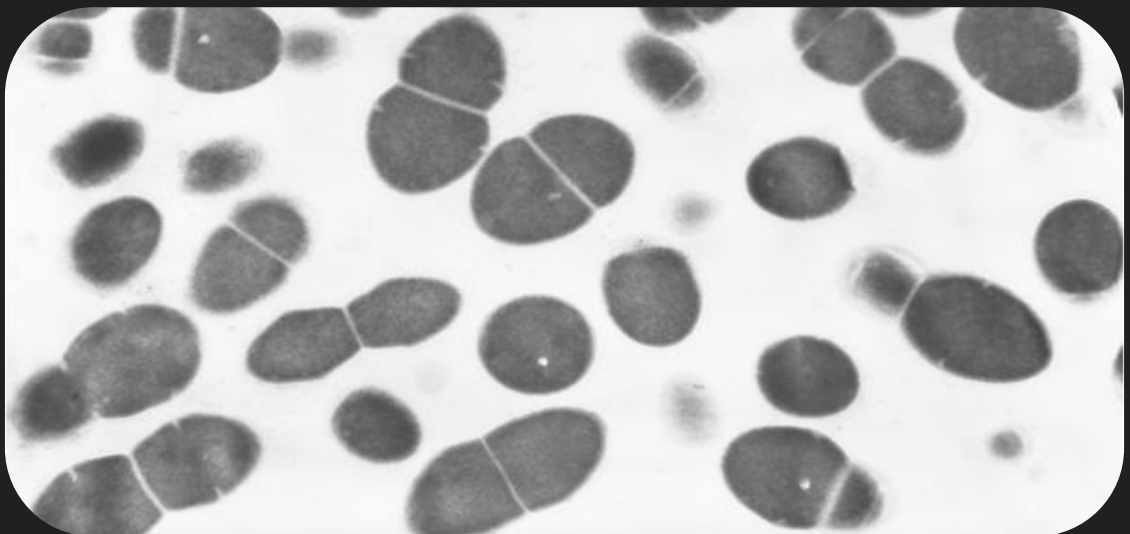


***Enterococcus* and biocides: mechanisms of tolerance and selection for vancomycin resistance.**

Teresa Marina Fonseca de Almeida Santos Braga



Dissertation presented to obtain the Ph.D degree in Biology
Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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And to Bruno, for everything...

Abstract

Biocides are chemical agents, generally with a broad-spectrum of activity, used to destroy, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism. Biocidal products comprise several chemical groups. Among the most commonly used are alcohols, aldehydes, biguanidines (e.g. chlorhexidine), phenols (e.g. triclosan) and quaternary ammonium compounds (e.g. benzalkonium chloride).

Although some of the biocides were discovered many years ago, their generalised use began only some decades ago. They are used for cleaning and/or disinfecting in many different environments, such as in hospital facilities, veterinary facilities, food and pharmaceutical industry sites and in our homes. Biocides are also incorporated in several products as preservatives, such as deodorants, body creams and soaps. Some of the environments where biocides are applied are shared with enterococci.

Enterococci are Gram-positive lactic acid bacteria. They are commensal bacteria and natural inhabitants of the gastro-intestinal tract of humans and other animals. *Enterococcus faecalis* and *E. faecium* are the most common species found in this habitat. Enterococci are robust bacteria which are able to grow under very different conditions of pH, temperature, etc. These characteristics allow them to grow/survive in many different environments, such as sand, plants, water, hospitals, food industry, cheese and meat. These bacteria are also responsible for nosocomial infections and have intrinsic resistance to some antibiotics (such as certain β -lactams, clindamycin, some aminoglycosides) and acquired resistance to others (such as chloramphenicol, erythromycin, tetracycline, high-level aminoglycoside and vancomycin). Vancomycin resistant enterococci (VRE) are of great concern as this antibiotic is used

as one of the last resort treatments for multiple antibiotic resistant enterococci.

The way these bacteria interact with biocides or respond to them has not been totally investigated. The studies so far published concerning this issue have essentially investigated the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values for such compounds.

In this work, three of the most commonly used biocides were studied: benzalkonium chloride (BC), chlorhexidine (CHX) and triclosan (TCS). We investigated the role of EFA0010 gene, annotated for *E. faecalis* V583 as a putative SMR transporter, as a tolerance mechanism to biocides. This gene, named *qacZ*, was constitutively expressed and it was involved in the response of *E. faecalis* V583 to BC, but not to CHX or ethidium bromide. The susceptibility of enterococci isolated from different environments (clinical, veterinary and dairy products) to biocides was also determined by MIC assay and the values obtained were similar to those already described in the literature. The dissemination of the *qacZ* gene was also investigated in these isolates. No correlation was found between the MIC values for BC and CHX and the presence of this gene.

Similarly, no association was observed between the MIC values for the same biocides, plus TCS, and resistance to vancomycin. Several experiments (such as growth curves in the presence of the biocides, relative fitness assays, exposure to biocides for a certain period of time) were performed in order to understand whether there was any survival advantage for a vancomycin-resistance enterococcus (VRE) in the presence of biocides or if these compounds can select VRE strains over vancomycin-susceptible enterococci (VSE) strains. From the obtained results, VRE strains were found to present no advantage in the presence of BC, CHX and TCS.

An unexplored environment, concerning biocides susceptibility patterns and enterococci, was analysed. Dust samples from Portuguese swine breeding facilities were collected and enterococci were isolated from them. The strains were identified and characterized regarding biocide tolerance to BC and CHX. From the same samples, VRE were also isolated, identified and characterized. Once more, no association was found between vancomycin resistance and tolerance to biocides.

The work herein presented offers a new approach to enterococci response to biocides. The first description of a gene involved in the response to a quaternary ammonium compound is given, as well as its dissemination in several environments where enterococci are found. The possible association between vancomycin resistance and biocides was thoroughly investigated. VRE had no survival advantage in the presence of these compounds when compared to VSE. Biocides are used in the different explored environments for cleaning and disinfection, but no connection between the MIC values for the tested compounds and vancomycin resistance was observed. The use of these compounds does not select VRE strains either in clinical or in veterinary environments.

Sumário

Os biocidas são agentes químicos, geralmente com um largo espectro de actividade, utilizados para eliminar, atenuar, impedir a acção de, ou controlar microrganismos prejudiciais. Agrupam-se em vários grupos químicos, dentro dos quais os mais comuns são: álcoois, aldeídos, biguanidas (ex.: clorexidina), fenóis (ex.: triclosan) e compostos quaternários de amónia (ex.: cloreto benzalcónio).

Embora muitos biocidas tenham sido descobertos há muitos anos atrás, o seu uso generalizado aumentou nas últimas décadas. São usados para limpar e/ou desinfectar ambientes muito diferentes, tais como instalações hospitalares, instalações veterinárias, indústria alimentar e farmacêutica e nas nossas casas. Os biocidas podem também ser incorporados como conservantes em alguns produtos tais como desodorizantes, cremes para o corpo e sabonetes. Alguns dos ambientes onde os biocidas são utilizados são partilhados com enterococos.

Os enterococos são bactérias Gram-positivas, ácido lácticas, comensais humanas e que habitam o tracto intestinal humano e de outros animais. As espécies mais comuns neste habitat são *Enterococcus faecalis* e *Enterococcus faecium*. Dada à sua robustez, os enterococos são capazes de crescer em diferentes condições de pH e temperatura, o que lhes permite crescer/sobreviver em ambientes muito diferentes, tais como areias, plantas, águas, hospitais, indústria alimentar, queijos e carne. Estas bactérias são também responsáveis por infecções nosocomiais, possuindo resistência intrínseca a alguns antibióticos (como por exemplo: alguns β -lactâmicos, clindamicina, alguns aminoglicosídeos) e resistência adquirida a outros (tais como cloranfenicol, eritromicina, tetraciclina, aminoglicosídeos de alto nível e vancomicina). A vancomicina constitui um dos últimos recursos no tratamento contra enterococos multi

resistentes, pelo que o aparecimento de enterococos resistentes à vancomicina constitui um factor de enorme preocupação.

Ainda não está totalmente esclarecido o modo como estas bactérias interagem ou respondem aos biocidas. Os estudos até agora publicados sobre este assunto debruçaram-se essencialmente sobre valores de concentração inibitória mínima (CIM) e de concentração bactericida mínima (CBM) para estes compostos.

Neste trabalho foram estudados três dos biocidas mais utilizados, cloreto benzalcónio, clorexidina e triclosan. Foi estudado o papel do gene EFA0010, anotado para *E. faecalis* V583 como um transportador putativo SMR, como um mecanismo implicado na tolerância desta bactéria aos biocidas. Este gene, nomeado *qacZ*, é expresso constitutivamente, está envolvido na resposta do *E. faecalis* V583 ao cloreto benzalcónio, mas não à clorexidina nem ao brometo de etídio. Foi determinada a susceptibilidade de enterococos isolados de diferentes ambientes (clínicos, veterinários e produtos lácteos) aos biocidas por ensaios de concentração inibitória mínima. Os valores obtidos foram muito semelhantes aos já descritos na literatura disponível. A disseminação do gene *qacZ* foi também investigada nestes isolados. Não foi encontrada nenhuma associação entre o valor de CIM para o cloreto benzalcónio e para a clorexidina e a presença deste gene.

Também não foi encontrada nenhuma associação entre os valores de CIM para o cloreto benzalcónio, a clorexidina e o triclosan, e a resistência à vancomicina em enterococos. Com o objectivo de compreender se enterococos resistentes à vancomicina têm alguma vantagem de sobrevivência na presença de biocidas ou se estes compostos podem estar a seleccionar estirpes de enterococos resistentes à vancomicina em relação a estirpes susceptíveis a este antibiótico, foram efectuadas vários ensaios, nomeadamente curvas de crescimento na presença dos biocidas, ensaios de “fitness” e exposição aos biocidas por

um determinado período de tempo. Os resultados obtidos permitem-nos concluir que as estirpes resistentes à vancomicina não apresentam qualquer vantagem na presença de cloreto benzalcónio, clorexidina ou triclosan.

Um ambiente inexplorado foi também analisado relativamente ao padrão de susceptibilidade a biocidas. Amostras de pó de suiniculturas Portuguesas foram recolhidas e destas isolaram-se enterococos. As estirpes foram identificadas e caracterizadas em relação à sua tolerância ao cloreto de benzalcónio e à clorexidina. Das mesmas amostras foram isolados e identificados enterococos resistentes à vancomicina, e caracterizados quanto à sua susceptibilidade aos biocidas. Mais uma vez, não foi encontrada nenhuma associação entre a resistência à vancomicina e a tolerância aos biocidas.

O trabalho aqui apresentado revela novos factos no que respeita à resposta dos enterococos aos biocidas. É feita a primeira descrição de um gene envolvido na resposta a um composto quaternário de amónia, assim como a sua disseminação em diversos ambientes onde os enterococos são encontrados. A possível associação entre resistência à vancomicina e biocidas foi também investigada. Enterococos resistentes a este antibiótico não apresentam qualquer vantagem de sobrevivência na presença destes compostos, quando comparados com enterococos susceptíveis à vancomicina. Os biocidas são usados para limpeza e desinfecção nos diferentes ambientes estudados, no entanto não foi encontrada nenhuma ligação entre o valor de CIM para os compostos estudados e a resistência à vancomicina. O uso destes compostos não está a seleccionar enterococos resistentes à vancomicina nem em ambiente clínicos, nem em ambientes veterinários.

List of publications

Corsetti A., Settanni L., **Braga T.M.**, Silva Lopes M.F., Suzz G.; An investigation on the bacteriocinogenic potential of lactic acid bacteria associated with wheat (*Triticum durum*) kernels and non-conventional flours. – Food Science and Technology, 2007, Sept, 41(7):1173-1182.

Marques J, **Braga T.M.**, Almeida Paz FA, Santos TM, Silva Lopes M.F., Braga S.S.; Cyclodextrins improve the antimicrobial activity of the chloride salt of Ruthenium(II) chloro-phenanthroline – trithiacyclononane. Biometals. 2009 Jun, 22(3):541-56

Braga T.M., Marujo P., Pomba C., Silva Lopes M. F.; Involvement and dissimination of the enterococcal small multidrug resistance transporter QacZ in resistance to quaternary ammonium compounds. Journal of Antimicrobial Chemotherapy. 2011, 66: 283-286.

Braga T.M., Pomba C. and Silva Lopes M.F.; Occurrence of enterococci and vancomycin-resistant enterococci in dust samples from pig breeding facilities and assessment of susceptibility to biocides. Submitted to Journal of Antimicrobial Chemotherapy.

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General Introduction

Chapter I

1. Biocides

1.1 Definitions

Biocide is a general term describing a chemical agent defined as active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, render harmless, prevent the action of, or otherwise exert controlling effect on any harmful organism by chemical or biological means (17). Biocides usually have a broad spectrum of activity in contrast to antibiotics, which have a narrower range of antimicrobial activity. The term is used in nonmedical applications (5,32). Because biocides range in antimicrobial activity, other terms may be more specific, including “-static”, referring to agents which inhibit growth (e.g., bacteriostatic, fungistatic and sporistatic) and “-cidal”, referring to agents which kills the target organism (e.g., sporicidal, virucidal and bacteriocidal). (31)

Biocides can be used as antiseptics – applied to the skin or mucous membranes to destroy or inhibit the growth of microorganisms (e.g. health care personnel hand washes and surgical scrubs); disinfectants – applied to inanimate objects or surfaces to destroy harmful microorganisms (can be sporostatic, but not necessary sporicidal); and preservatives – incorporated into medications or fluids and food to prevent microbial growth (31, 63).

1.2 History

Biocides (antiseptics, disinfectants and preservatives) and other antimicrobial agents have been used in various forms for centuries. Early empirical approaches stored potable water in copper and silver vessels; used vinegar and honey for cleaning injuries; balsams as natural preservatives in mummification; and drying, salting and spices for food preservation. Later, a quantitative method for chemical preservation was

developed; it compared various salt solutions with a standard (sea salt) for their capacity of preservation. Alcohol has been used for over 2000 years as an antimicrobial agent, although it was not recognized as such until recently. Wine was used to heal all kind of injuries, but its concentration was very low to be used as an antiseptic. Time passed by and the concentration of this compound in wine and other alcoholic drinks increased. There are some reports of brandy used to clean and disinfect wounds. The Portuguese surgeon A. M. Barbosa, reported a mortality rate of only 10% for 243 amputations using alcohol in a Lisbon hospital during 1855 and 1866 and only one death in 14 tight operations. Later in 1903, Harrington and Walker showed that a 60% to 70% alcohol solution was the most effective, although it did not kill bacterial spores (4).

There are reports from 19th and early 20th, of the use of iodine as a wound disinfectant, of chloride water in obstetrics, of phenol as a wound dressing and in antiseptic surgery, and the claimed sporicidal activity of mercuric chloride. Curiously, phenol and chloride were first used as deodorants to prevent the foul odors of sewage and garbage. Quaternary ammonium compounds (QACs) were introduced in the earliest 1900s, while chlorhexidine was more recently introduced. In 1945 the most common used biocides were phenolic compounds, organomercurials, iodine, alcohol, formaldehyde, hydrogen peroxide, silver compounds and dyes (Table 1). Some of these are still used nowadays. The most important biocides used after 1945 include QACs, biguanides (chlorhexidine), biphenols (triclosan), aldehydes (glutaraldehyde) paracetic acids, among others. (4, 52, 55).

Table 1. Biocides and their introduction into clinical practices

Biocide	Discovery or first description	Introduction/application
Alcohols	BC	Early AD
Chlorine	1774	1847
Sodium hypochlorite	1789	1827
Chlorine dioxide	1925	1946
Iodine	1812	1816
Iodophors	1949	1956
Hydrogen peroxide	1818	1891
Formaldehyde	1867	1894
Phenol	1834	1867
Triclosan	1906	Early 1970s
QACs	1856/1916	1933
Chlorhexidine	1946	1954
Glutaraldehyde	1904	1960s
Acridines		1913

Adapted from (52); BC - Before Christ; AD - Anno Domini (After Christ)

The 20th century was very rich in chemical advances and including the discovery and application of new biocides that we use in so many different ways. They protect our food from the action of microorganisms; preserve many of our products; protect our body from micro invaders.

Nowadays their use is generalized in the hospital environment, food industry, veterinary medicine and even in our homes.

1.3 Chemical groups and active molecules in biocidal products

The number of biocides in use is large. They can be used alone or in combination with a variety of products. During the research work for the elaboration of this thesis, the three biocides studied were benzalkonium chloride, chlorhexidine and triclosan. Their chemical group will be described in more detail; other important biocide chemical groups are summarised in Table 2.

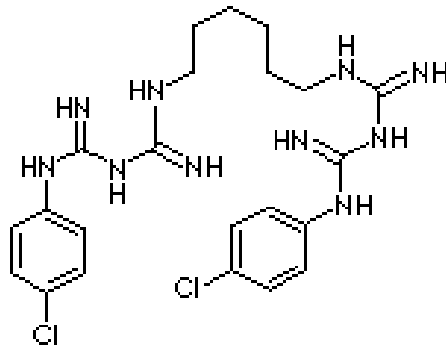
1.3.1 Biguanides

Biguanides are compounds that contain the C_2HN_7 ligand. The most common active molecules are:

Chlorhexidine
Alexidine
Polymeric biguanides

These compounds are used as antiseptics, disinfectants, preservatives and antiplaque agents. They have a broad-spectrum of activity, with rapid action against Gram-positive and Gram-negative bacteria; they are less active against fungi (including yeast and molds). They are not sporicidal, although they do inhibit the outgrowth of spores and some sporicidal activity can be achieved by combination with other biocides or at higher temperatures ($>70^\circ\text{C}$). Their activity against virus is also limited (31, 33, 36).

1.3.1.1 Chlorhexidine



Chlorhexidine (CHX) is the most commonly used biguanide with a broad range of application: antimicrobial soaps, wound dressings, mouth washes, hair care products, surface disinfectants and preservatives (for example, contact lens storage solution). It is used for surface disinfection at 0.5% to 4%, for antisepsis at 0.02% to 4% and for preservation at 0.0025% to 0.01% (31, 33). Chlorhexidine has a broad-spectrum of activity, produces minimal skin irritation and its substantivity on skin and mucous membranes. Although in few cases, some sensitivity to chlorhexidine has been described. Chlorhexidine plays an important role in the control of hand transmission pathogens in hospital. It has the ability to remain present at a bacteriostatic concentration on skin even after hand washing. Chlorhexidine and other biguanides can be inactivated by nonionic surfactants, in some soaps, hand creams and inorganic water contaminants (33).

Chlorhexidine activity is pH dependent and is largely reduced in the presence of organic matter. Its major target is the cytoplasmic (inner) membrane. Low concentrations of this compound affect the membrane integrity, while high concentrations coagulate the cytoplasm. Primarily, chlorhexidine acts on cell membranes, provoking loss of structure and function. It is rapidly absorbed through bacterial and fungal cell walls and damages the inner cell membrane, causing cytoplasm leakage and

precipitation of proteins and nucleic acids (31, 33). This biocide shows a rapid action against Gram-positive and Gram-negative bacteria. It is less effective against fungi, including yeast and molds. It has no sporicidal activity, although it can inhibit (but not the germination of) spores. Mycobacteria are generally less susceptible to chlorhexidine, *Mycobacterium avium-intracellulare* is considerably more tolerant than other mycobacteria. The antiviral activity of chlorhexidine is variable; it is more effective against lipid-enveloped viruses and cannot inactivate nonenveloped virus, such as rotaviruses, hepatitis A virus or polioviruses. It is believed that the outer coat of virus is the major target of chlorhexidine (31, 33).

1.3.2. Phenolics

Phenolics are a class of alcohol compounds with one or more hydroxyl groups attached to an aromatic hydrocarbon ring. A wide variety of phenolics are used for disinfection, antisepsis and preservation (31):

Coal tar

Cresol

Phenol

Xylenols

Naphthols

Non-coal tar phenols

2-Phenylphenol

4-Hexylresorcinol

Halogenated phenols

Chapter I

Chlorophenol

4-Chloro-3,5-dimethylphenol (chloroxylenol; para-chloro-meta-xyleneol; PCMX)

2-Nitrophenol

4- Nitrophenol

Bisphenols

Hexachlorophene

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether)

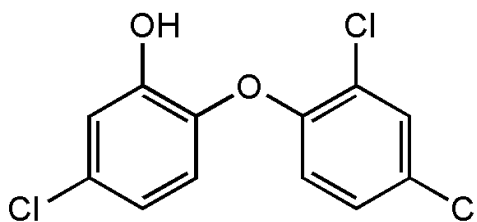
Other phenol derivatives

Salicylic acid

2,3-Diaminophenol

Most phenolic compounds have a broad-spectrum of activity against bacteria (generally more efficient against Gram-positive than Gram-negative), fungi and viruses. Tuberculocidal activity has also been observed. They are sporicidal, with little or no sporicidal activity. Phenolic compounds are cellular poisons, but they also have cell membrane-active properties. At low concentrations, they inactivate membrane enzyme functions and increase the cell wall permeability and they induce the progressive leakage of intracellular constituents. At high concentrations, phenols have multiple effects on cell wall, cell membrane and cytoplasmic components, including their coagulation, causing irreversible cellular damages (31, 33, 36).

1.3.2.1 Triclosan



Triclosan (TCS) is an antimicrobial agent used for several purposes for more than 20 years (57). It is commonly used in antiseptic soaps, hand rinses, lotions, cleaners, shampoos, deodorants, gels and antiacne washes. Concentrations range between 0.1 to 2%, but higher concentrations can be used in higher-risk applications (33). It has a cumulative and persistent effect on skin and is neither toxic nor irritant to skin and mucous membranes. Triclosan is also used in mouth rinses and antibacterial toothpastes. It is also used as a preservative in cosmetic and other products, usually in combination with other biocides. Due to its thermal and chemical stability, triclosan has also been incorporated into plastics and fabrics.

Triclosan as antiseptic can penetrate into and through skin without any toxic, allergenic or mutagenic effect. Its stability and activity is preserved in the presence of organic matter and high temperatures, allowing triclosan to be used as an antimicrobial barrier in textiles and plastics. Due to its stability and extensive use, triclosan has been found in the environment, raising some concern about its ecological effects (33, 36).

Triclosan has a broad-spectrum of activity, it is particularly efficient against Gram-positive bacteria, but it is also active against Gram-negative bacteria and yeasts. Lower activity is observed against some enveloped viruses, fungi and some pseudomonads. *Pseudomonas aeruginosa* is highly resistant to triclosan. At high concentrations, this compound cause rapid release of the cellular components and consequently cell death; at

low concentrations it has more specific targets, such as the fatty acid biosynthesis. Triclosan blocks lipid synthesis by interacting specifically with the substrate binding site of FabI enoyl reductase and simulating enzyme's natural substrate. It was proven that in *Escherichia coli* and mutations in or overexpression of the gene *fabI* (encoding for enoyl reductase) prevents this blockage (28, 34). This has also been observed in *S. aureus* and *Mycobacteria*.

1.3.3 Quaternary ammonium compounds

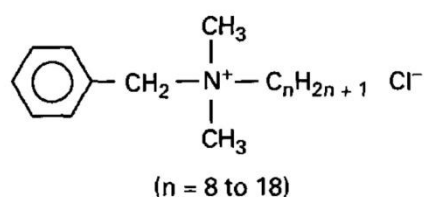
QACs are cationic surfactants (or "surface-active agents"), containing one quaternary nitrogen associated with at least one major hydrophobic substituent. The most common used are:

Benzalkonium chloride

Cetrimide

QACs are used for disinfection, antiseptis, preservation and cleaning (31). From an antimicrobial perspective, cationic surfactants, especially QACs, are the most commonly used.

1.3.3.1 Benzalkonium chloride



Benzalkonium chloride (BC) is a first-generation QAC. It consists of a mixture of alkyldimethylammonium chlorides and the length of alkyl

chains range between C₈ to C₁₈. It is used as household, industrial and health care general surface disinfectant, in high-level surgical instrument sterilizing and disinfecting solutions. Benzalkonium chloride can also be used as preservative (e.g. contact lens solutions and cosmetics at concentration of 0.001-0.01%) (36), fabric and laundry deodorization or softening.

