32^R	4	32^R	64	1	2048	$>32^R$	32^R	$>16^R$	5	0.56
H_2 4 B	*E. faecium*	256	32^R	4	64^R	32	1	2048	16^R	32^R	16^R	5	0.56
H_2 5 B	*E. faecium*	256	32^R	4	64^R	16	1	2048	16^R	64^R	8	4	0.44
H_3 6 B	*E. faecalis*	256	32^R	4	32^R	32	1	2048	16^R	64^R	$>16^R$	5	0.56
H_3 7 B	*E. faecium*	256	32^R	2	32^R	32	1	2048	16^R	64^R	16^R	5	0.56
H_3 8 B	*E. faecium*	128	$>32^R$	2	$>64^R$	128	4	2048	16^R	64^R	16^R	5	0.56
H_4 1 B	*E. faecium*	128	$>32^R$	4	$>64^R$	128	4	2048	16^R	64^R	$>16^R$	5	0.56
H_4 2 B	*E. faecium*	512	32^R	4	$>64^R$	32	1	4097	16^R	64^R	8	4	0.44
H_4 6 B	*E. faecium*	256	32^R	4	16^R	128	2	2048	16^R	64^R	16^R	5	0.56
IN 1 B'	*E. faecalis*	256	$>32^R$	4	64^R	128	2	2048	$>32^R$	64^R	16^R	5	0.56
IN 5 B'	*E. faecalis*	512	32^R	2	64^R	128	2	1024	$>32^R$	64^R	$>16^R$	5	0.56
H_2 4 B'	*E. faecalis*	512	32^R	2	16^R	64	1	2048	8	3	8	2	0.22
H_2 10 B'	*E. faecalis*	$>1024^R$	32^R	4	64^R	64	$>16^R$	2048	32^R	64^R	16^R	7	0.78
H_3 8 B'	*E. faecalis*	128	$>32^R$	4	64^R	16	1	2048	32^R	64^R	16^R	5	0.56
H_3 9 B'	*E. faecium*	512	32^R	2	$>64^R$	64	2	2048	32^R	64^R	$>16^R$	5	0.56
H_4 8 B'	*E. faecium*	128	32^R	2	16^R	16	$>16^R$	4096	32^R	64^R	16^R	6	0.67
IN 3 C	*E. faecium*	128	$>32^R$	4	64^R	32	$>16^R$	2048	16^R	32^R	16^R	6	0.67
IN 8 C	*E. faecium*	512	$>32^R$	4	64^R	64	$>16^R$	2048	16^R	64^R	16^R	6	0.67

IN 10 C	*E. faecalis*	>1024^{R}	>32^{R}	4	>64^{R}	128	>16^{R}	2048	16^{R}	64^{R}	16^{R}	7	0.78
IN 11 C	*E. faecium*	256	>32^{R}	4	64^{R}	64	2	4096	16^{R}	64^{R}	16^{R}	5	0.56
IN 12 C	*E. faecium*	128	>32^{R}	4	>64^{R}	64	1	2048	16^{R}	64^{R}	16^{R}	5	0.56
IN13 C	*E. faecium*	>1024^{R}	>32^{R}	4	2	64	>16^{R}	4097	16^{R}	>64^{R}	16^{R}	6	0.67
IN 15 C	*E. faecium*	512	>32^{R}	4	1	128	0,5	2048	4	64^{R}	4	2	0.22
H_2 8 C	*E. faecalis*	1024^{R}	>32^{R}	4	8^{R}	128	>16^{R}	2048	16^{R}	16	16^{R}	6	0.67
H_3 10 C	*E. faecium*	128	>32^{R}	2	4^{R}	16	1	2048	4	64^{R}	4	3	0.33
IN 2 C'	*E. faecium*	>1024^{R}	>32^{R}	4	>64^{R}	64	>16^{R}	2048	32^{R}	64^{R}	>16^{R}	7	0.78
IN 3 C'	*E. faecium*	256	>32^{R}	1	64^{R}	128	0,5	2048	16^{R}	64^{R}	16^{R}	5	0.56
IN 5 C'	*E. faecium*	512	>32^{R}	4	>64^{R}	64	2	2048	16^{R}	64^{R}	16^{R}	5	0.56
IN 11 C'	*E. faecium*	256	>32^{R}	1	64^{R}	128	0,5	2048	16^{R}	64^{R}	16^{R}	5	0.56
IN 17 C'	*E. faecium*	1024^{R}	>32^{R}	4	8^{R}	128	1	127	16^{R}	32^{R}	4	5	0.56
H_2 1 C'	*E. faecium*	512	>32^{R}	4	64^{R}	64	>16^{R}	2048	32^{R}	64^{R}	8	5	0.56
H_2 2 C'	*E. faecium*	256	>32^{R}	4	64^{R}	32	1	2048	16^{R}	>64^{R}	8	4	0.44
H_2 3 C'	*E. faecium*	256	>32^{R}	4	32^{R}	64	1	2048	32^{R}	64^{R}	16^{R}	5	0.56
H_2 5 C'	*E. faecium*	256	>32^{R}	4	64^{R}	32	1	2048	16^{R}	>64^{R}	8	4	0.44
H_2 6 C'	*E. faecium*	128	>32^{R}	4	16^{R}	64	0,5	2048	8	32^{R}	8	3	0.33
H_3 5 C'	*E. faecium*	512	>32^{R}	4	32^{R}	128	1	2048	16^{R}	32^{R}	16^{R}	5	0.56
H_3 7 C'	*E. faecium*	128	>32^{R}	4	64^{R}	32	0,5	2048	16^{R}	32^{R}	16^{R}	5	0.56
H_3 10 C'	*E. faecium*	512	>32^{R}	4	1	64	1	2048	4	16	4	1	0.11
H_4 4 C'	*E. faecium*	256	>32^{R}	4	32^{R}	32	1	2048	4	32^{R}	8	3	0.33
IN 2 D	*E. faecium*	256	>32^{R}	4	32^{R}	256^{R}	1	2048	>32^{R}	64^{R}	16^{R}	6	0.67
IN 3 D	*E. faecium*	256	>32^{R}	2	64^{R}	128	1	2048	8	32^{R}	8	3	0.33
IN 5 D	*E. faecium*	1024^{R}	>32^{R}	4	32^{R}	64	2	1024	8	64^{R}	>16^{R}	5	0.56

IN 9 D	*E. faecium*	128	>32^{R}	4	32^{R}	64	1	2048	32^{R}	64^{R}	16^{R}	5	0.56
IN 11 D	*E. faecium*	256	>32^{R}	4	32^{R}	128	2	2048	16^{R}	64^{R}	>16^{R}	5	0.56
H_2 1 D	*E. faecium*	512	>32^{R}	4	16^{R}	64	2	2048	16^{R}	64^{R}	16^{R}	5	0.56
H_2 2 D	*E. faecium*	128	>32^{R}	4	1	64	2	2048	16^{R}	64^{R}	>16^{R}	4	0.44
H_2 8 D	*E. faecium*	256	>32^{R}	2	>64^{R}	64	>16^{R}	2048	16^{R}	64^{R}	>16^{R}	6	0.67
H_2 9 D	*E. faecium*	256	>32^{R}	2	64^{R}	128	1	2048	16^{R}	64^{R}	16^{R}	5	0.56
H_3 1 D	*E. faecium*	256	>32^{R}	2	>64^{R}	32	1	2048	>32^{R}	64^{R}	>16^{R}	5	0.56
H_4 3 D	*E. faecalis*	512	>32^{R}	2	>64^{R}	32	1	2048	16^{R}	64^{R}	16^{R}	5	0.56
H_4 5 D	*E. faecalis*	1024^{R}	>32^{R}	4	16^{R}	16	0,5	127	>32^{R}	>64^{R}	8	5	0.56
H_4 9 D	*E. faecium*	256	>32^{R}	4	32^{R}	32	1	2048	16^{R}	64^{R}	>16^{R}	5	0.56
H_4 10 D	*E. faecium*	512	>32^{R}	4	64^{R}	256^{R}	2	4096	16^{R}	64^{R}	8	5	0.56
IN 1 D'	*E. faecium*	1024^{R}	>32^{R}	4	>64^{R}	128	2	2048	32^{R}	64^{R}	2	5	0.56
IN 2 D'	*E. hirae*	1024^{R}	>32^{R}	4	>64^{R}	128	2	2048	32^{R}	64^{R}	2	5	0.56
H_2 1 D'	*E. faecium*	>1024^{R}	>32^{R}	4	8^{R}	128	4	2048	>32^{R}	64^{R}	>16^{R}	6	0.67
H_2 2 D'	*E. faecium*	256	>32^{R}	2	64^{R}	128	0,5	4096	16^{R}	64^{R}	16^{R}	5	0.56
H_2 5 D'	*E. faecium*	512	>32^{R}	2	64^{R}	128	4	2048	32^{R}	64^{R}	>16^{R}	5	0.56
H_2 6 D'	*E. faecium*	256	>32^{R}	2	>64^{R}	64	1	2048	16^{R}	64^{R}	16^{R}	5	0.56
H_3 1 D'	*E. faecium*	256	>32^{R}	4	32^{R}	32	2	2048	16^{R}	64^{R}	16^{R}	5	0.56
H_4 1 D'	*E. faecium*	128	>32^{R}	4	>64^{R}	128	2	4096	16^{R}	64^{R}	16^{R}	5	0.56
H_4 2 D'	*E. faecium*	512	>32^{R}	4	64^{R}	64	4	4096	32^{R}	64^{R}	16^{R}	5	0.56
H_4 3 D'	*E. faecium*	128	>32^{R}	2	>64^{R}	32	1	4096	4	64^{R}	4	3	0.33
H_4 5 D'	*E. faecium*	256	>32^{R}	4	32^{R}	64	2	4096	32^{R}	64^{R}	16^{R}	5	0.56
H_4 6 D'	*E. faecalis*	1024^{R}	>32^{R}	4	64^{R}	256^{R}	4	4096	32^{R}	64^{R}	16^{R}	7	0.78
H_4 7 D'	*E. faecium*	512	>32^{R}	2	>64^{R}	64	2	4096	>32^{R}	64^{R}	16^{R}	5	0.56

