

PhD in Pharmaceutical Sciences
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Response of *Enterococcus* spp. to Cationic Biocides Across Dynamic Environments: Phenotypic and Genotypic Insights

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Response of *Enterococcus* spp. to Cationic Biocides Across Dynamic Environments: Phenotypic and Genotypic Insights

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Abstract

The widespread use of cationic biocides (CBs) in the healthcare, human domestic contexts, food chain, or veterinary is key to control the spread of pathogens but has been raising concerns about the potential selection of bacterial populations resistant to CBs and antibiotics. *Enterococcus faecium* and *Enterococcus faecalis* are major multidrug-resistant pathogens of global healthcare-associated infections. They are also part of the human and animal gut microbiota and are ubiquitous in other environments (e.g., food chain, sewage, soil), exposing them to varying CBs concentrations. Yet, the outcomes of this exposure on *Enterococcus*' response to CBs and antibiotics remain unclear. This study aimed to investigate the influence of CBs on the development of tolerance in *E. faecium* and *E. faecalis* populations, spanning different environments over recent decades, and their impact on resistance to clinically relevant antibiotics. To achieve this, we evaluated the susceptibility phenotypes and genotypes of 786 diverse strains to CBs using classical and advanced molecular microbiological techniques. The response of *Enterococcus* to chlorhexidine (CHX), benzalkonium chloride (BC), and didecyldimethylammonium chloride (DDAC), three common CBs representing a biguanide and two quaternary ammonium compounds (QACs), was assessed by determining their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). MICs and MBCs were notably lower than typical in-use CBs concentrations among *E. faecium* and *E. faecalis* with varied characteristics. However, an increased tolerance to CHX, BC, or DDAC was observed in particular *E. faecium* or *E. faecalis* populations from human infections or the food chain, suggesting a possible adaptation due to the growing CBs usage, warranting further monitoring. An in-depth genomic analysis was conducted to evaluate the occurrence of previously identified CBs genotypes within a broad range of *Enterococcus* populations and their correlation to susceptibility phenotypes. No correlation between mutations in the EfrEF multidrug transporter or its transcription regulator ChlR and CHX phenotypes was detected. However, low CHX MICs ($\text{MIC} \leq 1 \text{ mg/L}$) were observed in most *E. faecalis* and *E. faecium* strains lacking a functional EfrEF or ChlR. Notably, a truncated EfrE protein associated with ST40 *E. faecalis* with a human origin, from different time spans and geographical regions, reveals a divergent evolution within this clonal lineage, with still unclear underlying reasons. Acquired genes associated with CBs tolerance (CBTa), encoding efflux pumps in various bacterial species (e.g., *qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*, *qrg*, *bcrABC*, and *oqxAB*), were rare in *Enterococcus* published genomes (0.5%, $n=117$) and only found in one *E. faecium* and one *E. faecalis* from our collection. This suggests that *Enterococcus* are not significant hosts for CBTa. However, the presence of identical protein variants encoded by CBTa among *Enterococcus* and other bacterial species indicates their spread across diverse taxa. Moreover, the predominant isolation sources of *Enterococcus* and other taxa

carrying specific CBTa reflects the main settings in which these genes circulate. CBTa, often located on plasmids, are commonly co-located with metal and antibiotic resistance genes, facilitating co-selection events and spread. Yet, in our collection, the single *E. faecium* and *E. faecalis* strains with *qacZ* showed QACs phenotypes similar to those lacking the gene, implying that CBTa may not significantly impact *Enterococcus* susceptibility to CBs, thus justifying their scarcity. Of note, in examining the effect of diverse environmental conditions found in QACs usage settings, *E. faecium* and *E. faecalis* isolates, from diverse backgrounds, exhibited two to eightfold increases in MICs and MBCs to BC at 22°C and/or pH = 5, regardless of the presence of the *qacZ* gene. This highlights the influence of variable growth conditions on BC susceptibility and the potential for selecting more tolerant populations in settings where they occur. Finally, serial *in vitro* exposure to subinhibitory CHX concentrations was performed to assess their impact on CBs tolerance and antibiotic resistance. This resulted in strain-specific outcomes in six *E. faecium* isolates, possibly associated with known CHX genotypes. Notably, the P102H mutation in the DNA-binding response regulator ChtR was found in two of the three strains with increased CHX MIC and/or MBC, while the absence of the EfrEF transporter was observed in the sole strain showing increased CHX susceptibility post-exposure. However, these results also suggest the involvement of additional mechanisms, including unidentified genotypes or changes in gene expression, as one adapted isolate showed increased MIC and MBC without any known CHX tolerance genotypes. This emphasizes the need for future research with comprehensive transcriptomic analyses coupled with whole-genome sequencing before and after exposure to clarify this adaptation further. Subinhibitory CHX exposure did not increase antibiotic resistance in CBs adapted *E. faecium* strains but notably increased the transfer of plasmids carrying vancomycin or linezolid resistance genes by up to 12.5-fold among diverse strains. This effect was absent in one strain lacking a functional EfrEF. The CHX concentrations leading to adaptation or enhanced gene transfer resembled those found in contexts like patients' skin or sewage, where CBs may contribute to similar events.

In conclusion, this thesis offers critical insights into the susceptibility and adaptation dynamics of *E. faecium* and *E. faecalis* populations to CBs. Despite the effectiveness of these compounds against *Enterococcus*, the observed effects of widespread subinhibitory concentrations require careful consideration in future research to ensure the continued efficacy of both CBs and antibiotics, thereby safeguarding Public Health.

Keywords: *Enterococcus faecium*, *Enterococcus faecalis*, Cationic Biocides, Antimicrobial resistance, One Health

Resumo

O uso de biocidas catiónicos (CBs) é essencial para controlar a dispersão de agentes patogénicos em vários contextos, como em hospitais, clínicas veterinárias, ambiente doméstico ou cadeia alimentar. Contudo, têm emergido preocupações sobre o eventual impacto desses compostos na seleção de bactérias resistentes tanto aos CBs como a antibióticos. *Enterococcus faecium* e *Enterococcus faecalis* são importantes agentes multirresistentes de infeções nosocomiais a nível mundial. Fazem também parte da microbiota intestinal humana e animal e de outros ambientes (p. ex. cadeia alimentar, águas residuais, solo), expondo-os a concentrações variadas de CBs com efeitos ainda desconhecidos nestas bactérias. Assim, foi objetivo deste estudo investigar o impacto dos CBs na adaptação de *E. faecium* e *E. faecalis*, isolados de diversas origens ao longo de duas décadas, analisando a sua evolução na tolerância a esses compostos e resistência a antibióticos. Para isso, os fenótipos e genótipos de um total de 786 estirpes foram analisados através de métodos clássicos e avançados de microbiologia molecular. De modo a avaliar a resposta de *Enterococcus* a três dos CBs mais usados, foram determinadas as concentrações mínimas inibitórias (CMI) e concentrações mínimas bactericidas (CMB) de clorexidina (CHX), cloreto de benzalcónio (BC) e cloreto de didecildimetilamónio (DDAC), que correspondem a uma biguanida e dois compostos de amónio quaternário (QACs). As CMI e CMB de uma coleção diversa de *E. faecium* e *E. faecalis* foram muito mais baixas do que as concentrações de CBs tipicamente usadas em produtos biocidas. No entanto, foi detetada uma maior tolerância a CHX, BC ou DDAC em *E. faecium* ou *E. faecalis* de infeções humanas ou da cadeia alimentar, sugerindo uma eventual adaptação pelo aumento do uso de CBs nestes contextos, que requer monitorização no futuro. A correlação entre genótipos conhecidos de tolerância a CBs e fenótipos de suscetibilidade foi avaliada em diversas populações de *Enterococcus*. Mutações no transportador EfrEF ou no seu regulador de transcrição ChIR não se correlacionaram com o fenótipo à CHX. No entanto, a maioria de *E. faecalis* e *E. faecium* sem EfrEF ou ChIR funcionais apresentou CMI de CHX ≤ 1 mg/L. De notar que EfrE truncadas ocorreram em *E. faecalis* ST40 de origem humana, recuperados de diferentes anos e regiões geográficas, revelando uma evolução divergente nessa linhagem clonal ainda por clarificar. Genes adquiridos de tolerância a CBs (CBTa), que codificam para bombas de efluxo (*qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*, *qrg*, *bcrABC* e *oqxAB*) em vários taxóns bacterianos, são pouco frequentes em genomas públicos de *Enterococcus* (0,5%, n=117) e na nossa coleção (um *E. faecium* e um *E. faecalis*). Embora estes dados sugiram que *Enterococcus* não são hospedeiros relevantes de CBTa, a presença das mesmas variantes de proteínas codificadas por CBTa em *Enterococcus* e outros géneros indica a sua dispersão

entre diversos táxons bacterianos. Além disso, o nicho predominante de *Enterococcus* e outros táxons que contêm genótipos específicos reflete os diversos ambientes microbianos nos quais os genes circulam. Os CBTa, frequentemente localizados em plasmídeos, estão comumente co-localizados com genes de resistência a metais e antibióticos, facilitando eventos de co-seleção. No entanto, na nossa coleção, *E. faecium* e *E. faecalis* com *qacZ* apresentaram fenótipos aos QACs similares aos isolados sem gene, sugerindo que os CBTa podem não ter impacto considerável na suscetibilidade de *Enterococcus* aos CBs, justificando assim a sua escassez neste gênero. Ao analisar o efeito de diversas condições ambientais que ocorrem em contextos onde QACs são usados, *E. faecium* e *E. faecalis* com diferentes origens exibiram CMI e CMBs de BC duas a oito vezes mais altas a 22°C e/ou pH = 5, independentemente da presença do gene *qacZ*. Estes dados destacam a influência das condições ambientais na suscetibilidade ao BC e a potencial seleção de populações mais tolerantes em ambientes onde estas condições ocorrem. Por fim, o impacto da exposição consecutiva a concentrações subinibitórias de CHX na tolerância a CBs e na resistência a antibióticos foi avaliada *in vitro*. Os efeitos detetados para os seis *E. faecium* testados foram diversos e possivelmente associados a genótipos de tolerância à CHX. A mutação P102H no regulador ChtR foi encontrada em duas das três estirpes com aumento de CMI e/ou CMB de CHX após exposição, contrastando com um aumento da suscetibilidade à CHX na única estirpe sem EfrEF. Estes dados também indicam o potencial envolvimento de outros genótipos desconhecidos ou alterações na expressão genética, dado o aumento na CMI e CMB de um isolado sem genótipos conhecidos de tolerância à CHX, o que realça a necessidade de estudos futuros com transcriptômica. A exposição a concentrações subinibitórias de CHX não induziu resistência a antibióticos, mas aumentou, até 12,5×, a transferência horizontal de plasmídeos com genes de resistência à vancomicina ou linezolida em todos os *E. faecium*, com a exceção de um isolado sem um EfrEF funcional. De notar que as concentrações de CHX que resultaram em adaptação ou no aumento da transferência de genes de resistência a antibióticos são similares às descritas na pele de pacientes ou em águas residuais, onde os CBs podem contribuir para estes eventos. Concluindo, esta tese explora conceitos críticos da suscetibilidade e dinâmica de adaptação de *E. faecium* e *E. faecalis* aos CBs ao longo das últimas décadas. Embora a sua eficácia atual contra *Enterococcus* não seja questionada, as consequências identificadas da exposição a concentrações subinibitórias de CBs exigem considerável atenção em estudos futuros, visando garantir a eficácia contínua dos CBs e antibióticos e, assim, salvaguardar a Saúde Pública.

Palavras-chave: *Enterococcus faecium*, *Enterococcus faecalis*, Biocidas catiónicos, Resistência antimicrobiana, Uma Só Saúde

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List of Abbreviations

AFLP: Amplified Fragment Length Polymorphism
AmpR: Ampicillin-Resistant
AMR: Antimicrobial Resistance
ABC: ATP-Binding Cassette
ATCC: American Type Culture Collection
ATPase: Adenosine triphosphatase
BAPS: Bayesian Analysis of Population Structure
BC: Benzalkonium Chloride
BHI: Brain Heart Infusion
CB: Cationic Biocide
CC: Clonal Complex
CE: Cetrimide
CGE: Center for Genomic Epidemiology
cgMLST: core genome Multilocus Sequence Typing
CHX: Chlorhexidine
CLSI: Clinical & Laboratory Standards Institute
CPC: Cetylpyridinium Chloride
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
CT: Complex Types
DDAC: Didecyldimethylammonium Chloride
DNA: Deoxyribonucleic Acid
ECDC: European Centre for Disease Prevention and Control
ECHA: European Chemicals Agency
ECOFF: Epidemiological Cut-off
Efm: *Enterococcus faecium*
Efs: *Enterococcus faecalis*
EUCAST: European Committee on Antimicrobial Susceptibility Testing
GIT: Gastrointestinal Tract
HA-*Efm*: Hospital-associated *Enterococcus faecium*
HAI: Healthcare-Associated Infections
HGT: Horizontal Gene Transfer
HiRECCs: High-Risk Enterococcal Clonal Complexes
MDR: Multidrug-Resistant
MFS: Major Facilitator Superfamily
MGE: Mobile Genetic Element

MH: Mueller-Hinton
MHB: Mueller-Hinton Broth
MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration
MLST: Multilocus Sequence Typing
MRSA: Methicillin-Resistant *Staphylococcus aureus*
n: number
NCBI: National Center for Biotechnology Information
PBP5: Penicillin-Binding Protein 5
PCR: Polymerase Chain Reaction
PFGE: Pulsed-Field Gel Electrophoresis
PHMB: Polyhexamethylene Biguanides
QAC: Quaternary Ammonium Compound
RND: Resistance Nodulation Division family
ROS: Reactive Oxygen Species
SCENIHR: Scientific Committee on Emerging and Newly Identified Health Risks
SD: Standard Deviation
SMR: Small Multidrug Resistance family
SNP: Single Nucleotide Polymorphisms
ST: Sequence Type
UK: United Kingdom
USA: United States of America
VRE: Vancomycin-resistant *Enterococcus*
VREfm: Vancomycin-resistant *Enterococcus faecium*
WGS: Whole-Genome Sequencing
WHO: World Health Organization
2CS: Two-Component regulatory System



Chapter I

Introduction

“If I have seen further, it is by standing on the shoulders of giants”

Isaac Newton

**1. *Enterococcus* spp. Response to Cationic Biocides: Current
Insights to Lead Future Research**

***Enterococcus* spp. Response to Cationic Biocides: Current Insights to Lead Future Research**

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Abstract

Cationic biocides (CBs), such as quaternary ammonium compounds and biguanides, are critical for controlling the spread of bacterial pathogens like *Enterococcus* spp., a leading cause of multidrug-resistant healthcare-associated infections. The widespread use of CBs in recent decades has prompted concerns about the potential emergence of *Enterococcus* spp. populations exhibiting resistance to both biocides and antibiotics. Such concerns arise from their frequent exposure to subinhibitory concentrations of CBs in clinical, food chain and diverse environmental settings. This comprehensive narrative review aimed to explore the complexity of the *Enterococcus*' response to CBs and of their possible evolution towards resistance. To that end, CBs' activity against diverse *Enterococcus* spp. collections, the prevalence and roles of genes associated with tolerance to CBs, and the potential for co- and cross-resistance between CBs and antibiotics are reviewed. Significant methodological and knowledge gaps are identified, highlighting areas that future studies should address to enhance our comprehension of the impact of exposure to CBs on *Enterococcus* spp. populations' epidemiology. This knowledge is essential for developing effective One Health strategies that ensure the continued efficacy of these critical agents in safeguarding Public Health.

Keywords: *Enterococcus*, Biocides, Quaternary Ammonium Compounds, Biguanides, Tolerance, One Health

1. Introduction

The use of cationic biocides (CBs) is critical to control and prevent the dissemination of bacterial pathogens in the most diverse environments [1, 2]. They have been a cornerstone in the improvement of hygienic practices, preventing infections and, consequently, reducing the need for antibiotic use [1, 2]. The global antiseptics and disinfectants market, including CBs, has been continuously growing and is expected to reach \$13.3 billion by 2028, which is almost double the market size of \$7.5 billion in 2020 [3]. Thus, over the last decades, the extensive use of CBs and consequent high exposure of bacteria to these compounds has been raising concerns about the possibility of selection of strains resistant either to these or other antimicrobials, as antibiotics [4]. The assessment of the impact of biocides in the evolution of *Enterococcus* populations is of current concern, as this is one of the leading multidrug-resistant (MDR) healthcare-associated pathogens worldwide [5, 6]. Among the more than 60 validated *Enterococcus* species (<http://www.bacterio.net/enterococcus.html#r>), *Enterococcus faecalis* and *Enterococcus faecium* are the two predominantly implicated in human opportunistic infections and are among the most prevalent in the human gut microbiota [5-7]. They are intrinsically resistant to a broad spectrum of antibiotics and have rapidly acquired resistance to other critical ones, particularly to ampicillin or vancomycin among *E. faecium* [5, 6, 8]. Indeed, vancomycin-resistant *E. faecium* are categorized as high priority on the World Health Organization priority pathogens list for research and development of new antibiotics, causing infections with limited treatment options and associated with high mortality and health care costs [5, 6, 8, 9]. They are easily spread between patients and across their surrounding hospital environment through faecal contamination of the hands of patients, the healthcare staff and visitors, and of the medical equipment or other inanimate surfaces [10, 11]. Furthermore, due to their remarkable ability to survive harsh conditions, such as nutrient scarcity and desiccation, *Enterococcus* spp. might remain on these contaminated surfaces for extended periods, even years [10-14]. Effective antisepsis and disinfection practices are, therefore, crucial to break the chain of transmission, prevent spread of these microorganisms in the hospital environment and potential life-threatening MDR infections. *Enterococcus* spp. exposure to CBs, including at subinhibitory concentrations, extends beyond human clinical settings, as they are part of the natural microbiota of plants, soil, and the human and animals' gastrointestinal tract, and cause animal infections [2, 5-7, 15-17]. Therefore, CBs also play a key role in the control of their transmission in the veterinary, food chain (e.g., food industry, farms), or human domestic contexts [2, 5-7, 15-17]. In addition, *Enterococcus* spp. are exposed to CBs in sewage, aquatic systems, and soil/sediments, resulting from domestic, hospital, and industrial discharges [18-27].

Among CBs, the most common and to which the susceptibility of *Enterococcus* spp. has been increasingly studied in the last years, are the quaternary ammonium compounds (QACs) and

the biguanides [2, 4, 28]. However, the dispersed and sometimes contradictory information, coupled with limitations in study designs impacting their general conclusions, underscores the need for a comprehensive literature review to establish a standpoint on current data and address existing research gaps.