Benzalkonium chloride provides a good cleaning and disinfection, being noncorrosive and nonstaining on surfaces. It has a “clean” odor and is regarded as nontoxic under typical conditions of use. At high concentrations it can be irritant to skin and mucous membranes (33).

At concentrations above 1 mg/ml, it has a broad-spectrum of activity, being bactericidal (Gram-positive bacteria are usually more susceptible than Gram-negative bacteria) and fungicidal. Formulations using benzalkonium chloride and other QACs have been used to improve their activity against viruses and mycobacteria. This compound is sporistatic, but cannot inhibit the actual germination process. The primary target of this biocide is the bacterial and fungal cell walls and membranes. It is easily absorbed causing the disruption of cell wall and its structure. When in contact with the membrane, benzalkonium chloride reacts with its lipids and proteins (including enzymes), leading to loss of structure and function and leakage of cytoplasmic material. It also degrades nucleic acids (33).

Table 2: Biocides chemical groups and characteristics.

Chemical groups	Active molecules	Applications	Spectrum of activity
Alcohols	Ethyl alcohol (ethanol) Isopropyl alcohol (isopropanol) Methyl alcohol (methanol) Benzyl alcohol Phenylethanol (phenylethyl alcohol) Bronopol (2-bromo-2-nitro-1,3-diol) Phenoxyethanol (phenoxetol) Chlorbutanol (chlorbutol) 2,4-Dichlorobenzyl alcohol	Antisepsis Disinfection Preservation	Broad-spectrum activity against vegetative bacteria (including mycobacteria), viruses and fungi. Not sporicidal, however inhibit sporulation and spore germination.
Aldehydes	Glutaraldehyde (pentanedial) Formaldehyde (methanol) Ortho-phthalaldehyde	Sterilization Disinfection Preservation	Virucidal, bactericidal, mycobactericidal, fungicidal and also sporicidal (at low concentrations sporistatic).
Anillides	Triclocarban Salicylanilide Diphenylureas (carbanilides) Tribromsalan	Antisepsis Preservation	Bacteriostatic and fungistatic agents; more effective against Gram-positive bacteria than Gram-negative.

Table 2: *Continued.*

Chemical groups	Active molecules	Applications	Spectrum of activity
Diamidines	Propamidine Dibromopropamidine	Antisepsis Preservation	Effective against bacteria, fungi and parasites; Gram-positive are more susceptible to them than Gram-negative and fungi.
Halogens and Halogen-releasing agents	Chlorine compounds Iodine compounds Bromide compounds	Antisepsis Disinfection	Broad-spectrum activity against bacteria, fungi and virus; some are effective against mycobacteria.
Metals	Silver Copper	Antisepsis Disinfection Preservation	Effective against bacteria, fungi and viruses
Peroxygens	Hydrogen peroxide Paracetic acid Ozone	Sterilization Disinfection Antisepsis Preservation	Broad-spectrum of activity against bacteria, viruses, mycobacteria, fungi and bacteria spores; more effective against Gram-positive than Gram-negative.

Adapted from (31, 33, 36 54, 60).

1.4 Biocide activity

1.4.1 Mechanisms of action

The use of biocides has increased in the past years in very different environments, such as health-care and veterinary facilities, food- and pharmaceutical industries and even in our homes. Biocides may act at one or several generalized targets within the cell, while antibiotics have a specific target. This non-specificity of biocides reduces their selectivity and negates their use in therapy (23). Some years ago, it was discovered a biocide with a single target site (triclosan) (34). Used concentrations of biocides are generally high, so they cause membrane disruption or inactivate a broad range of enzymes. Lower concentrations (like residual concentrations that may be left after cleaning and disinfection of surfaces) may act in the same way as antibiotics, specifically affecting one or two cell targets (23). Biocides vary greatly in their chemical structures, as already mentioned. The precise mechanism(s) of action may reflect this variety, although the final damage may seem quite similar (when high or lethal concentrations are used) (Table 3).

When biocides and a microorganism “meet together” a sequence of events occurs. First the biocide binds to the cell surface, then it penetrates the cell wall and membrane, thus entering the cytoplasm, where it interacts with cellular proteins or nucleic acids. The lethal effect is due to cellular damage cause by the biocide at some stage of this process (27). Mechanisms of action depend on the chemical nature of the biocide, the microorganism used in their evaluation and the test conditions (60). Some biocides are chemically reactive and bind covalently to proteins and peptidoglycan in cell wall; others are membrane-active and by physical interaction with the membrane components disrupt the structure of the cell membrane, releasing cell contents. Some act upon enzymes in the membrane or cytoplasm or upon nucleic acids.

The action of a biocide may be defined according to the bacterial structure which it is acting. Therefore, three levels of interaction can be described: a) interaction with outer cell components (cell wall); b) interaction with cytoplasmic membrane and c) interaction with cytoplasmic constituents (14, 27, 30).

1.4.1.1 Interaction with outer cell components

In general, Gram-negative bacteria are less susceptible to biocides than Gram-positive, due to their outer membrane. There are several compounds that act specifically against this barrier, such as chlorhexidine, QACs, phenols, chlorine-releasing agents, by increasing its permeability. Some of these compounds are thought to damage the cell wall and outer membrane, thus promoting their own uptake and being able to reach their target site(s) at cytoplasmic membrane or within the cytoplasm. Ethylenediaminetetraacetic acid (EDTA) binds to divalent cations, especially Mg^{2+} , releasing lipopolysaccharides molecules and thus provoking loss of the permeability function of the outer membrane. Some aldehydes are very reactive molecules and their cross-link to the cell wall can result in a bactericidal effect. Hypochlorite induces lysis in Gram-negative bacteria, by apparently affecting the cell wall. Phenol, formalin and mercuric chloride also affect this cellular structure (14, 27, 30, 31, 60).

Table 3. Biocides: targets and mode of action.

Biocide	Target and mechanism(s) of action
Alcohols	Penetrating agents that cause loss of cellular membrane function, releasing intracellular components, denaturing proteins and inhibiting DNA, RNA, protein and peptidoglycan synthesis
Aldehydes	Cross-linking agents that interact with the unprotonated amines in outer cell wall, leading to loss of cell wall function. Inhibition of DNA and RNA synthesis as result of cross-linkage of thiol, sulphhydryl and amino groups.
Anillides	Adsorb to and destroy the semipermeable character of the cytoplasmic membrane.
Biguanides	Membrane-active agent that damage cell wall and outer membrane, collapsing the membrane potential and intracellular leakage. Enhanced passive diffusion mediates further uptake, leading to cytosol coagulation.
Diamidines	Interfere with cell membrane and structure; precipitation of DNA, RNA and nucleoside-containing compounds.
Halogens and halogen-realising agents	Highly active oxidizing agents that destroy cellular activity of proteins. Disrupts oxidative phosphorylation and membrane-associated activities. Iodine reacts with cysteine and methionine thiol groups, nucleotides and fatty acid, leading to cell death.
Metals	Cause protein desnaturation, DNA degradation and cell wall disruption.
Peroxygens	Hydrogen peroxide produces hydroxyl free radicals that function as oxidants which react with lipids, proteins and DNA. Cell permeability is increased due to the targeted of sulphhydryl groups and double bonds.
Phenols	Increase cytoplasmic membrane permeability, resulting in progressive leakage of intracellular constituents. Permeability to protons leads to dissipation of proton motive force and uncoupling of oxidative phoshorylation, coagulation of cytoplasm and cell lysis. In the particular case of triclosan, it binds to enoyl-acyl carrier protein reductase, inhibiting fatty acid biosynthesis.
QACs	Damage cell wall and cytoplasmic membrane, by binding to phospholipids and provoking loss of structural integrity of cytoplasmatic membrane. Enhances further uptake and induces leakage of intracellular components and cell lysis.

Adapted from (30, 31, 33 60).

1.4.1.2 Interactions with cytoplasmic membrane

The so called “membrane active agents” is applied to biocides active at the cytoplasmic membrane level. These include phenols, biguanides, QACs, alcohols, parabens and polymyxins, and due to their different chemical structures, their effect against cytoplasmic membrane must be different. We can group the effects of these biocides in three different categories: cytoplasmic membrane, proton motive force (PMF) and enzymatic systems.

Cytoplasmic membrane. Disruption of the cytoplasmic membrane usually implies leakage of intracellular components. Phenols, cresols and their chlorinated derivatives induce leakage of intracellular materials in bacteria. Chlorhexidine initially causes a high rate of leakage of intracellular components, but in higher concentrations it coagulates the cytosol. Polyhexamethylene biguanide causes domain formation of the acidic phospholipid, causing cytoplasmic membrane disruption. QACs, as well as chlorhexidine, also induce the leakage of intracellular components, and it is believed that they combine with the membrane phospholipids, thus causing cytoplasmic membrane disruption. Anionic agents, organic acids and esters, may also induce leakage of intracellular components, by causing membrane disruption. Ethanol causes release of intracellular components and disorganizes the membranes (14, 27, 31, 30, 60).

Proton motive force. Several agents cause dissipation of the proton motive force (PMF), which is the way the energized state of bacterial membranes is expressed. PMF is composed of an electrical potential and a proton gradient that bacteria maintain across their cytoplasmic membrane to drive vital processes such as active transport of solutes, oxidative phosphorylation and ATP synthesis. The proton motive force is generated by oxidation-reduction reactions occurring during electron transport. Several different types of biocides affect the proton motive force across the membrane, namely lipid-soluble phenols, phenoxyethanol, organic

acids and esters, by accelerating the movement of protons, inhibiting the active uptake of amino and oxo acids, changing pH and denaturing proteins. TCS and other cationic compounds also discharge the membrane potential component (14, 27, 31, 30, 60).

Enzymatic systems. Some biocides interact with enzymes embedded in the cytoplasmic membrane. Ethanol inhibits enzymes involved in glycolysis, fatty acid and phospholipid synthesis and solute uptake, causing membrane disruption. Other compounds such as metals (copper and silver), interact with the thiol group of proteins, producing cell inactivation or inhibition. High concentrations of chlorhexidine inhibit membrane-bound adenosine triphosphatase. Triclosan (in low concentrations) has effect upon fatty acid biosynthesis, by targeting the enoyl-acyl carrier protein reductase (FabI) in *E. coli* and *Mycobacterium smegmatis*. (28, 27, 30, 34).

1.4.1.3 Interactions with the cytoplasmic constituents

Compounds like dyes and acridines combine with DNA, by intercalation of one molecule between two layers of base pairs, and can also block protein synthesis. Ethylene oxide and formaldehyde affect purine nucleosides and nucleic acids, as they interfere with amino, sulphhydryl and hydroxyl groups. Dyes, such ethidium bromide, bind to nucleic acids and precipitate them. Hydrogen peroxide can damage ribosome, although these are not its primary target site. It also forms hydroxyl radicals with oxidized thiol groups in proteins and enzymes. Ethylene oxide, propylene oxide and β -propiolactone react with amino, carboxyl, sulphhydryl and hydroxyl groups in bacterial proteins and nucleic acids. Aldehydes are very reactive compounds which combine with proteins and nucleic acids. Chlorhexidine, QACs, halogens, copper and silver ions, and hydrogen peroxide can cause cytoplasmic protein coagulation (14, 27, 30, 31, 60).

1.4.2 Factors influencing biocide activity

Generally speaking, several factors influence the activity of biocides: period of contact, nature, number, location and condition of microorganism (bacteria, spores, yeast and molds, protozoa) or entity (prions, viruses), temperature, formulation effects, presence of organic matter or other interfering or enhancing materials/compounds, concentration and pH (51, 54). Other important aspects to take into account when studying the activity of biocides are related to the pre-treatment, during and post-treatment factors (6, 55).

1.4.2.1 Pre-treatment factors

Pre-treatment factors are related to culture growth conditions. Bacterial cell walls are variable structures which change in response to growth environment. In a batch-grown culture, there are some aspects to take into consideration (6, 55):

- growth medium: may influence the subsequent sensitivity of cells to biocides;
- growth phase: latter phase cultures contain a high proportion of dead cells that will protect the viable ones;
- pH of culture medium: cell walls of bacteria may be different according to the pH in which they were grown and this may lead to variations in response to biocides;
- incubation temperature: different temperatures may lead to changes in phospholipid content or composition of spores, thus leading to different responses to biocides;
- presence/absence of oxygen: few information is available on this parameter.

When performing an experimental assay in order to study the susceptibility of a microorganism to biocides, it is important to take these

factors into account. The cultures must be always incubated in the same conditions and the growth medium used must be the same (even from the manufacture).

1.4.2.2 During treatment factors

Several factors may affect the activity of an antibacterial agent during treatment. Their effects maybe on the bacterial cell itself or may result from direct effects on the agents. Herein, will be described the most important ones to take into account under experimental conditions.

Concentration

This is a major factor in biocidal activity. The concentration exponent (n or η) measures the effect of concentration, or dilution, on the activity of the biocide. To determine η , it is necessary to measure the time required to produce a comparable degree of death of a bacterial suspension at two different concentrations of the biocide.

$$\eta = (\log t_2 - \log t_1) / (\log C_1 - \log C_2)$$

Where C_1 and C_2 represent the two concentrations and t_1 and t_2 the respective times to reduce the viable population to a similar degree. Biocides with high η -values (e.g. phenolic compounds and alcohols) are largely affected by changes in concentration (or dilution), whereas those with low η -value (e.g. QACs, chlorhexidine, glutaraldehyde) are less influenced by dilution. Concentration exponent: benzalkonium chloride 3.5; chlorhexidine 1.9; ethanol 4.5 and phenol 5.8 (15). Thus, concentration is quite important in clinical and other environments, where residues of biocides remain and must be considered and in order to determine accurately the lethal activity of a biocide. "Wrong" concentration may lead to biocide failure. (6, 55)

Temperature

The activity of a biocide usually increases when the temperature at which it is acting is increased. The effect of temperature can be calculated from the following formula:

$$\theta^{(T_2 - T_1)} = k_2 / k_1$$

Where k_2 and k_1 are the death rate constants at temperatures T_2 and T_1 , respectively. The temperature coefficient (θ) refers to the effect of temperature per 1 °C increments, and is nearly always between 1.0 and 1.5. Due to that, is more common to use θ^{10} (or Q_{10}) value, which is the change of activity per 10 °C rise. Some temperature coefficients (Q_{10}) are: 2.9 to 5.8 for benzalkonium chloride; 3 to 16 for chlorhexidine; 45 for ethanol and 5 for phenol (15). Biocides respond differently to temperature variations, and this is an important factor to take into account when performing experimental assays or any cleaning and disinfection procedure (6, 55).

Number of microorganism cells

The activity of a biocide decreases when the inoculum size increases. This is dependent of the type of microorganism used and is particularly important in the production of various types of pharmaceutical and cosmetic products (6, 55).

Type of microorganism

An ideal biocide should have broad-spectrum activity against both Gram-negative and Gram-positive bacteria, molds and yeasts. In practice, biocide activity varies greatly between different types of microorganisms and it might also differ between different strains of the same species. Generally, Gram-positive bacteria, due to the composition of their cell

envelope, are more sensitive to biocides than Gram-negative and prions are on the top of the list in what concerns biocide low susceptibility (Figure 1).

Bacteria can be surrounded by a capsule and secreted materials. These may play an important role in decrease susceptibility to biocides. The outer membrane of Gram-negative and the cell wall of mycobacteria act as permeability barriers and are responsible for intrinsic resistance of these bacteria to some biocides (6, 30, 55, 49).

Environmental pH

The biocidal activity is influenced by environmental pH, as changes in ionic status alter the interaction with the target groups on the microbial cell. Several biocides have an optimum pH range of activity. Glutaraldehyde and cationic biocides (like chlorhexidine and QACs) are most effective at alkaline pH, whereas hypochlorites and phenols are most potent at acid pH. Some biocides optimal pH values are: 4-10 for benzalkonium chloride; 5-8 for chlorhexidine; <7 for ethanol and phenol (15). Changes in environmental pH can also produce changes in charge distribution of bacterial cell surface, thus affecting the uptake of charged biocide. A pH value increment, increases the number of negatively charged groups on the bacterial cell surface, thus promoting the degree of binding of positively charged molecules (e.g. QACs) (6, 55).

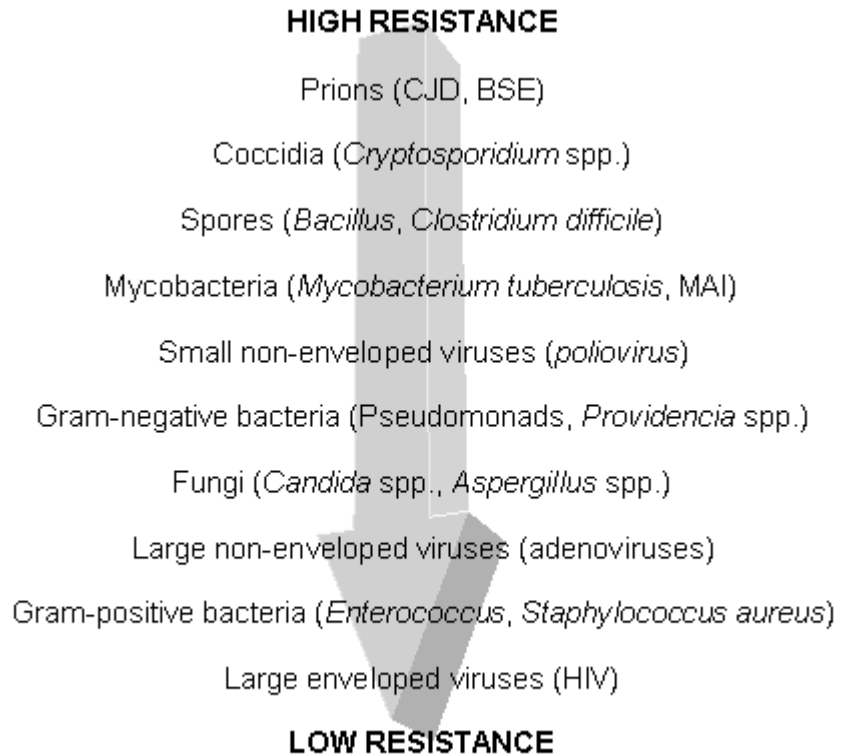


Figure 1. Classification of microorganisms according to their sensitivity to biocides. CJD – Creutzfeld-Jacob disease agent; BSE – bovine spongiform encephalopathy agent; MAI – *Mycobacterium avium intracellulare*; HIV – human immunodeficiency virus. Adapted from (30, 49).

Interfering substances

Organic matter (such as serum, blood, dirt, pus, food residues, etc) may interfere with the biocidal activity. This interference is usually due to reaction between the biocide and the organic matter, which reduces the biocide efficacy. Organic matter may also protect microorganism from the action of the biocide. In the food and dairy industries, organic matter may become a problem. A way to overcome this is by performing suitable pre-cleaning before using a disinfectant or by combination of a disinfectant with a detergent.

The presence of non-ionic agents can also reduce the activity of some biocides (e.g. QACs). Significant increases in concentration of the biocide are required in order to inhibit microorganism in the presence of these agents (6).

Humidity

Relative humidity has a great influence on the activity of gaseous disinfectants (e.g. formaldehyde). It is the most important factor influencing the activity of vapour-phase disinfectants (6, 55).

1.4.2.3 Pos-treatment factors

There are several factors that influence the recovery of microorganisms exposed to a biocide: composition and pH of recovery medium, removal of the biocide, temperature and period of incubation and composition of the diluents used for serial dilutions in viable cells counts (6).

1.5 Mechanisms of bacterial resistance to biocides

Bacterial response to biocides is usually dependent of its chemical nature and other factors already mentioned above. Mechanisms of bacterial resistance are divided in two main types: intrinsic and acquired. Intrinsic resistance is due to some inherent characteristic of the cells, while acquired resistance is obtained after a previous exposure of the sensitive cell to the antimicrobial agent (31, 47, 50).

1.5.1 Intrinsic resistance

Intrinsic resistance is defined as a natural (innate) chromosomally controlled property of a bacterial cell that enables it to circumvent the action of a biocide. It is more commonly found in Gram-negative bacteria,

mycobacteria and bacterial spores (46, 47). This intrinsic resistance has contributions from several cell compartments, namely the outer layers of the cell and lipopolysaccharides, acting as a permeability barrier; charge property of the cell surface, causing bacterial resistance to positively charge biocides; the presence of efflux pumps, which decreases the intracellular concentration of the biocides; and enzymatic transformations.

Gram-Positive bacteria such as enterococci, staphylococci and streptococci are generally more sensitive to biocides than Gram-negative bacteria. Enterococci are generally less sensitive to biocides than are staphylococci (31); although the exact mechanism remains unclear, but could involve reduced uptake of biocides. The outer membrane of Gram-negative bacteria acts as a barrier limiting or preventing the entry of several biocides, thus being responsible for confer intrinsic resistance. *Pseudomonas* are particularly resistant to some biocides (31). They tolerate high concentrations of QACs, chlorhexidine and triclosan. These bacteria have differences in the lipopolysaccharide composition and in the cationic content of the outer membrane. Members of the genus *Proteus* have high resistance to chlorhexidine, QACs, EDTA and diamides due to the presence of a less acidic type of outer membrane lipopolysaccharides (31).

1.5.1.1 Physiological (phenotypic) adaptation

Biofilms

Biofilms are defined as a group of microorganisms attached to a surface and organized within an extensive exopolysaccharide exopolymer (47). Biofilms can be communities of several different species, or mixed phenotypes of the same organism. Its formation is a multistage process in which microbial cells adhere to the surface (initial reversible attachment), while the subsequent production of an extracellular matrix (containing polysaccharides, proteins and DNA) results in a firmer attachment (61).

Bacteria residing in biofilms are 10- to 100-fold more resistant to biocides than planktonic bacteria. There are several reasons that may justify this: (i) difficult access of the biocide to the inner part of the biofilm; (ii) different physiology of bacteria at different parts of the biofilm due to different nutritional conditions; and consequently (iii) different growth rates within the depths of the biofilm (iv) chemical interaction between the biocide and the biofilm itself; (v) modulation of the microenvironment; (vi) production of degradative enzymes (and neutralizing chemicals); and (vii) genetic exchange between cells in a biofilm. However, biofilms can change according to the microorganism and biocide in question. Bacteria removed from a biofilm and recultured in a culture media, are generally as susceptible as the “normal” planktonic cells of that species (31, 47).

Biofilm formation and consequent decreased susceptibility to biocides, has important implication in clinical environment (biofilm formation in catheters, artificial joints and other medical devices) leading to patient infections, and in several industries (biofilm formation in surfaces of storage tanks, pipelines, filtrations systems and other machineries), leading to product contamination.

Biofilms provide an important example of how physiological adaptation can play a role in conferring intrinsic resistance.

1.5.2 Acquired resistance

Acquired resistance results from genetic changes in a cell and arises either by mutation or by the acquisition of genetic material (plasmids, transposons) from another cell (47). Acquired, non-plasmid-encoded resistance can appear when bacteria are exposed to a gradually increasing concentration of a biocide. Such resistance is usually unstable and may be lost if cells are grown in a biocide-free medium.

1.5.2.1 Plasmid-mediated resistance to biocides

There are some possible plasmid-mediated resistance mechanisms to biocides.

Inactivation

Biocide inactivated as result of an enzymatic modification. The most common example of biocide inactivation involves mercury and other organomercury compounds. Although, mercurials are no longer used as disinfectants, phenylmercuric salts and thiomersal are still used in some types of pharmaceutical products. The mechanism of resistance to mercury involves detoxifying reductase and hydrolase enzymes. Resistance to formaldehyde has also been reported in some bacteria as associated with formaldehyde dehydrogenase (provoking aldehyde degradation) (47, 48).

Impermeability and cell surface alterations

Biocide uptake and accumulation is reduced in case of impermeability and cell surface alterations. One example is the plasmid-mediated resistance to silver salts (still used as topical antimicrobial agents). Although, it is not totally understood, it is believed that the mechanism is by reducing the accumulation rather than silver reduction. This reduced uptake as also been studied for QACs and chlorhexidine, but results are not conclusive. There are some reports of plasmid-encoded changes in the outer membrane proteins of some Gram-negative bacteria leading to reduced susceptibility to formaldehyde and other industrial biocides (31, 48).