IN 12 E	*E. faecium*	128	>32[R]	4	1	64	1	2048	8	64[R]	>16[R]	3	0.33
IN 14 E	*E. faecium*	256	>32[R]	4	1	32	2	2048	16[R]	64[R]	16[R]	4	0.44
IN 17 E	*E. faecium*	256	>32[R]	2	64[R]	64	1	2048	16[R]	64[R]	16[R]	5	0.56
H_2 5 E	*E. faecium*	512	>32[R]	4	64[R]	64	1	1024	16[R]	32[R]	16[R]	5	0.56
H_2 6 E	*E. faecium*	256	>32[R]	4	64[R]	64	2	2048	32[R]	64[R]	16[R]	5	0.56
H_2 8 E	*E. faecium*	128	>32[R]	4	>64[R]	64	>16[R]	2048	16[R]	64[R]	16[R]	6	0.67
H_2 9 E	*E. faecium*	128	>32[R]	2	64[R]	64	>16[R]	4097	16[R]	>64[R]	16[R]	6	0.67
H_3 3 E	*E. faecalis*	1024[R]	>32[R]	4	32[R]	256[R]	2	2048	16[R]	>64[R]	>16[R]	7	0.78
H_3 9 E	*E. faecium*	128	>32[R]	4	64[R]	16	2	1024	16[R]	32[R]	8	4	0.44
H_3 10 E	*E. faecium*	256	>32[R]	4	64[R]	32	1	1024	32[R]	32[R]	>16[R]	5	0.56
H_4 5 E	*E. faecium*	128	>32[R]	2	8[R]	32	2	1024	4	64[R]	8	3	0.33
H_4 6 E	*E. faecalis*	>1024[R]	>32[R]	4	64[R]	>256[R]	>16[R]	4096	32[R]	64[R]	16[R]	8	0.89
H_4 7 E	*E. faecium*	1024[R]	>32[R]	4	64[R]	256[R]	2	2048	32[R]	64[R]	16[R]	7	0.78
H_4 8 E	*E. faecium*	128	>32[R]	4	>64[R]	64	4	2048	16[R]	64[R]	16[R]	5	0.56
IN 9 E'	*E. faecium*	256	>32[R]	2	32[R]	64	16[R]	2048	16[R]	32[R]	16[R]	6	0.67
IN 10 E'	*E. faecium*	512	>32[R]	2	1	128	1	2048	32[R]	64[R]	>16[R]	4	0.44
IN 11 E'	*E. faecium*	256	>32[R]	4	64[R]	128	>16[R]	2048	16[R]	32[R]	8	5	0.56
IN 14 E'	*E. faecium*	256	>32[R]	2	64[R]	32	>16[R]	2048	32[R]	32[R]	16[R]	6	0.67
IN 17 E'	*E. faecium*	256	>32[R]	4	4[R]	32	1	2048	16[R]	32[R]	8	4	0.44
IN 18 E'	*E. faecium*	128	>32[R]	4	>64[R]	32	1	2048	8	32[R]	8	3	0.33
H_2 2 E'	*E. faecium*	256	>32[R]	2	16[R]	32	4	4097	>32[R]	64[R]	8	4	0.44
H_2 9 E'	*E. faecium*	512	>32[R]	4	64[R]	128	2	2048	>32[R]	32[R]	>16[R]	5	0.56
H_2 10 E'	*E. faecium*	512	>32[R]	2	>64[R]	128	1	2048	32[R]	32[R]	>16[R]	5	0.56
H_3 1 E'	*E. faecium*	256	>32[R]	2	16[R]	64	2	2048	16[R]	32[R]	16[R]	5	0.56

H_3 4 E'	*E. faecium*	128	$>32^R$	2	$>64^R$	16	1	2048	16^R	32^R	16^R	5	0.56
H_3 6 E'	*E. faecalis*	$>1024^R$	$>32^R$	2	64^R	128	$>16^R$	2048	$>32^R$	64^R	$>16^R$	7	0.78
H_3 10 E'	*E. faecium*	256	$>32^R$	2	64^R	32	$>16^R$	2048	16^R	32^R	16^R	6	0.67
H_4 3 E'	*E. faecium*	512	$>32^R$	4	64^R	128	2	2048	$>32^R$	64^R	$>16^R$	5	0.56
H_4 8 E'	*E. faecium*	512	$>32^R$	2	1	128	1	2048	16^R	$>64^R$	$>16^R$	4	0.44
H_4 9 E'	*E. faecium*	1024^R	$>32^R$	1	$>64^R$	64	1	2048	$>32^R$	32^R	$>16^R$	6	0.67

*CLSI and EUCAST-approved clinical breakpoint and epidemiological cut-off values.
STRE: streptomycin; ERY: erythromycin; VAN: vancomycin; RIF: rifampicin; GEN: gentamicin; TET: tetracycline; SUL: sulfamethoxazole; AMP: ampicillin; KAN: kanamycin; CHL: chloramphenicol; PEN: penicillin G. Total resistance (number of antibiotics to which a strain showed resistance). MAR Index: as a/b, where a represents the number of antibiotic to which isolate is resistant, and b represents the number of antibiotics to which isolate was exposed. MAR values >0.2 indicate multidrug resistant (MDR) strain. R: resistantR: resistant.

Evaluating the multidrug resistance of strains, 70(54%) were resistant to 5 antibiotics, 16(12%) to 6 antibiotics, and 7(4%) to 7 antibiotics. Furthermore, high prevelence of MDR strains was detected in each sampling site. Considering the inlet, where the value of MDR isolates was 91%, a decrease was observed only in CW1 site (Table 4). The highest MAR index was 0.89, for one *E. faecalis* strain isolated from CW3 effluent, whereas the lowest (0.11) was detected for one *E. faecium* strain, isolated from CW2 effluent, that showed resistance exsclusively to erythromycin (Table 3). The MAR index considered for each sampling site was 0.52 for CWs influent and 0.50, 0.53 and 0.51 for CW1, CW2 and CW3 effluents, respectively. With the aim to find out an association between strain

resistance patterns and tested antibiotics, a correlation analysis was performed. The results revealed the clustering of enterococcal population into two large groups, highlihting a correlation among susceptibility to streptomycin, gentamicin and tetracycline. On the same time, the resistance to ampicillin was correlated to resistance to penicillin, rifampicin and chloramphenicol (data not shown).

Table 4. Frequency of antibiotic resistant enterococci and Multiple Antibiotic Resistance (MAR) index at different sites.