Here, we reflected on CBs' activity against *Enterococcus* spp. from different sources, geographical regions and years, while taking into consideration the current challenges associated with the study of biocide susceptibility. Moreover, we explore the evolution of tolerance to CBs reported for particular enterococcal populations of diverse epidemiological backgrounds as well as discuss the outcomes of *in vitro* exposure of *Enterococcus* spp. to subinhibitory concentrations of CBs. The prevalence of genes with a known or predicted role on CBs tolerance in *Enterococcus* spp. and their confirmed or possible link to reduced susceptibility phenotypes are also reviewed, as well as the potential risk of co- and cross-resistance between CBs and antibiotics. Finally, this review highlights the methodological and knowledge gaps that need to be addressed in future research to better understand the implications of CBs use on *Enterococcus* spp. evolution and ultimately contribute to the development of appropriate interventions in diverse sectors highly exposed to CBs.

2. Cationic Biocides

Cationic biocides (CBs) are broad-spectrum antimicrobial compounds widely employed in disinfectants, antiseptics, and preservatives [2]. Due to their low toxicity, these agents have been used in clinical and domestic settings, the food industry, agriculture, and other sectors since the 1930s [4, 29]. CBs primarily target the negatively charged cytoplasmic membrane of Gram-positive bacteria, such as *Enterococcus* spp., although the specific interaction of each CB with the membrane and subsequent concentration-dependent bacteriostatic or bactericidal mechanisms differ between the chemically diverse compounds (Figure 1) [4, 28, 29]. Indeed, these membrane-active agents fall into different classes with various chemical structures (Figure 1), whose intrinsic properties influence the CB's activity [4, 28, 29].

The most widely used CBs are the Quaternary Ammonium Compounds (QACs), often monocationic, and the biguanides, which have either two cationic groups or more, as is the case of polymeric biguanides (Figure 1) [4, 29]. These CB classes will be detailed in the next sections, specifically those molecules that have been, so far, the most studied against *Enterococcus* spp.

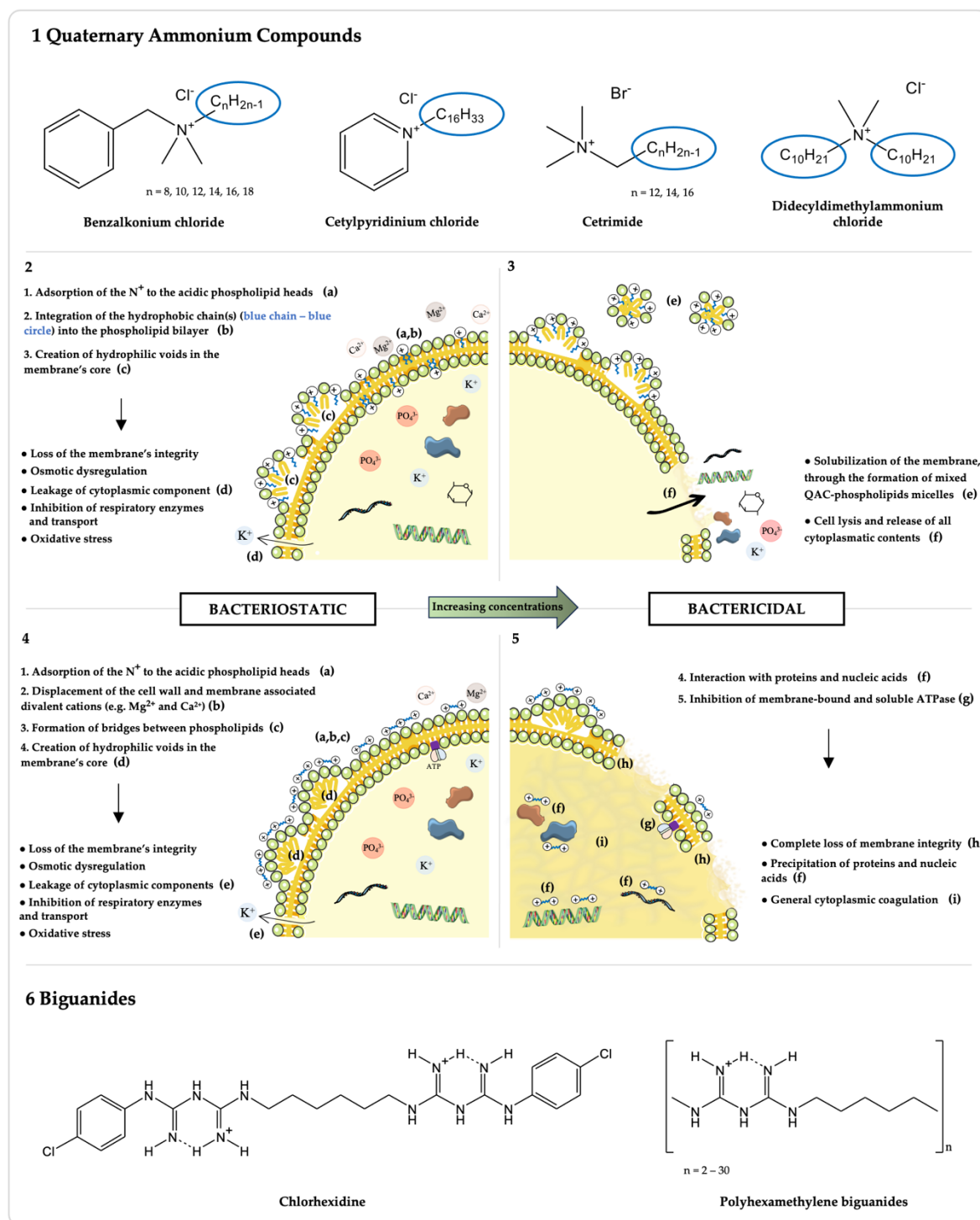


Figure 1. Chemical structures and mechanisms of action of Quaternary Ammonium Compounds (QACs) and Biguanides. The chemical structures of the most common QACs (1) and biguanides (6), whose activity against *Enterococcus* spp. has been studied, are presented along with their respective mechanisms of action for bacteriostatic (2: QACs; 4: biguanides) and bactericidal (3: QACs; 5: biguanides) concentrations. Molecules were drawn using ChemDraw v16.0 (<https://revvitysignals.com/products/research/chemdraw>). The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

2.1. Quaternary Ammonium Compounds

Over the past decades, and particularly in recent years, the use of QACs has been increasing in diverse fields of application [30-32]. Their general chemical structure is $N^+R_1R_2R_3R_4 X^-$, where R represents hydrogen atoms, alkyl or aryl groups, and X represents an anion, commonly Cl^- or Br^- (Figure 1-1) [15, 33]. The antimicrobial activity of QACs correlates with the n-alkyl chain length, which, against Gram-positive bacteria, such as *Enterococcus* spp., is optimal at n=12-14 [33]. This is because its hydrophobic tail(s) are directly involved in the interaction of QACs with the cytoplasmic membrane (Figure 1-2,3) [2, 29]. After the adsorption of the positively charged quaternary nitrogen to the acidic phospholipid heads within the membrane, the hydrophobic chain(s) interdigitates into the hydrophobic bilayer, creating hydrophilic voids in the membrane's core (Figure 1-2) [2, 29]. This results in a loss of the membrane's physical and ionic integrity, with leakage of cytoplasmic components, osmotic dysregulation, inhibition of respiratory enzymes and transport, and oxidative stress (Figure 1-2) [20, 29]. With increasing concentrations, QACs have a bactericidal action by solubilizing the hydrophobic membrane components, with the formation of mixed QAC-phospholipids micelles, lysing the cell and consequently releasing all cytoplasmic contents (Figure 1-3) [29].

A wide variety of QACs has been formulated over the years with increasing antimicrobial efficacy, lower toxicity and improved activity in adverse conditions (e.g., anionic residues, hard water) [31, 34, 35]. Among the most broadly used QACs, to which susceptibility of *Enterococcus* spp. has been studied, are benzalkonium chloride (BC), cetylpyridinium chloride (CPC), cetrimide (CE), and didecyltrimethylammonium chloride (DDAC) (Figure 1-1; Table S1) [33].

Benzalkonium chloride (BC) is a widely used mixture of n-alkyl-dimethyl-benzyl-ammonium chlorides, with variable n-alkyl chain lengths, typically ranging from 8 to 18 carbons (Figure 1-1) [29, 33, 34]. It has been used since the 1930s and its extensive applications span from personal care products (e.g., mouthwashes, shampoos and body lotions), to disinfectants and antiseptics in household, industrial, agricultural, and clinical environments, with concentrations ranging from 20 mg/L, in ophthalmologic formulations, to 20,000 mg/L, in wood preservation products [21, 43, 87, 88]. BC's concentrations of 100-3,000 mg/L are used for healthcare and household antiseptics and surface disinfection [2, 87].

Other QACs include cetylpyridinium chloride (CPC), also known as 1-hexadecylpyridinium chloride, corresponding to the chlorine salt of a positively charged pyridine bonded to a hexadecane lipophilic chain, and cetrimide (CE), which consists in a mixture of tetradecyltrimethylammonium, dodecyltrimethylammonium, and hexadecyltrimethylammonium bromides (Figure 1-1) [61, 89-91]. CPC, whose antimicrobial activity was first described in 1939, has been used for decades in dentistry, being

predominantly found in over-the-counter oral hygiene products, such as mouthwashes, toothpastes, and sprays, at 30-3000 mg/L, for the prevention and control of oral infections [61, 91-94]. In addition, it is approved by regulatory agencies of several countries for the sanitization of poultry carcasses in poultry processing plants, at concentrations of $\leq 1\%$ [95-98]. CE, in use since 1942, serves as a topical antiseptic for cleaning the skin, wounds and minor burns, in dentistry, and for the treatment of nappy rash, acne and seborrheicitis, in concentrations between 1,000-30,000 mg/L [99].

Didecyltrimethylammonium chloride (DDAC), a twin QAC developed in the 1960s, features two long-chain alkyl groups and two methyl substituents bonded to the positively charged nitrogen, along with the negatively charged chloride anion (Figure 1-1) [34, 100]. DDAC finds applications in antiseptics and disinfectants used in clinical, food chain, and domestic environments, in laundry, agricultural tools and vehicles, in swimming pools and water displays, and various indoor and outdoor hard surfaces (e.g., walls, floors), with concentrations ranging from 200 to 12,000 mg/L [2, 66, 100].

QACs may leave residues on treated surfaces and in the environment as they are hydrolytically and photolytically stable and have long half-lives (e.g., >150 days in pH ≥ 5) [87, 100-102]. They have been found in some types of food including fruits and nuts, vegetables or dairy products (up to 14.4 mg/kg), possibly through contact with disinfected surfaces [103-105]. Also, like most trace contaminants, QACs are not completely removed through wastewater treatment, being released into the environment and found in sewage and surface waters in different concentrations (0.0078 $\mu\text{g/L}$ to 6 mg/L) [18, 20, 21, 23, 106-108]. QACs are considered “very toxic to aquatic life with long-lasting effects” by the European Chemicals Agency (ECHA) [109, 110]. Moreover, QACs are highly biodegradable under aerobic conditions and known to adsorb strongly to the negatively charged surfaces of sludge, soil and sediments, because of their positive charge, interfering with their bioavailability and enabling the fluctuation of QACs’ concentrations that can impact local microbiota [20, 87, 106, 107]. Further investigation is needed to determine the influence of other factors promoting the environmental persistence of QACs, such as the emergent micropollutants like microplastics, to which QACs may potentially bind [111].

2.2. Biguanides

Biguanides correspond to a class of compounds that carry the functional moiety $\text{HN}(\text{C}(\text{NH})\text{NH}_2)_2$ (Figure 1-6), comprising antidiabetic (e.g., metformin), antimalarial (e.g., proguanil) and other antimicrobial compounds, some of which are included in the WHO List of Essential Medicines [29, 112-114]. The activity of the two most common biguanide biocides, chlorhexidine digluconate (CHX) and polyhexamethylene biguanides (PHMB), the latter also

known as polihexanide [113, 115], against *Enterococcus* spp. has been evaluated over the years, particularly for CHX (Figure 1-6; Table S1).

CHX is a bisbiguanide with extensive applications in the human and veterinary healthcare contexts as a hand, surgical site or wound antiseptic, and as a surface and instrument disinfectant, due to its broad-spectrum activity and safety and long-lasting residual activity when comparing to other biocides [2, 116-118]. Moreover, CHX daily bathing of intensive care unit patients has been increasingly adopted in order to reduce colonization and infection by MDR bacteria, such as vancomycin-resistant *Enterococcus* [80, 83, 117, 119-121]. Similarly, CHX is one of the most broadly used biocides in household antiseptics, especially in oral, pharmaceutical and handwashing products, disinfectants, and preservatives (e.g., cosmetics and personal care products), as well as in industrial manufacturing processes and products (e.g., food and paper industries) [116]. In-use concentrations range from 25-100 mg/L for preservation to 500-40,000 mg/L for antiseptics and disinfection purposes [2, 116, 122]. CHX's chemical structure consists in a symmetric bisbiguanide with two chloroguanide groups connected by a hydrophobic hexamethylene chain (Figure 1-6) [113, 123]. A key distinction between bisbiguanides and QACs' mechanisms of action lies in the solubilization of the hydrophobic regions (Figure 1). Whilst the hydrophobic chain of QACs integrate into the hydrophobic core of the cytoplasmatic membrane, CHX's, being only 6 carbons long, is not able to do so [29]. Instead, as a bacteriostatic, the two positively charged biguanide groups of CHX displace the cell wall and cytoplasmatic membrane associated divalent cations (e.g., Mg^{2+} and Ca^{2+}) and associate to the then exposed anionic sites, forming bridges between pairs of adjacent phospholipids (Figure 1-4) [29, 113]. This interaction disturbs the membrane's fluidity, osmoregulation and metabolism, with increased permeability and leakage of low molecular weight cytosolic components (e.g., potassium ions), and inhibition of transport and cellular respiration (Figure 1-4) [29, 113, 117]. At higher concentrations, CHX has a bactericidal mechanism of action through the complete loss of cytoplasmatic membrane integrity and, ultimately, the precipitation of proteins and nucleic acids and general cytoplasmic coagulation (Figure 1-5) [29, 113, 124]. Additionally, it seems to inhibit membrane-bound and soluble ATPase (Figure 1-5) [125, 126].

Similarly to QACs, CHX is not completely removed through wastewater treatment, with low concentrations detected in the treated effluent [18, 22, 24]. Residues of CHX have also been described in the skin of patients (<4.69 to 600 mg/L) after CHX bathing, as well as in milk from cows treated with CHX teat dips and sprays [19, 83]. CHX undergoes photodegradation but limited biodegradation and has a long half-life (e.g., 180-365 days in water, soil or sediment) [24, 116]. It tends to persist in water, suggesting potential for prolonged exposure far from the sources of discharge to the environment, and is "very toxic to aquatic life with long-lasting effects" according to ECHA [24, 116, 127]. However, its bioavailability reduces over time by

the adsorption of CHX to sediments and soil [24, 116], potentially with a decreased impact over local microbiota.

PHMB is a polymeric biguanide, composed by 2 to 30 repeats of hexamethylene biguanide units, and possesses a bacteriostatic and bactericidal mechanism of action similar to the one described for CHX (Figure 1-6), although with a distinct initial interaction with the bacterial cytoplasmatic membrane [29, 113, 128-130]. Given the polycationic nature of PHMB, the bridging occurs not only between pairs of adjacent phospholipids but rather there is the formation of a mosaic of single phospholipid type domains, each with different phase transition properties [29, 129]. The activity of PHMB increases with higher levels of oligomerization which allow for the establishment of larger domains and, consequently, increased permeability and disruption of the cytoplasmatic membrane and its associated proteins' function (Figure 1-4) [29, 114, 131]. It has been used predominantly in concentrations between 30-32,000 mg/L in recreational water (e.g. swimming pools, artificial fountains) treatment, as well as in wound and burn antisepsis, surfaces and instrument disinfection in hospitals, dentists, farms and food handling settings, and in contact lens solutions, personal care products and fabric softeners preservation [2, 113, 132, 133]. PHMB shows very low human toxicity or risk of adverse effects [2, 29, 114, 128].

PHMB is very persistent in water, also showing a long half-life in this context, which may constitute an issue in aquatic environments as it is classified by ECHA, like CHX and QACs, as “very toxic to aquatic life with long lasting effects” [132, 134, 135]. Also, it is considered to be hydrolytically and photolytically stable [135]. On the other hand, PHMB binds immediately to soils, except for sandy soil, and it is likely susceptible to some extent of biodegradation, although it is regarded as “non readily biodegradable” [25, 132, 135, 136].

3. *In Vitro* Testing of *Enterococcus* spp. Susceptibility to Cationic Biocides

A good antimicrobial activity of the most used CBs against *Enterococcus* spp. has consistently been reported since they were introduced decades ago. The assessment of *Enterococcus* spp. susceptibility to CBs has primarily relied on the *in vitro* determination of minimum inhibitory concentrations (MICs), mainly due to the methodology's ease of use [2, 15]. MIC is defined as the lowest antimicrobial concentration that inhibits the growth of the microorganisms, and it is usually measured in doubling dilutions [2, 15, 137, 138]. Additionally, some studies also include the determination of the minimal bactericidal concentrations (MBCs), corresponding to the minimum concentration that kills >99.9% of cells, which constitute a more suitable measure of susceptibility for most biocidal applications where the desired effect is to kill the microorganisms [2, 15, 28, 138].

Several studies have published *Enterococcus* spp. MICs and MBCs for QACs (BC, DDAC, CE, CPC) and biguanides (CHX and PHMB) (Table S1). MICs and MBCs varied, respectively, across the following ranges (MICs/MBCs): BC (0.05-256 mg/L / 1->256 mg/L), DDAC (0.14–4 mg/L / 1–4 mg/L), CE (<0.1–250 mg/L / 29 mg/L), CPC (<0.1–250 mg/L / 4 mg/L), CHX (<0.1-2,500 mg/L / 1–128 mg/L), and PHMB (0.3–80 mg/L / 7.3–166 mg/L).

However, prudence is necessary when comparing MIC/MBC data across different studies, given the absence of established standardized protocols for the study of biocide susceptibility in bacteria, making comprehensive epidemiological analysis difficult [28, 33]. A clear example of such issue is the broad range of CHX MICs available for the *E. faecalis* ATCC 29212, from <1 to 27 mg/L (Table S1), determined using different methodologies. Factors such as the incubation time, bacterial growth phase (e.g., exponential vs stationary), growth culture media or the type of plate plastic can influence the susceptibility to CBs *in vitro*, conducting to diverse outcomes for the same strains [138, 139]. Many studies use the microdilution broth protocol described by the CLSI guidelines for antibiotic susceptibility testing [137, 138], which has the advantage of allowing the comparison of data between analyses, but it remains to assess if such methodological adaptation remains the best choice to study microbes' biocide susceptibility. This protocol was designed primarily to assess the therapeutical success of antibiotics for infections' treatment, which is reflected in the bacterial growth conditions specified (37°C; pH = 7.3) [1, 28, 137]. Thus, several other factors (e.g., variable temperature or pH, oxygen limitation, starvation, lower or higher bacterial density, bacterial growth phase) that can affect the efficiency of biocides in real application contexts, namely by altering the cytoplasmic membrane or reducing the cell's metabolic activity, are not pondered [2, 12, 28, 140-144].