Efflux

Efflux or extrusion of cytotoxic drugs from the cell via membrane proteins is one of the most common employed resistance strategies in

biological systems. Those proteins act like bilge pumps and decrease the intracellular concentration of the drug. In prokaryotic organism the drug efflux process is largely conferred by pumps in which the drug extrusion is coupled to the influx of a proton (H^+). The pumps can be categorized into several families: major facilitator (MF) superfamily, the small multidrug resistance (SMR) family, resistance/nodulation/cell division (RND) family, and drug/metabolite efflux (DME) family. In other pumps, the drug efflux is coupled to Na^+ influx – multidrug and toxic compound extrusion (MATE) family; or gets energy from the hydrolysis of ATP – ATP-binding cassette (ABC) family (7, 29, 43).

Staphylococci are one of the most well studied bacteria concerning the genetic aspect of plasmid-mediated biocide resistance mechanisms. In these bacteria, known biocide exporters are members of the MF and SMR super families and include several determinants, namely: *qacA*, which encodes resistance to QACs, acridine, ethidium bromide and low-level resistance to chlorhexidine, *qacB*, which is similar but specifies resistance to QACs and intercalating dyes, *qacC* and *qacD*, which specify resistance to QACs and low-level resistance to ethidium bromide (47, 48). Biocide exporters in Gram-negative bacteria are in general chromosomally encoded, with the exception of *qacE*, *qacE Δ 1* and *qacF* genes (acting against compounds such as QACs and ethidium bromide) (10, 44).

1.5.3 Possible link between biocide use and antibiotic resistance

In the last decades, we have witnessed a huge increase in the use of biocides. Their generalised use and sometimes misuse is causing some concern if they are creating cross-resistance with antibiotics. It is quite difficult to get a clear response to this, as the data available focus on specific bacteria or specific compound. Moreover, there is always a difference between what happens *in vitro* and *in vivo*. Biocides usually

have multiple target sites and the mechanism of resistance to them is often concentration dependent. At high concentrations, multiple structural and metabolic targets are involved, but at low concentrations fewer targets are affected. In contrast, antibiotics usually have distinct structural and/or metabolic targets.

When bacteria are killed by biocides at in use concentrations, several target sites are involved (Figure 2a)). If very low concentrations of biocide are used, they may work as a nutrient. This can be useful in case the objective is biodegradation of the biocide. As concentration increases, bacteria will be affected in several targets and killed by various ways. This means, that at in use concentration, in order to become resistant, several different mechanisms would have to be involved and multiple targets resistance is unlikely to occur. If the biocide is diluted, as its concentration gets lower than its in use concentration, the susceptible targets will also be reduced (Figure 2b)). This may happen when a surface is cleaned or disinfected, as there will be areas in which bacteria will be exposed to sub inhibitory concentrations of biocide (22). This also happens in biofilms, as already mention. A gradient of concentrations is created and at some point there will probably be a selection pressure on a single target. If this single target happens to be shared with another antimicrobial agent, such as an antibiotic, cross resistance may occur.

Antibiotic resistance does not rely only on target change, efflux mechanisms are also involved. Efflux pumps can be induced when bacteria are exposed to sub-lethal concentrations of antibiotics (such as tetracycline), but also to some biocides such as QACs and triclosan (22). When bacteria are exposed to low concentration of these agents, cells that are able to pump out such compounds will be selected. For biocides, this will not influence treatment outcome (as high concentrations are usually applied), but the use of these compounds may be selecting antibiotic resistant bacteria. Cationic biocides, such as biguanides and

QACs, get into bacterial cells by the so called “self-promoted uptake”, the same mechanism of cell entry of aminoglycoside antibiotics. Changes in the permeability of the cell wall also occur in the presence of some antibiotics and biocides. Therefore, adaptations against these agents may lead to cross-resistance. Filament formation induced in Gram-negative bacteria by some antibiotics (e.g. β -lactams and fluoroquinolones) is also induced by biocides (such as acridines, phenoxyethanol and phenylethyl alcohol). The autolysis provoked by low concentrations of phenolics and inorganic and organic mercury is said to be also induced by penicillin (53). There is also possibility of genetic linkage between genes for biocide resistance and antibiotic resistance when biocide and antibiotic resistance genes are part of the same genetic element (44).

Biocides play an important role in our society. They are essential in preventing and controlling infection in several environments and it is important to take into account that their use creates a certain selective pressure. Some actions may be taken in order to try to avoid cross-resistance between biocides and antibiotics. Limit biocide usage to applications where there is demonstrable gain and a proven need for hygiene and prefer those that are more rapidly neutralized at point of use; biocides with low chemical reactivity, such as QACs and triclosan, are more difficult to be neutralized. Rotation of disinfectants in hospital and some industries (pharmaceutical and food) has been adopted in order to prevent the development of bacterial resistance. Ideally, one biocide should be replaced by another having a different mode of action.

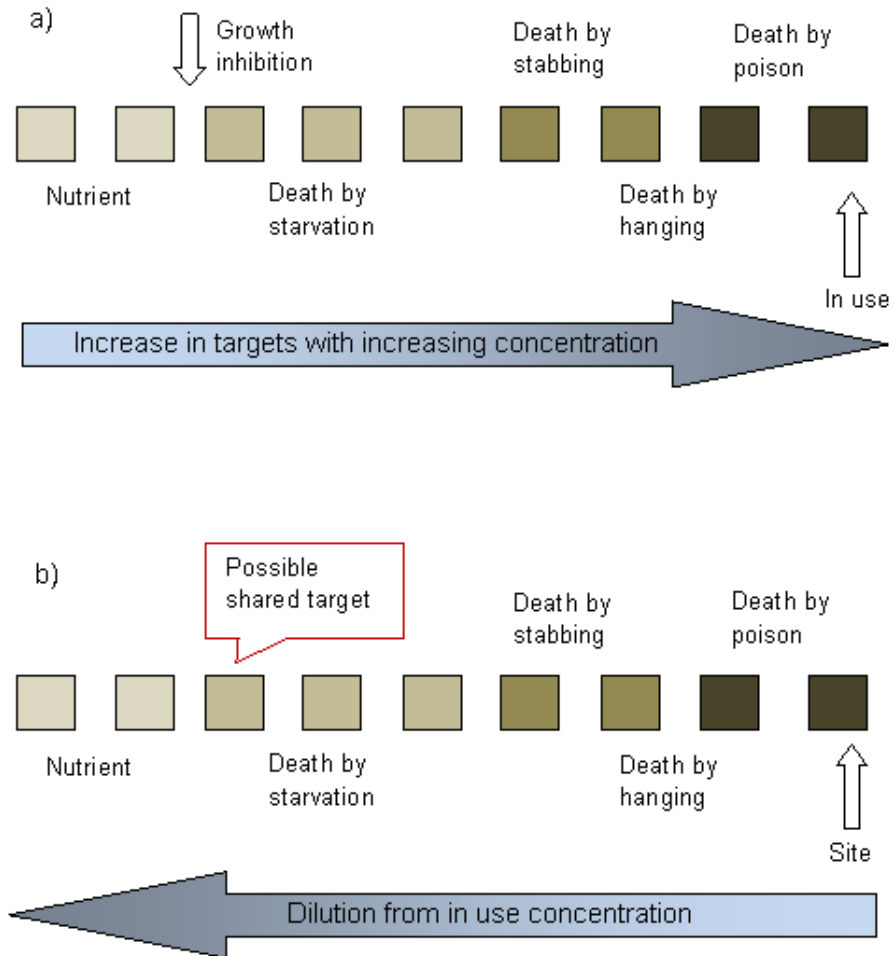


Figure 2. Each box represents a different target site. a) biocide mechanism: at in use concentration all targets are involved (multiplicity of target site), resistance is unlikely to occur. b) biocide dilution: a gradient of concentration is created, reduction in the number of target sites; at a certain point there will be selection of a single target, that may be shared with other antimicrobial agent. Adapted from (22).

1.5.4 Some resistance and cross-resistance described

Biocide resistance was first recognized in 1936 by Heathman *et al.* (18) who identified *Salmonella* Typhi resistant to chlorine. In 1952, the isolation of *Serratia marcescens* resistant to QACs was reported by Chaplin (9). Dance *et al.* (13) reported the outbreak of *Proteus mirabilis* resistant to chlorhexidine in a general hospital in the United Kingdom. These strains were also resistant to some antibiotics, like gentamicin, trimethoprim and ampicillin.

Some of the outbreaks described are related to contaminated antiseptics and disinfectants. These outbreaks usually occurred due to Gram-negative bacteria and mycobacteria. Some of outbreaks reported were related to chlorhexidine; most of them related to contaminated water used to prepared the solutions and/or reuse of bottles to dispense the biocide without adequate disinfection. There are also reports of outbreaks related to the use of QACs, especially benzalkonium chloride. Many of these were linked to the storage of this biocide with cotton or gauze, and others with improper dilution of its solution. Few outbreak reports are related to triclosan, but one was due to intrinsic contamination of the antiseptic soap. *Pseudomonas*, *Burkholderia* and *Ralstonia* were the genera usually involved in these outbreaks (63).

Triclosan, as already mentioned, inhibits the growth of some bacteria by targeting an enoyl reductase enzyme (*FabI* gene) in *E. coli*; for *Mycobacteria*, this enzyme is also the major target for the anti-tubercular drug isoniazid, hexachlorophene and some new antibiotics, the diazaborines. The high degree of homology between the enoyl reductase of *E.coli* and *S. aureus* might have implications in a future emergence of triclosan-resistant staphylococci; mutations in the *FabI* gene of staphylococci can confer triclosan resistance (25). Triclosan also cause expression of efflux pumps common to some antibiotics. Although the connection between triclosan and antibiotics is clearly established, what

happens in the real world is still unknown, namely how triclosan affects the microbial flora in all environments in which it is used. The widespread of this biocide, could lead to environments where *Pseudomonas* and other triclosan-resistant bacteria may prevail (58). Another mechanism for triclosan resistance occurs through multidrug efflux pumps in *E. coli* (AcrAB) and *P. aeruginosa* (Mex proteins); these pumps confer both resistance to triclosan and antibiotics.

Resistance to QACs, chlorhexidine, acridines and diamidines has been observed in staphylococcal strains, *Pseudomonas* spp. and Enterobacteriaceae. The resistance is encoded by several multidrug resistance determinants, of which *qacA*, *B*, *C*, *D* and *E* genes are included (10, 18, 48). These multidrug efflux pumps can efflux a variety of antibiotics (trimethoprim, sulphonamids, oxacillin and aminoglycosides) and biocides, as well as dyes such as ethidium bromide (18). Having in mind that several *S. aureus* are methicillin-resistant (MRSA), this could become a problem, especially in the hospital environment. Significant difference between the efficacy of these agents against MRSA and methicillin-susceptible *S. aureus* (MSSA) has already been described/found (31).

Enterococci are the second most frequently reported cause of surgical wound and nosocomial urinary tract infections and the third most frequently reported cause of bacteraemia (39). Little is known about their resistance to biocides.

2. The genus *Enterococcus*

The name “entérocoque” was first used by Thiercelin in 1899, to emphasize the intestinal origin of a new Gram-positive diplococcus. In

1906 Andrewes and Horder classified an organism isolated from a patient with endocarditis as *Streptococcus faecalis*; as it was very similar to other strains isolated from human intestine (19, 37). In 1933, Lancefield developed a serological typing system for streptococci, those of “faecal origin” had the group D antigen. Later Sherman proposed a classification scheme which separated streptococcus into four divisions: pyrogenic, viridians, lactic and enterococcus. The latter was used for those organisms able to grow at temperatures from 10 to 45 °C and most of them can survive for 30 min at 60 °C, in the presence of 6.5 % of NaCl and at pH 9.6 and are able to hydrolyze esculin. The enterococcus group included *S. faecalis*, *S. faecium*, *S. bovis* and *S. equinus*; *Streptococcus durans* was accepted either as a separate species or as a subspecies of *S. faecium*. As years passed by, more species were isolated, such as *S. avium*, *S. casseliflavus* and *S. gallinarum*. The use of nucleic acid relatedness helped clarified and expanded the classification of enterococci. In 1984, Schleifer and Kilpper-Bälz showed, by DNA-DNA and DNA-rRNA hybridization studies, that *S. faecalis* and *S. faecium* were distantly related to streptococci that they should be transferred to a new genus, the *Enterococcus*. There are more than 30 different species included in this genus (19, 37).

Enterococci are robust Gram-positive, catalase-negative, facultative anaerobic bacteria. They are ovoid in shape and grow in short chains, in pairs or single cells. They are able to grow in the presence of 40 % of bile salts (esculin hydrolyzation) at pH from 4.6 to 9.6 (37, 39). Due to these characteristics, they are able to grow/survive in many different environments, such as humans and animal intestinal tract, sand, plants, water, hospitals, food industry, cheese, meat and even in insects. In the human and other animal intestine, the most common species found are *E. faecalis* and *E. faecium*. These species are also found in plants and invertebrates, as well as *E. mundtii* and *E. casseliflavus* (16).

Since the beginning of the last century, enterococci have been recognized as opportunistic human pathogens. The earliest reports associating human diseases with enterococci were published in the beginning of the 20th century. Probably, many of these strains were not truly enterococci or could have been contaminated. As already mentioned, enterococci are the second most frequently cause of surgical wound and nosocomial urinary tract infections and the third most frequently cause of bacteraemia. Enterococci can also cause neonatal infections, central nervous system infections and intraabdominal and pelvic infections (39). They can also cause opportunistic infections in animals, such as septicaemia in chickens, bovine mastitis, endocarditis in cattle and lambs, and urinary-tract infections in dogs and cats (45). Enterococcal infections are predominantly caused by *E. faecalis* followed by *E. faecium*, *E. durans*, *E. gallinarum*, *E. casseliflavus* and *E. raffinosus* have also been responsible for infections (39).

In enterococcal nosocomial infections, it is hard to know if the microorganism comes from the patient's own microbiota or if it was acquired during hospitalization. The increased number of infections caused by these bacteria may be a consequence of use, abuse and misuse of antibiotics in the last decades. Enterococci have intrinsic resistance to some antibiotics (such as certain β -lactams, clindamycin, some aminoglycosides) and acquired resistance to others (such as chloramphenicol, erythromycin, tetracycline, high-level aminoglycoside and vancomycin) (37, 39). Vancomycin resistant enterococci (VRE) are of great concern as this antibiotic is used as one of the last resort treatment for multiple antibiotic resistant enterococci. VRE strains are also a cause of concern in the veterinary environment, more specifically in farm animals (1). The use of avoparcin, a glycopeptide antibiotic, is pointed as the cause of appearance of VRE strains in farms. The use of that antibiotic has been banished in all European countries in 1997 (64). However, VRE

strains are still isolated from farm animals and from the environment (8, 21, 26, 35). Enterococci also have virulence factors which are involved in attachment both to host cells and to extracellular matrix proteins (AS – aggregation substance; Esp – Enterococcal surface protein; EfaA – *E. faecalis* antigen A), in resistance to macrophages (AS; HypR – hydrogen peroxide regulator), in cell and tissue damage (Cyl - cytolysin; GelE – gelatinase; SprE – serine protease) and in immune system evasion (capsular polysaccharides) (39). Some of these virulence factors are plasmid encoded which can be transferred between enterococci.

Enterococci are not just “problematic” bacteria. They play an important role in the food industry as fermented foods and as probiotics. They are much used in several Southern European countries (Portugal included) to produce cheeses, contributing to the final organoleptic properties of the product. Enterococci have a positive influence in the traditional cheese ripening and the ability to produce bacteriocins (especially *E. faecalis* and *E. faecium*) against some bacteria (such as *Listeria monocytogenes*, *S. aureus*, *Clostridium botulium* and *Clostridium perfringens*) (11, 12). These technological and metabolic characteristics turn enterococci into good starter cultures for the cheese industry. Enterococci can also be used as probiotic for humans and farm animals. However, their use as such is still controversial, due to risk of transference of antibiotic resistance and virulence genes to human strains (19, 20, 42, 59).

3. Enterococci and biocides

The relationship between enterococci and biocides is not very well established. As mentioned above, enterococci can be found in different environments which are cleaned or disinfected with biocides. One of the most important environments is the clinical environment, where

enterococci play a critical role due to their ability to cause nosocomial infections. This environment is the most frequently studied and usually the susceptibility of enterococci is evaluated by measuring Minimal Inhibitory Concentration (MIC) or Minimal Bactericidal Concentration (MBC), either using surface carrier tests and/or suspension tests. The presence of enterococci in the human mouth and their ability to survive after some endodontic treatments is also well documented. Enterococci together with lactobacilli, actinomyces and streptococcus, constitute the best invaders of dentine and root canals. *E. faecalis* has been recovered from tooth canals when endodontic treatments failed. Several studies were performed on this area testing the efficacy of the most commonly used biocides in endodontic treatments (such as chlorhexidine, calcium hydroxide and sodium hypochlorite) against enterococci (3, 24, 40). Concentrations of 2 % for chlorhexidine and 5.25 % for sodium hypochlorite were able to eliminate enterococci. Although some studies have been performed in order to understand the susceptibility of enterococci to biocides, the way they respond to these compounds and which mechanisms are involved are a question that still need to be addressed.

3.1 Thesis outline

The main issue of this thesis was to understand the susceptibility of enterococci to three different biocides and if there was any relationship between that and resistance to vancomycin. Bacteria from this genus are able to grow in different environments where biocides are daily used; the most important are the clinical environment, the veterinary environment and the food industry environment. Isolates from these habitats were studied and characterized as well as new isolates from a never explored environment. Another issue was to open the door of which mechanisms enterococci use to respond to biocides: identification of a gene implicated

in the susceptibility to benzalkonium chloride and its dissemination through enterococci of the three different environments above mention.

E. faecalis V583 was the first enterococcal strain fully sequenced (41) and its EFA0010 was described as a putative multidrug resistance protein. The specific role of this gene was not clarified and to which drugs it confers “resistance” to was an open question. In chapter II it is discussed the role of EFA0010 in the susceptibility to benzalkonium chloride and the distribution of this gene in enterococci from different origins (clinical, veterinary and dairy isolates), as well as the susceptibility pattern of the same isolates to the two different biocides benzalkonium chloride and chlorhexidine.

One of the main questions concerning biocides and enterococci is if biocides are capable of eliminating VRE strains, which are responsible for many nosocomial infections, and if VRE strains are less susceptible to biocides than vancomycin susceptible enterococci (VSE) strains. VRE strains are capable of persisting on surfaces and hands, thus being easily transmitted and moved from one place to another (38). Therefore an appropriated cleaning and disinfection are required. There are some research works done in this field, usually comparing the MIC of VRE strains with VSE strains or testing the time needed to kill each with a certain biocide (2, 62). The conclusions are quite similar, and VRE are generally as susceptible to biocides as VSE. However, constant surveillance and more research are pointed as required. The same isolates discussed in chapter II were used to understand if their susceptibility/resistance to vancomycin was somehow related to their susceptibility pattern to the same biocides; this was investigated in chapter IV. Two isogenic strains (one VRE and the other VSE) were used in several assays, MIC, continuous exposure to biocides, growth curves in

the presence of biocides and fitness assays, in order to understand if one took advantage to the other under the tested conditions.

As previously mentioned, enterococci also play an important role in the veterinary environment, causing animal diseases and threatening biosecurity by entering into the human food chain. Animal facilities and instruments are cleaned and disinfected using biocides (e.g. chlorhexidine and benzalkonium chloride), but few or nothing is known about how these compounds are affecting or selecting bacteria present in these environments. Chapter III presents a complete study of enterococci isolated from the first time from breeding pig facilities dust. The isolates were identified to species level, characterized for their susceptibility to benzalkonium chloride and chlorhexidine. The presence of VRE isolates and their identification and characterization was investigated, as well as their susceptibility to the same biocides. Understand if there was any association between their presence and the use of these compounds was also discussed.

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Involvement, and dissemination, of the enterococcal small multidrug resistance transporter QacZ in resistance to quaternary ammonium compounds

Chapter II

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Teresa M. Braga, Paulo E. Marujo, Constança Pomba, M. Fátima Silva Lopes.

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The experimental work was performed by the author of this thesis.

Summary

The aim of this work was to investigate the role of a putative small multidrug-resistance transporter, annotated in *Enterococcus faecalis* V583 genome as EFA0010 (we will refer to this gene as *qacZ*), in decreased susceptibility to biocides. A derivative strain of V583, susceptible to erythromycin (V583ErmS), was complemented with pORI23 carrying *qacZ* gene (strain EF-SAVE1). MIC values for benzalkonium chloride, chlorhexidine and ethidium bromide were determined for the complemented strain and wild type. RT-PCR and ethidium bromide efflux assays were performed in order to fully understand the role and specificity of *qacZ* gene. The presence of *qacZ* in 73 enterococcal strains from different origins was investigated by PCR and MIC values for benzalkonium chloride and chlorhexidine were determined in the same strains. The complemented strain, EF-SAVE1, presented higher MIC value for benzalkonium chloride (8 µg/ml) than V583ErmS (4 µg/ml); for chlorhexidine and ethidium bromide the MIC values were the same in both cases, 4 µg/ml and 16 µg/ml respectively. Expression of *qacZ* was found to be higher in EF-SAVE1 strain and constitutive, i.e. not inducible by any of the three tested biocides. Overexpression of *qacZ* was not responsible for changes in ethidium bromide efflux. This gene was present in 52% of the enterococcal isolates studied and the MICs for benzalkonium chloride and chlorhexidine ranged between 2 to 8 µg/ml. Tolerance to biocides was also determined in *E. faecalis* V583, the V583ErmS wild type strain, from which V583ErmS was derived. The role of the *ermB* gene in the *E. faecalis* V583 cells was discussed.

We demonstrate the involvement of *qacZ* gene in tolerance towards the quaternary ammonium compound benzalkonium chloride, but not ethidium bromide. This work constitutes the first report of a biocide resistance

mechanism in *E. faecalis*, and reveals its dissemination amongst the genus *Enterococcus*.

Introduction

In the past few decades, the use of biocides as antiseptics, disinfectants and preservatives has been growing, not only in the hospital environment (sterilization of medical devices, disinfection of surfaces and water, skin antiseptics and preservation of various formulations) (11), but also in the food industry and more recently in our homes (to clean surfaces and hands, in oral and dental hygiene, dishwashing and more recently to disinfect food) (19). Introduction of biocides into clinical and other practice, has been suggested to contribute to selection of antibiotic-resistant bacteria, and may lead to decrease the number of solutions to control bacterial load in Hospital settings, in food industry and even at home (6). Taken together, the need to find the mechanisms responsible for biocide resistance in bacteria causing severe infections, namely in bacteria that can be found in different environments and, therefore, subject to biocidal action in these same environments, is obvious and urgent. Enterococci are Gram-positive commensal bacteria which inhabit the gastrointestinal tract of humans and animals. Their robust nature allows them to disseminate in a variety of other habitats, namely fermented foods, water, soil and plants. Members of this genus have intrinsic and acquired resistance to several antibiotics and thus became the second most frequently reported cause of surgical wound and nosocomial urinary tract infections and the third most frequently reported cause of bacteraemia (12). However, and despite their relevance as nosocomial multiresistant pathogens, there are no tolerance mechanisms to biocides described so far for the genus *Enterococcus*. Multidrug

resistance transporters are responsible for both antibiotic and biocide resistance in bacteria. Among secondary multidrug resistance transporters, which use proton electrochemical gradients as the driving force, the small multidrug resistance (SMR) transporter family has the simplest organization. Among *Staphylococcus* isolates from both clinical and food sources *qac* genes, encoding small multidrug resistance (SMR) transporters, mediate resistance to quaternary ammonium compounds (QAC) (15, 16). pTEF1 plasmid of *Enterococcus faecalis* V583 carries an orf, EFA0010, annotated as a putative SMR transporter. In this work we provide evidence which supports this activity of EFA0010 and we will thus refer to it as *qacZ* gene. For practical reasons, we used *E. faecalis* V583ErmS (a derivative of *E. faecalis* V583, susceptible to erythromycin due to deletion of the *ermB* gene (EFA0007), kindly supplied by Axel Hartke from Caen, France), as the plasmid used in the gene cloning carried resistance to erythromycin. During the course of this study, V583 and V583ErmS were also compared in what respects biocide susceptibility.