Site	Number of isolates	Frequency (%) of MDR isolates	MAR* index range of AR isolates	MAR** index of site
CWs Influent	43	91%	0.22-0.78	0.52
CW1 Effluent	36	86%	0.22-0.78	0.50
CW2 Effluent	26	96%	0.11-0.78	0.53
CW3 Effluent	25	100%	0.33-0.89	0.51

*MAR of strain is calculated as = a/b, where a represents the number of antibiotic to which isolate is resistant, and b represents the number of antibiotics to which isolate has been exposed. MAR values >0.2 indicate multidrug resistant (MDR) strain. **MAR index of site is calculated as = $c/(n \cdot b)$, where c represents the total number of resistance scored; n = number of isolates from the site; b represents the number of tested antibiotics. Site MAR values ≥0.2 indicate a site with a high risk of potential contamination by MDR strain

3.4 Clonal relatedness

PFGE profiles of the 130 strains showed 15–20 fragments ranging from approximately 48.5 to 291 kb in size, differentiating the isolates in very numerous different patterns, with Dice's coefficient of similarity (CS) higher than 95%. Among obtained pulso-types, over 40 PFGE patterns were unique, including strains isolated from different sampling points (data not shown). Within the ten clonal groups which included strains with comparable PFGE profiles, a certain pulso-type recurrence was observed in the different CW effluents (Fig. 1). In details, CW1 site showed a clone occurrence very similar to those detected into inlet site, whereas CW2 and CW3 sites showed a different pulso-type arrangement, with the dominance of clones not detected in influent (Fig. 1). In addition, different patterns of resistance were detected among the same *E. faecium* pulso-types (Fig. 1). In particular, within the ten most recurrent clone groups, six pulso-types exhibited different phenotypic resistance patterns, being resistant to a different number of antibiotics (Fig. 2) and to different specific antibiotic molecules (data not shown). Regarding the MIC rate, a different MIC value distribution was observed between CW influent and effluents. In details, as reported in Figure 3, among *E. faecium* strains, in all CW effluents, an increase of MIC values for rifampicin and for sulphametoxazole, and conversely a general decrease for tetracycline was registered. Overall, a higher variability was detected among *E. faecalis* strains with a remarkable different trend among CW effluents. In particular, higher MIC values were detected for streptomycin and lower values for rifampicin, in all CW effluents. In addition, higher MIC values for both vancomycin and gentamycin were found in CW2 and CW3

effluents, whereas decreased values were registered for ampicillin in CW1 and CW2, and for penicillin in CW1 and CW3 effluents (Fig. 3).

4. Discussion

The occurrence, diversity and persistence of AR resistant enterococci in three CWs effluents receiving the same influent were evaluated, and the antibiotic resistance profiling of strains was revealed. The counting results highlighted a mean enterococcal density of 4.04 log CFU/100 mL in influent, which slightly decrease in all effluents to about 3 log CFU/100 mL, in agreement with previous studies (Graves and Weaver 2010; Martins da Costa et al., 2006), confirming that conventional wastewater treatments are not able to efficiently remove these microorganisms. This result has been related to the attitude of enterococci to adapt and to persist in harsh conditions and/or to produce biofilms difficult to remove (Giebułtowicz et al., 2017; Mohamed and Huang, 2007; Vilanova et al., 2004). Looking at species composition, in the present work, the isolates mostly belonged to *E. faecium* and *E. faecalis* species, the most common causative agents of nosocomial infections. It is interesting to observe that prevalence of *E. faecium* has been rarely reported on environmental sources and domestic wastewater (Graves and Weaver, 2010). Hoverer the present findings are in agreement with the increasing dominance of the species, recently observed in environment samples in all around the world (Aarestrup et al., 2000). Although comparing antibiotic resistances in environmental samples is very difficult, given the absence of any standardized methods,

some trends could be outlined, at least within enterococcal population that represents the most investigated bacteria in UWTPs. Several authors, using distinct methodologies, indicated high resistance rates (20–44 %) for tetracycline, erythromycin and quinolones and considerably lower resistance for aminopenicillins and sulfonamides (1–7%) (Ferreira da Silva et al., 2006; Łuczkiewicz et al., 2010; Martins da Costa et al., 2006). In the present study, a high prevalence of resistances was observed, revealing a given multidrug resistance, mainly against erythromicin, chloramphenicol, rifampicin and ampicillin. In the present study, 93% of *E. faecalis* strains were found ampicillin resistant, even if this is considered as a rare trait in *E. faecalis* (EUCAST, 2018). Resistance to these antibiotics has been related to the frequent and continuous usage of antibiotics, like penicillin and aminoglycosides, for empirical treatment of infectious disease (Aarestrup et al., 2000; Arvanitodou et al., 2001; Devarajan et al., 2015), and to the widespread prevalence of resistance genes in the environment (Jamet et al., 2012; Kang et al., 2017). In the present study, vancomycin resistance was never detected, and this finding could be related to the distance (about 12 km) of the considered plant to the nearest hospital, located in Caltagirone town (38,500 inhabitants, in 2016). This result is in agreement with those recently reported that observed a low prevalence of VRE in a WWTP, and confirms that the presence of VRE may vary depending on the sampling site and on treatment policies of various regions (Oravcova et al., 2017; Taucer-Kapteijn et al., 2016). Although a reduced abundance of AR enterococci are reported in the effluents (Taučer-Kapteijn et al., 2016), in the present study MDR enterococci persisted in all three sampling sites, with the highest prevalence in CW3

effluents, where the highest number of *E. faecalis* strains was detected. The different persistence rates suggests a different enterococcal selection process in the CW sites, as previously observed (Ferreira da Silva et al., 2006; Łuczkiewicz et al., 2010; Martins da Costa et al., 2006). However the MAR indexing, determined for the different sites, results not correlated neither to the bacterial removal efficiency or system parameters. In the present study the MAR indexing determined for the different sites were higher than the arbitrary value of risk contamination, indicating the CW as relevant risk contamination sites (Krumperman, 1983). The concern for the high prevalence of MDR enterococci is alarming considering that bacteria with antibiotic resistance factors have a selective advantage over antibiotic sensitive forms in the natural environment. The genotyping identification, established by *Sma*I PFGE, showed a high genetic diversity. Several pulso-types with a unique pattern were detected, demonstrating the independent origin of the strains. The high diversity could be attributed to the reiterate exposure to environmental stresses that may have promoted evolutionary processes, such as mutation, selection and recombination, necessary for the adaptation of enterococcal population. The persistence of *E. faecalis* in CW units could be ascribed to the ability of this species to adhere to zooplankton and to persist in the environment for extended period of time. In the present study the persistence of the same *E. faecium* pulso-types in both different sites and sampling periods, suggests the common presence of some peculiar clone in the ecosystem. Within the same pulso-type, different phenotypic resistance patterns were observed, confirming the high-level of plasticity of the enterococcal genome and the role of the system in the dissemination of resistance (Rizzo et al.,

2013). Overall, the evidences on dissemination of clinically relevant antimicrobial resistance traits among enterococci in non-hospital environments supports the concept of exogenous acquisition of resistant determinants, creating an additional need to understand the fate of AR enterococci. Although the irrigation with reclaimed wastewater has been widely applied in different countries to mitigate the water shortage, the current state of knowledge alerts about AR enterococci, which are not completely eliminate by the most conventional wastewater treatment processes and for which neither Italian nor European legislation provides any limit.

5. Conclusions

The persistence of AR enterococci in UWTPs suggests their possible spreading to both surface waters and vegetables crops through irrigation, and could contribute to increase the risk of transmission via food chain. Although the CWs show different effects, the high prevalence of antibiotic resistance prompts a selective advantage in such a natural environment and the high MAR index in all sampling sites represents an alarming public health risk. In order to preserve the water quality of the receiving systems, the optimization of wastewater treatment parameters and the implementation of further technologies together with a strict management strategy are urgently required.

Conflict of interest

The Authors declare that there are no conflict of interest.

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References

Aarestrup, F.M., Agerso, Y., Gerner–Smidt, P., Madsen, M., Jensen, L.B. (2000). Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark Diagnostic Microbiology and Infectious Disease 37, 127–137.

Aiello, R., Bagarello, V., Barbagallo, S., Iovino, M., Marzo, A., Toscano, A. (2016). Evaluation of clogging in full-scale subsurface flow constructed wetlands. Ecol Eng, 95: 505-513.

APHA, American Public Health Association. (1998). Standard Methods for the Examination of Water and Wastewater. Washington, DC: American Public Health Association.

APHA, American Public Health Association. (2006). Standard Methods for the Examination of Water. American Public Health Association, New York.

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

Arvanitidou, M., Katsouyannopoulos, V., Tsakris, A. (2001). Antibiotic resistance patterns of enterococci isolated from coastal bathing waters. J Med Microbiol, Vol. 50; 1001-1005.

Batt, A.L., Bruce, I.B., Aga, D.S. (2006). Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. Environ Pollut, 142(2):295–302.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J. (2012). Enterococci in the environment. Microbiol Mol Biol Rev 76, 685-706.

Castorina, A., Consoli, S., Barbagallo, S., Branca, F., Farag, A., Licciardello, F., Cirelli, G.L. (2016). Assessing environmental impacts of constructed wetland effluents for vegetable crop irrigation. Int J Phytorem, 18: 626-633.

Cheng, S., McCleskey, F.K., Gress, M.J., Petroziello, J.M., Liu, R., Namdari, H., Beninga, K., Salmen, A. et al. (1997) A PCR assay for identification of *Enterococcus faecium*. J Clin Microbiol, 35, 1248–1250.