Recently, we learned that *E. faecium* and *E. faecalis* isolates from different epidemiological and clonal backgrounds exhibited decreased susceptibility to BC at 22°C and/or pH = 5 [42], confirming the influence of external growth conditions on CBs susceptibility. Furthermore, *Enterococcus* are also regularly found within multicellular communities such as biofilms, both on wet environments and dry surfaces, in various settings [145]. Biofilms are usually associated with a reduced susceptibility to biocides via several mechanisms such as persister cells and surrounding extracellular polymeric matrix that forms a barrier to the diffusion of biocides through the biofilm [28, 145, 146]. Although a good biocidal activity remains generally described for *Enterococcus* spp. biofilms [147-154], one study reported a reduced susceptibility, between 1.8-8 fold, in enterococcal biofilms compared to planktonic cells for BC, DDAC, CHX and PHMB [27]. Also, in a recent study, a BC concentration of 80 mg/L was not sufficient to eradicate *E. faecium* and *E. faecalis* biofilms for which respective planktonic cells' MBCs varied from 10 to 40 mg/L [47].

Although, in most susceptibility studies, MICs and MBCs are determined for unformulated CBs in simple aqueous solutions, such data may not be directly applicable for in-use biocidal formulations, even if the concentrations used correspond to those in the biocidal products [2, 27, 28]. These generally include other compounds that enhance CBs' activity or stability [2, 28]. Some studies of biocidal formulations against *Enterococcus* spp. are available, showing MIC and MBC values higher or within the ranges of those in Table S1 for unformulated CBs [4, 27, 155-160]. However, these were evaluated at the endpoint of the MIC determination protocols used, corresponding typically to 24h or sometimes longer (48h), instead of the usually recommended disinfectant contact times in different contexts of 3 to 10 min [35, 122, 161]. Hence, tests to assess CBs formulations' efficacy in such conditions against *Enterococcus* spp. from diverse epidemiological and genomic backgrounds are still lacking. Despite the limitations of data analysis related to the inconsistency of the methods used and the potential implications on the applicability and relevance of *in vitro* data in real environments, the diverse susceptibility testing methodologies (e.g., altered growth conditions, planktonic cells or biofilms, biocidal formulations vs unformulated CBs) may be valuable in different contexts, provided they are standardized. Currently, the assessment of MICs and MBCs following CLSI/EUCAST guidelines enables the comparison between different studies through a reproducible method. Meanwhile, more studies using biocidal formulations, along with the inclusion of biofilms or modified growth conditions simulating real scenarios, are crucial to ascertain the activity of CBs against *Enterococcus* populations and to conclude about the appropriate course of action.

Finally, another aspect needing standardization, also linked to susceptibility testing, is the terminology used to define the reduced susceptibility of bacteria to biocides [28]. One of the most used, which will be applied for the purposes of this review, is the term "tolerance" that refers to cases in which the in-use concentrations of a biocide remain higher than the *in vitro* MICs or MBCs [35, 162-164]. On the contrary, if the decreased susceptibility implies that the microorganisms are not inactivated by the in-use concentrations of a biocide, then the term "resistance" to the biocide is applied [35, 162-164].

4. Evolution of Susceptibility to Cationic Biocides of *Enterococcus* spp. from Diverse Epidemiological and Genomic Backgrounds

Available MICs and MBCs referring to *Enterococcus* spp. from diverse sources and time frames, are, mostly, way below the concentrations typically used for antiseptics and disinfection, with few exceptions (Table S1) [4, 38, 39, 44, 53, 54, 79]. However, more and more the wide use of antiseptics and disinfectants in particular environments (e.g., hospitals, the food chain) has been a cause of concern given the possibility that repeated exposure to

subinhibitory concentrations of these agents may progressively select for populations more tolerant to these antimicrobials [28, 165-167].

As a baseline to monitor the susceptibility evolution trends or the development of tolerance to CBs over the years, setting epidemiological cut-off (ECOFF) values could be a useful tool [49, 168, 169]. These correspond to the highest MIC of an antimicrobial against microorganisms with no phenotypically detectable resistance mechanisms [49, 168, 169]. Few individual analyses have proposed CBs' ECOFF values based on their *Enterococcus* spp. collection's MIC and MBC distributions, including isolates from different sources, geographical regions and time frames [43, 49, 74, 170]. For CHX, MIC ECOFFs of 8 mg/L and 32 mg/L and an MBC ECOFF of 64 mg/L have been proposed for *E. faecium*, whereas, for *E. faecalis*, MIC ECOFFs of 8 mg/L, 16 mg/L and 64 mg/L and MBC ECOFFs of 64 mg/L or higher have been recommended [43, 49, 74]. For BC, MIC ECOFFs of 8 mg/L and 16 mg/L and an MBC ECOFF of 16 mg/L have been estimated for both *E. faecium* and *E. faecalis* [43, 49]. However, the variation in ECOFFs proposed by different studies underscores the need for comprehensive analyses of MIC and MBC distributions across the various species-biocide pairs. These must be conducted using diverse *Enterococcus* spp. collections and laboratories to address potential biological, methodological, and interlaboratory variations. Such an approach aligns with EUCAST recommendations for antibiotics and is crucial for establishing definitive ECOFFs for biocides (123).

In one of the studies proposing ECOFFs to CHX for *E. faecalis*, even though the whole population was considered wild type by the statistical model recommended for the ECOFF estimation, differences in the susceptibility were detected among isolates from diverse sources and years [74]. Similarly, other authors have found significant differences across their *Enterococcus* spp. collections [42, 43, 57, 66, 74, 170]. Most found a higher occurrence of strains with higher tolerance to CHX, DDAC, or BC among clinical isolates comparing to *Enterococcus* from other origins included in the same study [42, 57, 66, 170, 171], although contradictory data has also been reported for BC [43, 57]. Moreover, an increase in the mean CHX MICs and MBCs of human infection *E. faecalis* isolates over the years, between 2001 and 2020, has been recently reported [74]. Beyond the clinical environment, a significant increasing trend in the BC MICs over time has also been detected in *E. faecium* isolated from the food chain, including food-animal production settings, meat of animal origin, and other food products [42]. Furthermore, within populations of *E. faecalis* and *E. faecium* from these settings, higher average CHX MICs and MBCs have been identified compared to other sources [43, 74]. Of note, most of these studies did not find any particular genotype justifying the evolution of phenotypes between sources or timeframes [42, 43, 66, 74].

Available data suggest an adaptation of *Enterococcus* populations in settings where they are exposed to CBs. Of note, an increase in the CHX MIC₅₀ (from 2 to 8 mg/L) and MIC₉₀ (from

16 to 32 mg/L) was detected in vancomycin-resistant *E. faecium* recovered from patients' infections or colonization after daily CHX bathing was instituted in the hospital ward, compared to isolates recovered before the intervention, suggesting that prolonged exposure to this biocide might indeed select for reduced susceptibility [80]. Nonetheless, large longitudinal metadata analyses of the populations' dynamics in such contexts are critically needed for more supported conclusions. Currently, most available CBs susceptibility studies lack objective data on local biocide consumption (e.g., type of compound, amount used, compliance with effective biocide application practices, "during use" concentrations) or on the occurrence of subinhibitory concentrations [28]. This hinders the establishment of a clear cause-and-effect relationship of biocide use and *Enterococcus* spp. evolved phenotypes. Most studies also use a limited number of *Enterococcus* strains, lack clonal or genotypic characterization, and show a low source diversity, making it challenging to have a global perspective on the evolution of tolerance to CBs in particular *Enterococcus* spp. populations or environments.

5. Effects of *in vitro* Serial Exposure to Subinhibitory Concentrations of Cationic Biocides in *Enterococcus* spp.

Despite unclear cause-and-effect relationships in the previously mentioned studies that detected CBs phenotypic evolutions among field *Enterococcus* spp. isolates from diverse sources or dates, the hypothesis that exposure to diverse subinhibitory concentration gradients of CBs could lead to reduced susceptibility has been tested *in vitro* [20, 26, 27]. Indeed, passages with BC, DDAC, CPC, CE, CHX, and PHMB have resulted in MIC increases of 1.2 to more than 100-fold, that were strain-specific within each species (Table S2). In most cases, MICs and MBCs of *Enterococcus* spp. adapted strains remained below the in-use concentrations of CBs. However, for one *E. casseliflavus* and one *E. faecalis* treated with increasing CE and PHMB concentrations, respectively, MICs and, for the *E. faecalis*, MBCs reached the in-use concentrations range and were stable or only partially reversed after several biocide-free subcultures (Table S2) [2, 27, 63, 99]. Also, for most CHX experiments, including different species, MICs reached the concentrations typically used for preservation (25-100 mg/L) after exposure (Table S2) [2, 116, 122].

The reduced bacterial susceptibility following the adaptation protocols may be explained by several factors such as changes in membrane fatty acid composition, differential expression or mutations in efflux pumps, induction of stress responses, among others [27]. Although some of the analyses revealed stable phenotypes, suggesting genotypic adaptation rather than noninheritable physiological or metabolic adaptation mechanisms [26], these were scarcely investigated. Bhardwaj et al. identified significant changes in membrane phospholipids and mutations in several genes with previously predicted or experimentally confirmed roles in CHX

tolerance, among CHX-adapted *E. faecium* strains [172]. In particular, all of them shared a mutation (A290V) in the gene *efrE*, which encodes one subunit of the heterodimeric ATP-binding cassette (ABC) transporter EfrEF whose deletion resulted in increased CHX susceptibility [172]. Additionally, increased surface hydrophobicity was detected in *E. faecalis* passaged in the presence of CPC as well as in those serially exposed to CHX for which a change in the protein profile was also found [62]. Findings such as these suggest the occurrence of complex bacterial adaptation mechanisms to CBs and underscore the importance of more in-depth analyses employing advanced technologies, like whole-genome sequencing (WGS) and transcriptomics, to identify possible drivers of *Enterococcus* spp. tolerance.

The effects of *in vitro* serial exposure to subinhibitory concentrations of CBs in *Enterococcus* spp. were not limited to the greater tolerance to that specific biocide. Considerable increases of several fold in the MICs of other biocides (2 to >100 fold), reaching in-use concentrations in many cases, have also been detected (Table S2) [38, 63, 172]. On the other hand, an increase in susceptibility to CE occurred in CPC-adapted *Enterococcus* spp. and BC-adapted *E. faecium*, *E. faecalis*, and *Enterococcus* spp., to BC in CPC-adapted *Enterococcus* spp., to didecyldimethylammonium bromide in CPC-adapted *Enterococcus* spp., to CPC in BC-adapted *Enterococcus* spp. and CHX-adapted *Enterococcus* spp., and to triclosan in CHX-adapted *E. casseliflavus* and CPC-adapted *Enterococcus* spp. [38, 63].

All in all, despite the *in vitro* research suggesting that bacteria can adapt to CBs exposure, evidence of such rapid adaptation in the environment is scarce [27, 28]. Multiple external factors specific of each setting (*e.g.*, physicochemical, wide range of CBs concentrations, presence of additional compounds with antimicrobial properties, presence of organic matter), the different physiological state of bacterial populations (*e.g.*, changes in metabolic activity and gene expression), among others, may not allow for the conditions required for *Enterococcus* bacteria to adapt [2, 28]. Also, another hypothesis that might explain the limited correlation data between biocide use and an evolution towards tolerance in real environmental contexts are the loss of the adaptation mechanism or the struggle of adapted populations to compete in their microbial communities when the CB stress is removed [27].

6. Cationic Biocides' Tolerance Genes among *Enterococcus* spp.

Increased tolerance to CBs in Gram-positive bacteria has been mainly attributed to efflux activity [2, 20]. *Enterococcus* spp. have been found to harbor several acquired genes encoding efflux pumps, including the well-known *qac*, *bcrABC* and *oqxAB* genes, all demonstrated to be implicated in tolerance to QACs by functional studies in Gram-positive or Gram-negative bacteria, and, in the case of some *qac* genes, also with tolerance to CHX. Intrinsic

heterodimeric ABC transporters, like EfrEF in *E. faecium* and *E. faecalis*, and mutations in regulatory genes, such as the DNA-binding response regulator (ChtR), have also been shown to impact CHX susceptibility.

However, as will be detailed throughout this chapter, for most CBs tolerance genotypes, their role in *Enterococcus* antimicrobial tolerance is not completely clear and requires further characterization, as their presence does not consistently correlate with an altered phenotype to CBs. Nonetheless, the potential gene exchange with diverse phyla, both of Gram-positive and Gram-negative bacteria, in contexts where CBs are present in a wide range of concentrations, is noteworthy and needs further exploration as the same genotypes in diverse genetic or epidemiological backgrounds may be associated with diverse outcomes of CBs tolerance.

6.1. *qac* genes

The *qac* genes detected in *Enterococcus* spp. are often plasmid-located and belong to two major classes of efflux pump systems: the major facilitator superfamily (MFS; e.g., *qacA/B*) and the small multidrug resistance family (SMR; e.g., *qacC*, *qacE*, *qacEΔ*, *qacG*, *qacJ*, *qacZ*) [42, 46, 131]. They are proton motive force-dependent efflux pumps integrated in the cytoplasmic membrane via transmembrane segments [131, 173-176].

QacA has been associated with tolerance to various cationic compounds including QACs, CHX, diamides, intercalating dyes, among others [173, 175-177]. It is encoded by the *qacA* gene which is closely related to *qacB*, with the encoded proteins differing at amino acid position 323 [131, 173, 178]. QacA features aspartic acid at this position, while QacB has alanine, impacting substrate recognition and binding and reducing QacB's efflux activity of divalent cations [131, 173, 177, 178]. Both genes are regulated by a TetR/CamR transcriptional regulator, QacR, that binds to the *qacA/B* promoter, inhibiting its expression [173, 176, 179]. When substrates of QacA/B directly bind to QacR, the regulator dissociates from the promoter and allows for expression of the efflux pump genes [173, 176, 179].

Previous studies have reported different occurrence rates of *qacA/B* in collections of *E. faecalis* and *E. faecium* isolates with diverse epidemiological backgrounds (Table S3) [43, 46, 65, 118]. These have shown a susceptible phenotype to DDAC, BC and CHX at concentrations much lower than those present in biocide-containing products. One of the *qacA/B*-carrying *E. faecalis*, recovered from human blood, had a higher DDAC MIC of 2.45–3.5 mg/L compared to isolates without this gene (MIC of 1.05 mg/L), whereas the other *qacA/B*-carrying *E. faecalis*, isolated from cattle, did not present an increased MIC value for DDAC (Table S1) [65]. The authors suggested the different phenotypes could be linked to the nucleotide polymorphisms between *qacA/B* sequences in the two isolates [65], but additional analyses to confirm the role of such mutations in DDAC increased tolerance are needed. In *E. faecium*,

an increased tolerance to CHX (MIC of 14 mg/L) was detected in a swine production chain strain (EA26; *qacA/B*-positive) compared to MICs of 4-10 mg/L for *qacA/B* negative isolates (Table S1) [46]. On the other hand, the BC MIC of *E. faecium* EA26 was within the range determined for isolates without *qacA/B* in this study (2-4 mg/L) (Table S1) [46]. In a more recent study including 647 *Enterococcus* spp. isolates from different sources in Iran, BC and CHX MIC₉₀ of *E. faecalis* and *E. faecium* harbouring or not *qacA/B* were similar [43]. Additionally, on publicly available genomes at the NCBI (until 28/4/2022), the gene *qacA/B* was also found among two *E. faecalis* and one of *E. faecium*, all from recent human infections in South Africa [42].

The *qacC* gene, also known as *ebr*, *smr* (for staphylococcal multidrug resistance) or *qacD*, confers tolerance to QACs and β -lactam antibiotics in Gram-positive and Gram-negative bacteria, and is usually located on conjugative or small rolling-circle replicating (nonconjugative) plasmids [131, 173, 176, 177, 200-202]. Its expression does not require a transcriptional regulator and the corresponding QacC protein is 107 amino acids long [173, 176, 200]. QacC has been found in six *E. faecalis* isolated from paediatric bloodstream infections, human stool and cheese (Table S3), as well as in 29 *E. faecalis* and seven *E. faecium* genomes available on NCBI (until 28/4/2022) [42, 65, 118]. The susceptibility to DDAC was tested for the two non-clinical isolates and showed that both had an MIC of 1.05 mg/L similar to the *qacC* negative isolates (Table S1) [65].

The genes *qacE* and the partially deleted derivative known as *qacE Δ 1*, resulting from the insertion of a DNA segment containing a sulfonamide resistance gene near the 3' end of the *qacE* gene, are commonly found in integrons of a broad range of Gram-negative bacteria [181, 203, 204]. They are associated with tolerance to QACs, with *qacE* associated with higher tolerance levels than *qacE Δ 1* [203]. In *Enterococcus*, *qacE* was found among three vancomycin-resistant *E. faecium* from patients' infections or colonization (Brazil, 2005-2009) (Table S3) but an association with CHX MICs was not established [80]. Similarly, *qacE Δ 1* has been detected in nine clinical *E. faecalis* isolates (Japan, 1996) and in 44 *E. faecalis* and 73 *E. faecium* from diverse sources in Iran (2018-2020) (Table S1 and S3), that did not show increased BC and CHX MIC₉₀ compared to *qacE Δ 1* negative isolates [43, 181].

The gene *qacG* has been detected in *E. faecium* 8D1-48 (NCBI; until 28/4/2022), a soil isolate, and *qacJ* in two *E. faecalis* strains from human infections (NCBI; until 28/4/2022) and cattle processed meat (Table S3) [42, 182]. QacG and QacJ have a high protein sequence identity between them (82.6%) and with QacC (>69%), belonging to the SMR family [173, 205, 206]. No phenotypic assays are available to assess their role in QACs tolerance in *Enterococcus* spp.

The *qacZ* and *qacH*, identified in *Enterococcus* spp. and *Staphylococcus* spp., respectively, share a high sequence similarity (98% nucleotide identity) but different substrates [37, 55].

The role of gene *qacZ* in *Enterococcus* spp. tolerance to QACs has been shown, but not to ethidium bromide or proflavine, which are also substrates of the efflux pump coded by *qacH* [37, 55]. Braga et al. found a high occurrence of *qacZ* among different *Enterococcus* spp. isolated from Portuguese clinical settings (63%) and dairy products (70%), although a correlation between the prevalence of the gene and the different BC and CHX MICs was not detected (Table S1 and S3) [37]. In a different collection, only one ST17 *E. faecium* (E241), recovered from hospital sewage in 2002, also in Portugal, carried *qacZ* (Table S3). Similarly, its BC and CHX MIC and MBC remained low and within the ranges also observed for isolates without such gene (Table S1 and S3) [42, 55, 170]. Twenty *E. faecalis* and 12 *E. faecium* *qacZ*-carrying genomes available on NCBI (until 28/4/2022), mostly from human infections in different countries and years (1987-2014), have also been reported [42].