Materials and Methods

Bacterial strains

A total of 73 enterococcal isolates were randomly selected from the Lab collection. All these isolates were already identified to the species level and typed by PFGE. None of the selected 73 isolates are clones. Our selection focused on three different environmental sources, namely Portuguese milk and cheese (27 dairy isolates), nosocomial infections (30 clinical isolates) (genito-urinary tract infections, bacteraemia/sepsis, catheter-associated infections), and animal infections (16 veterinary

isolates) (cats and dogs isolates from urinary tract and skin and soft tissue infections).

E. faecalis V583, *E. faecalis* V583ErmS, and EF-SAVE1 (a derivative of V583ErmS carrying the high-copy number plasmid pSAVE1, constructed in the present study).

Biocides

Three different biocides were used in this study: benzalkonium chloride (Sigma-Aldrich) (BC), chlorhexidine (Fluka) (CHX) and ethidium bromide (Sigma-Aldrich) (EtBr).

Susceptibility tests

Susceptibility to benzalkonium chloride, chlorhexidine and ethidium bromide, a QAC, a biguanidine and a dye, respectively, was measured by Minimal Inhibitory Concentration (MIC) assays (performed according to CLSI) (4). Strains were grown overnight in Brain Heart Infusion (BHI) broth from Oxoid and plated in BHI agar. Colonies were picked and incubated overnight in Muller-Hinton (MH) broth, also from Oxoid. The inoculum density was adjusted to 0.5 McFarland in fresh MH broth and diluted 1:100. On a 96 wells microplate, 100 µl of each bacterial culture diluted 1:100 were added to 100 µl solution of each compound (range from 0.25-256 µg/ml). Results were read after 24h. All incubations were performed at 37°C. Assays were repeated at least three times.

Dissemination of *qacZ* gene in *Enterococcus* population by PCR

The dissemination of *qacZ* in the 73 enterococcal isolates was addressed using PCR (primers *qac*-IF ATATTGCTTACGCAAGTTGG and *qac*-IR AGTGTGATGATCCGAATGTG); amplicon of 156 bp. The PCR consisted of 35 cycles of denaturation (94 °C, 30 s), annealing (51 °C, 30

s) and extension (72 °C, 30 s). The reaction was performed using MasterMix (5 Prime).

qacZ gene cloning

The gene was amplified using *qac* - EF and *qac* - ER primers from *E. faecalis* V583, GAATCGGATCCATCACTTAATAAAGGAGC and GAATCCTGCAGATACACTAAACAAAGTGGGC, respectively; *qac*-EF has a restriction site for *Bam*HI and *qac*-ER for *Pst*I, both underlined. The PCR product (480bp) obtained contains the *qacZ* ribosome binding site (RBS), but excludes the gene promoters. The reaction consisted in 35 cycles of denaturation (94 °C, 30 s), annealing (59 °C, 30 s) and extension (68 °C, 1 min), and was performed with Expand High Fidelity^{PLUS} PCR System (Roche). The amplicon and the pORI23 plasmid (14) were digested with *Bam*HI and *Pst*I enzymes (New England Biolabs) and ligated with T4 DNA ligase (New England Biolabs). The ligation product was introduced into the electrocompetent *E. coli* DH5 α cells. The transformants were selected on Luria-Bertani agar (LBA) (Sigma-Aldrich) containing 500 μ g/ml erythromycin (Sigma-Aldrich). Proper *qacZ* gene placement in the recombinant plasmid was (pSAVE1) confirmed by sequencing (Stabvida).

Complementation of the *qacZ* in *E. faecalis*

pSAVE1 was introduced into electrocompetent *E. faecalis* V583ErmS cells (prepared according to Frankenberg et al (8)). Transformants, named EF-SAVE1, were selected on Brain Heart Infusion agar (BHIA) (Oxoid) containing 500 μ g/ml erythromycin and confirmed by specific PCR using *pori* - F (GGATTGGATTAGTTCTTGTGG) and *pori* - R (TTGAGTGAGCTGATACCGC) primers. Size of the amplicon for “empty” plasmid was 408 bp and for pSAVE1 was 888 bp. The reaction consisted in 35 cycles of denaturation (94 °C, 30 s), annealing (51 °C, 30 s) and

extension (72 °C, 1 min). Plasmid pORI23, without any insertion, was introduced into V583ErmS cells and transformants (EF-SAVE2) were used as a control for expression assays by RT-PCR.

RNA extraction and cDNA synthesis

Total RNA was extracted from V583ErmS, EF-SAVE1 and EF-SAVE2 cells. Two different assays were performed: i) strains were grown in BHI added of 1 µg/ml BC, 2 and 4 µg/ml of EtBr until OD₆₀₀ 0.4 was reached (control was performed the same way, but only in BHI); ii) strains were grown in BHI until a 0.4 OD₆₀₀ was reached, at that time the biocides were added (for BC the concentrations used were 0.25, 1, 4 and 8 µg/ml; for EtBr the concentrations used were 2 and 8 µg/ml). In the first assay total RNA was extracted when the OD₆₀₀ 0.4 was reached and in the second assay 15 min after the addition of the biocides. Three ml of each culture were used and mixed with 6 ml of RNA Protect Bacteria Reagent (QIAGEN); this mixture was vortexed for 5 min and left 5 min at room temperature. It was then centrifugated for 10 min at 3500 r.p.m. at 4 °C and pellets were kept at 4°C until RNA extraction. Total RNA was then extracted following the RNeasy Mini kit (QIAGEN). RNA quality and quantity was determined by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm, respectively. Twenty-eight µl of total RNA from each sample was treated with 3.5 µl of RNase-free DNase I (Roche) and 3.5 µl of 10x DNase I buffer (Roche) and incubated at 37°C for 1 hour. Samples were further purified using RNeasy MinElute Cleanup kit (Qiagen) and quantified using the NanoDrop Spectrophotometer (Nanodrop Technologies). Prior to PCR reactions DNA contamination of the RNA samples was assessed by a standard PCR reaction with primers targeting a plasmidic *E. faecalis* V583 gene (EFA0014). The primers used were EFA0014-F (TTACTATTGGACAGCTTATCC) and EFA0014-R (TGCACTAAGGTTAATTAGGAC); the amplicon had 638 bp and the

reaction consisted in 35 cycles of denaturation (94 °C, 30 s), annealing (54 °C, 1min) and extension (72 °C, 2 min). The concentration of total RNA used for this PCR was around 500 to 1000 ng/μl.

cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Sample (Roche) according to the manufacturer's instructions, using 1.5 μg of RNA as template (the ideal concentration is 4 μg, but due to low concentrations of RNA obtained it was not possible to use such quantity) and random hexamer primer. Each sample was divided in two, one used as control with no addition of high fidelity reverse transcriptase (water was used instead) and the other with addition of the enzyme.

Reverse transcriptase PCR (RT-PCR)

Semi-quantitative RT-PCR was done from serial cDNA dilutions (1; 1:10 and 1:100), assuming that intensity of PCR product bands correlates to the amount of PCR product, and thus to the amount of template mRNA. Primers used for PCR reactions were qac-IF and qac-IR and the reaction was done as above mentioned for these primers. 23S rRNA was used as a control; primers 23S-F (AAAGAAATCAACCGAGATTC CCC) and 23S-R (AAACAAGTGCTCTACCCCGG) with an amplicon of 694 bp and the reaction consisted in 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 5 min).

EtBr efflux assays

EtBr efflux assays were performed according to Jonas et al. (9). Cells from overnight cultures of V583, V583ErmS and EF-SAVE1 were washed two times with 20mM HEPES buffer (Sigma-Aldrich) by centrifugation at 5000 rpm for 20 min and resuspended in 5 ml of the same buffer. After that, cells were loaded with EtBr (final concentration

2.5 μM) by shaking at 37°C and adding carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) (Sigma-Aldrich) at 40 μM (final concentration). Cells were incubated for one hour and after that they were washed three times with HEPES buffer containing EtBr (2.5 μM) and resuspended in 3 ml of the same buffer. Cells were stored in ice until efflux was initiated by addition of BHI. Efflux was measured using a Cary Eclipse Fluorescence Spectrofluorimeter Varian every 30 s for 40 min with an excitation wavelength of 500 nm and an emission wavelength of 590 nm. In the control situation no BHI was added. All measurements were performed in triplicate.

Protein Blast

Protein sequence of *qacZ* and homologous proteins in *Staphylococcus* were obtained from NCBI database and compared using NCBI BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis

Microsoft Office Excel 2007 was used to process the data on the prevalence of *qacZ* and MIC values obtained. The prevalence of the gene was compared using the Chi-squared (χ^2) test to determine statistically significant differences. All tests were two-tailed and tested at level of 0.05 for significance.

Growth curves

Growth curves of *E. faecalis* V583 and its isogenic V583ErmS were performed as follows: 100 μl of an overnight culture were incubated in 10 ml of BHI; optical density (OD) at 600 nm was measure hourly during six hours and at 24h of incubation.

Results

Susceptibility to biocides and presence of *qacZ*

In the present work, 73 enterococcal strains from different environments were investigated in what concerns their susceptibility to BC and CHX. For all strains the MICs values for CHX and BC ranged from 2 to 8 $\mu\text{g/ml}$. In what respects CHX, the majority of the strains studied presented a MIC value of 4 $\mu\text{g/ml}$. This value of MIC for CHX was present in 80 and 89 % of the clinical and dairy isolates, respectively; and in all veterinary isolates. Only in the clinical and dairy isolates was observed strains with MIC of 8 $\mu\text{g/ml}$. In the case of BC, all dairy isolates presented a MIC of 4 $\mu\text{g/ml}$; the majority of the clinical (67 %) and veterinary (69 %) isolates had MIC of 4 $\mu\text{g/ml}$, but some strains presented MIC of 2 and 8 $\mu\text{g/ml}$. Curiously, all strains with MIC 8 $\mu\text{g/ml}$ for BC were *E. faecium* (Table 1). *qacZ* was detected in 60 % and 70 % of the strains isolated from clinical settings and dairy products, respectively, and was absent from veterinary isolates. It was detected in five different enterococcal species and *E. faecalis* and *E. faecium* presented very similar percentages of *qacZ* carriage, 52% and 47%, respectively. There was no difference in the prevalence of *qacZ* between different MICs values ($p > 0.05$; χ^2). There was a statistical difference in the prevalence of *qacZ* and the isolates origin ($p < 0.05$; χ^2).

V583ErmS and EF-SAVE1 MIC determinations were repeated five times and gave consistent and reproducible values: 4 $\mu\text{g/ml}$ for CHX for both strains, 16 $\mu\text{g/ml}$ for EtBr for both strains; 4 $\mu\text{g/ml}$ for BC in V583ErmS and 8 $\mu\text{g/ml}$ in EF-SAVE1. Although the increase in MIC value for BC was small (from 4 to 8 $\mu\text{g/ml}$), it was consistent and reproducible.

Table 1. MIC values for CHX and BC and the presence of *qacZ* gene in enterococcal strains from different species and environments.

Strains	N° of strains with MIC (µg/ml) value											
	Benzalkonium chloride						Chlorohexidine					
	2		4		8		2		4		8	
	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻
Clinical isolates												
<i>E. faecalis</i>	1	3	11	3			2	1	10	5		
<i>E. faecium</i>			2	3	3	2	1		3	5	1	
<i>E. hirae</i>	1		1				1		1			
Veterinary isolates	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻
<i>E. faecalis</i>		3		8						11		
<i>E. faecium</i>				3		1				4		
<i>E. solitarius</i> ^a		1								1		
Dairy isolates	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻	<i>qac</i> ⁺	<i>qac</i> ⁻
<i>E. faecalis</i>			12	5					11	5	1	
<i>E. faecium</i>			3						3			
<i>E. durans</i>			2	3					2	2		1
<i>E. casseliflavus</i>			1						1			
<i>E. hirae</i>			1				1					

^a Reclassified in 2005 as *Tetragenococcus solitarius* (for more information www.taxonomicoutline.org).

Reverse-transcriptase PCR

RT-PCR was also performed in order to confirm if a higher copy number of EFA0010 orf in V583ErmS lead to a higher expression of this gene and consequently a higher MIC value for BC. *qacZ* was found to be constitutively expressed and not induced by biocides in V583ErmS strain and its expression seems smaller when BC at 1 and 4 $\mu\text{g/ml}$ and EtBr at 2 and 8 $\mu\text{g/ml}$ were added at OD_{600} 0.4. When more copies of the *qacZ* were present the expression became higher, but still not inducible. The presence of the pORI23 plasmid does not influence the expression of this gene, as can be seen in (Figure 1 a) and b)). The expression of the 23S rRNA gene was strong and similar for all tested conditions and strains (Figure 1).

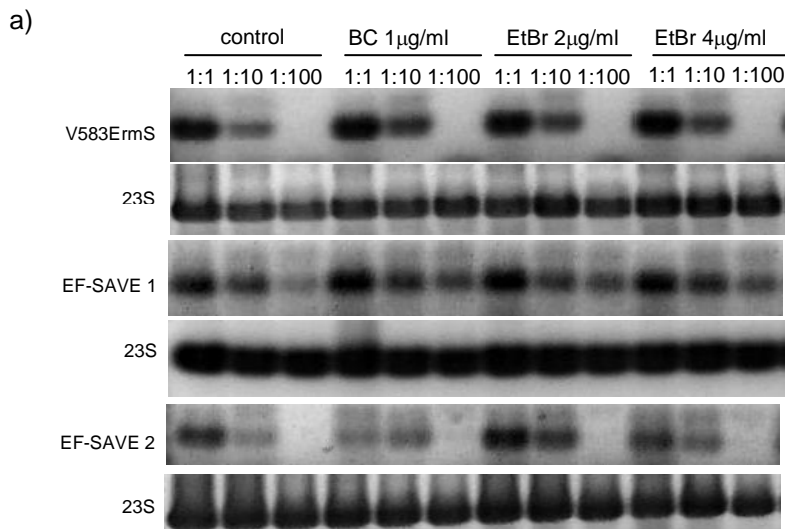


Figure 1. Analysis by semi-quantitative RT-PCR for *qacZ* expression and 23S rRNA.

a) biocide (when present) was added at the beginning of growth and RNA was extracted when OD_{600} 0.4 was reached.

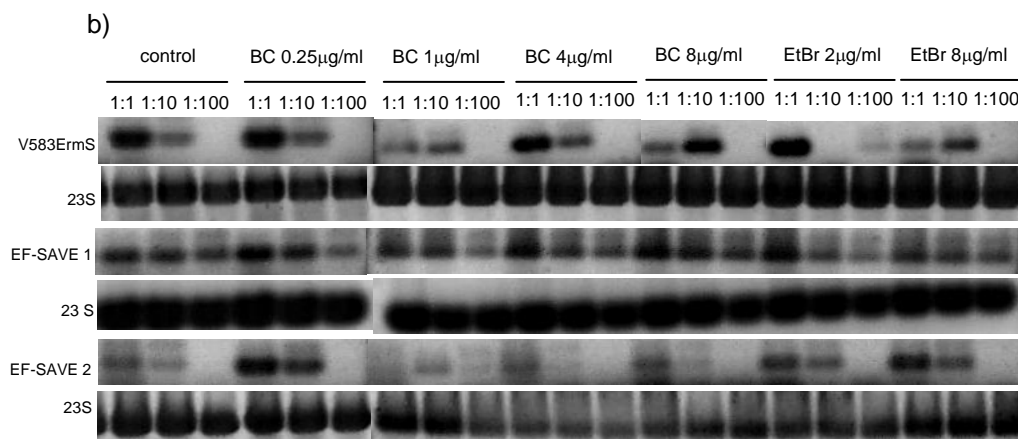


Figure 1 (cont.) . Analysis by semi-quantitative RT-PCR for *qacZ* expression and 23S rRNA. b) biocide (when present) was added when OD₆₀₀ 0.4 was reached and RNA was extracted 15 min after addition of biocide.

EtBr efflux assay

In order to understand if *qacZ* also played a role in the efflux of EtBr, efflux assays were performed under the same conditions for V583ErmS and EF-SAVE1 cells. Figure 2 shows that in the presence of BHI broth, both strains were able to efflux EtBr. However, without BHI broth, EtBr was not pumped out of the bacterial cells. After 30 min there was approximately 50% of difference in fluorescence between both strains with BHI broth and without the medium. No decrease in fluorescence was seen in both strains suspended in buffer only.

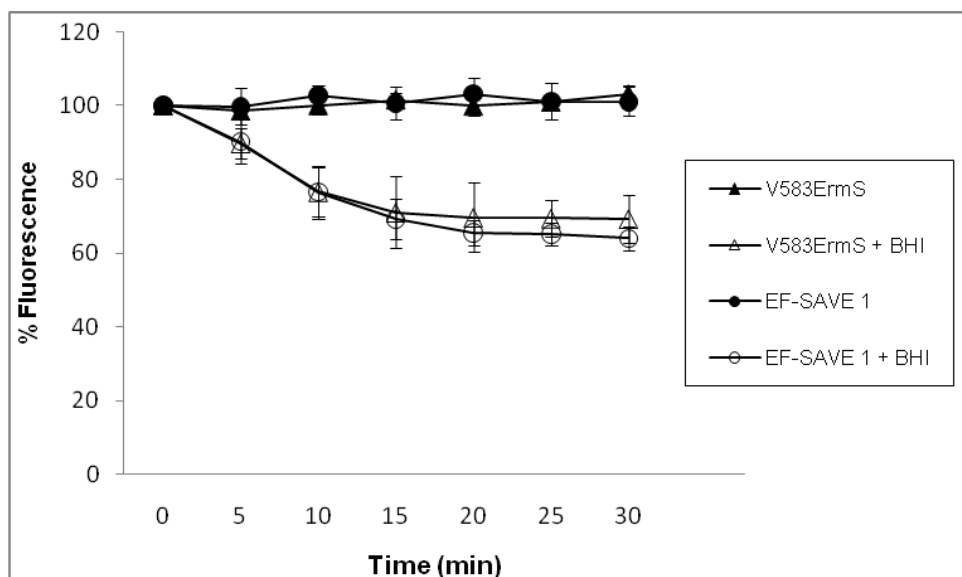


Figure 2. EtBr efflux assays in V583ErmS and EF-SAVE1. Efflux was initiated by the addition of BHI. Assays were performed in triplicate. Fluorescence units are expressed in % of the starting value. EtBr was used at 2.5 μ M and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 40 μ

Protein blast

From the alignment of QacZ with homologous proteins in the database (BLASTP, NCBI) with identities above 60%, it is possible to observe that QacZ has Glu-13 (E), which is conserved throughout the SMR family and is absolutely required for drug efflux activity (Figure 3). Putative transmembrane helix sequences of QacZ, a hydrophobic proteins (SOUSI 1.1.), are represented by horizontal lines above the aligned sequences. Conserved aminoacid residues are indicated by bold letters. NP_816940 is QacZ from *E. faecalis* V583, O87868 is QacH from *Staphylococcus saprophyticus*, NP_647561 is EtBr resistance determinant from *Staphylococcus epidermidis*, Q55339 is QacC from *Staphylococcus* sp. ST827, AAB47993 is a small multidrug resistance efflux pump from *S.*

aureus, NP_783299 is a QAC resistance protein from *S. aureus*, O87866 is QacG from *Staphylococcus* sp. ST94. Figure 3 also shows that cysteine 42, which is common in all Qac proteins, is substituted by a serine in QacZ.

	1	10	20	30	40
NP_816940	MPYLYLLLAIVSE	EVIGSAFLKSSD	GF	SKLYPTITTTIISYL	
O87868	MPYLYLLLSIVSE	EVIGSAFLKSSD	GF	SKLYPTITTTIISFL	
NP_647561	MPYIYLIIAISTE	EVIGSAFLKSSE	GF	SKFIPSLGTIISFG	
Q55339	MPYIYLIIISISTE	EVIGSAFLKSSE	GF	SKFIPSLGTIISFG	
AAB47993	MPYLYLVIAIITE	EIGTSFLKTAEG	FTKLWPTLGTLISFG		
NP_783299	MPYIYLLIAIITE	EIGTSFLKSAEG	FTKLWPTLGTLISFG		
O87866	MHYLYLFISIATE	EIGTSFLKTSE	GF	TKLWPTLGTLLSFG	
Consensus	MPYLYLLIAIITE	EVIGSAFLKSSE	GF	SKLWPTLGTIISFG	
	41	50	60	70	80
NP_816940	ISFYFLSKTMQHLPLN	IAYASWSGLGLVLT	TTIVSVLIFKE		
O87868	ICFYFLSKTMQHLPLN	IYASWAGLGLVLT	TTIVSVLIFKE		
NP_647561	ICFYFLSKTMQHLPLN	IYATWAGLGLVLT	TTVVSIIFKE		
Q55339	ICFYFLSKTMQHLPLN	IYATWAGLGLVLT	TTVVSIIFKE		
AAB47993	ICFYFLSVTMKYLPLN	VSYATWAGLGLVLT	TTIVSVVIFKE		
NP_783299	ICFYFLSTAMKYLPLN	ISYATWAGLGLVLT	TLVSVIIFKE		
O87866	ICFYFLSLTIKFLPLN	IYATWAGLGLVLT	TTIISVIVFKE		
Consensus	ICFYFLSKTMQHLPLN	IYATWAGLGLVLT	TTIVSVIIFKE		
	81	90	107		
NP_816940	QINLISIIISIIILII	IFGVVLLN	TFGSSH		
O87868	QINLISIIISIIILII	IFGVVLLN	TFGSSH		
NP_647561	QINLITIVSIVLII	IVGVVSLN	IFGTSH		
Q55339	QINLITIVSIVLII	IVGVVSLN	IFGTSH		
AAB47993	SVNLSIFSIILII	IIGVVLLN	VFGSSH		
NP_783299	NINLSIFSITLII	IIGVVLLN	VFGSGH		
O87866	NVNLSIISIGLIV	IIGVVLLN	VFGESH		
Consensus	QINLISIIISIIILII	IIGVVLLN	VFGSSH		

Figure 3. Alignment of QacZ (NP_816940) with homologous proteins in the database (BLASTP, NCBI), with identities above 60%.

Role of the *ermB* gene

The MIC value for BC and CHX for V583 was 8 and 4 $\mu\text{g/ml}$, respectively. The value obtained in the case of BC is higher than the one observed for V583ErmS and equal to the one obtained for EF-SAVE1, for the same compound. *E. faecalis* V583 was also able to efflux EtBr out of its cells when BHI was added (Figure 4). After 30 min there was approximately 20% of difference in fluorescence between both strains with BHI broth. The efflux capacity of V583 is higher than the one observed for V583ErmS (and consequently than EF-SAVE1).

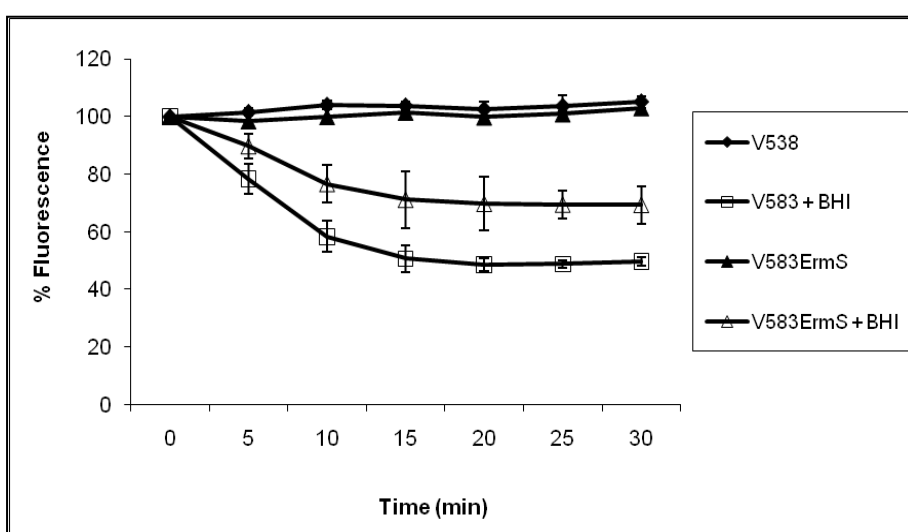


Figure 4. Efflux assay in *E. faecalis* V583 and *E. faecalis* V583ErmS.