Chief Medical Officer Annual Report (2011). Antimicrobial Resistance-GOV. UK. https://www.gov.uk/government/publications/chief-medical-officerannual-report-volume-2.

Cirelli, G.L., Consoli, S., Di Grande, V., Milani, M., Toscano, A. (2007). Subsurface constructed wetlands for wastewater treatment and reuse in agriculture: five years of experiences in Sicily, Italy. Water Sci Technol, 56:183-191.

CLSI, Clinical and Laboratory Standards Institute. (2016). Performance Standards for Antimicrobial Susceptibility Testing, 26th Edition CLSI supplement M100S, CLSI, Wayne, PA, USA.

Daniel, D.S., Leea, S.M., Gana, H.M., Dykesc, G.A., Rahmana, S. (2017). Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia. Journal of Infection and Public Health 10, 617–623.

Devarajan, N., Laffite, A., Graham, N.D., Meijer, M., Prabakar, K., Mubedi, J.I., Elongo, V., Mpiana, P.T., Ibelings, B.W., Wildi, W., Poté, J. (2015). Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in Central Europe. Environ Sci Technol, 49:6528-6537.

Doan, N.T.L., Van Hoorde, K., Cnockaert, M., De Brandt, E., Aerts, M., Le Thanh, B. Vandamme, P. (2012). Validation of MALDI-TOF MS for rapid classification and identification of lactic acid bacteria, with a focus on isolates from traditional fermented foods in Northern Vietnam. Letters in Applied Microbiology 55, 265–273.

EUCAST, European Committee on Antimicrobial Susceptibility Testing EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017. http://www.eucast.org.

Ferreira da Silva, M., Tiago, I., Veríssimo, A., Boaventura, R.A., Nunes, O.C., Manaia, C.M. (2006). Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. FEMS Microbiol Ecol, 55(2):322-9.

Gao, W., Howden, B.P., Stinear, T.P. (2018). Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. Current Opinion in Microbiology, 41:76–82.

Gatta, G., Libutti, A., Beneduce, L., Gagliardi, A., Disciglio, G., Lonigro, A., Tarantino, E. (2016). Reuse of treated municipal wastewater for globe artichoke irrigation: assessment of effects on morpho-quantitative parameters and microbial safety of yield. Sci Hortic 213, 55–65.

Giebułtowicz, J., Tyski, S., Wolinowska, R., Grzybowska, W., Zaręba, T., Drobniewska, A., Wroczyński, P., Nałęcz-Jawecki, G. (2017). Occurrence of antimicrobial agents, drug-resistant bacteria, and genes in the sewage-impacted Vistula River (Poland). Environ. Sci Pollut Res. Volume 25, Issue 6, pp 5788–5807.

Graves, A.K. and Weaver, R.W. (2010). Characterization of enterococci populations collected from a subsurface flow constructed wetland. Journal of Applied Microbiology 108, 1226-1234.

Hay, S.I., Rao, P.C., Dolecek, C., Day, N.P.J., Stergachis, A., Lopez, A.D., Murray, C.J.L. (2018). Measuring and mapping the global burden of antimicrobial resistance. BMC Medicine, 16:78.

ISO 9001:2008. Quality management systems– Requirements.

Jamet, E., Akary, E., Poisson, M. A., Chamba, J. F., Bertrand, X., & Serror, P. (2012). Prevalence and

characterization of antibiotic resistant *Enterococcus faecalis* in French cheeses. Food Microbiology, 31, 191–198.

Kang, B., Kim, D.H., Chon; J.W., Seo, K.H. (2017). Effect of microbial control measures on farmstead cheese-making and antimicrobial resistance of *Staphylococcus aureus* and *Enterococcus* spp. isolates. J Food Saf, e12432.

Kim, J., Lee, S., Choi, S. (2012). Copper resistance and its relationship to erythromycin resistance in *Enterococcus* isolates from bovine milk samples in Korea. Journal of Microbiology (Seoul, Korea), 50, 540–543.

Kim, S., Jensen, J. N., Aga, D.S., Weber, A.S. (2007). Tetracycline as a selector for resistant bacteria in activated sludge. Chemosphere 66, 1643–1651.

Krumperman, P.H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl Environ Microbiol, 46(1): 165–170.

Luczkiewicz, A., Fudala-Ksiazek, S., Jankowska, K., Quant, B., Olańczuk-Neyman, K. (2010). Diversity of fecal coliforms and their antimicrobial resistance patterns in wastewater treatment model plant. Water Sci Technol, 61(6):1383-92.

Martínez, J.L. and Baquero, F. (2014). Emergence and spread of antibiotic resistance: setting a parameter space. Upsala Journal of Medical Sciences; 119: 68–77.

Martins da Costa, P,, Vaz-Pires, P., Bernardo, F. (2006). Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. Water Res, 40(8):1735-40.

Mohamed, J.A. and Huang, D.B. (2007). Biofilm formation by enterococci. J Med Microbiol, 56, 1581-1588.

Novais, C., Coque, T.M., Sousa, J.C., Baquero, F., Peixe, et al. (2004). Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. Antimicrob. Agents Chemother, 48, 3613-3617.

Oravcova, V., Peixeb L., Coque, T.M., Novais, C., Francia, M.V., Literáka, I., Freitas A.R. (2018). Wild corvid birds colonized with vancomycin-resistant *Enterococcus faecium* of human origin harbor epidemic vanA plasmids. Environment International 118; 125–133.

Oravcova, V., Mihalcin, M., Zakova, J., Pospisilova, L., Masarikova, M., Literak, I. (2017). Vancomycin-resistant enterococci with vanA gene in treated municipal wastewater and their association with human hospital strains. Sci Total Environ, 609:633-643.

Pinto, B., Pierotti, R., Canale, G., Reali, D. (1999). Characterization of 'faecal streptococci' as indicators of faecal pollution and distribution in the environment. Letters in Applied Microbiology, 29, 258–263.

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sanchez- Melsiò, A., Borrego, C.M.,

Barcelò, D., Balcazar, J.L. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res, 69, 234-242.

Rosenberg Goldstein, R.E., Micallef, S.A., Gibbs, S.G., George, A., Claye, E., Sapkota, A., Joseph, S,W, Sapkota, A.R. (2014). Detection of vancomycin-resistant enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. Sci Total Environ, 466-467:404-11.

Russo, N., Caggia, C., Pino, A., Coque, M.T., Arioli, S., Randazzo, C.L. (2018). *Enterococcus* spp. in Ragusano PDO and Pecorino Siciliano cheese types: A snapshot of their antibiotic resistance distribution. Food and Chemical Toxicology 120: 277-286.

Sadowy, E., and Luczkiewicz, A. (2014). Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. BMC Microbiol, 14: 66.

Sayd, M., Dev, A., Sahr, Z., Sajisha, S., Safna, M., Anu, Ahmmad, B. (2017). Prevalence Of High Multiple Antibiotic Resistance Index (Mar) Bacterial Isolates From Mangroves With Reference To Calicut Zones, Kerala. International Journal of Advances in Science Engineering and Technology, ISSN: 2321-9009.

Signoretto, C., Burlacchini, G., Pruzzo, C., Canepari, P. (2005). Persistence of *Enterococcus faecalis* in aquatic

environments via surface interactions with copepods. Appl Environ Microbiol, 71:2756–2761.

Taučer-Kapteijn M., Hoogenboezem, W., Heiliegers, L., de Bolster, D., Medema, G. (2016). Screening municipal wastewater effluent and surface water used for drinking water production for the presence of ampicillin and vancomycin resistant enterococci. Int J Hyg Environ Health, 219 (4-5):437-42.

Tenover, F.C., Arbei,t R.D., Goering, R.V., Mickelson, P.A., Murray, B.E., Persing, D.H. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol, 33:2233–2239.

Varela, A.R., Ferro, G., Vredenburg, J., Yanık, M., Vieira, L., Rizzo, L., Lameiras, C., Manaia, C.M. (2013). Vancomycin resistant enterococci: from the hospital effluent to the urban wastewater treatment plant. Sci Total Environ, 450-451:155-61.

Vignaroli, C., Pasquaroli, S., Citterio, B., Di Cesare, A., Mangiaterra, G., Fattorini, D., Biavasco, F. (2018). Antibiotic and heavy metal resistance in enterococci from coastal marine sediment. Environ Pollut, 237: 406-413.

Vignaroli, C., Zandri, G., Aquilanti, L., Pasquaroli, S., Biavasco, F. (2011). Multidrug-resistant enterococci in animal meat and faeces and co-transfer of resistance from an *Enterococcus durans* to a human *Enterococcus faecium*. Curr Microbiol, 62(5):1438-47.

Vilanova, X., Manero, A., Cerdà-Cuéllar, M., Blanch, A.R. (2004). The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants. J Appl Microbiol, 96(2):279-88.