Generally, similar phenotypes to CBs have been described for *Enterococcus* with or without *qac* genes, suggesting that their activity may not have an impactful outcome in the tolerance to CBs, that other mechanisms such as the presence or differential regulation of other efflux pumps may also influence the resulting phenotypes, or that the necessary conditions for the expression of tolerant genotypes are not satisfied by the methodologies used to evaluate susceptibility [37, 42, 43, 65, 170]. Also, with few exceptions [37, 43, 118, 181], a low occurrence of *qac* genes among *Enterococcus* spp. isolates and genomes (available on NCBI) has been reported [42, 46, 55-57, 65, 66, 170]. These data suggest that *qac* genes may not have a significant impact on the response of *Enterococcus* spp. to CBs exposure, although such conclusion may be biased by the few published studies, by the *Enterococcus* collections included in such analyses or the public genomes available. Of note is the detection of *qac* genes on transferable plasmids, potentially facilitating their transmission, via horizontal gene transfer, within the microbial communities and settings *Enterococcus* spp. are part of [42, 55, 173, 176]. Indeed, the sequences of QacA/B, QacC, QacG, QacJ and QacZ, which were predominantly identified in human *Enterococcus*, were mainly shared with *Staphylococcus* isolates associated with human colonization and infection, in which they have been primarily and mostly described [42, 173].

6.2. *qrg*

The *qrg* gene belongs to the SMR family, as most *qac* genes, and has been shown to encode a fourfold reduced susceptibility to cetyltrimethylammonium bromide in one *Streptococcus oralis* isolate from the human oral cavity, in which the gene was first described [207]. Among *Enterococcus*, *qrg* was harbored only by one ST6 *E. faecalis* DVT_1043 (available on NCBI; until 28/4/2022), a human infection strain isolated in the USA in 2020 [42]. *Streptococcus* spp. was the predominant genus (98%, n = 183/187) sharing an identical Qrg sequence with the DVT_1043 strain, among the five genera (14 species) in which it was found [42]. CBs

susceptibility studies on *qrg*-carrying *Enterococcus* have not yet been published, with the impact of this gene in *Enterococcus* tolerance to QACs still to explore.

6.3. *bcrABC* cassette

The efflux system encoded by the *bcrABC* cassette is associated with tolerance to QACs and it is predominantly harbored by plasmids of *Listeria* spp., although a chromosomal location is also possible [208-211]. It is composed by a putative transcriptional regulator of the TetR family, *bcrA*, and two SMR genes, *bcrB* and *bcrC* [208, 209].

Although, in a previous study, *bcrABC* genes were not detected among a collection of over 200 *E. faecium* and *E. faecalis* from human, animal, food and aquatic sources from eight countries and spanning 25 years (Table S3), these were the most prevalent CBs tolerance genes among *Enterococcus* spp. genomes available at the NCBI database (n=22,428; until 28/04/2022), when compared to *qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*, *qrg*, *bcrABC*, and the *oqxAB* genes [42]. They were more frequent in *E. faecalis* than in *E. faecium* or *E. lactis*, as well as in the food chain compared to other sources, probably as a reflection of the microbial communities and settings in which these tolerance genes circulate [42]. Indeed, an identical *bcrABC* gene cluster to that found in *Enterococcus* was mostly detected in the food pathogen *Listeria monocytogenes* (97% among the 10 species, corresponding to six different genera, in which it was identified) [42]. While the increased CB tolerance has been confirmed for *Listeria* spp. [208], phenotype studies in *bcrABC*-carrying *Enterococcus* to support their role in this different host are needed.

6.4. *oqxAB* genes

The multidrug efflux pump encoded by the *oqxAB* cassette belongs to the resistance nodulation division family (RND) and has been described to reduce the susceptibility to the CBs BC, CE and CHX, as well as to multiple antibiotics [212, 213]. It is most commonly found on the chromosome and/or conjugative plasmids in *Enterobacterales* [212].

Among the rare studies in which *Enterococcus* isolates were screened for the presence of *oqxAB* (Table S3), high percentages of strains harboring these efflux pump genes were detected in manure of food-producing animals in China (*oqxA*: 79%; *oqxB*: 66%), which may be due to the extensive use of quinoxalines in animal husbandry in this country as suggested by the authors [183]. However, similarly to *qrg* or *bcrABC*, the susceptibility to CBs of any *oqxAB*-positive *Enterococcus* isolates has not yet been determined. The *oqxAB* genes seem more prevalent in *E. faecalis* than in *E. faecium*, and filter-mating experiments showed their transferability between *E. faecalis* strains [183]. The *OqxAB* variant found in *Enterococcus* has been identified in *E. coli* and *Salmonella enterica* available at the NCBI database (until 28/04/2022), recovered predominantly from the food chain [42].

6.5. *emeA*

EmeA is an enterococcal multidrug-resistant efflux pump, homolog to *S. aureus* NorA (32% identity), that belongs to the MFS and has been associated with tolerance to QACs, dyes and different antibiotics, such as fluoroquinolones [187, 214, 215]. Several studies screening the presence of the gene *emeA* among *Enterococcus* spp. isolates have been published (Table S3). In *E. faecalis* it is considered to contribute to intrinsic drug resistance, being identified in all complete and annotated *E. faecalis* genomes from GenBank on September 1st of 2020 [191]. However, in the few analysis where its impact on susceptibility to CBs has been assessed, no increased tolerance was observed for *emeA* positive *Enterococcus* spp. compared to isolates without such gene [43, 46], except in one case in which the presence of this gene was significantly associated with reduced susceptibility to CHX but not to BC [57].

6.6. *efrAB*

EfrAB is an *Enterococcus* heterodimeric ABC multidrug efflux pump, chromosomally encoded by the *efrA* and *efrB* genes, that transports multiple dyes and antibiotics, including fluoroquinolones, and with a suspected role in tolerance to CBs [48, 57, 216-218]. It has been reported in *Enterococcus* spp. in different percentages (Table S3), which may be related to a low sensitivity of detection methods to identify potential gene variability that remains to be assessed. On the other hand, it has been consistently found in a high occurrence (50 – 100%) in *E. faecalis* collections (Table S3). Lavilla Lerma et al. described that all the *E. faecalis* with higher tolerance to CHX included in their study harbored *efrAB*, although, in tolerant *E. faecium* strains, only 12% carried such genes [48]. Recently, also Sobhanipoor et al. revealed a significant association between the presence of these genes and *Enterococcus* reduced susceptibility to CHX, but not to BC, similar to what was observed for *emeA* [57]. Nevertheless, functional gene studies are lacking to confirm the role of *efrAB* in *Enterococcus* spp. tolerance to CBs.

6.7. *chlR-efrEF*

EfrEF is another intrinsic heterodimeric multidrug ABC transporter, encoded by the genes *efrE* and *efrF*, with a drug efflux profile similar to that of EfrAB [125, 218]. Furthermore, EfrEF's role in CHX tolerance has been shown both in *E. faecium* and *E. faecalis*, by deletion and complementation experiments [125, 172]. Using transcriptomic analysis to identify the genes upregulated in *E. faecalis* V583 under CHX exposure, Li et al. detected *efrEF* to be the most highly upregulated genes [125]. Their overexpression was mediated by ChlR, a putative MerR family transcription regulator, encoded by *chlR* located upstream of *efrEF* [125]. Deletion of the *chlR* gene resulted in increased susceptibility to CHX and was reduced in the complemented strain, as for *efrE* or *efrF* [125].

Following the observations made in these previous studies with a restricted set of strains, a large collection of 666 *E. faecalis* genomes from diverse epidemiological and clonal backgrounds was screened for the occurrence and variability of the *chlR-efrEF* genes [74]. The *efrEF* operon was detected in all but one isolate (Table S3), with 5% carrying genes coding for incomplete ChlR, EfrE or EfrF proteins [74]. Most of these corresponded to EfrE-truncated *E. faecalis* identified as ST40 and were predominantly recovered from humans [74]. Isolates harboring incomplete ChlR-EfrEF had consistently low MICs (≤ 1 mg/L, with rare exceptions) contrasting with those with complete operons (MIC=2-8 mg/L, with 2 exceptions), whereas MBCs remained similar to those of non-truncated *E. faecalis* [74]. A broad range of mutations was identified among the isolates with complete ChlR-EfrEF proteins, but no correlation between specific mutations and CHX susceptibility was recognized [74].

Similarly, *efrE* and *efrF* *E. faecium* orthologs were also upregulated in *E. faecium* 1,231,410 in response to CHX exposure [157]. Deletion of the *efrEF* operon rendered this strain more susceptible to the biocide, whereas complementation restored the CHX phenotype [172]. Moreover, *in vitro* serial exposure of *E. faecium* 1,231,410 to subinhibitory concentrations of CHX selected for a mutation in *efrE* (A290V) that was shown to confer decreased susceptibility to CHX [172]. Several ChlR-EfrEF and promoter mutations were detected among 33 *E. faecium* and 4 *E. lactis* (former *E. faecium* Clade B) isolates from various sources and years (Table S3), but not the A290V associated with CHX tolerance, which was also absent in 980 *E. faecium* genomes from field isolates coding for a complete EfrE protein published in the GenBank database in December 2018 [170].

6.8. *chtRS*

The conserved DNA-binding response regulator, ChtR, along with histidine kinase sensor ChtS, form a putative two-component regulatory system (2CS) that has been implicated in CHX tolerance in *E. faecium* [170, 171]. Strains with deleted *chtR* and *chtS* showed a compromised growth and morphology under CHX exposure that was reverted when the mutations were complemented in trans [171]. Additionally, a nonsynonymous single nucleotide polymorphism in *chtR*, leading to an amino acid substitution (P102H), predominantly found in clinical isolates, was linked to a CHX tolerant phenotype [170, 171]. The P102H mutation is located in the dimerization interface of the signal receiver domain of ChtR, which might affect the activation and function of the response regulator [171]. The genes *chtR* and *chtS* are predicted to be part of an operon, named 2CS-CHX^T, also composed by genes related to sugar and amino acid transport [170]. Several other 2CS-CHX^T operon mutations have been detected among *E. faecium* and *E. lactis* strains, but their role in CHX tolerance has not been investigated [170].

The action of 2CSs is to regulate the expression of effector genes in response to environmental cues [171]. The regulon associated with the 2CS-CHX^T operon in *E. faecium* Aus0004 has been predicted to include genes involved in peptidoglycan homeostasis, protein, glycerol, or amino sugars metabolism, protection against cationic compounds, and oxidative stress response, among others [170], although confirmatory laboratory studies are lacking. Thus, a diverse multi-process cellular response may be prompted by CHX stress [170].

7. Co- and Cross-Resistance Between Cationic Biocides and Other Antimicrobials

Although there are several differences between CBs and antibiotics (e.g., spectrum and mechanism of action, ratio of in-use concentration per microorganisms' MICs, commercialized formulations), both have been used for their antimicrobial activity for decades now and CBs have been critical for limiting the need of antibiotic use [2]. However, research has been suggesting that exposure to biocides may directly or indirectly select for bacterial populations with particular genotypes or phenotypes leading to co- or cross-resistance to antibiotics [16]. *Enterococcus*' response after exposure to CBs has been shown not only to alter the expression of genes involved in multidrug efflux, bacterial metabolism, and cell wall permeability, which may affect the susceptibility of the cells to very diverse antimicrobials, but also to upregulate genes directly associated with resistance to antibiotics [125, 157]. Bhardwaj et al. found that CHX stress induced the expression of the VanA-type vancomycin resistance genes and genes associated with reduced susceptibility to daptomycin (*liaXYZ*) in *E. faecium* [172]. However, vancomycin susceptibility was actually increased for the VanA-positive *E. faecium* in the presence of subinhibitory concentrations of CHX, revealing a CHX-vancomycin synergy [172, 219]. Excess of D-lactate contributed to this synergism, whereas the deletion of the gene *ddcP*, encoding a membrane-bound carboxypeptidase, and a mutation (S199L) on an ATPase of phosphate-specific transporter, encoded by gene *pstB*, conferred a survival advantage in the presence of both antimicrobials [172, 219]. Nonetheless, the occurrence of a possible cross-resistance between vancomycin and CBs, including CHX, is still controversial. MICs and MBCs of CHX and BC of vancomycin-resistant *Enterococcus* isolates significantly higher than those of vancomycin-susceptible *Enterococcus* spp. have been described among diverse collections [51, 56, 57]. On the contrary, a higher tolerance in vancomycin-susceptible *Enterococcus* compared to vancomycin-resistant *Enterococcus* as well as no significant difference between the two groups has also been found for the biocides BC, CHX, DDAC or CPC [36, 41, 57, 60, 75, 81, 84]. Such disparities among studies might be associated with the different methodologies used, various levels of previous exposure to CBs by the bacteria, or with other genetic or metabolic properties specific to each of the local bacterial populations studied.

A possible link between antibiotic resistance and CBs tolerance has also been observed for other antibiotics. A reduced susceptibility to CHX and DDAC was detected in *Enterococcus* that were ampicillin resistant or with a high-level of resistance to the aminoglycosides gentamycin and streptomycin [57, 66, 67]. Despite the suggestion of a correlation by these results, no genetic or cellular changes supporting such co-occurring phenotypes have been explored yet.

The selection of antibiotic resistant subpopulations after serial exposure to subinhibitory concentrations of several CBs has been identified (Table S2) [38, 63, 172]. Adaptation to CHX, CPC, BC or CE of *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. saccharolyticus* or other *Enterococcus* spp. led to cross-resistance to the clinically-relevant ampicillin, ciprofloxacin, daptomycin, imipenem and tetracycline (Table S2) [38, 63, 172]. On the other hand, there was also evidence of loss of resistance to ampicillin in CHX-adapted *E. faecalis*, to ciprofloxacin in CE-adapted *E. faecium*, and to ceftazidime and/or cefotaxime in BC-adapted *E. faecium*, CPC-adapted *Enterococcus* spp., CE-adapted *E. casseliflavus*, *E. faecium* and *Enterococcus* spp., and CHX-adapted *E. faecium* [38, 63]. The genetic mechanisms or phenotypic expression events behind the increase or loss of antibiotic resistance detailed in these studies are mostly unknown, but they were mainly transient and may be associated with non-specific membrane permeability increase, metabolic changes (e.g., decreased growth rate), or others [38, 63]. For *E. faecium* 1,231,410, in which reduced susceptibility to daptomycin arose with passages in increasing CHX concentrations, Bhardwaj et al. detected physiological and genetic alterations in the adapted strains compared to the parental strain [172]. These included significantly lower growth rates, changes in cellular membrane phospholipid and glycolipid content, overexpression of the three-component regulatory system encoded *liaXYZ* involved in cell envelope homeostasis, and mutations in genes associated with global nutritional stress response (*relA*), nucleotide metabolism (*cmk*), multidrug efflux (*efrE*), phosphate acquisition (*phoU*), and glycolipid biosynthesis (*bgsB*) [172]. Daptomycin is a lipopeptide antibiotic often used to treat vancomycin-resistant *Enterococcus* spp. infections, that interacts primarily with the bacterial cell membrane, as part of a daptomycin-calcium complex, ultimately leading to cell death [172, 220]. Thus, the selection of potential structural changes in the biophysical properties of the cell wall or membrane and in the cellular stress responses by the biocide are strongly associated with reduced daptomycin susceptibility in *Enterococcus* [172, 220]. Since this antibiotic is used for the treatment of serious *Enterococcus* infections that lack other therapeutic alternatives, and CBs are extensively used in hospitals, these results may have serious clinical implications and deserve further studies [172]. However, as previously discussed, it must be taken into consideration that *in vitro* adaptation experiments may not accurately mimic real environments' conditions in the different contexts.

Of note, CBs, antibiotics, and other antimicrobials such as metals may co-exist as selective agents in many ecosystems, not only in human or animal clinical contexts, the community or food production, but also in wastewater and others [1, 221, 222]. In a report produced by SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks), in 2009, it was stated that ‘biocides are likely to contribute to maintaining selective pressure allowing the presence of mobile genetic elements harboring specific genes involved in the resistance to biocides and antibiotics’, and recommended the surveillance of levels of biocide resistance [223]. Indeed, co-location of genetic determinants conferring CBs and metal tolerance and antibiotic resistance on the same plasmid or other mobile genetic element has been observed among diverse *Enterococcus* spp. strains [42, 55, 183, 221]. In previous studies, the analyzed genetic contexts of several CBs tolerance genes (*qacA/B*, *qacC*, *qacZ*, *oqxAB*) in *Enterococcus* spp., from different sources, geographical regions, or dates of isolation, harbored genes conferring resistance to aminoglycosides, beta-lactams, macrolides, lincosamides, streptogramin of group B, or trimethoprim [42, 55, 183]. Metal tolerance genes, namely to copper and cadmium, were also detected within the vicinity of *qacA/B* genes in two clinical *E. faecalis* [42]. The genetic contexts of *qacA/B*, *qacC*, *qacJ*, *qacZ*, *qrg*, *bcrABC* and *oqxAB* detected in *Enterococcus* were compared with those from other taxa and found to be generally very diverse, probably resulting from a high number of recombination events, as suggested by the abundance of insertion sequences and recombinases detected [42]. Also, the co-location of these genes on mobile genetic elements such as plasmids, often carrying toxin-antitoxin systems that contribute to their maintenance in the bacterial populations, may facilitate their spread and, thus, mechanisms of co-selection [2, 28, 42, 55, 183, 221].

Besides co-location of diverse antimicrobial resistance genes on the same genetic contexts, the occurrence of cross-resistance, where a single resistance mechanism to a certain antimicrobial also affects additional compounds, has also been hypothesized in *Enterococcus* spp. [221]. CBs tolerance genes such as *emeA*, *efrEF*, *oqxAB* and *chtR* are known to be involved in antibiotic resistance, as previously mentioned [183, 214, 216-218, 224]. For instance, the *chtR* and *chtS* *E. faecium* deletion mutants showed an increased susceptibility to both CHX and the antibiotic bacitracin [171]. Additionally, Yuan et al. proposed that the extensive use of quinoxalines in animal husbandry in China could be selecting for a local high prevalence of the *oqxAB* genes, known to confer tolerance to such antibiotics and CBs, among *Enterococcus* spp. [183].

Recently, a few studies have proposed another process in which CBs exposure could contribute to antibiotic resistance spread [225-229]. In these, subinhibitory concentrations of different CBs, including QACs and CHX, have been shown to increase horizontal gene transfer via conjugation, through multiple cellular processes such as increased reactive oxygen species (ROS) production, upregulated stress and SOS response, enhanced cell membrane

permeability, and changes in the expression of conjugative transfer genes, among others [225-229]. However, this has not yet been studied for *Enterococcus* spp.

All these examples identify possible overlaps between responses to different biocides and antibiotics and the potential for the development of co- and cross-resistance among antimicrobials in various environments, with impact in diverse Public Health contexts. However, it is crucial to recognize the large limitations of the research on this topic. Although some studies support the associations detected between CBs tolerance and resistance to specific antibiotics through robust methodological approaches, for most, caution is required in interpreting the correlations made as they may be linked to independent, co-occurring events of cellular response to diverse antimicrobials. Thus, the underlying processes by which exposure to one substance may lead to reduced susceptibility to another remain unclear.