The expression of *qacZ* in *E. faecalis* V583 was constitutive and seemed not to be induced by the presence of BC nor EtBr (Figure 5). The major differences found were in samples where biocides were added when OD_{600} 0.4 was reached and the total RNA extracted 15 min later. When comparing both controls made, the expression of *qacZ* was lower 15 min after reaching OD_{600} 0.4. The lowest concentrations of BC tested (0.25 and 1 $\mu\text{g/ml}$) may induce more activity of this gene, as well as 8

$\mu\text{g/ml}$ of EtBr. V583ErmS presented a lower expression of *qacZ* when compared with the wild-type; the levels of expression of the wild-type were similar to those obtained for EF-SAVE1, where multi copies of the *qacZ* were present. The amplification and expression of the 23S rRNA was strong and similar for all tested conditions and strains (Figure 5).

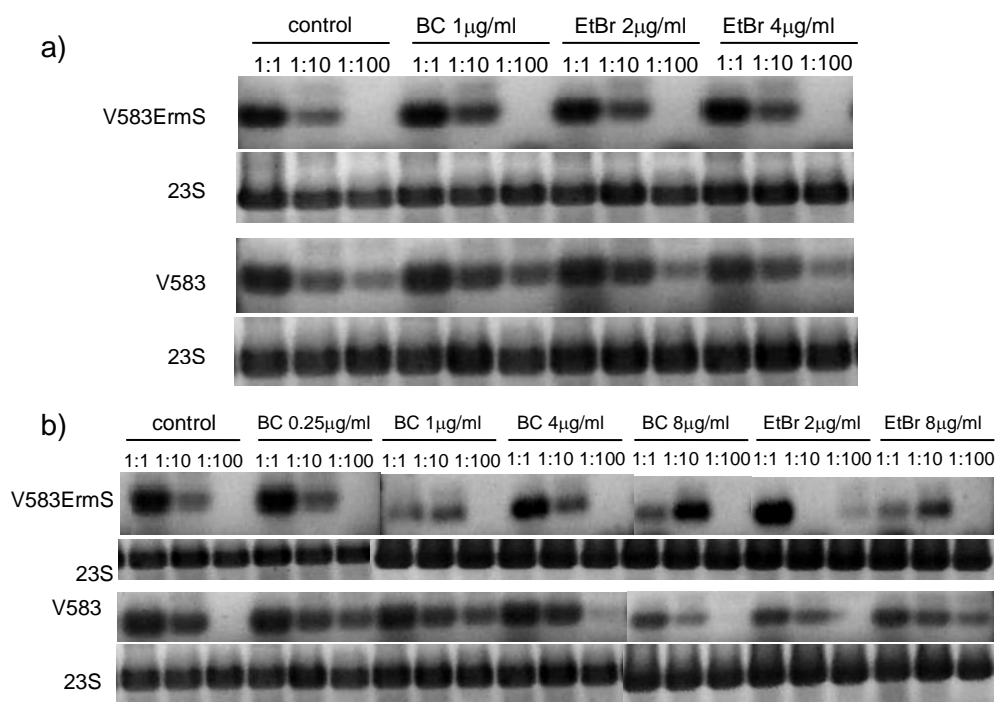


Figure 5. Analysis by semi-quantitative RT-PCR of *qacZ* expression and 23S rRNA.

a) biocide (when present) was added at the beginning of growth and RNA was extracted when OD_{600} 0.4 was reached; b) biocide (when present) was added when OD_{600} 0.4 was reached and RNA was extracted 15 min after addition of biocide.

When the growth of the two strains was compared, great differences were observed (Figure 6). The lack of the *ermB* gene reduces the growth of V583 and the final OD reached by V583ErmS is much lower than the observed in the wild-type.

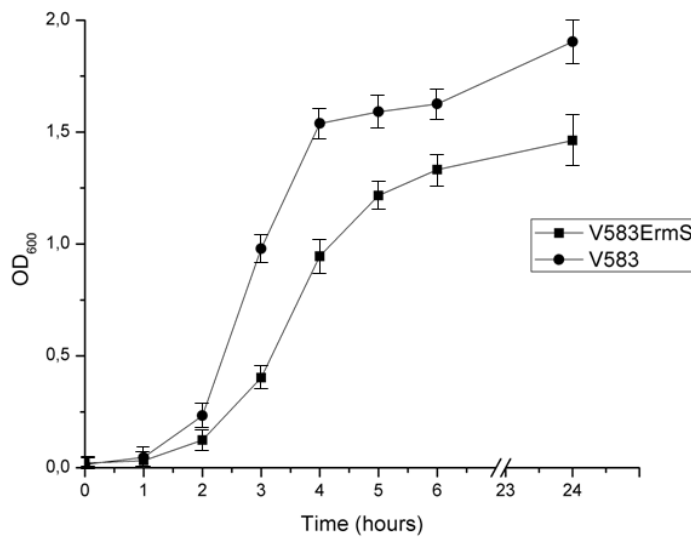


Figure 6. Growth curves of *E. faecalis* V583 and V583ErmS in BHI broth.

Discussion

Some studies have been performed in order to test enterococci susceptibility to biocides, namely CHX and BC. Strains from clinical environment (VRE and VSE) are the most common studied, as well as food isolates. The MICs values found for CHX ranged from 0.5 to 16 $\mu\text{g/ml}$ (2, 3, 10, 18). In the case of BC the MICs values found in literature varied between 0.08 and 16 $\mu\text{g/ml}$ (2, 7, 18). In another study (17), the authors examined 500 strains of food isolated *Enterococcus* and the MICs found were all < 30 $\mu\text{g/ml}$. Although, the results for the isolates herein tested are similar to the ones described by the above mention authors and the major MIC value found was of 4 $\mu\text{g/ml}$, *qacZ* was detected in 63% and 73% of the strains isolated from clinical settings and dairy products, respectively, and was absent from veterinary isolates. These values are quite high, and

worthy of some concern, considering that these species are simultaneously the most relevant in clinical settings and abundant in food products. No difference was found between MIC values and the presence of *qacZ* ($p > 0.05$; χ^2), suggesting that there are other mechanisms, probably other multidrug efflux pumps, involved in tolerance to BC and CHX, as already evidenced for staphylococcal isolates (5). However, when comparing the presence of the *qacZ* gene and source of isolates, statistical difference was found ($p < 0.05$; χ^2), as no veterinary isolate presented this gene. It is not possible to rule out the hypothesis that *qacZ* is less disseminated in the veterinary clinical environment. A search with more strains is advisable to confirm this finding. The increase in MIC value for BC observed for EF-SAVE1 when compared to V583ErmS was small (from 4 to 8 $\mu\text{g/ml}$). This was a consistent and reproducible result that evidences the involvement of *qacZ* in decreased susceptibility to QAC, and not to CHX nor to EtBr (as the MIC values for these compounds remain the same).

RT-PCR confirmed a higher expression of *qacZ* in strain EF-SAVE1 and consequently a higher MIC value for BC, and that *qacZ* expression was not induced by the presence of BC and EtBr. The latest compound is highly fluorescent when it is bound to DNA inside the cell. When the EtBr is effluxed from the cell by way of an efflux pump, the fluorescence will decrease and this can be measured with a fluorescence spectrophotometer. The fluorescence of EtBr was used to measure the efflux of the compound from bacterial cells. The bacterial cells were starved and loaded with EtBr after dissipation of the membrane potential with the protonophore CCCP. Without an energy source the efflux pumps cannot function; therefore, the EtBr cannot be effluxed and the cells become loaded. Efflux of EtBr was initiated by the addition of a carbon source (BHI broth) that allows reconstitution of the membrane potential. V583ErmS and EF-SAVE1 have the same EtBr efflux ability, consistent

with QacZ being unable to provide resistance to EtBr. Figure 3 shows that cysteine 42, which is common in all Qac proteins, is substituted by a serine in QacZ. Paulsen et al (13) demonstrated that substitution C42S leads to a huge decrease in ability of QacC to provide resistance to EtBr (13). S42 in QacZ can thus provide us with a reason for its specificity to BC.

From data obtained using the wild-type strain, it is possible to understand that the *ermB* gene may play a role that goes further than resistance to erythromycin.

There were some differences detected between the wild-type strain *E. faecalis* V583 and its isogenic without the *ermB* gene. This gene codes for a ribosomal RNA adenine dimethylase family protein. The rRNA methylation causes conformational changes in the P site of the rRNA and prevention of macrolide binding (1). The lack of this gene seems to affect the cell in a lot of processes besides less resistance to erythromycin: growth is affected, the expression of *qacZ* and efflux of EtBr are smaller. The MIC values for CHX and EtBr were the same for V583ErmS and the wild type, as well as for EF-SAVE. The exception was for BC; wild-type presented a MIC of 8 µg/ml for this compound, while V583ErmS had a lower MIC (4 µg/ml). When more copies of the *qacZ* gene were introduced in V583ErmS, the phenotype was reverted. This demonstrates that *qacZ* is involved in the susceptibility to BC and its expression is affected by the presence/absence of *ermB* gene. Some other genes maybe down regulated by the absence of this methylase and/or it may play a role in the V583 cell that goes further than just erythromycin resistance. Future work must be done in order to address these questions.

In summary, we provide evidence for the role of QacZ in tolerance to BC in enterococci. The wide spread and high prevalence of *qacZ* among the *Enterococcus* genus, bacteria presenting a dual role and a

transversal environmental dissemination, is a cause of concern and urges future studies.

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**Occurrence of enterococci and
vancomycin-resistant enterococci in dust
samples from pig breeding facilities and
assessment of susceptibility to biocides**

Chapter III

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Teresa M. Braga, Constança Pomba, M. Fátima Silva Lopes

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The isolation and first identification of enterococci was performed at Faculdade de Medicina Veterinária, Technical University of Lisbon. The remaining experimental work was performed at ITQB, all by the author of the thesis.

Summary

Bio-security measures in pig facilities include disinfection with biocides to avoid the dissemination of opportunistic pathogenic bacteria, namely enterococci and vancomycin-resistant enterococci (VRE). In order to evaluate if the use of biocides is selecting for less tolerant enterococci and for VRE, we sampled dust from 171 breeding pig facilities, representative of the total of the Portuguese pig population. A total of 191 enterococcal isolates were recovered with ($n=41$) or without ($n=150$) vancomycin selection and screened for susceptibility towards benzalkonium chloride (BC) and chlorhexidine (CHX). *Enterococcus faecalis* and *Enterococcus hirae* were the most prevalent species detected. Ten isolates with high-level vancomycin-resistance were found. Amongst these, four *Enterococcus faecium* carrying *vanA* gene were detected and by Multi Locus Sequence Typing (MLST) determined to have four different sequence types (139, 522, 523 and 524). Based on the MLST these *E. faecium* isolates were found to be closely related to pig and human isolates from European countries and Brazil. Minimal Inhibitory Concentration values for CHX and BC were 8 $\mu\text{g/ml}$ and 4 $\mu\text{g/ml}$, respectively. No association was found between less susceptibility to biocides and vancomycin-resistance, suggesting that biocides are not selecting for vancomycin-resistant enterococci. Overall, this work reports the presence of enterococci and VRE in dust from pig facilities. We also evidence that the *vanA E. faecium* found are genetically related to strains found in humans, demonstrating that the transmission of VRE into humans may occur through inhalation of dust. This work suggests constant surveillance in order to avoid animal and human colonization with VRE.

Introduction

Enterococci are natural inhabitants of the gastro-intestinal tract of humans and other animals. They also rank now as leading nosocomial infectious agents worldwide, likely as a consequence of the increased use of antibiotics both in hospitals and in agriculture, as animal growth promoters. Enterococci constitute the third most prevalent pathogen isolated from bloodstream infections, and represent the most frequent cause of surgical-site infections in intensive care units (35). A new wave of concern about enterococcal infections comes from the dissemination of vancomycin resistance amongst enterococcal nosocomial isolates. This impairs the use of vancomycin to treat infections caused by multi-resistant Gram-positive bacteria. In Europe, vancomycin-resistant enterococci (VRE) are disseminated also outside the nosocomial environment. The use of growth-promoting drugs in food animals has caused an increase of antibiotic resistance in enterococci of animal origin. These bacteria are widely accepted as an indicator for the detection of the prevalence of resistance due to the use of growth-promoting antimicrobial agents (7). Several studies have described the correlation between the use of avoparcin as a growth promoter in farm animals and the occurrence of VRE (42). Although avoparcin has been banned in all European countries since 1997, the presence of VRE is still detected and therefore these bacteria may enter the food chain.

Thus, it is important to keep animal facilities under constant surveillance for VRE assessment. Enterococci are able to survive outside their hosts, and are thus found viable in soil, water and surfaces (16, 18). These environments can constitute dissemination vehicles for resistance mechanisms and resistant enterococcal strains between man and animals. Dust is also an important transmission vehicle for microorganisms, as they can easily go from one place to another and can

also be inhaled by humans or animals, thus transmitting virulence and antibiotic resistance genes that can be present in those microorganisms. A proper cleaning and disinfection of the animal facilities is thus an important way to avoid animal infections. Biocides are usually used to (i) clean and disinfect the farm building, (ii) create barriers (ex.: foot dips), (iii) disinfect vehicles and materials during outbreaks of infectious diseases, (iv) direct application to animal surfaces and (v) preservation of specific products (36). For that, the use of biocides, such as biguanides, quaternary ammonium compounds (QACs), triclosan and others, has been increasing in the last few decades. Chlorhexidine (CHX) is usually used for cleansing wounds, skin, instruments and equipment. It is also used on teat dips and sprays in mastitis control (11). Benzalkonium chloride (BC) is usually used for cleansing and disinfection of stables and animal transports, but can also be used as an injectable formulation, eye lotion and dermal spray (10). The increased use of biocides has raised the question if they could be selecting for antibiotic resistant bacteria (5).

In this study, enterococcal strains were, for the first time, isolated from dust samples collected from Portuguese breeding pig facilities. Their susceptibility to CHX and BC, a biguanide and a QAC respectively, was studied. We also investigated the presence of VRE on these samples and their susceptibility to the same biocides. Nasal mucosa of some veterinary swine practitioners was also searched for the presence of these bacteria and its characterization.

Materials and Methods

Sample collection

Dust samples were collected in 171 Portuguese breeding pig facilities. The collection was performed during October, November and December 2008. The survey was carried out on holdings harboring at least 80 % of the breeding pig population in Portugal. Preferentially holdings having 50 breeding pigs or more were sampled. Smaller holdings with less than 50 breeding pigs were also sampled because 80 % of the national herd of breeding pigs did not contain holdings having 50 breeding pigs or more. In each selected breeding herd and production herd taking into account an annual expected prevalence of 50 % of enterococci, five dust samples were gathered using five dry sterile swabs of about 500 cm² each (Sodibox) from five of the 10 pens selected for sampling. These five pens were chosen in a way that breeding pigs in different production stages were included (including gestation and farrowing). For each pen dorsal surfaces of pen partition walls were swabbed. In case there was not enough dust present, then ventilator ducts, etc. were sampled in addition. After use, the soiled swabs were placed in a sterile plastic bag. Samples were kept at room temperature during transportation and at $\pm 4^{\circ}\text{C}$ during storage. The samples were sent to the laboratory as quickly as possible and reached the laboratory no later than 10 days after sampling. There, the European base-line study for methicillin-resistant *Staphylococcus aureus* was being done according to the European Commission (EC) Decision 2008/55/EC, and enterococci were isolated from the enrichment media used for the EC study (9).

Enterococci were also isolated from the nose of six veterinary doctors of three different pig facilities, using a cotton swab that was placed into modified Amies' transport medium (Deltalab).

Isolation of enterococci and putative VRE

A sterile plastic bag containing a pool with the five dust swabs from each holding was filled with 100 ml of Mueller-Hinton broth (MH) (Oxoid) supplemented with 6.5 % NaCl and incubated at 37 °C for 16-20 h. The cotton swabs from nasal sampling were pooled into 10 ml of the same medium and incubated under the same conditions. 100 µl of each culture were spread onto Enterococcosel agar (Becton) and another 100 µl onto Enterococcosel agar supplemented with 16 µg/ml of vancomycin. Both plates were incubated for 48 hours at 37°C. From each plate one single colony (small, translucent with brownish-black to black zones) was selected, streaked onto Brain Heart Infusion (BHI) (Oxoid) agar plate and incubated for 24 hours at 37°C. If pure, the culture was gathered with a sterile cotton swab and stored in cryovials containing glycerol with BHI (20% v/v) at -80°C for further identification and characterization.

Identification and characterization of isolates

Identification to the species level was performed by multiplex PCR (Biometra T3000 Thermocycler) according to Danish Integrated Antimicrobial resistance Monitoring and Research Programme (DANMAP) (13) targeting specific genes (Table 1). Each multiplex PCR contained: 2 µl of each primer (10 pmol), 9 µl of MgCl₂ (25mM), 5 µl of 10x Taq Buffer (Fermentas), and 0.5 µl of Taq DNAPolimerase (5U/µl) (Fermentas). Isolates, for which the multiplex PCR was unable to identify the species, were further characterized using other species specific primers (Table 1). For isolates from plates with vancomycin, detection of *vanA* and *vanB* genes was performed by PCR (Table1).

Susceptibility testing

Susceptibility to biocides was assessed for all isolates in this study. Minimal inhibitory concentration (MIC) for BC (Sigma-Aldrich) and CHX (Fluka) were determined by microdilution according to Clinical and Laboratory Standards Institute (CLSI) (8). Isolates were grown overnight in BHI broth (Oxoid) and plated onto BHI agar. Colonies were picked and incubated overnight in Muller-Hinton (MH) broth (Oxoid). The inoculum density was adjusted to 0.5 McFarland in fresh MH broth and diluted 1:100. On a 96 wells microplate, 100 μ l of each bacterial culture diluted 1:100 were added to 100 μ l solution of each compound (range from 0.25-256 μ g/ml). Results were read after 24h. Assays were repeated at least three times. MIC was the lowest concentration of biocide at which the tested microorganism did not show visible growth. All incubations were performed at 37°C. All vancomycin-resistant isolates were assayed using E-test (Biomérieux), according to manufacturer instruction, for vancomycin MIC determination. Isolates with high-level vancomycin resistance were also assayed for susceptibility to teicoplanin by E-test (Biomérieux).

Table 1: Primers used for identification of enterococcal isolates and of vancomycin resistance genotype.

Target gene	Species	Type strain	Amplicon size (bp)	Nucleotide sequence (5' – 3')	Annealing temperature (°C) /time (s)	Ref.
<i>ddl</i> _{<i>E. faecalis</i>}	<i>E. faecalis</i>	DSMZ 20478	550	Fwd ATC AAG TAC AGT TAG TCT T Rev ACG ATT CAA AGC TAA CTG		
<i>ddl</i> _{<i>E. faecium</i>}	<i>E. faecium</i>	DSMZ 20477	941	Fwd CCA AGG CTT CTT AGA GA Rev CAT CGT GTA AGC TAA CTT C		
<i>vanC1</i>	<i>E. gallinarum</i>	DSMZ 20628	329	Fwd TCT CCA GAA TAC TCA GTG T Rev ACA TGG CAA CCA ACA TAA	55/ 30	(13)
<i>vanC2/3</i>	<i>E. casseliflavus</i>	DSMZ 20680	448	Fwd CCT CAA AAG GGA TCA CTA A Rev TCT TGA TAG GAT AAG CCG A		
16S	<i>Enterococcus</i> spp.		708	Fwd GGT TTC TTA AGT CTG ATG T Rev GAA GCT TTA AGA GAT TAG C		
<i>mur-2</i>	<i>E. hirae</i>	DSMZ 20160	521	Fwd CGT CAG TAC CCT TCT TTT GCA GAG TC Rev GCA TTA TTA CCA GTG TTA GTG GTT G	60/15	(3)
<i>mur-2_{ed}</i>	<i>E. durans</i>	DSMZ 20633	177	Fwd AAC AGC TTA CTT GAC TGG ACG C Rev GTA TTG GCG CTA CTA CCC GTA TC	55/15	(3)

Table 1: *Continued.*

Target gene	Species	Type strain	Amplicon size (bp)	Nucleotide sequence (5' – 3')	Annealing temperature (°C) /time (s)	Ref.
<i>sodA</i>	<i>E. raffinosus</i>	DSMZ 5633	287	Fwd GTC ACG AAC TTG AAT GAA GTT Rev AAT GGG CTA TCT TGA TTC GCG	50/30	(24)
<i>sodA</i>	<i>E. dispar</i>	DSMZ 6630	284	Fwd GAA CTA GCA GAA AAA AGT GTG Rev GAT AAT TTA CCG TTA TTT ACC	45/15	(24)
<i>sodA</i>	<i>E. mundtii</i>	DSMZ 4838	98	Fwd CAG ACA TGG ATG CTA TTC CAT CT Rev GCC ATG ATT TTC CAG AAG AAT	60/15	(24)
<i>sodA</i>	<i>E. flavescens</i>	DSMZ 7370	284	Fwd GAA TTA GGT GAA AAA AAG TT Rev GCT AGT TTA CCG TCT TTA ACG	45/30	(24)
<i>vanA</i>	<i>E. faecium</i>	BM 4147	731	Fwd GGA AAA CGA CAA TTG CTA TT Rev GTA CAA TGC GGC CGT TA	55/30	(13)
<i>vanB</i>	<i>E. faecalis</i>	V583	175	Fwd ATC GGC CTA CAT TCT TAC A Rev AGC GTT TAG TTC TTC CGT	55/30	(13)

Multilocus Sequence Typing (MLST)

Genetic relatedness of *E. faecium* vancomycin resistant isolates was determined by MLST. Internal fragments of seven housekeeping genes were amplified by PCR with the set of primers and conditions described by Holam *et al.* (22). Briefly, 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 30s and 72°C for 30s; and extension at 72°C for 5 min. Each PCR was performed using 8 µl 2x DyNzyme MasterMix (Finnzymes) in a 20 µl reaction. Sequencing of amplicons was performed by Applied Biosystems 3730xl DNA Analyzer using the Sanger method with BidDye Terminator V3.1 reagent (Applied Biosystems) (StabVida) and sequence types were determined and compared using the MLST database for *E. faecium* (<http://efaecium.mlst.net/>). A dendrogram was created with data retrieved by the allelic profile query from the MLST database.

Results

Identification of enterococcal isolates and of putative VRE

A total of 171 Portuguese pig facilities were sampled in this study and *Enterococcus* were isolated and identified in 88% (150 facilities) of those (Figure 1). These 171 pig facilities are representative of the pig breeder's population of the national territory. The most prevalent species found was *E. faecalis*, followed by *E. hirae* (Table 2). Twenty-five isolates were not identified to the species level, despite attempts using primers for the species mentioned in Table 1.

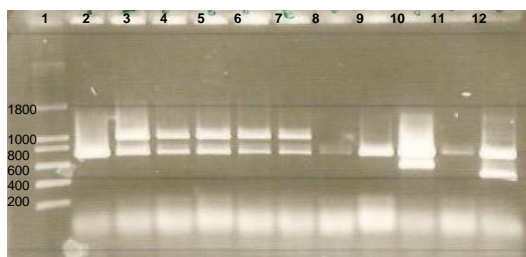


Figure 1: Identification by PCR of some of the isolated enterococci. Lines: 1 – NZY DNA ladder I in bp (NZYTech); 2, 8, 9 and 11 – *Enterococcus* spp.; 3 to 7 – *E. faecalis*; 10 – *E. faecium*; 12 – *E. casseliflavus*.