Werner, G., Coque, T.M., Franz, C.M., Grohmann, E., Hegstad, K., van Schaik, W., Weaver, K. (2013). Antibiotic resistant enterococci-tales of a drug resistance gene trafficker. Int J Med Microbiol, 303, 360–379.

Werner, G., Coque, T.M., Hammerum, A.M., Hope, R., Hryniewicz, W., Johnson, **A. et al.** (2008). Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull, 13.

WHO, World Health Organization, (2017). Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics. http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistantbacteria/en/.

WHO, World Health Organization. Antimicrobial Resistance: Global Report on Surveillance 2014. http://www.who.int/drugresistance/documents/ surveillancereport/en/.

Zarb, P., Coignard, B., Griskeviciene, J., Muller, A., Vankerckhoven, V., Weist, K. (2012). The European Centre for Disease Prevention and Control (ECDC) pilot point prevalence survey of healthcare-associated infections and antimicrobial use. Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull, 17.

Zhang, S., Han, B., Gu, J., Wang, C., Wang, P., Ma, Y., Cao, J., He, Z. (2015). Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes. Chemosphere 135, 138-145.

Integrative discussions

The prevalence of AR resistant enterococci in various settings has increased dramatically in the last decade, generating great concern. The controversial nature of these microorganisms has prompted an enormous increase in scientific papers and reviews in recent years (Foulquié Moreno et al., 2006). However, further studies are essential to determine their population structure and their role in food. Enterococci are omnipresent in several traditional fermented foods. Their ubiquity, mainly in milk products from different countries (Nieto-Arribas et al., 2011; Suzzi et al., 2000), on the wooden equipment used for traditional cheese making (Scatassa et al., 2015) and in the animal rennet used in milk curdling during the production of these cheese types (Cruciata et al., 2014), is the result of their high adaptability to the different parameters applied during food processing and storage conditions.

Our results highlighted the presence of enterococci into two Sicilian cheese types at different ripening stages, with *E. durans*, *E. faecalis*, and *E. faecium* being the predominant species. All strains, belonging to the different species showed a high rate of resistance to antibiotics, with the possible acquisition of resistance proven by the multimodal distribution of MIC values detected for some antimicrobials. Co- and/or cross-selection phenomena within the microbial population have been demonstrated by the different phenotypic resistance patterns obtained for the same pulso-type, highlighting the genomic high plasticity of the genus. In Ragusano PDO, at early stage of ripening, a conspicuous presence of the *E. durans* species, with a marked gene heterogeneity, was observed. The selective effect of the production process on the enterococcal

population was confirmed by the persistence of some clones at the different stage of ripening studied. In the light of the results obtained it is clear that the role of enterococci in cheese ripening remains questionable, mostly for *E. durans*, for which less information are available. The high percentage of multi-resistance and the multimodal distribution of the MIC values found, reinforce the need for constant monitoring and active surveillance of the species. Finally, the absence of the vancomycin resistance genes in the van-resistant strains lays on resistance mechanisms that should be investigated.

The study of the fate of AR bacteria and ARGs during wastewater treatment is complex, influenced by a myriad of external factors, difficult to control and monitor in real world system. This is probably the reason why contradictory findings are found in literature and many questions still remain unanswered, albeit the numerous important efforts are making around the world (Manaia et al., 2018).

Constructed Wetlands (CW) have been proposed as an economically and energitically efficient unit process to treat domestic wastewater for reuse purpose, and are tacking on a pivotal role in the protection of the environment. As demonstrated by several studies on removal efficiency of these systems, the WWTP treatment process had insufficient effect in removing pathogenic bacteria (Al-Gheethi et al., 2018; Edokpayi et al., 2015). Although the discrepancies observed in results may be attributed to the differences in the treatment policies of various regions, the total removal of faecal indicators has been rarely detected. From the microbiological point of view, results of the present work highlighted that H-SSF CW produces effluent not able to comply with Italian standards on wastewater

reuse in agriculture. Furthermore, although numerous tertiary tretaments has been proposed and regarded as a measures to obtain final treated wastewater of high quality (EEA, 2017), in the present study the combination of CWs with UV treatment produced highly effective results, meeting both the strict Italian legislation and the new European proposal. The same effect was not found for enterococci, for which neither Italian nor European legislation provides a limit (Iweriebor et al., 2015; Oravcova et al., 2017; Taučer-Kapteij et al., 2016). In the present study a clear majority of the detected isolates belonged to *E. faecium*. Our results are in agreement with other studies, where *E. faecium* was the predominant species. High prevalence of resistant to different class of antimicrobials was observed, but interesting, no VRE was founded. The persistence of enterococci along the continuum suggests the risk of their spreading from WWTPs to both surface waters and vegetables crops through irrigation, increasing, at the end, their transmission via food chain (Blanch et al., 2003). The present study also confirmed that WWTPs could be considered hotspots for AR resistance spread to non resistant bacteria, contributing to significantly increase the AR levels in receiving ecosystems. In order to preserve the water quality of the receiving systems, thus limiting the risk for human health, the application of new technologies in wastewater treatment and the implementation of watershed management strategies aiming to control the AR spread is a big challenge that needs to be faced in next years.

Conclusions and perspectives

In conclusion, the present study demonstred the high risk of spreading of enterococci antibiotic resistance through the food chain. We reinforced the need of a constant monitoring and an active surveillance of food of animal origins, in particular fermented products, where enteroccal population has referred as natural microbiota. Finally, we revealed the necessity to deepen the knowledge on non-*feacium* and non-*faecalis* species, resulted also resistant, in order to obtain a complete picture of the virulence level of these species often found in food.

Regarding the water-associated enterococci, we demonstrated that multiresistant and potentially pathogenic enterococci occurred in all sampled effluents. We revealed that the WWTP treatment process had insufficient effect in removing faecal indicators and that treated urban effluents could disseminate clinically important antibiotic-resistant enterococci into the environment. We assessed that further studies are needed to improve the WWTPs removal efficiency of AR bacteria and AR genes, in order to reduce the costs of advanced technologies and strategies for the mitigation of AR spread into the environment.

In the final analysis, however, the problem of antibiotic resistance will not be solved with the creation of many more, or stronger, bactericidal antimicrobials. In fact, microorganisms will consistently continue to adapt to their environment by developing resistance to newer antibiotics and serious infections could be caused by these bacteria, posing a major challenge to the practicing clinician (Alanis et al., 2015). A collaborative effort among industry, academia and government to combact AR bacteria is needed. An effort which will include the implementation of

several strategies simultaneously such as a more rational use of antibiotics and the creation and broader use of vaccines will be able in preventing infections with some of these multi-resistant bacteria (Alanis et al., 2015).

References

Alanis, A.J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? Arch Med Res, 36(6):697-705.

Al-Gheethi, A.A., Efaq A.N., Bala, J.D., Norli, I., Abdel-Monem, M.O., Ab. Kadir, M.O. (2018). Removal of pathogenic bacteria from sewage-treated effluent and biosolids for agricultural purposes. Applied Water Science, 8:74.

Blanch, A.R., Caplin, J.L., Iversen, A., Kühn, I., Manero, A., Taylor, H.D., Vilanova, X. (2003). Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. Journal of Applied Microbiology, 94(6):994 – 1002.

Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., De Filippis, F., Mancuso, I., La Storia, A., Moschetti, G., Settanni, L. (2014). Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 80, 2050–2061.

Edokpayi, J.N., Odiyo, J.O., Popoola, E.O., 2, Msagati, T.A.M. (2018). Evaluation of Microbiological and Physicochemical Parameters of Alternative Source of Drinking Water: A Case Study of Nzhelele River, South Africa. ISSN: 1874-2858-Volume 12.

EEA, European Environmental Agency. (2017). European waters Assessment of status and pressures 2018. ISSN 1977-8449.

Foulquié-Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L. (2006). The role and application of enterococci in food and health. Int. J. Food Microbiol.106, (1):1-24.

Iweriebor, B.C., Obi, L.C. Okoh, A.I. (2016). Macrolide, glycopeptide resistance and virulence genes in *Enterococcus* species isolates from dairy cattle. Journal of Medical Microbiology, 65, 641–648.

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Nunes, O.C. et al. (2018). Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environment International. 115, pp. 312-324.

Nieto-Arribas, P., Seseña, S., Poveda, J.M., Chicón, R., Cabezas, L., Palop, L. (2011). *Enterococcu*s populations in artisanal Manchego cheese: Biodiversity, technological and safety aspects. Food Microbiology 28, 891-899.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G. (2015). Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41.

Suzzi, G., Caruso, M., Gardini, F., Lombardi, A., Vannini, L., Guerzoni, M., Andrighetto, C., Lanorte, M.T., 2000. A survey of the enterococci isolated from an artisanal Italian goat's cheese (semicotto caprino). J. Appl. Microbiol. 89, 267–274.