8. Conclusions

Currently, the available literature offers valuable insights concerning the state of the genotypic and phenotypic *Enterococcus* spp. susceptibility to CBs, pointing to an effective biocidal activity with still no descriptions of resistance to CB's typical in-use concentrations. However, it also reveals several key research gaps that need to be tackled in future investigations.

Urgent priorities include standardizing biocide susceptibility methodologies that are of relevance to real-world scenarios, facilitating direct study comparisons and the establishment of surveillance protocols applicable across diverse environments with high selective pressures associated with CB's use. The study of large and well-characterized *Enterococcus* spp. collections from various epidemiological and clonal backgrounds is key to identify a possible evolution towards tolerance in particular populations. Conducting functional genetic assays, such as the deletion and complementation of genes accompanied by the observation of phenotype changes, is crucial for understanding the role of genes with predicted effect on susceptibility to CBs in *Enterococcus* spp. Moreover, it is imperative to develop strategies aimed at identifying and controlling bacteria harboring these genotypes, particularly in settings where CBs are heavily used. Finally, understanding the long-term consequences of exposure to subinhibitory concentrations of CBs and the mechanisms involved in *Enterococcus* adaptation to these compounds or in the co-selection or cross-resistance with antibiotics also requires thorough investigation. Thus, large longitudinal metadata analyses, incorporating WGS and transcriptomic approaches, are critical for clarifying dynamics of *Enterococcus* spp. populations exposed to CBs.

Fulfilling these methodological and knowledge gaps will provide more accurate insights into the impact of CBs exposure on *Enterococcus* spp. antimicrobial resistance patterns and

overall epidemiological evolution, enabling the development of effective One Health strategies to ensure the continued efficacy of these critical agents in safeguarding Public Health.