We were able to recover isolates from plates containing vancomycin from 41 pig facilities of the 171 sampled. Among these 41 isolates, the most common species were *E. faecium* and *E. casseliflavus*, followed by *E. hirae* (Table 2). These putative VRE isolates were further analyzed using E-test for vancomycin MIC determination. Eleven out of the forty one had MIC values classified as susceptible ($\leq 4\mu\text{g/ml}$). Among these, three were identified as *E. hirae*, three as *E. faecalis* and five as *E. faecium*. Twenty putative VRE strains were classified as intermediate (low-level resistance) to vancomycin (6-24 $\mu\text{g/ml}$). In this group, ten were identified as *E. casseliflavus*, five as *E. gallinarum*, four as *E. faecalis* and one as *E. faecium*. Ten enterococcal strains presented high-level resistance to vancomycin (MIC > 256 $\mu\text{g/ml}$), four were *E. faecium* and six were *E. hirae*. From these ten, two *E. faecium* were resistant also to teicoplanin, as well as one *E. hirae*. The *vanA* gene was detected in all *E. faecium* with high-level resistance to vancomycin; the *vanB* gene was present in two of the six high-level vancomycin resistant *E. hirae* and in one *E. gallinarum* with low-level resistance to vancomycin (MIC 16 $\mu\text{g/ml}$).

Table 2: Enterococcal isolates (n=150) and putative VRE isolates (n=41).

Species	Total	Percentage (%)
Enterococcal isolates (n=150)		
<i>E. faecalis</i>	53	35
<i>E. hirae</i>	39	26
<i>E. faecium</i>	22	15
<i>E. casseliflavus</i>	7	5
<i>E. gallinarum</i>	2	1
<i>E. raffinosus</i>	2	1
<i>Enterococcus</i> spp. ^a	25	17
Putative VRE isolates (n=41)		
<i>E. faecium</i>	10	24
<i>E. casseliflavus</i>	10	24
<i>E. hirae</i>	9	22
<i>E. faecalis</i>	7	17
<i>E. gallinarum</i>	5	13

^a These isolates are not from none of the species mention in Table 1.

Multilocus sequence typing

MLST typing was performed for the four *E. faecium* found to be high-level resistant to vancomycin (VREF), all carrying *vanA* and the seven house-keeping genes.

Results obtained in the MLST analyses are presented in Table 3.

Table 3: MLST results for the VREF isolates.

Strain name	Allele number for gene loci:							ST
	<i>atpA</i>	<i>ddl</i>	<i>gdh</i>	<i>purK</i>	<i>gyd</i>	<i>pstS</i>	<i>adK</i>	
T2706	57 ^a	2	1	3	1	1	5	522 ^b
E1777	3	5	1	3	1	1	5	139
F2010	9	5	12	3	1	20	1	523 ^b
C2511	4	5	12	3	1	20	1	524 ^b

^a new allele; ^b new ST

A new allele for *atpA* gene was found for strain *E. faecium* T2706. Three new STs were described, 522, 523 and 524. We found ST139 (strain E1777), which was already found in a pig from Belgium (15). The closest ST to the new ST 522 is 184, detected in a strain isolated from a pig, also in Belgium (A0047). These two STs differ only in the *atpA* allele. For ST 523 and 524 we built a dendrogram, presented in Figure 1. The closest ST to both is 153 (strain E0684). The closest strain to the group formed by ST 523 and ST 153 is A1769, isolated in Felgueiras, Portugal, from a pig (Figure 2 a)) (19). There are three other STs related with the group formed by ST 524 and ST 153, ST 97, ST 183 and ST 272, all detected only in human isolates. ST 183 was detected in one strain, while the other two STs were found in 3 strains each (Figure 2 b)).

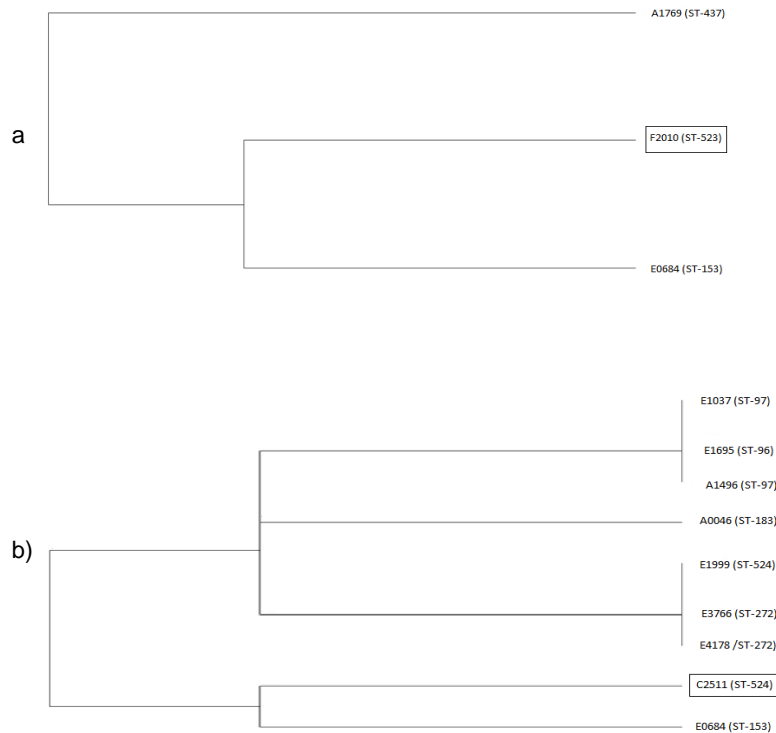


Figure 2: Dendogram for VREF ST 523 (a) and ST 524 (b). Both strains inside rectangular square.

a) Strain E0684: isolated from a pig in Spain. Differs from F2010 in *atpA* allele; Strain A1769: isolated from a pig in Portugal. Differs from F2010 in *gdh* allele and of E0684 in *gdh* and *atpA* alleles.

b) Strain E0684: isolated from a pig in Spain. Differs from C2511 in *atpA* allele; Strain E4178: isolated from non-hospitalized person in France. Differs from C2511 in *gdh* allele and of E0684 in *gdh* and *atpA* alleles; Strains E3766 and E1999: isolated from hospitalized patient in Netherlands and Berlin, respectively. Both differ from C2511 in *gdh* allele and of E0684 in *gdh* and *atpA* alleles; Strain A0046: isolated from non-hospitalized person in Belgium; differs from C2511, E4178, E3766 and E1999 in *gdh* allele and from E0684 in *gdh* and *atpA* alleles; Strains A1496 and E1695: isolated from hospitalized patient in Germany and Brazil, respectively; Differ from C2511 in *gdh* allele, as well as, from all the other above mention, with the exception of E0684; Strain E1037: isolated from non-hospitalized person in Netherlands; differs from the others in the same way as A1496 and E1995.

Susceptibility to biocides

All isolates from dust samples were tested for their susceptibility to two chemically different biocides, BC and CHX. For BC, MIC values had a maximum of 4 µg/ml and for CHX of 8 µg/ml. Results are presented in Figure 3 for enterococcal isolates ($n=150$) and in Figure 4 for putative VRE isolates ($n=41$). In the enterococcal isolates sampled, MIC of 4 µg/ml was the highest and the most common value found for BC. 87% and 82% of the *E. faecalis* and *E. faecium*, respectively, presented this MIC value for BC, while it was only registered in 28% of the *E. hirae*. In this species, the most common MIC value for BC was 2 µg/ml, which was present in 64% of *E. hirae*. In the same isolates ($n=150$), the highest MIC value obtained for CHX was 8 µg/ml and was only detected in *E. faecalis*. MIC of 4 µg/ml was the most common value for this compound; it was detected in 78%, 28% and 18% of *E. faecalis*, *E. hirae* and *E. faecium* isolates, respectively. In the last two species, *E. hirae* and *E. faecium*, the most common MIC value for CHX was 1 µg/ml (in 41% of this specie isolates) and 2 µg/ml (in 46% of this specie isolates), respectively.

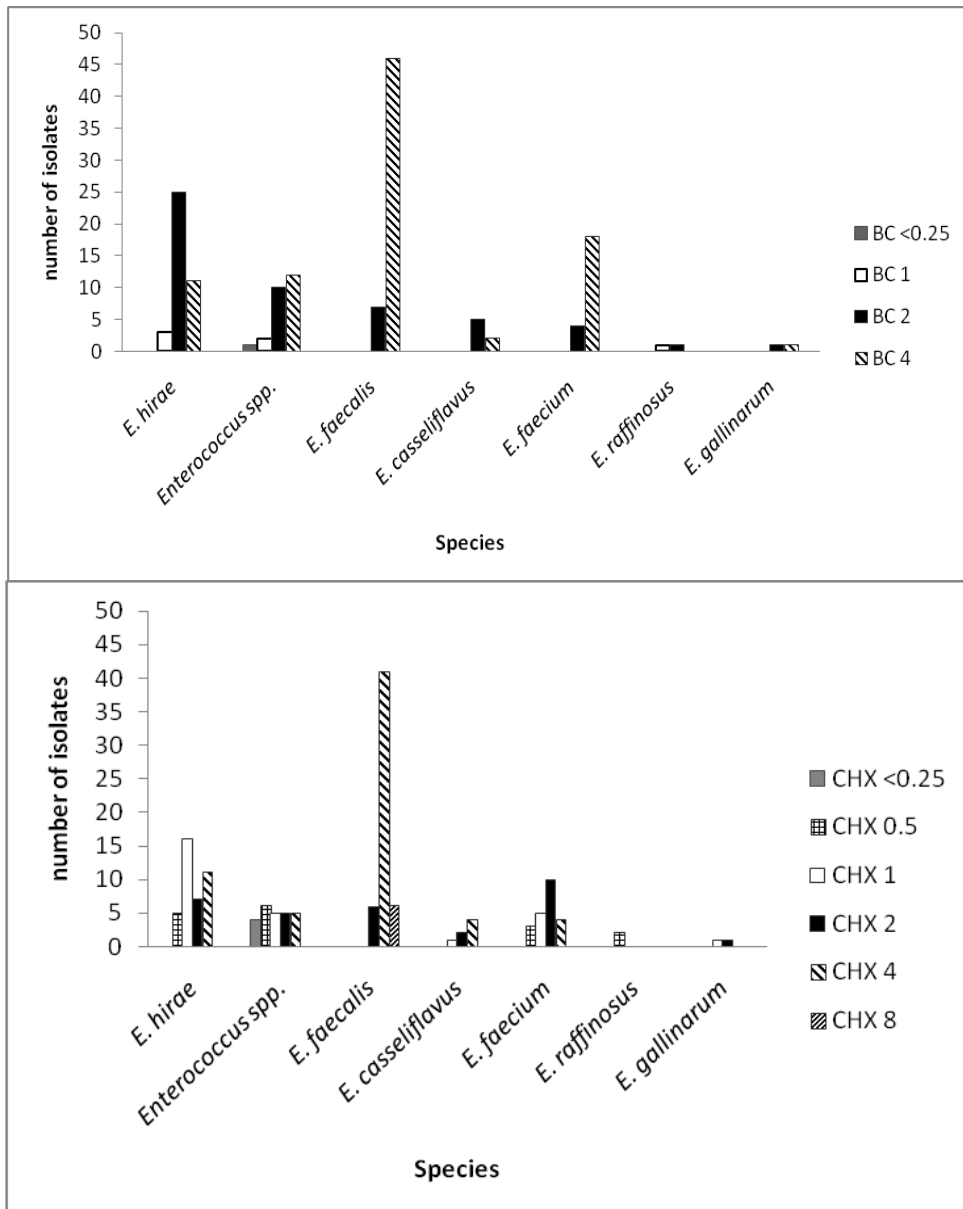


Figure 3: Distribution of MIC values (in $\mu\text{g/ml}$) for BC (top) and CHX (bottom), by species of enterococcal isolates from dust samples (n=150).

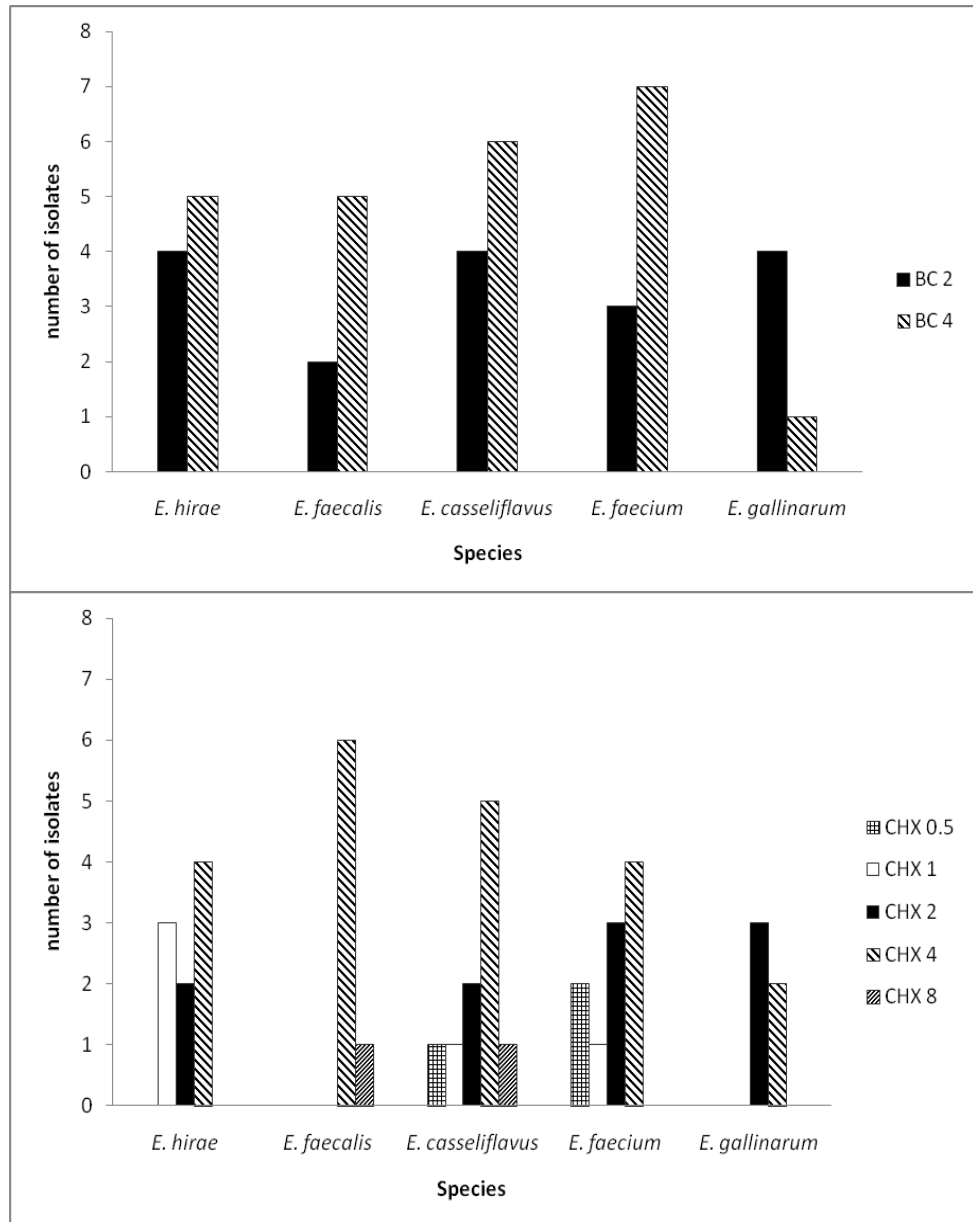


Figure 4: Distribution of MIC values (in $\mu\text{g/ml}$) for BC (top) and CHX (bottom), by species of putative VRE isolates (n=41).

In Table 4 we present the association between the MIC values for both biocides and vancomycin for all VRE isolates. The majority of VRE isolates presented low-level resistance to vancomycin, and it was in this group that the highest MIC for CHX (8 µg/ml) was detected. Half of the high-level resistant isolates had a MIC of 2 µg/ml for BC and the other half 4 µg/ml.

Table 4. Distribution of the MIC values (in µg/ml) for BC, CHX and Vancomycin in putative VRE isolates (n=41).

MIC for VA ^a	Species distribution according to CHX MIC					MIC for BC
	0.5	1	2	4	8	
2	hi		hi			2
						4
3						2
	hi					4
4	fl					2
	fc (n=3)		fl (n=2); fc (n=2)			4
6	fl					2
						4
8						2
				fl (n=2)	fl	4
12	ca		ca	ga		2
	ca (n=2)					ca
16	ga					2
	ca		ca (n=2); fc; ga			4
24	ca		ga (2)			2
						4
> 256	fc (n=2)		fc	hi (n=2)		2
	hi (n=2)		hi	hi; fc		4

^a None of the isolates had MIC value of 32, 64 or 128 µg/ml for vancomycin. hi – *E. hirae*; ca – *E. casseliflavus*; ga – *E. gallinarum*; fl – *E. faecalis*; fc – *E. faecium*. VA – vancomycin; CHX – chlorhexidine; BC – benzalkonium chloride.

In these isolates the MIC values for CHX are quite disperse (from 0.5 to 4 µg/ml), and none of them had MIC of 8 µg/ml. MIC of 8 µg/ml for CHX was only present in two strains, one *E. faecalis* and one *E. casseliflavus*, both low-level resistant to vancomycin.

All six swine veterinarian practitioners tested positive for the presence of enterococci. *E. faecalis* was identified in three of the six samples, *E. faecium* in two of them and *E. hirae* in one. The MICs for BC ranged between 2 and 4 µg/ml. For CHX all strains presented a MIC of 4 µg/ml, with the exception of one *E. faecium* (0.5 µg/ml). There were no isolates recovered from selective plates with vancomycin.

Discussion

Pig facilities as well as pigs, play an important role in transmission of enterococci, from animals to humans, through the food chain (20). It is thus reasonable to assume that dust from pig facilities are partners in this transmission. However, dust has never been sampled for screening the presence of enterococci or VRE. This is the first step to evaluate its role in transmission of enterococci between food and humans. Assuming that dust is cleaned using biocides, it is also logical to ask if the strains recovered from dust are more resistant to biocides. In order to better understand these two issues, we sampled 171 pig facilities, a representative sample of Portuguese breeding pig facilities. Our results will thus give an idea of the diversity of enterococci in this environment in Portugal. Two groups of isolates were studied: the total enterococcal isolates ($n=150$) and the putative VRE ($n=41$). *E. faecalis* was the most frequent species in the larger group ($n=150$) and *E. faecium* the most frequent among putative VRE isolates ($n=41$). Some work has been published concerning the presence of enterococci in pigs and related

environments. These works have focused on feces, manure, meat, soil and waste water. The most common species found were *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae*, *E. gallinarum*, *E. casseliflavus*, *E. solitarius*, *E. avium* and *E. mundtii* (6, 25-28, 30, 33, 38). In general, we can say that the species found in dust are the same found in other environments related with pigs and pig facilities.

Our protocol for isolation of putative VRE recovered 41 isolates from the 171 pig facilities sampled. After performing the E-test for this antibiotic, 30 of these isolates were confirmed as VRE. The prevalence of VRE present in dust samples of Portuguese breeding pig facilities is thus 17.5 %. However, if we took in account all the isolates that grew in plates with vancomycin, the prevalence of VRE would be 24 %. Despite the 6.5 % difference in the prevalence, we can say that the method used allows isolation of low-level vancomycin resistant species. There is no data available concerning VRE prevalence in the same type of samples and thus we cannot compare them. The only reports on the prevalence of VRE in similar environments are from superficial waters, solid waste, air and others (26%) (32), and from environmental swabs from broiler farms (20). The reported prevalence of VRE in pigs and pig environments is highly variable. In pig feces, values ranges from 85 % in a Greek study (29) to 7.1 % in a Spanish publication (21); 3.5 % in samples from large intestine after incision with a scalpel in a European study (14); and 17 %, 2 % and 6 % of VREF prevalence in Denmark, Spain and Sweden, respectively (1). In a recent publication, enterococci were screened in several animal and human feces and VRE were only recovered in pig samples (in six samples out of 55) (17). VRE prevalence of 8.5 and 12.5 % in fecal sample of pig farmers and non-farmers respectively, was described by Aubry-Damon *et al.* (4); in our study, no VRE were detected in veterinary medical doctors, although a direct comparison, as our samples were from nasal mucosa, is

not possible. The relevant finding is that VRE can be found in dust from pig facilities, with a high frequency – this is relevant and worrisome.

Resistance to teicoplanin was also investigated for all high-level vancomycin resistant isolates. The *vanA* genotype is described to confer resistance to teicoplanin, while the *vanB* genotype does not (12). In our work, three isolates presented resistance to both vancomycin and teicoplanin and two of them carry *vanA* (*E. faecium* E1777 and F2010). All the other isolates showing high-level resistance to vancomycin (including two *E. faecium* carrying *vanA*) were susceptible to teicoplanin. This has also been reported in other studies: one *E. faecium* of fecal swine origin (carrying *vanA*) (34); one *E. faecalis* and one *E. faecium*, both hospital isolates and carrying the *vanA* gene (31). Isolates with the *vanA* genotype, but susceptible to teicoplanin, corresponding to a VanB phenotype, have also been reported by others (43); as well as, the other way around, strains of *vanB* genotype may have co-resistance to vancomycin and teicoplanin (VanA phenotype) (41). Therefore, for unusual isolates, such as the ones from this study, phenotype cannot be inferred from the genotype. Two, out of six *E. hirae* high-level resistant to vancomycin, carried the *vanB* gene. Curiously, in Spanish fecal samples of pigs, an *E. hirae* carrying the *vanB2* gene was found, although this had not high-level resistance to vancomycin (40). One *E. gallinarum* carried the *vanB* gene and presented a MIC of 16 µg/ml to vancomycin. This is not the first report of an *E. gallinarum* carrying *vanB*. Such a case has been reported previously by Ishii *et al.* (23), but from pus obtained from a periproctal abscess on a patient with acute myeloid leukemia. The vancomycin MIC of this strain was also low (6.25 µg/ml).

MLST data from VREF from our samples also revealed relevant results. Three new STs were found (ST 522, 523 and 524) and another that was already described (ST 139) (15). Sequence types were compared with the ones already reported in the *E. faecium* MLST

database. As expected, the closest STs to the new ones are all of animal origin (source: pig). This finding means that animals may be infecting its environment and/or that dust may be carrying enterococci that are colonizing the animals. It is also interesting to notice that some of the STs found in this study are closely related with STs detected in strains isolated from humans (hospitalized and community) in six different countries, from Europe and South America. This result strongly suggests that these bacteria can also be transmitted from humans to animals and vice-versa.

A proper cleaning and disinfection of any animal facility plays an important role in protecting animals and workers. Benzalkonium chloride and chlorhexidine are usually used in the farm environment. No resistance to any of these biocides was detected in the 150 enterococcal isolates as the highest MIC values were 4 µg/ml for BC and 8 µg/ml for CHX. There are several studies concerning resistance to biocides in clinical enterococcal isolates, but very few reports regarding veterinary isolates. The results herein presented are very similar to those reported by Aarestrup and Hasman (2). These authors studied susceptibility to BC and CHX in 52 *E. faecalis* and 78 *E. faecium* of animal origin (including pigs) from the Danish Institute for Food and Veterinary Research collection. In the case of BC, the authors obtained MIC values from 2 to 8 µg/ml and 2 to 16 µg/ml for *E. faecalis* and *E. faecium*, respectively. In that study, and in contrast with ours, the MIC values were higher and 88 % of *E. faecalis* had MIC of 8 mg/ml; while 27 % and 49 % of *E. faecium* had MIC of 16 and 8 µg/ml, respectively. For CHX, these authors obtained MIC values between 0.5 and 8 µg/ml, for both species. Curiously, the majority of the *E. faecalis* (88 %) had MIC of 8 µg/ml. The greater part of *E. faecium* (60 %) had MIC of 4 µg/ml for CHX. In another research work (37), 500 *Enterococcus* spp. from animal origin (broilers, cattle and pig) presented

MIC to BC below 30 µg/ml. In clinical isolates the MICs found for CHX and BC are quite similar to those obtained in our study (39).

For the putative VRE isolates ($n=41$), the MIC values for BC and CHX were in the same range of those obtained for the *Enterococcus* isolates ($n=150$). For BC all strains presented MIC of 2 or 4 µg/ml, with no special incidence in any type of enterococcal strain. Regarding CHX, the MIC values ranged between 0.5 and 8 µg/ml; only two strains had MIC of 8 µg/ml, one *E. faecium* and one *E. casseliflavus*. From these results, there seems to be no association between VRE and less susceptibility to a biocide, nor evidence that the frequent use of a biocide may be selecting VRE strains.