Taučer-Kapteijna, M., Hoogenboezema, W., Heiliegersa, L., de Bolstera, D., Medemab, G. (2016). Screening municipal wastewater effluent and surface water used fordrinking water production for the presence of ampicillin and vancomycin resistant enterococci. International Journal of Hygiene and Environmental Health, 219, 437–442.

Annex

Food and Chemical Toxicology 120 (2018) 277–286

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Enterococcus spp. in Ragusano PDO and Pecorino Siciliano cheese types: A snapshot of their antibiotic resistance distribution

Nunziatina Russo[a], Cinzia Caggia[a,*], Alessandra Pino[a], Teresa M. Coque[b,c,d], Stefania Arioli[e], Cinzia L. Randazzo[a]

Food and Chemical Toxicology 115 (2018) 491–498

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Effects of selected bacterial cultures on safety and sensory traits of Nocellara Etnea olives produced at large factory scale

Cinzia L. Randazzo[a], Nunziatina Russo[a], Alessandra Pino[a], Agata Mazzaglia[a], Margherita Ferrante[b], Gea Oliveri Conti[b], Cinzia Caggia[a,*]

Food Microbiology 61 (2017) 126–135

Contents lists available at ScienceDirect

Food Microbiology

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

Influence of PDO Ragusano cheese biofilm microbiota on flavour compounds formation

Stefania Carpino [a], Cinzia L. Randazzo [b, *], Alessandra Pino [b], Nunziatina Russo [a], Teresa Rapisarda [a], Gianni Belvedere [a], Cinzia Caggia [b]

International Journal of Food Microbiology 252 (2017) 42–52

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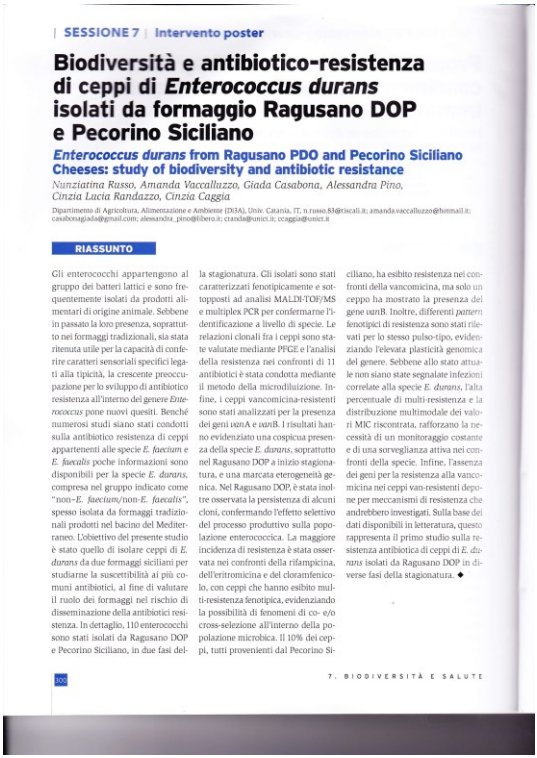
International Journal of Food Microbiology

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

Survival of potential probiotic lactobacilli used as adjunct cultures on Pecorino Siciliano cheese ripening and passage through the gastrointestinal tract of healthy volunteers

Alessandra Pino[a], Koenraad Van Hoorde[b], Iole Pitino[a], Nunziatina Russo[a], Stefania Carpino[c], Cinzia Caggia[a,*], Cinzia L. Randazzo[a]

Poster presentation



| SESSIONE 7 | Intervento poster

Biodiversità e antibiotico-resistenza di ceppi di *Enterococcus durans* isolati da formaggio Ragusano DOP e Pecorino Siciliano

***Enterococcus durans* from Ragusano PDO and Pecorino Siciliano Cheeses: study of biodiversity and antibiotic resistance**

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RIASSUNTO

Gli enterococchi appartengono al gruppo dei batteri lattici e sono frequentemente isolati da prodotti alimentari di origine animale. Sebbene in passato la loro presenza, soprattutto nei formaggi tradizionali, sia stata ritenuta utile per la capacità di conferire caratteri sensoriali specifici legati alla tipicità, la crescente preoccupazione per lo sviluppo di antibiotico resistenza all'interno del genere *Enterococcus* pone nuovi quesiti. Benché numerosi studi siano stati condotti sulla antibiotico resistenza di ceppi appartenenti alle specie *E. faecium* e *E. faecalis* poche informazioni sono disponibili per la specie *E. durans*, compresa nel gruppo indicato come "non-*E. faecium*/non-*E. faecalis*", spesso isolata da formaggi tradizionali prodotti nel bacino del Mediterraneo. L'obiettivo del presente studio è stato quello di isolare ceppi di *E. durans* da due formaggi siciliani per studiarne la suscettibilità ai più comuni antibiotici, al fine di valutare il ruolo dei formaggi nel rischio di disseminazione della antibiotici resistenza. In dettaglio, 110 enterococchi sono stati isolati da Ragusano DOP e Pecorino Siciliano, in due fasi della stagionatura. Gli isolati sono stati caratterizzati fenotipicamente e sottoposti ad analisi MALDI-TOF/MS e multiplex PCR per confermarne l'identificazione a livello di specie. Le relazioni clonali fra i ceppi sono state valutate mediante PFGE e l'analisi della resistenza nei confronti di 11 antibiotici è stata condotta mediante il metodo della microdiluizione. Infine, i ceppi vancomicina-resistenti sono stati analizzati per la presenza dei geni *vanA* e *vanB*. I risultati hanno evidenziato una cospicua presenza della specie *E. durans*, soprattutto nel Ragusano DOP a inizio stagionatura, e una marcata eterogeneità genica. Nel Ragusano DOP, è stata inoltre osservata la persistenza di alcuni cloni, confermando l'effetto selettivo del processo produttivo sulla popolazione enterococcica. La maggiore incidenza di resistenza è stata osservata nei confronti della rifampicina, dell'eritromicina e del cloramfenicolo, con ceppi che hanno esibito multi-resistenza fenotipica, evidenziando la possibilità di fenomeni di co- e/o cross-selezione all'interno della popolazione microbica. Il 10% dei ceppi, tutti provenienti dal Pecorino Siciliano, ha esibito resistenza nei confronti della vancomicina, ma solo un ceppo ha mostrato la presenza del gene *vanB*. Inoltre, differenti *pattern* fenotipici di resistenza sono stati rilevati per lo stesso pulso-tipo, evidenziando l'elevata plasticità genomica del genere. Sebbene allo stato attuale non siano state segnalate infezioni correlate alla specie *E. durans*, l'alta percentuale di multi-resistenza e la distribuzione multimodale dei valori MIC riscontrata, rafforzano la necessità di un monitoraggio costante e di una sorveglianza attiva nei confronti della specie. Infine, l'assenza dei geni per la resistenza alla vancomicina nei ceppi van-resistenti depone per meccanismi di resistenza che andrebbero investigati. Sulla base dei dati disponibili in letteratura, questo rappresenta il primo studio sulla resistenza antibiotica di ceppi di *E. durans* isolati da Ragusano DOP in diverse fasi della stagionatura. ◆

Russo N., Vaccalluzzo A., Casabona G., Pino A., Randazzo C.L., Caggia C. (2018). Biodiversità e antibiotico-resistenza di ceppi di *Enterococcus durans* isolati da formaggio Ragusano DOP e Pecorino Siciliano. XII Convegno Nazionale Biodiversità, Ambienti e Salute. Universita' degli Studi di Teramo. Campus A. Saliceti 13-15 Giugno 2018. Poster Sessione Biodiversità e Salute. P. 300. Edagricole, Ciserano (BG).