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Supplementary material

Table S1 - Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Benzalkonium chloride	<i>E. casseliflavus</i>	7	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	2 - 4		[36]
		<i>E. casseliflavus</i>	1	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. casseliflavus</i>	2	Food chain (organic foods)	Spain				BM	0.1 - 0.2		[38]
		<i>E. durans</i>	2	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. durans</i>	3	Food chain (milk, cheese)	Portugal				BM (CLSI)	4		[37]
		<i>E. durans</i>	1	Food chain (organic foods)	Spain				BM	0.5		[38]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (CLSI)	8	16	[39]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM	8		[40]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (CLSI)	2 - 4		[41]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (CLSI)	1 - 2	2 - 4	[42]
		<i>E. faecalis</i> V583	1	Human infection	USA	1987	6	<i>qacZ</i>	BM (CLSI)	4	4	[42]
		<i>E. faecalis</i>	26	Human infection	Portugal, Tunisia	1999-2020	2, 6, 9, 16, 22, 25, 30, 55, 59, 64, 116, 133, 159, 191, 200, 631, 1105, 1165		BM (CLSI)	1 - 4	2 - 4	[42]
		<i>E. faecalis</i>	55	Human infection	Iran	2018-2020			AD (CLSI)	4 - 16		[43]
		<i>E. faecalis</i>	12	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal			<i>qacZ</i>	BM (CLSI)	2 - 4		[37]
		<i>E. faecalis</i>	6	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal				BM (CLSI)	2 - 4		[37]
		<i>E. faecalis</i> B281	1	Human infection (child dental carie)	Tunisia				BM (CLSI)	256	>256	[39]
		<i>E. faecalis</i> 19-47-23	1	Human infection	Germany				BM (CLSI)	4 - 8		[41]
		<i>E. faecalis</i>	3	Human colonization (faeces of patients at hospital admission)	Brasil	2015-2016	6, 525		BM (CLSI)	2	2 - 4	[42]
		<i>E. faecalis</i>	4	Human colonization (faeces of long-term care patients)	Portugal	2015-2016	25, 143, 398, 679		BM (CLSI)	2	2 - 4	[42]
		<i>E. faecalis</i>	21	Human colonization (healthy humans' faeces, urinary tract, breast milk)	Portugal, Angola	2001-2018	21, 30, 40, 63, 64, 81, 116, 179, 191, 200, 206, 209, 275, 308, 394		BM (CLSI)	1 - 2	2 - 4	[42]
		<i>E. faecalis</i>	45	Human colonization (healthy humans)	Iran	2018-2020			AD (CLSI)	2 - 16		[43]
		<i>E. faecalis</i>	49	Pet infection (dogs, cats)	Germany	2017-2019			BM	1.25 - 5		[44]
		<i>E. faecalis</i>	11	Pet infection (dogs, cats)	Portugal				BM (CLSI)	2 - 4		[37]
		<i>E. faecalis</i>	2	Pet colonization (cat and bird faeces)	Tunisia	2014-2015	21, 116		BM (CLSI)	1	2 - 4	[42]
		<i>E. faecalis</i>	22	Food chain (poultry carcass, trout, bovine and goat milk, bovine meat, raw meat frozen pet food)	Portugal, Tunisia	1999-2020	21, 27, 32, 34, 40, 49, 117, 141, 200, 202, 206, 209, 227, 288, 436, 674, 721, 843, 860, 1008, 1106		BM (CLSI)	1 - 2	2 - 4	[42]
		<i>E. faecalis</i>	14	Food chain (aquaculture, piggery, poultry, ovine faeces)	Portugal, Tunisia	2006-2015	21, 22, 35, 40, 59, 65, 139, 200, 209, 330, 386, 631, 749, 872		BM (CLSI)	1 - 2	2 - 4	[42]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹⁾)	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Benzalkonium chloride	<i>E. faecalis</i>	53	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	2 - 4		[36]
		<i>E. faecalis</i>	52	Food chain (pigs and broilers faeces)	Denmark				BM (CLSI)	2 - 8		[45]
		<i>E. faecalis</i>	5	Food chain (swine meat production chain)	Italy			<i>emeA</i>	BM (CLSI)	2 - 4		[46]
		<i>E. faecalis</i>	4	Food chain (swine meat production chain)	Italy				BM (CLSI)	4		[46]
		<i>E. faecalis</i>	8	Food chain (cheese)	Portugal	2022			BM (CLSI)	5 - 20	10 - 20	[47]
		<i>E. faecalis</i>	12	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. faecalis</i>	5	Food chain (milk, cheese)	Portugal				BM (CLSI)	4		[37]
		<i>E. faecalis</i>	38	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of Congo				BM (CLSI)	<0.1		[48]
		<i>E. faecalis</i>	2	Food chain (organic foods)	Spain				BM	0.05 - 0.5		[38]
		<i>E. faecalis</i>	4	Food chain (ready-to-eat salads)	Portugal	2010	141, 165, 309, 594		BM (CLSI)	1 - 2	4	[42]
		<i>E. faecalis</i>	3	Environment (hospital sewage)	Portugal	2002	16, 35, 49		BM (CLSI)	2	2 - 4	[42]
		<i>E. faecalis</i>	34	Environment (hospital sewage)	Iran	2018-2020			AD (CLSI)	4 - 16		[43]
		<i>E. faecalis</i>	3	Environment (urban wastewater treatment plant)	Tunisia	2014-2015	23, 86, 117		BM (CLSI)	1 - 2	2 - 4	[42]
		<i>E. faecalis</i>	21	Environment (municipal sewage)	Iran	2018-2020			AD (CLSI)	8 - 16		[43]
		<i>E. faecalis</i>	38	Environment (poultry sewage)	Iran	2018-2020			AD (CLSI)	2 - 16		[43]
		<i>E. faecalis</i>	29	Environment (livestock sewage)	Iran	2018-2020			AD (CLSI)	4 - 16		[43]
		<i>E. faecalis</i>	2	Environment (river)	Portugal	2003	1, 4		BM (CLSI)	2	2 - 4	[42]
		<i>E. faecalis</i>	56		Spain	2001-2009			BM (CLSI)	1 - 8	2 - 16	[49]
		<i>E. faecalis</i>	1						BM	2	3.3	[27]
		<i>E. faecium</i> Aus0004	1	Human infection (blood)	Australia	1998			BM (EUCAST)	4	8	[50]
		<i>E. faecium</i>	26	Human infection	Portugal, Spain, Tunisia	1996-2020	17, 18, 22, 78, 80, 117, 190, 280, 391, 412, 419, 494, 515, 780		BM (CLSI)	1 - 4	2 - 4	[42]
		<i>E. faecium</i>	12	Human infection (blood)	Denmark	2004	22, 95, 178, 296, 361, 372, 697, 1277, 1279		BM (CLSI)	2 - 8	2 - 8	[51]
		<i>E. faecium</i>	37	Human infection (blood, urine, tissue)	Denmark	2012-2013	18, 80, 117, 203		BM (CLSI)	4 - 8	4 - 16	[51]
		<i>E. faecium</i>	17	Human infection	Iran	2018-2020			AD (CLSI)	8 - 16		[43]
		<i>E. faecium</i>	5	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal			<i>qacZ</i>	BM (CLSI)	4 - 8		[37]
		<i>E. faecium</i>	5	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal				BM (CLSI)	4 - 8		[37]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Benzalkonium chloride	<i>E. faecium</i>	3	Human infection (blood)	Germany				BM (CLSI)	8 - 16		[41]
		<i>E. faecium</i>	90	Human infection (blood cultures, swabs, urine, faeces)	Germany				BM (Vacuum Dried Biocide Microtiter Plates)	1 - 8	8 - 16	[41]
		<i>E. faecium</i>	4	Human colonization (faeces of long-term care patients)	Portugal	2015-2016	80, 117, 262		BM (CLSI)	2 - 4	2 - 4	[42]
		<i>E. faecium</i>	15	Human colonization (healthy humans' faeces, vagina)	Portugal, Angola	2001-2017	6, 8, 18, 22, 32, 89, 147, 845, 1051, 2095		BM (CLSI)	1 - 4	1 - 4	[42]
		<i>E. faecium</i>	219	Human colonization (healthy humans)	Iran	2018-2020			AD (CLSI)	4 - 32		[43]
		<i>E. faecium</i>	37	Pet infections (dogs, cats)	Germany	2017-2019			BM	1.25 - 5		[44]
		<i>E. faecium</i>	4	Pet infections (dogs, cats)	Portugal				BM (CLSI)	4 - 8		[37]
		<i>E. faecium</i>	27	Food chain (poultry carcass, trout, bovine milk, bovine meat, raw meat frozen pet food)	Portugal, Tunisia	1999-2020	9, 12, 17, 18, 25, 27, 32, 80, 157, 203, 245, 264, 451, 683, 1058, 1059, 1091, 1263, 2092		BM (CLSI)	1 - 4	1 - 4	[42]
		<i>E. faecium</i>	18	Food chain (aquaculture, piggery, poultry faeces)	Portugal, Angola	2001-2020	5, 6, 29, 30, 94, 101, 132, 185, 264, 393, 430, 434, 891, 970, 971		BM (CLSI)	0.5 - 2	1 - 4	[42]
		<i>E. faecium</i>	22	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	2 - 4		[36]
		<i>E. faecium</i>	78	Food chain (pigs, broilers and cattle faeces)	Denmark				BM (CLSI)	2 - 16		[45]
		<i>E. faecium</i> CF3 1.3	1	Food chain (ceca of an adult broiler chicken)					BM (CLSI)	4		[52]
		<i>E. faecium</i>	79	Food chain (farm animal's faeces)					BM	25		[53]
		<i>E. faecium</i>	1	Food chain (swine meat production chain)	Italy			<i>qacA/B, emcA</i>	BM (CLSI)	4		[46]
		<i>E. faecium</i>	8	Food chain (swine meat production chain)	Italy			<i>emcA</i>	BM (CLSI)	2 - 4		[46]
		<i>E. faecium</i>	3	Food chain (swine meat production chain)	Italy				BM (CLSI)	2 - 4		[46]
		<i>E. faecium</i>	8	Food chain (cheese)	Portugal	2022			BM (CLSI)	5 - 20	10 - 40	[47]
		<i>E. faecium</i>	3	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. faecium</i>	84	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of Congo				BM (CLSI)	<0.1		[48]
		<i>E. faecium</i>	13	Food chain (organic foods)	Spain				BM	0.1 - 0.5		[38]
		<i>E. faecium</i>	4	Food chain (ready-to-eat salads)	Portugal	2010	352, 640, 666		BM (CLSI)	1 - 2	1 - 4	[42]
		<i>E. faecium</i>	20	Food chain (fresh produce)	Spain		22, 26, 43, 46, 55, 94, 296		BM	12.5 - 50		[54]
		<i>E. faecium</i>	2	Environment (hospital sewage)	Portugal	2001-2002	368, 2091		BM (CLSI)	2	2	[42]
		<i>E. faecium</i> E241	1	Environment (hospital sewage)	Portugal	2002	17	<i>qacZ</i>	BM (CLSI)	4	4	[42]
		<i>E. faecium</i> E241	1	Environment (hospital sewage)	Portugal	2002	17	<i>qacZ</i>	AD (CLSI)	9		[55]
		<i>E. faecium</i>	63	Environment (hospital sewage)	Iran	2018-2020			AD (CLSI)	2 - 32		[43]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Benzalkonium chloride	<i>E. faecium</i>	3	Environment (urban wastewater treatment plant)	Tunisia	2014-2015	666		BM (CLSI)	1 - 2	2	[42]
		<i>E. faecium</i>	42	Environment (municipal sewage)	Iran	2018-2020			AD (CLSI)	4 - 16		[43]
		<i>E. faecium</i>	50	Environment (community waste water)	USA				BM (CLSI)	2 - 8		[52]
		<i>E. faecium</i>	39	Environment (poultry sewage)	Iran	2018-2020			AD (CLSI)	2 - 16		[43]
		<i>E. faecium</i>	45	Environment (livestock sewage)	Iran	2018-2020			AD (CLSI)	4 - 16		[43]
		<i>E. faecium</i>	3	Environment (river)	Portugal	2003	22, 132, 2093		BM (CLSI)	2	2 - 4	[42]
		<i>E. faecium</i>	2	Others (wild birds)	Germany, Canada	2011-2013	78, 448		BM (CLSI)	1 - 4	2 - 4	[42]
		<i>E. faecium</i>	53			1986-2009			BM (CLSI)	1 - 8	2 - 16	[49]
		<i>E. gallinarum</i>	2	Food chain (dust sample from breeding pig facilities)	Portugal	2008			BM (CLSI)	2 - 4		[36]
		<i>E. hirae</i> ATCC 10541	1						BM	8		[40]
		<i>E. hirae</i>	2	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal			<i>qacZ</i>	BM (CLSI)	2 - 4		[37]
		<i>E. hirae</i>	39	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	1 - 4		[36]
		<i>E. hirae</i>	1	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. raffinosus</i>	2	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	1 - 2		[36]
		<i>E. solitarius</i>	1	Pet infection (dogs, cats)	Portugal				BM (CLSI)	2		[37]
		<i>Enterococcus</i> spp.	69	Human infection (blood, cerebrospinal fluid, urine, abscess, catheter tips, nasal secretions, endotracheal aspiration fluid)		2010-2011			AD (CLSI)	8 - 16		[56]
		<i>Enterococcus</i> spp.	59	Human infection					BM	25 - 250		[53]
		<i>Enterococcus</i> spp.	54	Human infection					BM	2 - 8		[57]
		<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)					BM	2 - 16		[57]
		<i>Enterococcus</i> spp.	500	Food chain (broilers, cattle and pigs)	Denmark	1995-1996			BM	<30		[58]
		<i>Enterococcus</i> spp.	25	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	<0.25 - 4		[36]
		<i>Enterococcus</i> spp.	25	Food chain (seafood)					BM	25		[53]
		<i>Enterococcus</i> spp.	40	Food chain (meat and dairy products)					BM	2.5 - 250		[53]
		<i>Enterococcus</i> spp.	6	Food chain (organic foods)	Spain				BM	0.1 - 0.5		[38]
		<i>Enterococcus</i> spp.	22	Food chain (vegetables)					BM	25		[53]
		<i>Enterococcus</i> spp.	5	Environment (samples of homes of antibacterial product users)	USA, UK				BM (CLSI)	0.426 - 0.851		[59]
		<i>Enterococcus</i> spp.	6	Environment (samples of homes of antibacterial product nonusers)	USA, UK				BM (CLSI)	0.426 - 0.851		[59]
		<i>Enterococcus</i> spp.	47	Others (wildflowers)					BM	25 - 250		[53]
		<i>Enterococcus</i> spp.	5						AD	5 - 6		[60]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Cetylpyridinium chloride	<i>E. casseliflavus</i>	2	Food chain (organic foods)	Spain				BM	0.5 - 1		[38]
		<i>E. durans</i>	1	Food chain (organic foods)	Spain				BM	5		[38]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (CLSI)	2 - 4	-	[41]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM	1	4	[61]
		<i>E. faecalis</i> 19-47-23	1	Human infection	Germany				BM (CLSI)	2 - 4	-	[41]
		<i>E. faecalis</i>	38	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of Congo				BM (CLSI)	<0.1		[48]
		<i>E. faecalis</i>	2	Food chain (organic foods)	Spain				BM	1 - 5		[38]
		<i>E. faecalis</i> SS497	1						BM	2		[62]
		<i>E. faecium</i>	3	Human infection (blood)	Germany				BM (CLSI)	2 - 8	-	[41]
		<i>E. faecium</i>	90	Human infection (blood cultures, swabs, urine, faeces)	Germany				BM (Vacuum Dried Biocide Microtiter Plates)	≤1 - 4	4	[41]
		<i>E. faecium</i>	79	Food chain (farm animal's faeces)					BM	2.5 - 25		[53]
		<i>E. faecium</i>	84	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of				BM (CLSI)	<0.1		[48]
		<i>E. faecium</i>	20	Food chain (fresh produce)	Spain		22, 26, 43, 46, 55, 94, 296		BM	1 - 2	-	[54]
		<i>E. faecium</i>	13	Food chain (organic foods)	Spain				BM	0.5 - 5		[38]
		<i>Enterococcus</i> spp.	59	Human infection					BM	2.5 - 250		[53]
		<i>Enterococcus</i> spp.	40	Food chain (meat and dairy foods)					BM	2.5 - 25		[53]
		<i>Enterococcus</i> spp.	25	Food chain (seafood)					BM	2.5 - 25		[53]
		<i>Enterococcus</i> spp.	22	Food chain (vegetables)					BM	2.5 - 25		[53]
		<i>Enterococcus</i> spp.	6	Food chain (organic foods)	Spain				BM	1 - 50		[38]
		<i>Enterococcus</i> spp.	47	Others (wildflowers)					BM	2.5 - 25		[53]
		<i>Enterococcus</i> spp.	5						AD	5 - 6		[60]
Cetrimide		<i>E. casseliflavus</i>	2	Food chain (organic foods)	Spain				BM	5 - 10		[63]
		<i>E. durans</i>	1	Food chain (organic foods)	Spain				BM	1		[63]
		<i>E. faecalis</i> WIBG 1.1	1	Human infection (wound)					BM	12.1	29	[64]
		<i>E. faecalis</i>	38	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of Congo				BM (CLSI)	<0.1		[48]
		<i>E. faecalis</i>	2	Food chain (organic foods)	Spain				BM	0.5 - 5		[63]
		<i>E. faecium</i>	79	Food chain (farm animal's faeces)					BM	2.5 - 25		[53]
		<i>E. faecium</i>	84	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of Congo				BM (CLSI)	<0.1		[48]
		<i>E. faecium</i>	20	Food chain (fresh produce)	Spain		22, 26, 43, 46, 55, 94, 296		BM	25 - 75	-	[54]
		<i>E. faecium</i>	13	Food chain (organic foods)	Spain				BM	0.5 - 10		[63]
		<i>Enterococcus</i> spp.	59	Human infection					BM	2.5 - 250		[53]
		<i>Enterococcus</i> spp.	25	Food chain (seafood)					BM	2.5 - 25		[53]
		<i>Enterococcus</i> spp.	40	Food chain (meat and dairy foods)					BM	2.5 - 25		[53]
		<i>Enterococcus</i> spp.	22	Food chain (vegetables)					BM	2.5 - 25		[53]
		<i>Enterococcus</i> spp.	6	Food chain (organic foods)	Spain				BM	0.1 - 10		[63]
		<i>Enterococcus</i> spp.	47	Others (wildflowers)					BM	2.5 - 25		[53]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Didecyltrimethylammonium chloride	<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (CLSI)	2		[41]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (CLSI)	0.5 - 1	1 - 2	[42]
		<i>E. faecalis</i> V583	1	Human infection	USA	1987	6	<i>qacZ</i>	BM (CLSI)	2	2	[42]
		<i>E. faecalis</i>	26	Human infection	Portugal, Tunisia	1999-2020	2, 6, 9, 16, 22, 25, 30, 55, 59, 64, 116, 133, 159, 191, 200, 631, 1105, 1165		BM (CLSI)	1 - 2	1 - 4	[42]
		<i>E. faecalis</i>	42	Human infection (blood)	Germany	2008-2009			BM	1.05		[65]
		<i>E. faecalis</i> Efa-AB-2	1	Human infection (blood)	Germany	2008-2009		<i>qacA/B</i>	BM	2.45 - 3.5		[65]
		<i>E. faecalis</i>	88	Human infection (blood)	Germany				BM	1.05 - 3.5		[66]
		<i>E. faecalis</i> 19-47-23	1	Human infection	Germany				BM (CLSI)	2 - 4	-	[41]
		<i>E. faecalis</i>	109	Human colonization (hospitalized patient's faeces)	Germany	2008-2009			BM	1.05		[65]
		<i>E. faecalis</i> Efa-smr-1	1	Human colonization (hospitalized patient's faeces)	Germany	2008-2009		<i>qacC</i>	BM	1.05		[65]
		<i>E. faecalis</i>	210	Human colonization (hospitalized patient's faeces)	Germany				BM	1.05 - 3.5		[66]
		<i>E. faecalis</i>	3	Human colonization (faeces of patients at hospital admission)	Brasil	2015-2016	6, 525		BM (CLSI)	1	2	[42]
		<i>E. faecalis</i>	102	Human colonization (outpatients' faeces)	Germany				BM	1.05 - 3.5		[66]
		<i>E. faecalis</i>	4	Human colonization (faeces of long-term care patients)	Portugal	2015-2016	25, 143, 398, 679		BM (CLSI)	1	2 - 4	[42]
		<i>E. faecalis</i>	21	Human colonization (healthy humans' faeces, urinary tract, breast milk)	Portugal, Angola	2001-2018	21, 30, 40, 63, 64, 81, 116, 179, 191, 200, 206, 209, 275, 308, 394		BM (CLSI)	1	1 - 4	[42]
		<i>E. faecalis</i>	2	Pet colonization (faeces of cats and birds)	Tunisia	2014-2015	21, 116		BM (CLSI)	1	1 - 2	[42]
		<i>E. faecalis</i>	22	Food chain (poultry carcass, trout, bovine and goat milk, bovine meat, raw meat frozen pet food)	Portugal, Tunisia	1999-2020	21, 27, 32, 34, 40, 49, 117, 141, 200, 202, 206, 209, 227, 288, 436, 674, 721, 843, 860, 1008, 1106		BM (CLSI)	1 - 2	2 - 4	[42]
		<i>E. faecalis</i>	14	Food chain (aquaculture, piggery, poultry, ovine faeces)	Portugal, Tunisia	2006-2015	21, 22, 35, 40, 59, 65, 139, 200, 209, 330, 386, 631, 749, 872		BM (CLSI)	1 - 2	2 - 4	[42]
		<i>E. faecalis</i> Efa-AB-1	1	Food chain (cattle)	Germany			<i>qacA/B</i>	BM	1.05		[65]
		<i>E. faecalis</i>	112	Food chain (meat products)	Germany				BM	1.05		[65]
		<i>E. faecalis</i>	226	Food chain (faeces of farm animals)	Germany				BM	1.05		[65]
		<i>E. faecalis</i>	54	Food chain (poultry)	Germany				BM	1.05 - 2.1		[66]
		<i>E. faecalis</i>	30	Food chain (beef)	Germany				BM	1.05 - 2.1		[66]
		<i>E. faecalis</i>	28	Food chain (pork)	Germany				BM	1.05 - 2.1		[66]
		<i>E. faecalis</i>	50	Food chain (cattle, matistis milk)	Germany				BM	1.05		[66]
		<i>E. faecalis</i>	76	Food chain (swine faeces)	Germany				BM	1.05		[66]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines) ¹	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Didecyltrimethylammonium chloride	<i>E. faecalis</i>	50	Food chain (cattle faeces)	Germany				BM	1.05		[66]
		<i>E. faecalis</i>	50	Food chain (chicken faeces)	Germany				BM	1.05 - 1.75		[66]
		<i>E. faecalis</i>	56	Food chain (poultry caecum, cloacal swab, faeces)	Germany				MD (German Veterinary Society guidelines)	0.14 - 1.44		[67]
		<i>E. faecalis</i>	12	Food chain (cloacal swabs of hunted chicken)	Finland				MD (German Veterinary Society guidelines)	0.36 - 1.44		[67]
		<i>E. faecalis</i>	96	Food chain (dairy products)	Germany				BM	1.05		[65]
		<i>E. faecalis</i> Efa-smr-2	1	Food chain (cheese “Camembert”)	Germany			<i>qacC</i>	BM	1.05		[65]
		<i>E. faecalis</i>	96	Food chain (dairy products)	Germany				BM	1.05 - 3.5		[66]
		<i>E. faecalis</i>	4	Food chain (ready-to-eat salads)	Portugal	2010	141, 165, 309, 594		BM (CLSI)	1	2 - 4	[42]
		<i>E. faecalis</i>	3	Environment (hospital sewage)	Portugal	2002	16, 35, 49		BM (CLSI)	1	2 - 4	[42]
		<i>E. faecalis</i>	3	Environment (urban wastewater treatment plant)	Tunisia	2014-2015	23, 86, 117		BM (CLSI)	1	2 - 4	[42]
		<i>E. faecalis</i>	2	Environment (river)	Portugal	2003	1, 4		BM (CLSI)	1	2 - 4	[42]
		<i>E. faecalis</i>	1						BM	1	1	[27]
		<i>E. faecium</i>	26	Human infection	Portugal, Spain, Tunisia	1996-2020	17, 18, 22, 78, 80, 117, 190, 280, 391, 412, 419, 494, 515, 780		BM (CLSI)	1 - 2	1 - 4	[42]
		<i>E. faecium</i>	45	Human infection (blood)	Germany				BM	1.05 - 1.75		[66]
		<i>E. faecium</i>	90	Human infection (blood cultures, swabs, urine, faeces)	Germany				BM (Vacuum Dried Biocide Microtiter Plates)	≤0.5 - 2	1 - 2	[41]
		<i>E. faecium</i>	3	Human infection (blood)	Germany				BM (CLSI)	2 - 4	-	[41]
		<i>E. faecium</i>	52	Human colonization (hospitalized patient's faeces)	Germany				BM	1.05 - 1.75		[66]
		<i>E. faecium</i>	38	Human colonization (outpatients' faeces)	Germany				BM	1.05 - 2.8		[66]
		<i>E. faecium</i>	4	Human colonization (faeces of long-term care patients)	Portugal	2015-2016	80, 117, 262		BM (CLSI)	1 - 2	2 - 4	[42]
		<i>E. faecium</i>	15	Human colonization (healthy humans' faeces, vagina)	Portugal, Angola	2001-2017	6, 8, 18, 22, 32, 89, 147, 845, 1051, 2095		BM (CLSI)	1 - 2	1 - 2	[42]
		<i>E. faecium</i>	27	Food chain (poultry carcass, trout, bovine milk, bovine meat, raw meat frozen pet food)	Portugal, Tunisia	1999-2020	9, 12, 17, 18, 25, 27, 32, 80, 157, 203, 245, 264, 451, 683, 1058, 1059, 1091, 1263, 2092		BM (CLSI)	1 - 2	1 - 4	[42]
		<i>E. faecium</i>	18	Food chain (aquaculture, piggery, poultry faeces)	Portugal, Angola	2001-2020	5, 6, 29, 30, 94, 101, 132, 185, 264, 393, 430, 434, 891, 970, 971		BM (CLSI)	0.5 - 1	1 - 2	[42]
		<i>E. faecium</i> CF3 1.3	1	Food chain (ceca of an adult broiler chicken)					BM (CLSI)	0.5		[52]
		<i>E. faecium</i>	64	Food chain (poultry caecum and faeces)	Germany				MD (German Veterinary Society guidelines)	0.14 - 1.44		[67]
		<i>E. faecium</i>	17	Food chain (cloacal swabs of hunted chicken)	Finland				MD (German Veterinary Society guidelines)	0.36 - 1.44		[67]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Quaternary Ammonium Compounds	Didecyltrimethylammonium chloride	<i>E. faecium</i>	4	Food chain (ready-to-eat salads)	Portugal	2010	352, 640, 666		BM (CLSI)	0.5 - 1	1 - 2	[42]
		<i>E. faecium</i>	2	Environment (hospital sewage)	Portugal	2001-2002	368, 2091		BM (CLSI)	1 - 2	2	[42]
		<i>E. faecium</i> E241	1	Environment (hospital sewage)	Portugal	2002	17	<i>qacZ</i>	BM (CLSI)	2	2	[42]
		<i>E. faecium</i>	3	Environment (urban wastewater treatment plant)	Tunisia	2014-2015	666		BM (CLSI)	1	2	[42]
		<i>E. faecium</i>	50	Environment (community waste water)	USA				BM (CLSI)	0.5 - 2		[52]
		<i>E. faecium</i>	3	Environment (river)	Portugal	2003	22, 132, 2093		BM (CLSI)	1	2	[42]
		<i>E. faecium</i>	2	Others (wild birds)	Germany, Canada	2011-2013	78, 448		BM (CLSI)	1 - 2	2	[42]
Biguanides	Chlorhexidine	<i>E. avium</i>	1	Human infection					BM (CLSI)	1		[68]
		<i>E. casseliflavus</i>	1	Human infection					BM (CLSI)	2		[68]
		<i>E. casseliflavus</i>	7	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	1 - 4		[36]
		<i>E. casseliflavus</i>	1	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. casseliflavus</i>	2	Food chain (organic foods)	Spain				BM	5		[63]
		<i>E. durans</i>	2	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. durans</i>	3	Food chain (milk, cheese)	Portugal				BM (CLSI)	4 - 8		[37]
		<i>E. durans</i>	1	Food chain (organic foods)	Spain				BM	5		[63]
		<i>E. faecalis</i> ATCC 29211	1	Human infection (urine)	USA		30		BM (CLSI)	<1		[68]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (DIN:58940-7, 58940-8)	16	16	[69]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		MD (CLSI)	32	256	[70]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM	2.5	10	[71]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM	27		[40]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (CLSI)	8		[41]
		<i>E. faecalis</i> ATCC 4083	1						BM	3.9		[72]
		<i>E. faecalis</i> NCTC 775	1						BM (EUCAST)	2	4	[73]
		<i>E. faecalis</i>	47	Human infection	Portugal, Tunisia	1996-2019	2, 6, 9, 16, 21, 22, 25, 30, 40, 41, 55, 59, 64, 97, 116, 126, 133, 159, 191, 200, 275, 286, 319, 397, 631, 679, 1105, 1164, 1165		BM (CLSI)	1 - 8	4 - 64	[74]
		<i>E. faecalis</i>	55	Human infection	Iran	2018-2020			AD (CLSI)	2 - 8		[43]
		<i>E. faecalis</i>	10	Human infection					MD (CLSI)	4 ²	24 ²	[75]
		<i>E. faecalis</i>	2	Human infection					BM (CLSI)	<1 - 2		[68]
		<i>E. faecalis</i>	1	Human infection					BM (DIN:58940-7, 58940-8)	16	32	[69]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Biguanides	Chlorhexidine	<i>E. faecalis</i>	12	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal			<i>qacZ</i>	BM (CLSI)	2 - 4		[37]
		<i>E. faecalis</i>	6	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal				BM (CLSI)	2 - 4		[37]
		<i>E. faecalis</i> WIBG 1.1	1	Human infection (wound)					BM	3.6	26.6	[64]
		<i>E. faecalis</i> 19-47-23	1	Human infection	Germany				BM (CLSI)	8 - 16		[41]
		<i>E. faecalis</i>	3	Human colonization (faeces of patients at hospital admission)	Brasil	2015-2016	6, 525		BM (CLSI)	4 - 8	8 - 16	[74]
		<i>E. faecalis</i>	7	Human colonization (faeces of long-term care patients)	Portugal	2015-2016	25, 26, 40, 143, 191, 398, 679		BM (CLSI)	1 - 8	8 - 64	[74]
		<i>E. faecalis</i>	49	Pet infection (dogs, cats)	Germany	2017-2019			BM	0.3 - 10		[44]
		<i>E. faecalis</i>	11	Pet infection (dogs, cats)	Portugal				BM (CLSI)	4		[37]
		<i>E. faecalis</i>	3	Pet colonization (dog, cat, bird)	Tunisia	2014-2015	21, 116		BM (CLSI)	4	8 - 32	[74]
		<i>E. faecalis</i>	44	Human colonization (healthy humans' faeces, urinary tract, vagina, breast milk)	Portugal, Angola	2001-2018	16, 21, 23, 30, 40, 55, 56, 63, 64, 81, 96, 116, 117, 168, 179, 191, 200, 206, 209, 275, 308, 394		BM (CLSI)	0.5 - 8	1 - 64	[74]
		<i>E. faecalis</i>	45	Human colonization (healthy humans)	Iran	2018-2020			AD (CLSI)	0.5 - 8		[43]
		<i>E. faecalis</i>	36	Food chain (poultry carcass, trout, bovine and goat milk, bovine meat, raw meat frozen pet food)	Portugal, Tunisia	1999-2020	4, 16, 21, 22, 25, 27, 30, 32, 34, 40, 49, 68, 82, 117, 141, 200, 202, 206, 209, 227, 249, 288, 436, 648, 674, 721, 843, 860, 1106, 1107,		BM (CLSI)	0.5 - 8	8 - 64	[74]
		<i>E. faecalis</i>	23	Food chain (aquaculture, piggery, poultry, ovine faeces)	Portugal, Tunisia	2006-2015	21, 22, 35, 40, 59, 65, 100, 139, 200, 209, 330, 386, 445, 631, 749, 872		BM (CLSI)	1 - 8	8 - 64	[74]
		<i>E. faecalis</i>	53	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	2 - 8		[36]
		<i>E. faecalis</i>	52	Food chain (pigs and broilers faeces)	Denmark				BM (CLSI)	0.5 - 8		[45]
		<i>E. faecalis</i>	5	Food chain (swine meat production chain)	Italy			<i>emeA</i>	BM (CLSI)	8 - 12		[46]
		<i>E. faecalis</i>	4	Food chain (swine meat production chain)	Italy				BM (CLSI)	8 - 10		[46]
		<i>E. faecalis</i>	12	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4 - 8		[37]
		<i>E. faecalis</i>	5	Food chain (milk, cheese)	Portugal				BM (CLSI)	4		[37]
		<i>E. faecalis</i>	38	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of Congo				BM (CLSI)	<0.1-2.5		[48]
		<i>E. faecalis</i>	6	Food chain (ready-to-eat salads)	Portugal	2010	100, 141, 165, 309, 579, 594		BM (CLSI)	4	8 - 32	[74]
		<i>E. faecalis</i>	2	Food chain (organic foods)	Spain				BM	5		[63]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Biguanides	Chlorhexidine	<i>E. faecalis</i>	6	Environment (hospital sewage)	Portugal	2001-2002	6, 16, 21, 35, 49, 206		BM (CLSI)	2 - 8	4 - 16	[74]
		<i>E. faecalis</i>	34	Environment (hospital sewage)	Iran	2018-2020			AD (CLSI)	0.5 - 8		[43]
		<i>E. faecalis</i>	4	Environment (urban wastewater treatment plant)	Tunisia	2014-2015	4, 23, 86, 117		BM (CLSI)	4 - 8	16	[74]
		<i>E. faecalis</i>	21	Environment (municipal sewage)	Iran	2018-2020			AD (CLSI)	2 - 8		[43]
		<i>E. faecalis</i>	38	Environment (poultry sewage)	Iran	2018-2020			AD (CLSI)	0.5 - 8		[43]
		<i>E. faecalis</i>	29	Environment (livestock sewage)	Iran	2018-2020			AD (CLSI)	2 - 8		[43]
		<i>E. faecalis</i>	2	Environment (river)	Portugal	2003	1, 4		BM (CLSI)	4	16	[74]
		<i>E. faecalis</i>	56		Spain	2001-2009			BM (CLSI)	8 - 64	32 - >64	[49]
		<i>E. faecalis</i>	2						AD	0.39 - 1.56		[76]
		<i>E. faecalis</i>	8						AD	4 - 64		[77]
		<i>E. faecalis</i>	11						AD	2 - 32		[78]
		<i>E. faecalis</i>	1						BM	3.9	14.3	[27]
		<i>E. faecalis</i> SS497	1						BM	3		[62]
		<i>E. faecalis</i>	1						BM	3,300		[79]
		<i>E. faecium</i> Aus0004	1	Human infection (blood)	Australia	1998			BM (EUCAST)	4	4	[50]
		<i>E. faecium</i>	12	Human infection (blood)	Denmark	2004	22, 95, 178, 296, 361, 372, 697, 1277, 1279		BM (CLSI)	1 - 4	1 - 5	[51]
		<i>E. faecium</i>	28	Human infection and colonization (patients without CHX bathing)	Brasil	2005-2013		<i>qacE</i> (3 isolates)	AD	1 - 32		[80]
		<i>E. faecium</i>	20	Human infection and colonization (patients with CHX bathing)	Brasil	2005-2013			AD	4 - 32		[80]
		<i>E. faecium</i>	37	Human infection (blood, urine, tissue)	Denmark	2012-2013	18, 80, 117, 203		BM (CLSI)	1 - 8	1 - 8	[51]
		<i>E. faecium</i>	17	Human infection	Iran	2018-2020			AD (CLSI)	4 - 16		[43]
		<i>E. faecium</i>	45	Human infection					AD	2 - 4		[81]
		<i>E. faecium</i>	8	Human infection					BM (CLSI)	<1 - 16		[68]
		<i>E. faecium</i>	5	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal			<i>qacZ</i>	BM (CLSI)	2 - 8		[37]
		<i>E. faecium</i>	5	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal				BM (CLSI)	4		[37]
		<i>E. faecium</i>	3	Human infection (blood)	Germany				BM (CLSI)	4 - 16		[41]
		<i>E. faecium</i>	90	Human infection (blood cultures, swabs, urine, faeces)	Germany				BM (Vacuum Dried Biocide Microtiter Plates)	0.5 - 4	2 - 16	[41]
		<i>E. faecium</i>	219	Human colonization (healthy humans)	Iran	2018-2020			AD (CLSI)	0.5 - 8		[43]
		<i>E. faecium</i>	37	Pet infection (dogs, cats)	Germany	2017-2019			BM	0.3 - 2.5		[44]
		<i>E. faecium</i>	4	Pet infection (dogs, cats)	Portugal				BM (CLSI)	4		[37]
		<i>E. faecium</i>	22	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	0.5 - 4		[36]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Biguanides	Chlorhexidine	<i>E. faecium</i>	78	Food chain (pigs, broilers and cattle faeces)	Denmark				BM (CLSI)	0.5 - 8		[45]
		<i>E. faecium</i> CF3 1.3	1	Food chain (ceca of an adult broiler chicken)					BM (CLSI)	1		[52]
		<i>E. faecium</i>	79	Food chain (farm animal's faeces)					BM	2.5 - 250		[53]
		<i>E. faecium</i>	1	Food chain (swine meat production chain)	Italy			<i>qacA/B, emeA</i>	BM (CLSI)	14		[46]
		<i>E. faecium</i>	8	Food chain (swine meat production chain)	Italy			<i>emeA</i>	BM (CLSI)	4 - 10		[46]
		<i>E. faecium</i>	3	Food chain (swine meat production chain)	Italy				BM (CLSI)	8 - 10		[46]
		<i>E. faecium</i>	3	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	4		[37]
		<i>E. faecium</i>	84	Food chain (traditional fermented foods)	Morocco, Spain, and Republic of Congo				BM (CLSI)	<0.1-2.5		[48]
		<i>E. faecium</i>	20	Food chain (fresh produce)	Spain		22, 26, 43, 46, 55, 94, 296		BM	75		[54]
		<i>E. faecium</i>	13	Food chain (organic foods)	Spain				BM	5		[63]
		<i>E. faecium</i>	63	Environment (hospital sewage)	Iran	2018-2020			AD (CLSI)	0.5 - 8		[43]
		<i>E. faecium</i>	42	Environment (municipal sewage)	Iran	2018-2020			AD (CLSI)	1 - 16		[43]
		<i>E. faecium</i>	50	Environment (community waste water)	USA				BM (CLSI)	0.5 - 2		[52]
		<i>E. faecium</i>	39	Environment (poultry sewage)	Iran	2018-2020			AD (CLSI)	0.5 - 8		[43]
		<i>E. faecium</i>	45	Environment (livestock sewage)	Iran	2018-2020			AD (CLSI)	0.5 - 8		[43]
		<i>E. faecium</i>	53			1986-2009			BM (CLSI)	2 - 32	4 - 64	[49]
		<i>E. gallinarum</i>	1	Human infection					BM (CLSI)	2		[68]
		<i>E. gallinarum</i>	2	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	1 - 2		[36]
		<i>E. hirae</i> ATCC 10541	1						BM	16		[40]
		<i>E. hirae</i>	2	Human infection (urinary, vagina, blood culture, pus, catheter, sputum)	Portugal			<i>qacZ</i>	BM (CLSI)	2 - 4		[37]
		<i>E. hirae</i>	39	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	0.5 - 4		[36]
		<i>E. hirae</i>	1	Food chain (milk, cheese)	Portugal			<i>qacZ</i>	BM (CLSI)	2		[37]
		<i>E. raffinosus</i>	2	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	0.5		[36]
		<i>E. saccharolyticus</i> MBRG 9.16	1	Environment (domestic drain microcosm)	UK				BM	7.8	62.5	[4]
		<i>E. solitarius</i>	1	Pet infection (dogs, cats)	Portugal				BM (CLSI)	4		[37]