Dust from pig facilities is an important vehicle for dissemination of bacteria, as they can be inhaled by workers and animals and go from a place to another. Good practices of cleaning and disinfection may be a good way to eliminate the majority of these microorganisms. In this study, we tried to understand if the use of biocides was being efficient in the Portuguese breeding pig facilities and if it was selecting bacteria (in our case *Enterococcus*) resistant to this type of compounds and/or vancomycin. For that we used a new methodology which allows the isolation of different enterococcal strains from samples with high diversity of microorganisms (dust), and is also a good approach for the isolation of VRE strains, as low-level resistant enterococci were also selected. The prevalence of VRE found (17.5 %) is still a cause of concern, as animals maybe carrying resistance genes into the food chain. The ban of avoparcin usage in the pig industry in the European Union is still recent and may reflect the high VRE prevalence. In the case of biocides, the MIC values distribution is similar to other environments, which is good for the farm bio-security. There seems to be no correlation between the use of these compounds and VRE presence. Enterococci isolated from

Portuguese breeding pig facilities: a case of concern? Above all, it is a reason for implementing constant surveillance.

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**Vancomycin resistance in enterococci
does not fit and is not selected under
biocide challenge.**

Chapter IV

This chapter contains data that will soon be submitted for publication.

All the experimental work was performed by the author of this thesis.

Summary

Enterococci can be found in many different environments, such as hospitals, veterinary facilities and food industry. These environments are cleaned and disinfected every day with biocides. Their use has been rising in the past decades and little is known about how enterococci respond to these chemical agents. The presence of vancomycin-resistance enterococci (VRE) in the above mentioned environments constitutes a cause of concern. VRE ability to tolerate biocides, when compared to vancomycin-susceptible enterococci (VSE), has not been well understood or evaluated. In order to address the questions are VRE strains more tolerant or have more survival advantages in the presence of three different biocides (benzalkonium chloride, chlorhexidine and triclosan) than VSE? Tolerance to these biocidal compounds was compared in an enterococcal population from clinical and veterinary environments and from dairy products and no correlation was found between the MIC value for the biocide and susceptibility to vancomycin. To address the second question, we used two isogenic strains (one VRE and one VSE). Strains were grown alone or together, and growth rates in the presence of biocides were determined, as well as relative fitness. When growing alone, growth was significantly affected by the highest concentration of biocide used in both strains. When growing together, the VRE strain was much more affected than the VSE strain and its relative fitness very low in the presence of benzalkonium chloride. Changes in MIC values for biocides, vancomycin and other antibiotics were measured after exposing both strains to different concentrations of benzalkonium chloride, chlorhexidine and triclosan. Results were similar for both strains. In particular, exposure to biocides induced an increment in susceptibility to penicillin. VRE strains had no survival advantage in the presence of biocides nor are more

tolerant to these compounds than VSE, suggesting that the use of these compounds is not acting as a pressure for selecting VRE strains.

Introduction

Enterococci are Gram-positive commensal bacteria which inhabit the gastro-intestinal tract of humans and other animals. Members of this genus have intrinsic and acquired resistance to several antibiotics and constitute the third most prevalent nosocomial pathogens isolated from bloodstream infections, and represent the most frequent cause of surgical-site infections in intensive care units (26). Nosocomial infections caused by vancomycin-resistant enterococci (VRE) have been recognized as a problem in hospitals worldwide (12, 21, 23, 25).

The use of biocides in the health care environment is a potential approach to controlling rates of VRE infections in hospitals. Enterococci harsh nature allows them to disseminate in a variety of other habitats, namely fermented foods, water, soil and plants. Due to this, the use of biocides also plays an important role in eradicating these bacteria in the food industry, agriculture and veterinary setting and in our homes. Biocides have been incorporated into diverse items such as surgical scrubs, laundry soaps, hand washes, cosmetics, toothpastes, mouthwashes and even in toys. Unlike antibiotics, biocides have multiple, non-specific modes of action to destroy bacteria and are used as antiseptics, disinfectants or preservatives. Usually, biocides are used at concentrations high enough to kill all organisms, including the resistant ones. Although, there is a concern related to the way they are used (for example surface cleaning and disinfection), as there will be areas in which bacteria will be exposed to sub-inhibitory concentrations (gradients of biocides). In this scenario, the risk of selecting bacteria less susceptible to

the biocidal agent used or to other antimicrobial agents increases. Moreover, if the nonsusceptible organism is cross-resistant to an antibiotic, an undesirable outcome is created as a result of biocide use: selection of an antibiotic-resistant microorganism (30, 34).

Many studies have approached two questions: is the use of biocides selecting for antibiotic-resistant microorganisms?, and are antibiotic-resistant microorganisms less susceptible to biocides than antibiotic-susceptible ones? (for review 7, 15, 27). This problematic has special importance in the hospital environment, where both biocides and antibiotics are used more frequently and where it is easier to find antibiotic-resistant bacteria. Surfaces are frequently contaminated with VRE and other antibiotic-resistant bacteria, health care workers can easily contaminate their hands by touching these surfaces and therefore transport pathogens to other patients/environments. Biocides have to be effective against VRE, methicillin-resistant *Staphylococcus aureus* (MRSA) and many other bacteria. Three of the most commonly used biocides are chlorhexidine (CHX), used as a surgical scrub and skin disinfectant, triclosan (TCS), used for skin and hands disinfection, and benzalkonium chloride (BC), used for disinfection of surfaces. A concern that rises from the use of these compounds is the possibility that antibiotic-resistant bacteria, namely VRE, could be co-resistant to these disinfectants.

In this work, the correlation between susceptibility to biocides and vancomycin resistance was investigated. Despite other previous works have addressed the same question, we believe it is important to compare VSE and VRE isogenic strains. First MIC values for BC, CHX and TCS of several enterococcal isolates from different origins were measured and correlated with susceptibility/resistance to vancomycin of the same isolates. Second, for the first time, two isogenic strains (one VRE and one

VSE) were used in competition assays, prolonged exposure to biocides and fitness assays.

Materials and Methods

Bacterial strains

A total of 83 enterococcal isolates from our collection were studied: 35 clinical isolates, 16 veterinary isolates and 32 dairy isolates. All isolates have been previously identified to the species level and typed by PFGE. None of the 83 isolates are clones and were isolated from three different environments, namely Portuguese milk hard cheese, nosocomial infections (genito-urinary tract infections, bacteraemia/sepsis, catheter-associated infections), and animal infections (cats and dogs isolates from urinary tract and skin and soft tissue infections). *E. faecalis* V583 Δ ermB (Van^R) (vancomycin-resistant and erythromycin susceptible; kindly provided by Axel Hartke) and *E. faecalis* V583 Δ vanB (Van^S) (vancomycin and erythromycin susceptible) were also used. Van^R has a MIC value for vancomycin > 256 μ g/ml, while Van^S as a MIC of 6 μ g/ml for the same antibiotic (both determined by E-test).

Biocides and antibiotics

Three different biocides were used in this study: benzalkonium chloride (Sigma-Aldrich) (BC), chlorhexidine (Fluka) (CHX) and triclosan (Sigma-Aldrich) (TCS). Vancomycin E-test (bioMérieux) and antibiotic disks (Oxoid) were used: erythromycin (E) 15 μ g, penicillin G (P) 10U, chloramphenicol (C) 30 μ g and methicillin (MET) 5 μ g.

Susceptibility tests

Minimum inhibitory concentration (MIC) for TCS was determined by micro dilution for the 83 strains according to CLSI (10). For BC and CHX MIC values were already determined by us in a previous work (8) with the exception of five clinical isolates (four *E. faecalis* and one *Enterococcus* spp.) and five dairy isolates (two *E. faecium*, one *E. durans* and one *Enterococcus* spp.). Isolates obtained after prolonged exposure to biocides were also tested for their susceptibility to vancomycin, by E-test, and to E, P, C and MET by disk diffusion method according to bioMérieux (for E-test) and CLSI (for disk diffusion test) instructions. Strains were classified as susceptible, intermediate or resistance according to the bioMérieux criteria for E-tests and to CLSI criteria for the remaining antibiotic disks.

Continuous exposure to vancomycin and biocides

Van^R and Van^S were grown separately in 5 ml of Brain Heart Infusion (BHI, Oxoid) during 14 days in the presence of each antimicrobial agent separately. Each day, 1% of the previous overnight culture was passed into fresh medium containing the respective agent. At day 7 and day 14, the culture was stored at -20°C for future use. Two types of assays were performed, one in which the antimicrobial agent concentration was constant (CC) in all passages and another in which the concentration was increasing (IC) through time. A control experiment was performed by passing the strains into fresh BHI without antimicrobial agent, every day during the same time period, using the same percentage of inoculum. In the case of biocides, the increases were of 0.5 µg/ml each day until 4 µg/ml and 7 µg/ml, for BC (initial concentration 1 µg/ml), and 3 µg/ml and 6.5 µg/ml, for CHX and TCS (initial concentration 0.25 µg/ml for both), at day 7 and day 14, respectively. The constant concentration used was 3 µg/ml for each biocide. For vancomycin, increases were of 2 µg/ml

each day until a final concentration of 12 µg/ml at day 7, and 24 µg/ml at day 14 (initial concentration 1 µg/ml). The constant concentration used for this antibiotic was 8 µg/ml.

Detection of the *vanB* gene

The presence of the *vanB* gene was detected using primer Fwd ATC GGC CTA CAT TCT TAC A and primer Rev AGC GTT TAG TTC TTC CGT (11).

Growth curves

100 µl of an overnight culture of Van^R and of Van^S were incubated separately in 10 ml of BHI supplemented with different concentrations (0.25 µg/ml, 0.5 µg/ml, 1 µg/ml and 2 µg/ml) of the three tested biocides. Optical density (OD) at 600 nm was measure along time. For each biocide and each concentration, the biocide was either added at the begging of the assay or added when the culture reached an OD of 0.4. Controls were performed in the same way but without any biocide. Growth rates were determined at the beginning of exponential phase when biocide was added at the begging of the assay in at least three independent experiments. Strains were also incubated together in 200 ml of BHI, in 1:1 proportion, supplemented with 1 µg/ml or 2 µg/ml of each biocide. OD and colony-forming unit (cfu) were monitorized every hour during 6h and after 24h of incubation. In all assays, controls were performed incubating both strains only in BHI.

Fitness assays

Competition assays were performed in 500 ml Erlenmeyers containing 200 ml of BHI incubated with 1 ml of an overnight culture of Van^R and 1 ml of an overnight culture of Van^S, for 24 hours at 37°C, with agitation. In the control situation no biocide was added to the medium. BC, TCS and CHX were tested separately under the same conditions and 2 µg/ml was used for each compound. At time 0h and at the end of this competition assay (time 24h), appropriate dilutions were plated onto BHI agar plates. Plates were incubated for 24h at 37°C and 100 colonies were picked into BHI agar plates, without and with vancomycin (32 µg/ml). These plates were also incubated under the same conditions and colonies were count after 24h. Fitness was calculated according to Turner and Chao (32). Fitness (W) was defined as a relative change on the ratio VRE:VSE or $W = (R_t/R_0)/(S_t/S_0)$; being R_0 and S_0 the cfu of VRE and VSE, respectively, at the beginning of the assay and R_t and S_t the cfu of VRE and VSE, respectively, at the end of the assay (24h later).

Statistical analysis

Microsoft Office Excel 2007 was used to process data on the frequency of vancomycin-resistance and MIC values obtained. The prevalence of vancomycin-resistance was compared using the Chi-squared (χ^2) test to determine statistically significant differences. Student's t-test was performed using Microsoft Office Excel 2007 to compare the fitness value obtained in the control situation with the expected value and to compare the growth rate of VRE strain and VSE strain exposed to biocides with the control situation. All tests were two-tailed and tested at level of 0.05 for significance.

Results

Susceptibility to biocides

In order to understand if there was any association between susceptibility to BC, CHX and TCS, and vancomycin resistance, we studied several enterococcal isolates from different environments. MIC values for CHX and BC ranged between 1 and 8 $\mu\text{g/ml}$ (8). For TCS, MIC value was 8 $\mu\text{g/ml}$ for all isolates, regardless of the environment, species or resistance to vancomycin (Table 1). The majority of isolates from the three environments had MIC values of 4 $\mu\text{g/ml}$ for both BC and CHX. There was no difference between *E. faecalis* or *E. faecium* (the two most abundant species) in susceptibility to any of the three biocides tested. The same pattern was observed for both VRE and VSE isolates: 87% and 90% of the VSE isolates presented a MIC value ≥ 4 $\mu\text{g/ml}$ for BC and CHX, respectively; while 73% and 91% of the VRE isolates presented MIC value ≥ 4 $\mu\text{g/ml}$ for the same biocides, respectively. There was no association between MIC values and vancomycin-resistance ($p > 0.05$; χ^2) for any of the biocides tested.

Table 1: Distribution of enterococcal isolates from different species and environments amongst the different MIC values ($\mu\text{g/ml}$) for BC, CHX and TCS and susceptibility to vancomycin.

Species	Number of isolates																							
	MIC BC ^a						MIC CHX ^a						MIC TCS											
	1			2			4			8			8											
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R						
Clinical isolates																								
<i>E. faecalis</i>				2	2	1	4	8	5				1	2		5	8	4		2		6	10	6
<i>E. faecium</i>							4	1		5			1			7	1		1			9	1	
<i>E. hirae</i>				1			1						1			1						2		
<i>Enterococcus</i> spp								1									1						1	
Veterinary isolates																								
<i>E. faecalis</i>				3			5	3								8	3					8	3	
<i>E. faecium</i>							3			1						4						4		
<i>E. solitarius</i>				1												1						1		

Table 1: Continued.

Species	Number of isolates																											
	MIC BC ^a						MIC CHX ^a						MIC TCS															
	1			2			4			8			2			4			8			8						
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R				
Dairy isolates																												
<i>E. faecalis</i>								11	4	2									9	4	2	2				11	4	2
<i>E. faecium</i>						1		2	1	1				1					2	1	1				2	1	2	
<i>E. durans</i>			1					5											4		1	1			5		1	
<i>E. casseliflavus</i>								1											1						1			
<i>E. hirae</i>								1						1											1			
<i>Enterococcus</i> spp.								2						1					1						2			
Total	0	0	1	7	2	2	39	18	8	6	0	0	5	2	1	43	18	8	4	0	2	52	20	11				

S – susceptible to vancomycin; I – intermediate to vancomycin; R – resistant to vancomycin. According to CLSI criteria (unpublished data from Fátima Lopes Lab).

^a Data from Braga et al (8).

Continuous exposure to vancomycin and biocides

Van^R and Van^S had the same MIC values for BC (4 µg/ml), CHX (4 µg/ml) and TCS (8 µg/ml). Table 2 shows the MIC values for BC, CHX, TCS and vancomycin of those two strains after exposure to the tested antimicrobial agents. When exposed to BC and CHX, both strains had a MIC increment of four times the initial MIC value, synonymous of decreasing susceptibility to these two biocides. No effect in the MIC value was observed in the case of exposure to TCS. The susceptibility to vancomycin of the studied strains was not changed upon exposure to BC and CHX. In contrast, after continuous exposure to TCS, Van^R lost its resistance to vancomycin (Figure 1). *vanB* gene was thus searched by PCR and confirmed to be present in the adapted Van^R strains (data not shown). The exposure of Van^S strain to any of the three biocides did not lead to a decrease in vancomycin susceptibility. Exposure to vancomycin induced an increase in MIC values for BC and CHX, but an opposite effect in TCS MIC was observed in some cases, regardless of being a VRE or VSE strain. The exposure of Van^S to vancomycin turned this strain back to the vancomycin resistant phenotype.

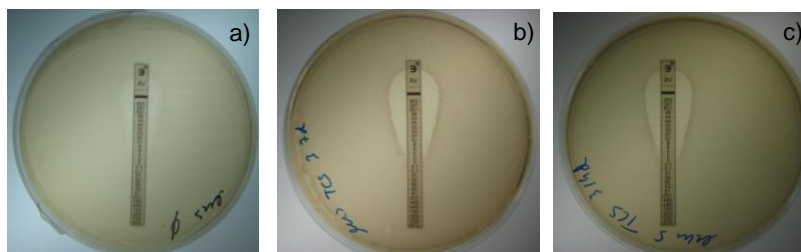


Figure 1: Van^R susceptibility to vancomycin after growth in: a) BHI (control); b) BHI plus 3 µg/ml of TCS for 7 days; c) BHI plus 3 µg/ml of TCS for 14 days.

Table 2: MIC values for BC, CHX, TCS and VA for the two strains exposed to different antimicrobial agents.

Strains	Antimicrobial agent assay	MIC ($\mu\text{g/ml}$)				
		BC	CHX	TCS	VA	
Van ^R	Control	BHI ^a	4	4	8	>256
		BHI 7d	4	4	8	>256
		BHI 14 d	8	4	8	> 256
	BC	CC-3-7d	8			>256
		IC-4-7d	8			>256
		CC-3-14d	16			>256
		IC-7-14 d	16			>256
	CHX	CC-3-7d		16		>256
		IC-3-14d		16		>256
		CC-3-14d		16		>256
		IC-6.5-14d		16		>256
	TCS	CC-3-7d			8	6
IC-3-7d				8	6	
CC-3-14d				8	6	
IC-6.5-14d				8	6	
VA	CC-8-7d	8	16	8	>256	
	IC-12-7d	8	16	4	>256	
	CC-8-14d	8	16	8	>256	
	IC-24-14d	8	8	4	>256	

Table 2: Continued.

Strains	Antimicrobial agent assay	MIC ($\mu\text{g/ml}$)				
		BC	CHX	TCS	VA	
Van ^S	Control	BHI ^a	4	4	8	6
		BHI 7d	4	4	8	6
		BHI 14 d	4	4	8	6
	BC	CC-3-7d	8			6
		IC-4-7d	8			6
		CC-3-14d	16			6
		IC-7-14 d	16			6
	CHX	CC-3-7d		16		6
		IC-3-14d		16		6
		CC-3-14d		16		6
		IC-6.5-14d		16		6
	TCS	CC-3-7d			8	6
		IC-3-7d			8	6
		CC-3-14d			8	6
		IC-6.5-14d			8	6
	VA	CC-8-7d	8	16	8	>256
		IC-12-7d	8	16	8	>256
		CC-8-14d	8	16	8	>256
		IC-24-14d	8	16	4	>256

^a Results obtained from MIC determination performed from fresh inoculum.

We also investigated if exposure to biocides induced any change in the susceptibility to erythromycin, penicillin G, chloramphenicol and methicillin. Both tested strains were susceptible to chloramphenicol and resistant to methicillin and exposure to the three biocides did not change these susceptibilities (data not shown). Differences were found in the case of erythromycin and penicillin G (Table 3). Exposure of both Van^R and Van^S strains to any of the biocides tested induced susceptibility to penicillin. TCS induced resistance to erythromycin in the VSE strain. Van^S strain was able to keep its susceptibility to erythromycin after exposure to BC and CHX, as opposed to the Van^R strain. However, in this strain, the increased resistance to erythromycin was not a result of exposure to biocides, as BHI cultures alone lead to erythromycin resistance by an unknown mechanism.

Growth curves

In order to understand if the presence of biocides induced changes in growth, the two isogenic strains were grown in separate in the presence of different concentrations of each biocide. When biocides were added at OD 0.4 (middle exponential phase), no differences in growth were observed, for any biocide nor any concentration tested (data not shown). When biocides were added at the beginning of the growth many differences were observed (Figure 2). Maximum growth rates (μ_{max}) were calculated and values obtained are reported in Table 4.

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Table 3: Susceptibility of Van^R and Van^S strains to erythromycin and penicillin assessed by disk inhibition method after exposure to BC, CHX and TCS.

Strains	Antimicrobial agent assay	Antibiotic		
		Erythromycin	Penicillin	
		Halo (mm)	Halo (mm)	
Van ^R	Control	BHI ^a	16	13.6
		BHI 7d	0	11.5
		BHI 14 d	0	11.7
	BC	CC-3-7d	0	21.6
		IC-4-7d	0	21.1
		CC-3-14d	0	20.8
		IC-7-14 d	0	20.6
	CHX	CC-3-7d	0	15.2
		IC-3-14d	0	21.6
		CC-3-14d	0	20.5
		IC-6.5-14d	0	20.8
	TCS	CC-3-7d	0	17.9
		IC-3-7d	0	18.2
		CC-3-14d	0	19.9
		IC-6.5-14d	0	27.1
	Van ^S	Control	BHI ^a	16.3
BHI 7d			16	13.1
BHI 14 d			14.5	11.4
BC		CC-3-7d	18	21.3
		IC-4-7d	18.5	21.1
		CC-3-14d	16.6	19.8
		IC-7-14 d	17.4	19.8
CHX		CC-3-7d	14.9	15.4
		IC-3-14d	18.5	15.8
		CC-3-14d	12.4	21.7
		IC-6.5-14d	17	20.1
TCS		CC-3-7d	0	17.8
		IC-3-7d	0	19
		CC-3-14d	0	22.6
		IC-6.5-14d	0	18.8

^a Results of disk assays performed from fresh inoculum.

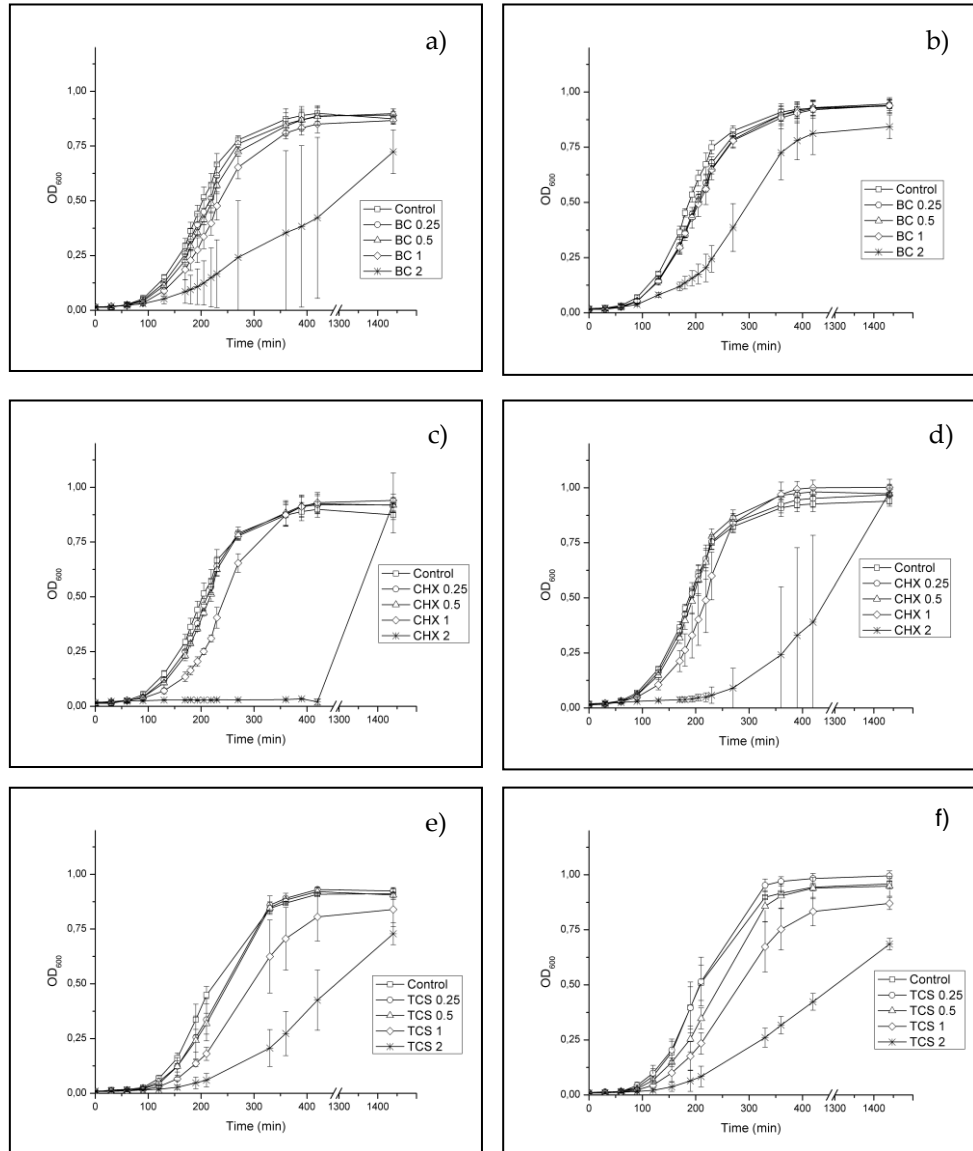


Figure 2: Growth curves for Van^R and Van^S strains when biocides were added at the beginning of the assay. a), c) and e): growth curves of Van^R in the presence of BC, CHX and TCS, respectively; b), d) and f): growth curves of Van^S in the presence of BC, CHX and TCS, respectively.