SESSIONE 4.4 | Contributo poster

Il pastazzo di agrumi come ammendante

Potential of citrus wastes as soil emended

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RIASSUNTO

Il pastazzo di agrumi (buccia e polpa di arancia residuati) è il principale sottoprodotto dell'industria di trasformazione agrumaria. Per la sua composizione, caratterizzata da elevata concentrazione di carboidrati, vitamine e nutrienti, rappresenta un'alternativa agli ammendanti organici naturali, impiegati per incrementare e mantenere la fertilità organica del suolo. Diversi studi hanno evidenziato il ruolo del pastazzo nel miglioramento delle caratteristiche di aereazione del suolo e nella promozione dell'attività microbica. Tuttavia, le prestazioni dell'attuale processo di compostaggio non compensano i costi di produzione e ne rendono proibitivo l'utilizzo. Il presente lavoro ha avuto l'obiettivo di valutare la sostenibilità dell'utilizzo del pastazzo fresco di agrumi come ammendante dei suoli agrari, al fine di predisporre delle linee guida per una revisione del D.lgs. n.75 del 2010. L'attività sperimentale è stata condotta presso un'area test dell'Azienda Agraria dell'Ateneo di Catania, un agrumeto di circa 400 m², comprendente 20 piante ventennali di Tarocco comune. Il sito di sperimentazione è stato suddiviso in 4 tesi, sulla base del quantitativo di pastazzo somministrato, e l'attività di monitoraggio ha previsto la valutazione degli effetti sulle caratteristiche fisiche, chimiche, microbiologiche e idrauliche del suolo e la valutazione delle caratteristiche fisiologiche, delle rese produttive e qualitative delle specie arboree considerate. Dal punto di vista chimico-fisico, i campioni di suolo, diversamente trattati, hanno mostrato una bassa conducibilità elettrica e una buona dotazione in carbonio organico e in azoto totale. I valori ottenuti di C/N hanno dimostrato la presenza di sostanza organica matura nel suolo stabile nel tempo. La capacità di scambio cationico e la dotazione in fosforo assimilabile sono risultate ottimali in tutte le tesi analizzate. Infine, una riduzione (circa un ordine di grandezza) è stata osservata per i valori di conducibilità idraulica e di saturazione nei campioni sperimentali. Dal punto di vista microbiologico, i risultati ottenuti hanno mostrato l'assenza di *Escherichia coli*, di Salmonella e di coliformi fecali su tutte le unità campionarie sia di pastazzo sia di suolo prima dell'ammendamento, in conformità alla normativa vigente. Assenza di *E. coli* e di Salmonella è stata rilevata anche in tutti i campioni sperimentali di suolo per tutti i tempi di osservazione. Incrementi della densità cellulare di enterococchi ed *Enterobacteriaceae*, nei diversi tempi di studio, sono stati osservati nei campioni di suolo sperimentali.

Dal punto di vista fisiologico le piante non hanno mostrato rilevanti differenze tra le tesi, né per quanto riguarda l'andamento della conduttanza stomatica, né della traspirazione fogliare. Differenze nelle produzioni, ma non imputabili alle diverse dosi di pastazzo somministrate, sono state rilevate. Infine, nessuna differenza statisticamente significativa è stata osservata per i parametri di produzione qualitativi considerati. I risultati ottenuti depongono per supportare la predisposizione di un regolamento per l'utilizzazione agronomica del pastazzo di agrumi. ◆

Caggia C., Randazzo C.L., Consoli S., Russo N., Baglieri A., Continella A., Barbagallo S. (2018). Il pastazzo di agrumi come ammendante. XII Convegno Nazionale Biodiversità, Ambienti e Salute. Universita' degli Studi di Teramo. Campus A. Saliceti 13-15 Giugno 2018. Poster Biodiversità microbica e micotica negli ecosistemi e nei processi agroindustriali. P. 223 Edagricole, Ciserano (BG).

SESSIONE 4.3 | Contributo poster

Impiego di lattobacilli probiotici e β-glucosidasi positivi per la messa a punto di olive da tavola funzionali a basso tenore di sodio

Functional low salt table olives setup using probiotic and β-glucosidase positive lactobacilli strains

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RIASSUNTO

Le olive da tavola rappresentano i vegetali fermentati più diffusi nell'area del Mediterraneo. Il trend di consumo e' fortemente in crescita sia nei paesi dell'Unione Europea (UE) sia nelle aree geografiche non UE, grazie alla crescente attenzione verso la Dieta Mediterranea. In Sicilia, il metodo di produzione più diffuso prevede la deamarizzazione naturale, ossia l'immersione diretta dei frutti nella salamoia e il processo fermentativo avviene ad opera del microbiota autoctono, responsabile anche dell'idrolisi dell'oleuropeina. Ai fini della standardizzazione del processo fermentativo, numerosi starter sono già presenti in commercio e l'impiego di ceppi ß-glucosidasi positivi rappresenta un vantaggio sia per la garanzia della sicurezza microbiologica e sia per ridurre la durata del processo produttivo. Nel presente studio, ceppi di batteri lattici, ascritti alla specie *Lactobacillus plantarum*, isolati da olive della cultivar Nocellara Etnea in fermentazione naturale, precedentemente identificati, sono stati sottoposti a screening per le più rilevanti proprietà tecnologiche e utilizzati come starter per la messa a punto di olive da tavola a ridotto contenuto di sodio. In dettaglio, le performance di crescita dei ceppi sono state valutate a diverse concentrazioni di sale (4.0, 5.0 e 6.0% di NaCl), di pH (4.5 e 5.5) e di temperatura (16 °C e 32 °C) e la capacità di deamarizzazione e' stata verificata attraverso la ricerca del gene che codifica per l'enzima β-glucosidasi. I ceppi con la migliore combinazione di caratteri sono stati impiegati in co-coltura con il ceppo probiotico *Lactobacillus paracasei* N24, in fermentazioni sperimentali condotte a 4.0, 5.0 e 6.0% di NaCl. Fermentazioni spontanee sono state allestite come controllo. Il processo fermentativo è stato monitorato per 120 giorni attraverso analisi chimico-fisiche, microbiologiche e sensoriali e la sopravvivenza del ceppo N24 è stata valutata attraverso metodi convenzionali e molecolari. I risultati hanno rivelato che 5 dei 13 ceppi testati sono stati in grado di crescere a tutte le condizioni sperimentali e 2 sono risultati positivi per la presenza del gene che codifica per la β-glucosidasi. Le fermentazioni sperimentali hanno evidenziato una rapida acidificazione e deamarizzazione. Le olive ottenute, sottoposte ad analisi sensoriale, hanno esibito tratti sensoriali maggiormente apprezzati rispetto al controllo. Inoltre, il ceppo probiotico impiegato ha evidenziato una buona sopravvivenza a fine fermentazione. I risultati del presente lavoro confermano che l'impiego di colture starter costituite da ceppi di lattobacilli β-glucosidasi positivi e probiotici rappresenta una promettente strategia per la produzione di olive da tavola funzionali. ◆

Pino A., Vaccalluzzo A., Casabona G., Russo N., Caggia C., Randazzo C.L. (2018). Impiego di lattobacilli probiotici e β-glucosidasi positivi per la messa a punto di olive da tavola funzionali a basso tenore di sodio. 2018. XII Convegno Nazionale Biodiversità, Ambienti e Salute. Universita' degli Studi di Teramo. Campus A. Saliceti 13-15 Giugno 2018. Poster Sessione Biodiversità microbica e microbica negli ecosistemi e nei processi agroindutriali. P. 204. Edagricole, Ciserano (BG).

Microbial Diversity 2017
DRIVERS OF MICROBIAL DIVERSITY
MD 2017

PS3-5 PAG. 355

Snapshot of species diversity within enteroccoccal population and antibiotic resistance in constructed wetland and traditional cheese

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Enterococci are microorganisms highly widespread in nature, being present in gastrointestinal tract of humans and animals, in soil, in water and in food, such as meat, milk and cheese. Recently some enterococci have been described as opportunistic pathogens, able to carrier virulence factors, and disseminate and/or acquiring antibiotic resistant determinants. The aim of the present study was to investigate the prevalence of antibiotic resistant (AR) enterococci isolated from Constructed Wetland (CW) and from a traditional Sicilian cheese.
Overall, 349 presumptive *Enterococcus* spp. were isolated from CW both from influent and effluents (209) and from traditional cheese at early and late stage of ripening (140),

and subjected to PCR-RFLP of the 16S rRNA gene and MALDI-TOF MS analyses. Moreover, isolates were tested for the AR to clinical antibiotics, by micro-dilution method, according to CLSI and EUCAST guidelines.
In details, 210 isolates were identified as *Enterococcus* spp., and among the water-associated enterococci *Enterococcus faecium* was mainly identified (91% occurrence). Among the cheese-associated enterococci, *E. durans* and *E. faecalis*, were the most frequently isolated species at early and late stage of ripening (83% and 80% occurrence, respectively).
Based on the AR data, high prevalence of resistance to the most tested antibiotics was revealed among water-associated enterococci, while significant differences were depicted among food-associated enterococci, with the highest incidence for rifampicin and erythromycin.
Results highlight that wastewater treatment sites could provide a selection for multi-resistant enterococci and, based on the bimodal distribution of MICs values, it is possible to suppose horizontal gene transfer events. The occurrence of prevalence of high number of resistant enterococci in traditional cheeses necessarily require a constant risk management to reduce the human exposure.