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Table S1 (continued)- Distribution of cationic biocides MICs and MBCs for *Enterococcus* spp. with different epidemiological and genetic backgrounds.

Biocide class	Biocide	Species	N of isolates	Source	Country	Year	ST	Tolerance genes	Methodology (guidelines ¹)	MIC (mg/L)	MBC (mg/L)	Reference
Biguanides	Chlorhexidine	<i>Enterococcus</i> spp.	69	Human infection (blood, cerebrospinal fluid, urine, abscess, catheter tips, nasal secretions, endotracheal aspiration fluid)		2010-2011			AD (CLSI)	6 - 12		[56]
		<i>Enterococcus</i> spp.	11	Human infection (central line-associated bloodstream infection in patients with daily CHX bathing)	USA	2012-2013			BM	2 - 16	16 - >32	[82]
		<i>Enterococcus</i> spp.	19	Human infection (central line-associated bloodstream infection in patients without daily CHX bathing)	USA	2012-2013			BM	2 - 16	16 - >32	[82]
		<i>Enterococcus</i> spp.	59	Human infection					BM	2.5 - 2,500		[53]
		<i>Enterococcus</i> spp.	54	Human infection					BM	16 - 128		[57]
		<i>Enterococcus</i> spp.	18	Human colonization (skin of medical intensive care unit patients receiving CHX daily bathing)	USA	2007			BM	1 - 4		[83]
		<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)					BM	8 - 128		[57]
		<i>Enterococcus</i> spp.	25	Food chain (dust samples from breeding pig facilities)	Portugal	2008			BM (CLSI)	<0.25 - 4		[36]
		<i>Enterococcus</i> spp.	25	Food chain (seafood)					BM	2.5 - 2,500		[53]
		<i>Enterococcus</i> spp.	40	Food chain (meat and dairy products)					BM	2.5 - 250		[53]
		<i>Enterococcus</i> spp.	22	Food chain (vegetables)					BM	2.5 - 2,500		[53]
		<i>Enterococcus</i> spp.	6	Food chain (organic foods)	Spain				BM	5		[63]
		<i>Enterococcus</i> spp.	47	Others (wildflowers)					BM	25 - 2,500		[53]
		<i>Enterococcus</i> spp.	107						BM (CLSI)	1.56 - 12.5		[84]
		<i>Enterococcus</i> spp.	5						AD	4 - 6		[60]
		<i>Enterococcus</i> spp.	32						AD	1 - 32		[85]
	Polyhexamethylene biguanides	<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (DIN:58940-7, 58940-8)	2	16	[69]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM	8		[40]
		<i>E. faecalis</i> ATCC 29212	1	Human infection (urine)	USA		30		BM (DIN:58940-7, 58940-8)	2		[86]
		<i>E. faecalis</i>	1	Human infection					BM (DIN:58940-7, 58940-8)	4	32	[69]
		<i>E. faecalis</i> WIBG 1.1	1	Human infection (wound)					BM	1.8	7.3	[64]
		<i>E. faecalis</i>	49	Pet infection (dogs, cats)	Germany	2017-2019			BM	1.25 - 80		[44]
		<i>E. faecalis</i>	1						BM	7.8	7.8	[27]
		<i>E. faecium</i>	37	Pet infection (dogs, cats)	Germany	2017-2019			BM	0.3 - 5		[44]
		<i>E. faecium</i>	20	Food chain (fresh produce)	Spain		22, 26, 43, 46, 55, 94, 296		BM	8 - 60	-	[54]
		<i>E. hirne</i> ATCC 10541	1						BM	21		[40]
		<i>E. saccharolyticus</i> MBRG 9.16	1	Environment (domestic drain microcosm)	UK				BM	31.2	166	[4]

1 - Guidelines followed in each study were included when available.

2 - Median MIC or MBC.

Abbreviations: AD, agar dilution; ATCC, American Type Culture Collection; BC, benzalkonium chloride; BM, broth microdilution; CE, cetrimide; CLSI, Clinical & Laboratory Standards Institute; CHX, chlorhexidine; CPC, cetylpyridinium chloride; DDAC, didecyltrimethylammonium chloride; DIN, *Deutsches Institut für Normung*; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MD, microdilution; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; N, number; PHMB, polyhexamethylene biguanides; ST, sequence type; UK, United Kingdom; USA, United States of America.

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Table S2 - Effects of *in vitro* Serial Exposure to Subinhibitory Concentrations of Cationic Biocides in *Enterococcus* spp.

Biocide class	Biocide	Species	N of isolates	Source	Exposure (N of passages when available)	Initial MIC (mg/L)	Final MIC (mg/L)	Fold MIC increase	Stability in biocide-free medium	MBC changes	Increased biocide tolerance phenotypes	Increased antibiotic resistance phenotypes ¹	Other associated changes	Reference
Quaternary Ammonium Compounds	Benzalkonium chloride	<i>E. casseliflavus</i>	2	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.1 - 0.2	2	10 - 20	Not stable for 20 subcultures		MIC: CPC (10 fold), CE (2 fold), CHX (100 - >100 fold), TC (100 - >100 fold), CF (>100 fold), DDAB (2 - 10 fold)	AMP		[38]
		<i>E. durans</i>	1	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.5	2	4	Not stable for 20 subcultures		MIC: CPC (10 fold), CE (5 fold), CHX (100 fold), TC (>100 fold), CF (>100 fold), DDAB (10 fold)	AMP		[38]
		<i>E. faecalis</i>	2	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.05 - 0.5	2.5	5 - 50	Not stable for 20 subcultures		MIC: CPC (10 - 50 fold), CHX (10 - 100 fold), TC (20 - 100 fold), CF (20 - >100 fold), DDAB (20 - 40 fold)			[38]
		<i>E. faecalis</i>	1		Passages with 100-fold concentration gradient (14) on Tryptone Soy Agar	2	7.8	4	Partially reversed after 14 subcultures (3.9 mg/L)	MBC increased from 3.9 mg/L to 7.8 mg/L and was stable for 14 biocide-free subcultures				[27]
		<i>E. faecium</i>	13	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.1 - 0.5	0.7 - 7	4 - 50	Not stable for 20 subcultures		MIC: CPC (5 - >100 fold), CE (2 - 20 fold), CHX (10 - 200 fold), TC (40 - >100 fold), CF (10 - >100 fold), DDAB (2 - 20 fold)	AMP, CIP, TE		[38]
		<i>Enterococcus</i> spp.	6	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.1 - 0.5	1 - 7	4 - 35	Not stable for 20 subcultures		MIC: CPC (20 - 100 fold), CE (2 - >100 fold), CHX (40 - 100 fold), TC (50 - 100 fold), CF (10 - >100 fold), DDAB (10 fold)	AMP		[38]
Cetylpyridinium chloride		<i>E. faecalis</i> SS497	1		Passages with a concentration gradient (10) in Brain Heart Infusion Broth	2	2	-	-				Increased surface hydrophobicity	[62]
		<i>Enterococcus</i> spp.	4	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	5 - 50	100 - >500	2 - >100	Stable for 20 subcultures for 1 strain		MIC: BC (>100 fold), CE (5 - 10 fold), CHX (10 - >100 fold), TC (5 - 100 fold), CF (10 - >100 fold), DDAB (2 - 10 fold)	AMP		[38]
Cetrimide		<i>E. casseliflavus</i>	2	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	5 - 10	200 - >1,000	20 - >100	Stable for 20 subcultures for 1 strain		MIC: BC (>100 fold), CPC (20 - >100 fold), CHX (40 - 100 fold), TC (10 fold), CF (>100 fold), DDAB (10 - 40 fold)	AMP		[63]
		<i>E. faecalis</i> WIBG 1.1	1	Human infection (wound)	Passages with 100-fold concentration gradient (10) on Mueller-Hinton Agar	12.1	14.5	1.2	Stable for 10 subcultures	MBC increased from 29 mg/L to 38.7 mg/L and continuously increased after 10 biocide-free subcultures (58 mg/L)				[64]
		<i>E. faecalis</i>	2	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.5 - 5	2 - 200	4 - 40	Not stable for 20 subcultures		MIC: BC (>100 fold), CPC (10 - >100 fold), CHX (100 fold), TC (2 fold), CF (100 - >100 fold), DDAB (10 - 20 fold)	AMP, TE		[63]

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Table S2 (continued) - Effects of *in vitro* Serial Exposure to Subinhibitory Concentrations of Cationic Biocides in *Enterococcus* spp.

Biocide class	Biocide	Species	N of isolates	Source	Exposure (N of passages when available)	Initial MIC (mg/L)	Final MIC (mg/L)	Fold MIC increase	Stability in biocide-free medium	MBC changes	Increased biocide tolerance phenotypes	Increased antibiotic resistance phenotypes ^a	Other associated changes	Reference
Quaternary Ammonium Compounds	Cetrimide	<i>E. faecium</i>	7	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.5 - 10	>50 - 100	10 - >100	Not stable for 20 subcultures		MIC: BC (4 - >100 fold), CPC (40 - >100 fold), CHX (40 - >100 fold), TC (10 - 100 fold), CF (100 - >100 fold), DDAB (100 fold)	AMP		[63]
		<i>Enterococcus</i> spp.	5	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	0.1 - 10	10 - 500	2 - >100	Stable for 20 subcultures for 1 strain		MIC: BC (100 - >100 fold), CPC (10 - >100 fold), CHX (40 - 100 fold), TC (2 - 10 fold), CF (100 - >100 fold), DDAB (10 - 40 fold)	AMP, TE		[63]
	Didecyltrimethylammonium chloride	<i>E. faecalis</i>	4	Human	Passages with increasing concentrations (70) in Tryptone Soya Broth	0.7 - 2.7	21.9	8 - 31						[66]
		<i>E. faecalis</i>	1		Passages with 100-fold concentration gradient (14) on Tryptone Soy Agar	1	2	2	Stable for 14 subcultures	MBC increased from 1 mg/L to 2 mg/L and was stable for 14 biocide-free subcultures				[27]
Biguanides	Chlorhexidine	<i>E. casseliflavus</i>	3	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	5	40 - 100	8 - 20	Stable for 20 subcultures for 1 strain		MIC: BC (30 - >100 fold), CPC (6 - 40 fold), CE (6 - 60 fold), TC (>100 fold), CF (20 - >30 fold), DDAB (2 - 6 fold)	IPM, TE		[63]
		<i>E. durans</i>	1	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	5	50	10	Not stable for 20 subcultures		MIC: BC (>100 fold), CPC (20 fold), CE (>100 fold), TC (10 fold), CF (8 fold), DDAB (16 fold)	AMP, IPM		[63]
		<i>E. faecalis</i> ATCC 4083	1		Passages with a concentration gradient (10) in Brain Heart Infusion Broth	3.9	15.6	4	-				-	[72]
		<i>E. faecalis</i> WIBG 1.1	1	Human infection (wound)	Passages with 100-fold concentration gradient (10) on Mueller-Hinton Agar	3.6	24.2	7	Reversed after 10 subcultures	MBC increased from 26.6 mg/L to 58 mg/L and partially reversed after 10 biocide-free subcultures (29 mg/L)				[64]
		<i>E. faecalis</i>	1	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	5	50	10	Not stable for 20 subcultures		MIC: BC (80 fold), CPC (16 fold), CE (>100 fold), TC (2 fold), CF (30 fold), DDAB (8 fold)	IPM		[63]
		<i>E. faecalis</i> SS497	1		Passages with a concentration gradient (10) in Brain Heart Infusion Broth	3	11	4	-				Increased surface hydrophobicity	[62]
		<i>E. faecalis</i>	1		Passages with 100-fold concentration gradient (14) on Tryptone Soy Agar	3.9	7.8	2	Continuously increased after 14 subcultures (15.6 mg/L)	MBC increased from 14.3 mg/L to 31.3 mg/L and was stable for 14 biocide-free subcultures				[27]
		<i>E. faecium</i>	9	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	5	10 - 80	2 - 16	Stable for 20 subcultures for 1 strain		MIC: BC (10 - >100 fold), CPC (5 - 30 fold), CE (4 - >100 fold), TC (2 - >100 fold), CF (4 - 30 fold), DDAB (4 - 8 fold)	AMP, IPM, TE		[63]

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Table S2 (continued) - Effects of *in vitro* Serial Exposure to Subinhibitory Concentrations of Cationic Biocides in *Enterococcus* spp.

Biocide class	Biocide	Species	N of isolates	Source	Exposure (N of passages when available)	Initial MIC (mg/L)	Final MIC (mg/L)	Fold MIC increase	Stability in biocide-free medium	MBC changes	Increased biocide tolerance phenotypes	Increased antibiotic resistance phenotypes	Other associated changes	Reference
Biguanides	Chlorhexidine	<i>E. saccharolyticus</i> MBRG 9.16	1	Environment (domestic drain microcosm, UK)	Passages with 100-fold concentration gradient (14) on R2A agar	7.8	1.9	-	-	MBC decreased from 62.5 mg/L to 15.6 mg/L				[4]
		<i>Enterococcus</i> spp.	6	Food chain (organic foods, Spain)	Passages with increasing concentrations in Trypticase Soya Broth	5	10 - 50	2 - 10	Not stable for 20 subcultures		MIC: BC (30 - >100 fold), CPC (5 - 20 fold), CE (6 - 60 fold), TC (3 - 15 fold), CF (5 - 30 fold), DDAB (2 - 6 fold)	IPM, TE		[63]
Polyhexamethylene biguanides		<i>E. faecalis</i> WIBG 1.1	1	Human infection (wound)	Passages with 100-fold concentration gradient (10) on Mueller-Hinton Agar	1.8	14.5	7	Partially reversed after 10 subcultures (9.7 mg/L)	MBC increased from 7.3 mg/L to 29 mg/L and reversed after 10 biocide-free subcultures				[64]
		<i>E. faecalis</i>	1		Passages with 100-fold concentration gradient (14) on Tryptone Soy Agar	7.8	31.3	4	Partially reversed after 14 subcultures (15.6 mg/L)	MBC increased from 7.8 mg/L to 125 mg/L and partially reversed after 14 biocide-free subcultures (15.6 mg/L)				[27]
		<i>E. saccharolyticus</i> MBRG 9.16	1	Environment (domestic drain microcosm, UK)	Passages with 100-fold concentration gradient (14) on R2A agar	31.2	20.8	-	-	MBC increased from 166 mg/L to 208 mg/L				[4]

1 - Among the antibiotics tested in each study that *Enterococcus* spp. are not intrinsically resistant to.