Table 4: Maximum growth rate (μ_{\max}) calculated when biocides were added in the beginning of growth.

		μ_{\max} (h^{-1})	
		Van ^R	Van ^S
Control		1.53 ± 0.21	1,56 ± 0.24
BC			
	0.25	1.19 ± 0.06*	1.51 ± 0.27
	0.5	1.24 ± 0.08*	1.40 ± 0.25
	1	1.22 ± 0.11*	1.31 ± 0.19
	2	0.61 ± 0.18*	0.79 ± 0.23*
CHX			
	0.25	1.19 ± 0.15*	1.62 ± 0.31
	0.5	1.14 ± 0.07*	1.44 ± 0.21
	1	0.97 ± 0.08*	1.25 ± 0.26
	2	nd	nd
TCS			
	0.25	1.62 ± 0.29	1.72 ± 0.42
	0.5	1.43 ± 0.22	1.65 ± 0.08
	1	1.40 ± 0.27	1.53 ± 0.08
	2	0.80 ± 0.21*	0.86 ± 0.28*

* Statistically different ($p < 0.05$) using Student's t-test, when compared to the control situation.

nd – not determined

Both Van^R and Van^S had similar growth rates when growing in BHI (control situation). The growth of Van^R was affected by all concentrations of BC and CHX. For TCS, only 2 $\mu\text{g/ml}$, the highest tested concentration, was able to induce an effect upon μ_{\max} of Van^R strain. Growth of Van^S strain was affected only in the presence of 2 $\mu\text{g/ml}$ of all tested biocide. The μ_{\max} for 2 $\mu\text{g/ml}$ of CHX was not determined, as the lag phase lasted around seven hours.

Relative fitness

We then decided to grow both strains together in order to measure their relative fitness in the presence of each biocide, as this is a scenario closer to reality. Strains growing together behaved similarly to growth separately in the presence of CHX and TCS (Figure 3). However, for 2 $\mu\text{g/ml}$ of BC, when Van^R and Van^S were together this concentration did not affect growth of the culture, as opposed to what was seen when strains grew separately in the same BC concentration (Figure 2).

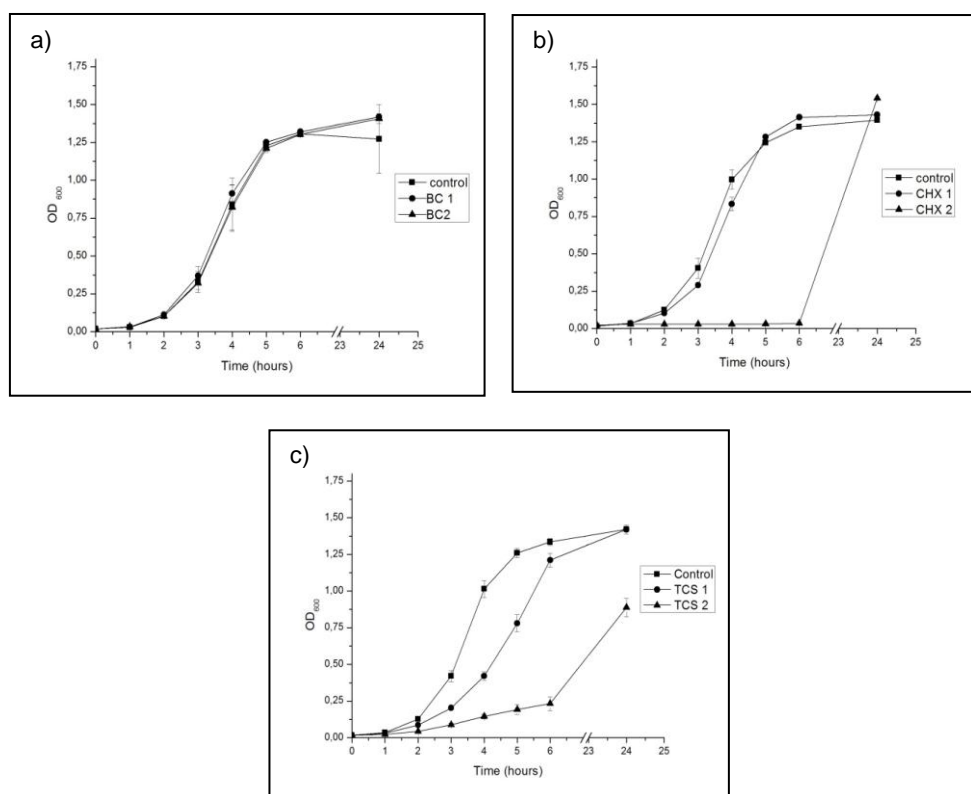


Figure 3: Growth curves of Van^R and Van^S growing together in the presence of the each biocide: a) benzalkonium chloride; b) chlorhexidine; c) triclosan.

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Fitness assays were also performed, but only for 2 µg/ml of each biocide, as it was the concentration with which stronger effects on growth were observed. In the control situation, where no biocide was added, there was no prevalence of any strain over the other ($W=1.31$; $p > 0.05$ when compared with a $W=1$). When biocides were used, Van^S had always a higher fitness, specially in the presence of BC and CHX (Table 5).

Table 5: CFU obtained for VRE strain and VSE strain at time 0h and time 24h when grown together in different conditions and respective average fitness value and standard deviation.

Conditions	CFU				Relative fitness
	VRE		VSE		
	t0	t24	t0	t24	
Control	$3.66 \times 10^6 \pm$	$3.99 \times 10^8 \pm$	$5.57 \times 10^6 \pm$	4.71×10^8	$1.31 \pm$
	1.16×10^6	0.91×10^8	0.93×10^6	$\pm 0.60 \times 10^8$	0.16
BC 2 µg/ml	$3,70 \times 10^6 \pm$	$6,50 \times 10^7 \pm$	$6.24 \times 10^6 \pm$	$5.45 \times 10^8 \pm$	$0.248 \pm$
	1.98×10^6	8.23×10^7	0.52×10^6	1.49×10^8	0.29
CHX 2 µg/ml	$4.37 \times 10^6 \pm$	$2.76 \times 10^7 \pm$	$7.35 \times 10^6 \pm$	$3.60 \times 10^8 \pm$	0.064
	0.21×10^6	4.54×10^7	0.31×10^6	4.17×10^8	± 0.07
TCS 2 µg/ml	$5.10 \times 10^6 \pm$	$7.88 \times 10^7 \pm$	$6.98 \times 10^6 \pm$	$1.35 \times 10^8 \pm$	$0.791 \pm$
	0.84×10^6	2.18×10^7	1.22×10^6	0.31×10^8	0.01

Discussion

In this study, a total of 83 enterococci from our collection were studied: 35 clinical isolates, 16 veterinary isolates and 32 dairy isolates. The issue of VRE spread is of great concern, as this may render ineffective one of the last resort antibiotic, vancomycin. Several studies have thus addressed the question if VRE are being selected or are more resistant to biocides. Our results demonstrated that vancomycin resistance carries no advantage in tolerance to the biocides herein tested. Other authors (4, 9, 16, 20, 31) have also studied different VRE and VSE clinical isolates and concluded that antibiotic resistance does not affect the utility of the biocide. In another study (5) environmental isolates of vancomycin-resistant *E. faecium* from human wastewater effluents were characterized for susceptibility to biocides and antibiotics and compared with VSE strains. No correlation was found between antibiotic resistance and disinfectant susceptibility patterns. Baillie *et al.* (3) compared *E. faecium* sensitive to both vancomycin and gentamicin with vancomycin-resistant and gentamicin-resistant strains and the results showed no increase in resistance to chlorhexidine as indicated by MIC. The majority of the studies published so far used clinical isolates, herein we compared clinical isolates with veterinary isolates and dairy food isolates. Although, all these studies, this one included, compared VRE and VSE strains from different genetic backgrounds and thus the differences could not be attributed, for sure, to differences in vancomycin resistance, as genomes were different.

In our study, for the first time, two isogenic strains (one VRE and the other VSE) were used in order to address the question if VRE strains are more tolerant or have more survival advantages in the presence of biocides than VSE. Several assays were performed with these two strains and conclusions were quite consistent: VRE strain has no advantages in

what respects biocide tolerance when compared with its isogenic VSE. When both Van^R and Van^S were exposed to different concentration of each tested biocide for 7 and 14 days, their MIC values suffered the same increment; with the exception of TCS that did not have any effect on the MIC value of both strains. Fraise (13) created a BC mutant of VRE strains, exposing them to non-inhibitory concentrations of the biocide, centrifuging and applying the pellet in agar plates containing range of inhibitory levels of biocide. He also observed a MIC value increment for this compound (it duplicated). The MIC increases observed in our study may be due to changes in the cell membrane, activation of efflux pumps and other systems that are turned on during exposure. However, the exposure to these biocides increased the MIC values to themselves but not to vancomycin. It seems that the mechanisms induced in the cell by BC and CHX are not responsible for co-resistance to vancomycin. They probably do not affect the peptidoglycan structure and synthesis. Van^S strain remained susceptible to this antibiotic even after exposure to the three biocides. Van^R was resistant to vancomycin, yet after exposure to TCS lost its resistance to that antibiotic (MIC value >256 µg/ml became 6 µg/ml). Triclosan inhibits bacterial enoyl-acyl carrier protein reductase (22), thus reducing fatty acid synthesis and consequently lipid synthesis. Triclosan also has a membranotropic effect, it is incorporated into the phospholipic membranes, probably aligning itself with the phospholipid acyl chains, interacting and affecting phospholipid membranes without cell lysis and inducing the formation of perturbed membrane structures (33). This may explain why Van^R became susceptible to vancomycin after being exposed to TCS and also why the exposure to this biocide did not change the MIC value for itself. When the two strains were exposed to different concentrations of vancomycin, MIC values increased for BC and CHX but not to TCS. Vancomycin bound to the C-terminal D-Ala-D-Ala of the late peptidoglycan precursors may be delaying the action of BC and CHX and

may not interfere or even help the action of TCS (in some cases, a MIC reduction was observed). The VSE strain became resistant to vancomycin after being exposed to it. It is important to notice that this only happens to this VSE strain, but not for an original VSE strain, namely JH2-2 (results not shown). Could it be a mutation or compensating deletion of *vanB*? At the moment we have no explanation for this, further experiences are needed to understand this result. However, important is to mention that incubation with biocide does not have the same effect and that JH2-2 also does not become VRE after incubation with vancomycin.

Changes in tolerance to other antibiotics after being exposed to biocides were also investigated using the two isogenic strains. The exposure to the biocides did not change the susceptibility pattern of any of the strains to chloramphenicol and methicillin, but this was not observed for penicillin and erythromycin. In the first case, both strains became susceptible to penicillin. These results suggest that exposure to biocides sensitize VRE and VSE to penicillin, and likely *E. faecalis*. These changes may be explained by changes in the affinity for penicillin-binding proteins (PBPs). Enterococci are known to have low affinity PBPs (24), which grants these bacteria resistance, or less susceptible to β -lactam antibiotics. Biocides may change affinity of PBPs to penicillin, eventually leading to bacteria less resistance to penicillin. In the case of erythromycin, no inhibition halo appeared for Van^R, either in the control situation (for 7d and 14d) or when exposed to biocides. Van^S presented no inhibition halo after treatment with TCS. These phenomena are difficult to explain, as both strains (Van^R and Van^S) do not contain the gene responsible for erythromycin resistance. However, other genes (such as those encoding for cell envelope, transport and binding proteins) are important for the survival and growth maintenance of V583 treated with this antibiotic (1) and those maybe playing an important role in the resistant patterns obtained. Future work should try to analyse if any of the

genes referred by Aakra *et al.* interfere or are activated by the use of this biocides. In the case of Van^R, we believe that the result obtained has little to do with the exposure to the biocide, as the strain presented an inhibition halo of 16 mm when the assay was performed with fresh inoculum, but after being for 7 and 14 days in BHI no halo was observed.

In order to understand if VRE had any advantage over VSE during growth in the presence of biocides, isogenic strains were grown alone and together in the presence of each tested compound. When they grew alone, the VRE strain was more affected by the presence of biocides than its isogenic VSE strain, but both were specially affected by the highest concentration tested, 2 µg/ml. When Van^R and Van^S were grown together, their behavior in the presence of these biocides was quite similar to the one observed when growing alone, with the exception of BC, as growth was not affected.

Crossing our data from Table 4 and Table 5 gave us a new perspective. When both strains were together and in the absence of the biocides, a two log increment was observed in 24 hours. In the presence of all biocides at 2 µg/ml, the VSE strain was able to keep this log increment, while the VRE strain could not. Although, VSE μ_{max} was significantly affect in the presence of all biocides for the above mentioned concentration. It seems like the VRE strain is promoting or “protecting” the VSE strain from the biocides. This can also explain why in the presence of BC the growth curves were so different when strains were alone or together, and growth was not affected.

The dynamics between VRE and VSE have also been approached in other studies. Foucault *et al.* (14) recently demonstrated that the inactivated or inducible Tn1549-encodded *vanB* operon was not costly to the enterococcal host in the absence of vancomycin. Resistant strains may persist for a long time if allowed to adapt to the environment (18). In the present study, VRE had no advantage to VSE when grown in the

absence of any antimicrobial agent tested, but in the presence of biocides it could not prevail. CHX and BC killed or inhibited VRE more than VSE, while TCS had a similar effect on both strains. Relative fitness of VRE to VSE was smaller in the presence of BC than in the presence of CHX and TCS. It seems that the advantage gained by VRE strains in the presence of vancomycin is not transferred when in the presence of biocides. Although, this was the first time that a fitness approach was applied to this problematic, more studies are required.

Other research works have approach the problematic of VRE and VSE and their tolerance to biocides and commercial disinfectants, using other techniques such as bactericidal activity (19, 29), suspension test (2, 28), used-dilution (17), surface test (6) and they concluded almost all the same. No differences between VRE and VSE strains in what concerns biocide tolerance. As already mentioned, all these results were obtained from strains with different genomes. This kind of approach is also present in our work and the same conclusions were taken: in what concerns BC, CHX and TCS, VRE strains have no surviving advantage when compared to VSE. We decided to go one step forward and for the first time, two isogenic strains (one carrying the *vanB* gene and the other without it) were studied in order to understand if the presence of this gene gave any advantage to the VRE strain in what concerns biocide tolerance. From our data, it is possible to conclude that a VRE strain has no survival advantage in the presence of biocides herein tested. Exposure to biocides increase MIC values to themselves, but does not increase resistance to vancomycin. Exposure to this antibiotic may have an effect on biocides tolerance. However, the use of biocides is not responsible for the prevalence of VRE in hospitals, as VRE are not more fitted to survive in the presence of these compounds. Constant surveillance may be required in hospital environment, veterinary facilities and other places where antibiotics and biocides are used in order to prevent future risk situations.

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General discussion

Chapter V

The present work represents a new approach to the interaction and response of enterococci to biocides. Enterococci, due to their robust nature, are able to survive in many different environments. During this research, three environments where enterococci play an important role were studied: clinical, veterinary and dairy food. An unexplored environment was screened for the presence of these bacteria: dust from breeding pig facilities. The biocides tested were chosen among the most commonly used for cleaning and disinfection of the mentioned environments. They were: benzalkonium chloride, chlorhexidine and triclosan.

First the MIC value for the three biocides was investigated for all the isolates above described. This would allow understanding if in any of the environments in study, enterococci were more tolerant to biocides. Unlike antibiotics, there are no guidelines for MIC values in respect to biocides. Although, this methodology is still a good approach to establish a reference level as it gives a quantitative value, results can be easily reproduced and compared and it is not expensive. It was also chosen as other studies already used it, so a comparative analysis could be easily done. The values obtained for benzalkonium chloride and chlorhexidine for the clinical, veterinary and dairy food isolates, ranged between 2 and 8 µg/ml and for triclosan all isolates presented a MIC of 8 µg/ml. Enterococci isolated from dust samples, presented MIC values from < 0.25 to 4 µg/ml for benzalkonium chloride and from < 0.25 to 8 µg/ml for chlorhexidine. Despite different origins, the MIC values obtained were very similar for the tested compounds. In the case of benzalkonium chloride and chlorhexidine, the predominant MIC value found for all isolates was 4 µg/ml. In general and independently from the environment, enterococci did not present tolerance to the biocides tested and the MIC values found were similar to those already described in the literature (3, 4, 7, 11, 12). It is interesting to notice that as time passed the MIC values recorded in different research works are similar and did not suffer an increment. In

general, for enterococci, there are no MIC values for these three compounds above 16 µg/ml. It would be important to periodically monitorize environments where biocides are daily used, such as clinical and veterinary facilities, in order to observe if the MIC values tend to increase with the use of these compounds. Besides MIC tests, the monitorization work could also include time kill curves, surface tests and/or suspension tests. It would be also important to screen new environments where biocides and enterococci co-habit, such as homes and schools.

An issue poorly explored concerns which mechanisms are present in the response of enterococci to biocides. Residual concentrations of biocides can be a problem, as already mention in this work, as a single mechanism can be responding to the biocide. The *Enterococcus faecalis* V583 pTEF1 orf EFA0010, annotated as a putative SMR transporter, was named during this thesis as *qacZ* and its role in the enterococci response to benzalkonium chloride, chlorhexidine and ethidium bromide was determined. This gene was constitutively expressed and was not induced by the presence of the biocides. The introduction of a high copy number plasmid containing this gene into V583ErmS, represented a MIC increment for benzalkonium chloride, but not for chlorhexidine or ethidium bromide. *qacZ* gene is involved in the response of enterococci to benzalkonium chloride, but not to ethidium bromide, as proven by the efflux assay performed (see chapter II, Figure 2). This is explained by the substitution of the cysteine 42 in the QacZ protein by a serine, as described by Paulsen et al (10) who demonstrated this substitution leads to a huge decrease in ability of QacC to provide resistance to EtBr. For the first time, for the genus *Enterococcus*, it was described a gene that is involved in the response to a QAC, benzalkonium chloride. The presence of this gene was investigated in the clinical, veterinary and dairy food

isolates, as well as a possible correlation between it and the MIC value for the above mentioned compounds. This gene was only found in clinical and dairy food isolates, which means that it may not yet be disseminated in the veterinary environment. Although, there was no association between the MIC value of the isolates for tested biocides and the presence/absence of *qacZ*.

It would be interesting to screen the presence of the *qacZ* gene in more isolates, especially veterinary ones, to investigate if this gene is or is not yet disseminated in this environment. From this work, the presence of this gene is not selecting enterococci more tolerant to biocides, in particular to benzalkonium chloride. To understand if its presence is not selecting for enterococci more tolerant to other antimicrobial agents, such as antibiotics would be interesting. A starting point could be to investigate if the enterococci isolates from dust of breeding pig facilities have this gene, what is its prevalence and if those who carry the *qacZ* are more tolerant to antibiotics used in veterinary practice. The above mentioned monitoring of environments such as clinical and veterinary facilities and new ones, should also contemplate the screening of the presence/absence of *qacZ* and the study of the relationship between MIC values for biocides and the prevalence of this gene. Future research should look for other enterococci mechanisms of response to biocides, how they are disseminated and if they are related or not to *qacZ*.

In the clinical, veterinary and dairy food isolates was also investigated if there was any association between their MIC value for the three biocides tested and their susceptibility/resistance to vancomycin. Once more, no association was found. So, as far as it was studied during this thesis, the MIC value for a certain biocide is not associated neither with the presence/absence of *qacZ* gene or resistance to vancomycin.

Resistance to vancomycin in enterococci raises another problem in the matter of biocides use: if VRE are more able to survive than VSE when these compounds are used or if the use of biocides selects for VRE strains. This issue was already explored by others investigators by comparing the susceptibility of several VRE and VSE isolates to biocides; MIC testing and time-kill determination were the most common approaches used (2, 3, 5, 7, 12) and where, in general, no differences were registered. A different and new approach of this problem was done during this research work, two isogenic strains were used, one carrying the *vanB* gene and the other without it. These two strains were submitted to different experiments, such as, growth in the presence of biocides, continuous exposure to biocides and relative fitness assays. Data obtained from these experiments, lead to a conclusion where the fact of being a VRE does not bring any survival advantage in the presence of biocides and its relative fitness decrease when compared to VSE. In fact, the opposite effect was registered, as the VRE strain appeared to be more affected by the presence of biocides during its growth. The exposure to biocides did not turn VRE or VSE strains more resistant to penicillin G, chloramphenicol, meticillin and vancomycin. In fact, the exposure of the VRE strain to triclosan changed its MIC value for the last antibiotic; the strain became susceptible to vancomycin. Although, both strains became less tolerant to each biocide after being exposed to it for 7 and 14 days. Which mechanisms are involved in this response and is this less tolerance reversible, are question that should be answered in the future.

This issue was also taken into account when the dust samples from breeding pigs were investigated. Two groups of isolates were screened during this study, one where enterococci were isolated using vancomycin in the medium and another where no antibiotic was used. For both groups identification to species and characterization for susceptibility to benzalkonium chloride and chlorhexidine was performed. The VRE

prevalence in the 171 Portuguese breeding pig facilities was of 17.5 %. This value is high, but it is inside the range of value recorded in pig feces and pig large intestine samples from different European countries (1, 6, 8). Similar environments such as superficial waters, solid waste, air and others presented a VRE prevalence of 26 % (9). VRE strains isolated in this work were also characterized for their susceptibility to biocides and once more no association was found between these two factors (MIC value for biocides and vancomycin low and high level resistance).

Three new ST of *E. faecium* were discovery in dust sampled, and implemented in the MLST *E. faecium* database. The closest STs to the new ones were of animal origin (pig), which means that animals may be contaminated by the environment and/or that dust may be carrying enterococci that colonizes animals. Some STs from human isolates were also correlated with the new ones.

Enterococci can also be transmitted from humans to animals and vice-versa. Although the growth promoter avoparcin is no longer used in veterinary practices, VRE strains are still present in breeding pig facilities in particular in dust. This is a question of concern, as dust can be spread and so disseminate VRE strains. It would be important to eradicate as much as possible VRE strains from animals environment and create monitorization practices as it is a question of bio-security to keep VRE out of the human food-chain. In what respects biocides, the clean and disinfection of pig facilities seems not to be selecting enterococci tolerant to benzalkonium chloride or to chlorhexidine. As far as this work can concern, dust from breeding pig facilities is not responsible for the dissemination of enterococci tolerant to any of the biocides tested.

During the development of this research work, I was confronted with an interesting issue: the role of the *ermB* gene (EFA0007) in the enterococci normal growth and consequently its response to biocides.

This gene, a ribosomal RNA adenine dimethylase, is responsible for *E. faecalis* V583 resistance to erythromycin. Although, its lack in the genome of this strain not only causes lost of resistance to the mention antibiotic, but also delays its growth. It also affects the expression of the *qacZ* gene as proven. Understanding what else happens in the cell in the absence of this gene and how it affects enterococci response to biocides would be an interesting challenge. Another interesting event was the changing of resistance to erythromycin of the isogenic VRE and VSE strains when they were grown for 7 and 14 days in BHI. Which changes occurred in the cell to turn a strain without the *ermB* gene resistant to erythromycin and if somehow this changes can cause any difference in the response to biocides is another open question.

Enterococci have a “good relationship” with biocides. The MIC values described for biocides cannot be considered high when compared to the usually in use concentration of biocides and as far as it is known, these bacteria do not present tolerance to these compounds. No association was found between the MIC values for biocides and vancomycin resistance and VRE strains did not proved to have any survival advantage or be more tolerant to biocides than VSE strains. Although, as biocides are part of the daily clean and disinfection routine of several environment where enterococci inhabit, constant surveillance and more screening investigations must be required.

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Tel (+351) 214 469 100
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