Microbial Diversity 2015
THE CHALLENGE OF COMPLEXITY
MD 2015

SESSION I: METABOLIC COMPLEXITY OF AGRICULTURAL AND NATURAL ENVIRONMENTS

Poster

TINA BIOFILMS AS SOURCE OF MICROORGANISMS INVOLVED IN VOLATILE ORGANIC COMPOUNDS PRODUCTION OF RAGUSANO CHEESE

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This study aimed at investigating the composition of biofilm microbiota of *Tina* used for Ragusano cheese-making. In addition, the ability of biofilm microbiota of *Tina* to generate volatile organic compounds (VOCs) in

milk samples was evaluated by incubating milk in the *Tina* under Ragusano cheese making conditions. *Tina* biofilms from eleven facilities producing Ragusano cheese under traditional conditions were analyzed. A polyphasic approach, based on culture–dependent and independent methods was used. VOCs were identified and quantified by GC/MS-SPME, coupled to Smart Nose and GC/O. All biofilm samples showed a significantly higher (P<0.05) counts of streptococci, compared to other microbial groups. *Streptococcus thermophilus* was the dominant species in both biofilm and in milk samples incubated with *Tina* biofilms. Other lactic acid bacteria genera (*Lactobacillus, Lactococcus, Enterococcus* and *Leuconostoc*) were variously identified within the eleven facilities. Low levels of *Pseudomonas* spp. and yeasts counts were detected, whereas coliforms and presumptive pathogens (*Listeria monocytogenes* and *Salmonella* spp.), were not detected. Several strong correlations (r>0.6; P<0.05) were found between some lactic acid bacteria of *Tina* biofilms and VOCs produced during incubation of milk samples under Ragusano cheese-making conditions. In details, positive correlations were found between *Enterococcus hirae* and alcohols, *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus delbrueckii* and aldeydes, and *Lactobacillus fermentum*, *Lactobacillus helveticus* and *Lactobacillus hilgardii* and ketones. The findings of this study demonstrate that *Tina* biofilm represents a source of lactic acid bacteria, which significantly contributes to the synthesis of VOCs in Ragusano cheese.

EVALUATION OF ANTIBIOTIC-RESISTANT ENTEROCOCCI IN CONSTRUCTED WETLANDS SYSTEM FOR WASTEWATER REUSE IN AGRICULTURE

Poster-Session I

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Constructed wetlands (CWs) are engineered systems which reproduce the natural water remediation processes occurring in nature in order to reuse the final effluent for agricultural purposes. The aims of this study were to monitor the sanitation efficiency of a full-scale horizontal sub-surface flow CW combined with UV treatment and to evaluate the Antibiotic-Resistance (AR) in enterococci, isolated, both in influent and in three different effluents, in 12 consecutive months. For this purpose, the microbiological indicators *Escherichia coli*, total coliforms and enterococci were monitored by plating counts. Moreover, the levels of enterococci sensitivity to different antibiotics molecules were measured using microdilution assay. The results

showed that the levels of microbiological indicators were significantly lowered in water samples collected downstream the CW system, compared to the water samples collected upstream the CW system. While the CW system allowed an efficient reduction in *E. coli* population, reaching a final value below the Italian limits required for wastewater reuse in agriculture, a moderate reduction was achieved for enterococci count. The taxonomic identification of isolates reveled the dominance of the species *Enterococcus faecalis* both in influent ad effluents, followed by *Enterococcus faecium* and *Enterococcus hirae*. Most of enterococci isolates showed high AR to most of the tested antibiotics and variable sensibility to glycopeptides and beta-lactam both in influent and effluent samples. Strong differences were also observed among sampling times with a higher AR levels monitored in the period from April to June. In conclusion, while the CW system considered in the present study is suitable for the water reuse in agriculture, according to the Italian legislation, the presence of enterococci at high concentrations underlined the persistence of this bacterial group in water environment with a potential risk to serve as a genetic reservoir of transferable AR, a matter of concern for public health.

SESSION II: THE COMPLEXITY OF FOOD ECOSYSTEMS: PHYSIOLOGY OF SINGLE STRAINS IN PURE COLTURE VS. COMPLEX CONSORTIA

Poster

LACTIC ACID BACTERIA FROM PIACENTINU ENNESE *CHEESE: CHARACTERIZATION AND IDENTIFICATION THROUGH A POLYPHASIC APPROACH*

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It is generally accepted that lactic acid bacteria (LAB) play a significant role during the cheese ripening, especially in those produced under traditional procedures. Among them, *Piacentinu Ennese* is an artisanal cheese produced in the Enna area of Sicily (Italy) using raw cow milk without addition of any starter cultures. The typical properties of this artisanal cheese are mainly due to their richer indigenous NSLAB population. In the present study, almost one-hundred lactic acid bacteria (LAB) strains were isolated

from fresh, 6 and 8 months ripened *Piacentinu Ennese* cheeses, provided from 3 different farms, in order to select strains to be use as starter cultures . Firstly, the LAB strain were screened for technological and safety aspects i.e. acidifying activity, sensitivity to different class of antibiotics, biogenic amine production and antimicrobial activity versus different pathogens (*Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Pseudomonas* spp). Then the strains were characterized and identified using a polyphasic approach, which combine a molecular fingerprinting technique (GTG5-PCR), a proteomics approach (MALDI-TOF MS) and sequencing analysis of the 16S rRNA gene. Results revealed that 90% of the strains exhibited good acidifying activity in skim milk; most of the strains (78%) showed variable resistance to the antibiotics tested and only few strains exhibited high resistance. All strains were not able to produce biogenic amines and 65% of the strains showed antimicrobial activity against the pathogens tested. Molecular characterization revealed that most of the strains isolated from fresh cheese were ascribed to *Lactococcus lactis* species, while several NSLAB were detected in the 6 and 8 ripened cheese. In detail, most strains consisted of *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus paracasei* an *Lactobacillus rhamnosus*. In addition to these dominant groups, some isolates identified as *Lactobacillus pentosus* and *Streptococcus macedonicus* were retrieved from the old cheese samples.

Manuscripts under review

Effect of molasses and dried orange pulp as sheep dietary supplementation on physico-chemical, microbiological and fatty acid profile of Comisana ewe's milk and cheese

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Abstract

The use of agro-industrial by-products for ruminant feed represents an economical and environmental convenient way for reducing waste discharge and waste management costs for food industries. Large amounts of waste from citrus processing industries are available in Sicily, Italy. In the present study the effect of dried citrus pulp as sheep dietary supplementation was evaluated on physico-chemical, microbiological and fatty acid composition of resulting milk and cheese. In detail, pelleted feed integrated with molasses and blond orange pulp replacing cane molasses and beet pulp and part of the maize and sunflower in ration, were administrated to ewes as experimental treatment. The experiment involved sixty Comisana breed sheep's, divided into two groups and two feeding trials (experimental and control). Ewe's milk and cheese samples were collected from January to April and analysed for physico-chemical, microbiological and fatty acid profile composition. Results suggested that both experimental milk and cheese were significantly different from controls. In particular, an increase of milk yield and fat content was registered and cheese samples exhibited a significant decrease of pH values and an increase in fat and

protein contents. In addition, a higher value of unsaturated fatty acids, polyunsaturated fatty acid and conjugated linoleic acids and a significant improvement of the oxidative stability were observed in experimental cheese samples, indicating the beneficial effect of dietary supplementation. Furthermore, no difference among the main microbial groups was detected, between experimental and control samples, with the exception of streptococci found higher in experimental cheeses and staphylococci, which were significantly reduced by experimental feed. Moreover, the application of culture-independent methods highlighted the dominance of *Lactobacillus rhamnosus*/*casei* group in the experimental cheese, suggesting a driving role of the dietary supplementation in the cheese microbiota composition. The present study demonstrated that the inclusion of citrus by-products in the diet of small dairy ruminants had positive effects on milk composition and cheese manufacture.

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Employ of citrus by-product as fat replacer ingredient for bakery confectionery products

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Abstract

Citrus fruits processing is one of the foremost industrial activities in Sicily and the main residual by-product, consisting of peels and seed, is called 'pastazzo'. Traditionally this by-product has been used for different purposes, and only most recently, has been described as source of a wide range of healthy bioactive compounds and dietary fibers. In the present work, a debittered food grade orange fiber (DOF), extracted from orange juice by-product, was produced in a pilot scale. The DOF showed high total fiber content, low activity water and a high water binding capacity, and was used as fat-replacer at different percentages (30, 50 and 70%) in bakery confectionery products (brioches). The obtained bakery products were characterized for nutritional, technological and microbiological parameters through storage at room temperature. Data showed that the addition of DOF was compatible with bakery processing resulting in final products with increased moisture content after one day of storage, and good textural proprieties. In addition, the fat strategy replacing, at different levels of DOF, resulted in final products with lowered fat content, increased content of dietary fiber and protein, and an invariable carbohydrates content. In particular, the replacement at 50% level of fat allowed obtaining

brioches with improved technological properties and with desirable microbiological traits, mostly within the first 24 hours from production and up to five days of storage.

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Specialty Section: Nutrition and Food Science Technology