Abbreviations: AMP, ampicillin; ATCC, American Type Culture Collection; BC, benzalkonium chloride; CE, cetrinide; CF, hexachlorophene [2,2,0-methylenebis(3,4,6-trichlorophenol)]; CHX, chlorhexidine; CIP, ciprofloxacin; CPC, cetylpyridinium chloride; DDAB, didecyldimethylammonium bromide; DDAC, didecyldimethylammonium chloride; IPM, imipenem; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; N, number; PHMB, polyhexamethylene biguanides; TC, triclosan; TE, tetracycline; UK, United Kingdom.

Table S3 - Prevalence of cationic biocides tolerance genes among collections of *Enterococcus* spp. with different epidemiological backgrounds.

Gene	Species	N of isolates	Source	Country	Year	Percentage of gene-carrying isolates (N of positive isolates)	Reference
<i>qacA/B</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	16	Human infection (pediatric bloodstream)	USA	2016-2017	43.8 (7)	[118]
	<i>E. faecalis</i>	222	Human infection, Human colonization (healthy humans), Environment (hospital, municipal, livestock and poultry wastewater)	Iran	2018-2020	19.4 (43)	[43]
	<i>E. faecalis</i>	585	Human infection, Human colonization (hospitalized patients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0.34 (2)	[65]
	<i>E. faecalis</i>	39	Human infection, Human colonization (hospitalized patients and outpatients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		5.12 (2, same isolates previously reported by Bischoff, 2012)	[66]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	9	Food chain (swine meat production chain)	Italy		0 (0)	[46]
	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	0 (0)	[170]
	<i>E. faecium</i>	48	Human infection, Human colonization	Brazil	2005-2009	0 (0)	[80]
	<i>E. faecium</i>	3	Human infection (pediatric bloodstream)	USA	2016-2017	0 (0)	[118]
	<i>E. faecium</i>	425	Human infection, Human colonization (healthy humans' faeces), Environment (hospital, municipal livestock and poultry wastewater)	Iran	2018-2021	10.3 (44)	[43]
	<i>E. faecium</i>	7	Human infection, Human colonization (hospitalized patients and outpatients' faeces)	Germany		0 (0)	[66]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecium</i>	12	Food chain (swine meat production chain)	Italy		8.3 (1)	[46]
	<i>Enterococcus</i> spp.	918	Human infection, Human colonization (healthy humans' faeces), Food chain (piggeries, aquaculture, animal/vegetable food products), Environment (hospital sewage, river)	Portugal	1996–2012	0 (0)	[55]
	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0 (0)	[42]
	<i>Enterococcus</i> spp.	69	Human infection		2010-2011	0 (0)	[56]
	<i>Enterococcus</i> spp.	54	Human infection			0 (0)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			0 (0)	[57]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]
<i>qacC</i> (<i>smr</i> , <i>abr</i> , <i>qacD</i>)	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	16	Human infection (pediatric bloodstream)	USA	2016-2017	25 (4)	[118]
	<i>E. faecalis</i>	585	Human infection, Human colonization (hospitalized patients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0.34 (2)	[65]
	<i>E. faecalis</i>	39	Human infection, Human colonization (hospitalized patients and outpatients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		5.12 (2, same isolates previously reported by Bischoff, 2012)	[66]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[280]
	<i>E. faecalis</i>	9	Food chain (swine meat production chain)	Italy		0 (0)	[46]

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Table S3 (continued) - Prevalence of cationic biocides tolerance genes among collections of *Enterococcus* spp. with different epidemiological backgrounds.

Gene	Species	N of isolates	Source	Country	Year	Percentage of gene-carrying isolates (N of positive isolates)	Reference
<i>qacC</i> (<i>smr</i> , <i>abr</i> , <i>qacD</i>)	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	0 (0)	[270]
	<i>E. faecium</i>	3	Human infection (pediatric bloodstream)	USA	2016-2017	0 (0)	[118]
	<i>E. faecium</i>	7	Human infection, Human colonization (hospitalized patients and outpatients' faeces)	Germany		0 (0)	[66]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecium</i>	12	Food chain (swine meat production chain)	Italy		0 (0)	[46]
	<i>Enterococcus</i> spp.	918	Human infection, Human colonization (healthy humans' faeces), Food chain (piggeries, aquaculture, animal/vegetable food products), Environment (hospital sewage, river)	Portugal	1996–2012	0 (0)	[55]
	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0 (0)	[42]
	<i>Enterococcus</i> spp.	69	Human infection		2010-2011	0 (0)	[56]
	<i>Enterococcus</i> spp.	54	Human infection			0 (0)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			0 (0)	[57]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]
<i>qacE</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	45	Human infection	Japan	1996	0 (0)	[181]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	9	Food chain (swine meat production chain)	Italy		0 (0)	[46]
	<i>E. faecium</i>	48	Human infection, Human colonization	Brazil	2005-2009	10.7 (3)	[80]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecium</i>	12	Food chain (swine meat production chain)	Italy		0 (0)	[46]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]
<i>qacEΔ1</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	45	Human infection	Japan	1996	20 (9)	[181]
	<i>E. faecalis</i>	222	Human infection, Human colonization (healthy humans' faeces), Environment (hospital, municipal livestock and poultry wastewater)	Iran	2018-2021	19.8 (44)	[43]
	<i>E. faecalis</i>	585	Human infection, Human colonization (hospitalized patients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[65]
	<i>E. faecalis</i>	39	Human infection, Human colonization (hospitalized patients and outpatients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[66]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	9	Food chain (swine meat production chain)	Italy		0 (0)	[46]
	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	0 (0)	[170]
	<i>E. faecium</i>	425	Human infection, Human colonization (healthy humans' faeces), Environment (hospital, municipal livestock and poultry wastewater)	Iran	2018-2021	17.2 (73)	[43]
	<i>E. faecium</i>	7	Human infection, Human colonization (hospitalized patients and outpatients' faeces)	Germany		0 (0)	[66]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[280]
	<i>E. faecium</i>	12	Food chain (swine meat production chain)	Italy		0 (0)	[46]

Table S3 (continued) - Prevalence of cationic biocides tolerance genes among collections of *Enterococcus* spp. with different epidemiological backgrounds.

Gene	Species	N of isolates	Source	Country	Year	Percentage of gene-carrying isolates (N of positive isolates)	Reference
<i>qacEA1</i>	<i>Enterococcus</i> spp.	918	Human infection, Human colonization (healthy humans' faeces), Food chain (piggeries, aquaculture, animal/vegetable food products), Environment (hospital sewage, river)	Portugal	1996–2012	0 (0)	[55]
	<i>Enterococcus</i> spp.	54	Human infection			0 (0)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			0 (0)	[57]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]
<i>qacG</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	585	Human infection, Human colonization (hospitalized patients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[65]
	<i>E. faecalis</i>	39	Human infection, Human colonization (hospitalized patients and outpatients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[66]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	0 (0)	[170]
	<i>E. faecium</i>	7	Human infection, Human colonization (hospitalized patients and outpatients' faeces)	Germany		0 (0)	[66]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[180]
	<i>Enterococcus</i> spp.	918	Human infection, Human colonization (healthy humans' faeces), Food chain (piggeries, aquaculture, animal/vegetable food products), Environment (hospital sewage, river)	Portugal	1996–2012	0 (0)	[55]
	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0 (0)	[42]
	<i>Enterococcus</i> spp.	69	Human infection		2010-2011	0 (0)	[56]
	<i>Enterococcus</i> spp.	54	Human infection			0 (0)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			0 (0)	[57]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]
<i>qacH</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	585	Human infection, Human colonization (hospitalized patients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[65]
	<i>E. faecalis</i>	39	Human infection, Human colonization (hospitalized patients and outpatients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[66]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecium</i>	7	Human infection, Human colonization (hospitalized patients and outpatients' faeces)	Germany		0 (0)	[66]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[180]
	<i>Enterococcus</i> spp.	918	Human infection, Human colonization (healthy humans' faeces), Food chain (piggeries, aquaculture, animal/vegetable food products), Environment (hospital sewage, river)	Portugal	1996–2012	0 (0)	[55]
	<i>Enterococcus</i> spp.	69	Human infection		2010-2011	0 (0)	[56]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]

Table S3 (continued) - Prevalence of cationic biocides tolerance genes among collections of *Enterococcus* spp. with different epidemiological backgrounds.

Gene	Species	N of isolates	Source	Country	Year	Percentage of gene-carrying isolates (N of positive isolates)	Reference
<i>qacJ</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	585	Human infection, Human colonization (hospitalized patients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[65]
	<i>E. faecalis</i>	39	Human infection, Human colonization (hospitalized patients and outpatients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[66]
	<i>E. faecalis</i>	8	Food chain (raw processed beef meat)	South Africa	2015-2016	12.5 (1)	[182]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	0 (0)	[170]
	<i>E. faecium</i>	7	Human infection, Human colonization (hospitalized patients and outpatients' faeces)	Germany		0 (0)	[66]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[180]
	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0 (0)	[42]
	<i>Enterococcus</i> spp.	69	Human infection		2010-2011	0 (0)	[56]
	<i>Enterococcus</i> spp.	54	Human infection			0 (0)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			0 (0)	[57]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]
<i>qacZ</i>	<i>E. faecalis</i>	39	Human infection, Human colonization (hospitalized patients and outpatients' faeces), Food chain (milk and dairy products, meat products, faeces of farm animals)	Germany		0 (0)	[66]
	<i>E. faecalis</i>	8	Food chain (raw processed beef meat)	South Africa	2015-2016	12.5 (1)	[182]
	<i>E. faecium</i>	73	Human infection, Human colonization (hospitalized patients and outpatients' faeces)	Germany		0 (0)	[66]
	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	2.7 (1, same isolate previously reported by Silveira 2015)	[170]
	<i>Enterococcus</i> spp.	918	Human infection, Human colonization (healthy humans' faeces), Food chain (piggeries, aquaculture, animal/vegetable food products), Environment (hospital sewage, river)	Portugal	1996–2012	0.11 (1)	[55]
	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0.48 (1, same isolate previously reported by Silveira 2015)	[42]
	<i>Enterococcus</i> spp.	54	Human infection			0 (0)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			0 (0)	[57]
	<i>Enterococcus</i> spp.	73	Human infection (urinary, vagina, blood culture, pus, catheter and sputum), Pet infection (dogs and cats), Food chain (milk and cheese)			52 (38)	[37]
	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	0 (0)	[170]
<i>qrg</i>	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	0 (0)	[170]

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Table S3 (continued) - Prevalence of cationic biocides tolerance genes among collections of *Enterococcus* spp. with different epidemiological backgrounds.

Gene	Species	N of isolates	Source	Country	Year	Percentage of gene-carrying isolates (N of positive isolates)	Reference
<i>qrg</i>	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0 (0)	[42]
	<i>Enterococcus</i> spp.	918	Human infection, Human colonization (healthy humans' faeces), Food chain (piggeries, aquaculture, animal/vegetable food products), Environment (hospital sewage, river)	Portugal	1996–2012	0 (0)	[55]
<i>bcrABC</i>	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0 (0)	[42]
<i>oqxAB</i> ¹	<i>E. faecalis</i>	51	Food chain (swine manure)	China	2010-2012	86.3 (44) <i>oqxA</i> + 70.6 (36) <i>oqxB</i> +	[183]
	<i>E. faecium</i>	36	Food chain (swine manure)	China	2010-2012	69.4 (25) <i>oqxA</i> + 58.3 (21) <i>oqxB</i> +	[183]
	<i>E. faecium</i>	120	Human infection (urine)	China	2017-2018	1.7 (2) <i>oqxA</i> + 0 (0) <i>oqxB</i> +	[184]
	<i>Enterococcus</i> spp.	210	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pets, Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, USA	1996–2020	0 (0)	[42]
	<i>Enterococcus</i> spp.	30	Human infection			0 (0)	[185]
	<i>Enterococcus</i> spp.	54	Human infection			0 (0)	[57]
	<i>Enterococcus</i> spp.	7	Human colonization (healthy humans' faeces)			0 (0)	[186]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			0 (0)	[57]
<i>emeA</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0)	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	38	Human infection	China	2010-2012	26.3 (10)	[187]
	<i>E. faecalis</i>	26	Human infection	China	2011-2015	100 (26)	[188]
	<i>E. faecalis</i>	222	Human infection, Human colonization (healthy humans' faeces), Environment (hospital, municipal livestock and poultry wastewater)	Iran	2018-2021	42.8 (95)	[43]
	<i>E. faecalis</i>	78	Human infection (blood, urine), Human colonization (faeces, oral cavity), Food chain (dairy products), Water, Others			100 (78)	[189]
	<i>E. faecalis</i>	22	Human infection (urinary tract)	Iran		100 (22)	[190]
	<i>E. faecalis</i>	42	Human, Animal, Food chain, Environment			100 (42)	[191]
	<i>E. faecalis</i>	369	Food chain (healthy chickens and pigs faeces)	Taiwan	2006-2007	100 (369)	[192]
	<i>E. faecalis</i>	47	Food chain (commercial beef processing facility and retail ground beef)	Canada	2014-2016	100 (47)	[193]
	<i>E. faecalis</i>	28	Food chain (retail chicken meat)	South Korea	2016-2017	100 (28)	[194]
	<i>E. faecium</i>	93	Food chain (retail meat)	South Korea	2016-2018	100 (93)	[195]
	<i>E. faecalis</i>	2	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecalis</i>	9	Food chain (swine meat production chain)	Italy		55.6 (5)	[46]
	<i>E. faecalis</i>	42	Human, Animal, Food chain, Environment			100 (42)	[191]
	<i>E. faecalis</i>	8	Food chain (raw processed beef meat)	South Africa	2015-2016	100 (8)	[182]
	<i>E. faecium</i>	62	Human infection	China	2010-2013	72.6 (45)	[187]
	<i>E. faecium</i>	120	Human infection (urine)	China	2017-2018	10 (12)	[184]
	<i>E. faecium</i>	88	Human infection	Iran	2017-2018	54.5 (48)	[196]
	<i>E. faecium</i>	425	Human infection, Human colonization (healthy humans' faeces), Environment (hospital, municipal livestock and poultry wastewater)	Iran	2018-2021	27.8 (118)	[43]
	<i>E. faecium</i>	1	Food chain (retail chicken meat)	South Korea	2016-2018	0 (0)	[194]
	<i>E. faecium</i>	13	Food chain (organic foods)			0 (0)	[180]
	<i>E. faecium</i>	12	Food chain (swine meat production chain)	Italy		66.7 (8)	[46]

Table S3 (continued) - Prevalence of cationic biocides tolerance genes among collections of *Enterococcus* spp. with different epidemiological backgrounds.

Gene	Species	N of isolates	Source	Country	Year	Percentage of gene-carrying isolates (N of positive isolates)	Reference
<i>emeA</i>	<i>Enterococcus</i> spp.	54	Human infection			57.4 (31)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			32 (16)	[57]
	<i>Enterococcus</i> spp.	19	Chicken litter and soil samples	South Africa	2018-2019	21 (4)	[197]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			0 (0)	[180]
<i>efrAB</i>	<i>E. casseliflavus</i>	3	Food chain (organic foods)			0 (0) <i>efrA</i> + 33.3 (1) <i>efrB</i> +	[180]
	<i>E. durans</i>	1	Food chain (organic foods)			0 (0) <i>efrA</i> + 100 (1) <i>efrB</i> +	[180]
	<i>E. faecalis</i>	80	Human infection (outpatients' urinary tract infections)	Iran	2014-2015	100 (80)	[198]
	<i>E. faecalis</i>	22	Human infection (urinary tract)	Iran		100 (22)	[190]
	<i>E. faecalis</i>	78	Human infection (blood, urine), Human colonization (faeces, oral cavity), Food chain (dairy products), Water, Others			100 (78)	[189]
	<i>E. faecalis</i>	42	Human, Animal, Food chain, Environment			100 (42)	[191]
	<i>E. faecalis</i>	47	Food chain (commercial beef processing facility and retail ground beef)	Canada	2014-2016	100 (47)	[193]
	<i>E. faecalis</i>	8	Food chain (raw processed beef meat)	South Africa	2015-2016	100 (8)	[182]
	<i>E. faecalis</i>	26	Food chain (traditional fermented foodstuffs of both animal and vegetable origins), Water	Morocco, Spain, Republic of Congo		96 (25)	[199]
	<i>E. faecalis</i>	8	Food chain (traditional fermented foods)	Morocco, Spain, Republic of Congo		100 (8)	[48]
	<i>E. faecalis</i>	2	Food chain (organic foods)			50 (1) <i>efrAB</i> + 50 (1) <i>efrB</i> +	[180]
	<i>E. faecium</i>	120	Human infection (urine)	China	2017-2018	10 (12) <i>efrA</i> + 15.8 (19) <i>efrB</i> +	[184]
	<i>E. faecium</i>	88	Human infection (blood, urine, wound, tracheal, fluids, sonde urinaire, sputum, fistula)	Iran	2017-2018	51 (45)	[196]
	<i>E. faecium</i>	30	Food chain (traditional fermented foodstuffs of both animal and vegetable origins), Water	Morocco, Spain, Republic of Congo		13 (4)	[199]
	<i>E. faecium</i>	49	Food chain (traditional fermented foods)	Morocco, Spain, Republic of Congo		12 (6)	[48]
	<i>E. faecium</i>	13	Food chain (organic foods)			30.8 (4) <i>efrAB</i> + 7.7 (1) <i>efrB</i> +	[180]
	<i>Enterococcus</i> spp.	54	Human infection			66.7 (36)	[57]
	<i>Enterococcus</i> spp.	50	Human colonization (healthy humans' faeces)			40 (20)	[57]
	<i>Enterococcus</i> spp.	6	Food chain (organic foods)			16.7 (1) <i>efrAB</i> + 33.3 (2) <i>efrB</i> +	[180]
<i>chlR-efrEF</i>	<i>E. faecalis</i>	666	Human infection, Human colonization, Food chain (food-animal production settings, meat of animal origin, and other food products), Pet faeces, Aquatic environment	Portugal, Tunisia, Angola, and Brazil	1996-2020	99.8 (665)	[74]
	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	100 (37)	[170]
<i>chtRS</i>	<i>E. faecium</i>	37	Human infection, Human colonization, Food chain (food-animal production settings and food of animal origin), Wild birds, Aquatic environment	Portugal, Tunisia, Angola, Spain	1998-2016	100 (37) ChtR-P102H mutation in 70 (26)	[170]
	<i>E. faecium</i>	8	Human infection (bloodstream), Human colonization (faeces of non-hospitalized individuals)			100 (8) ChtR-P102H mutation in 37.5 (3)	[171]

1 - Whilst the nucleotide sequence of the *oqxA* gene is highly conserved, the sequence of *oqxB* is variable and its detection by PCR may be compromised by point mutations in primer-binding sites [183].

Abbreviations: N, number; USA, United States of America.

2. Multidrug-resistant high-risk *Enterococcus faecium* clones: can we really define them?

Multidrug-resistant high-risk *Enterococcus faecium* clones: can we really define them?

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Review

Multidrug-resistant high-risk *Enterococcus faecium* clones: can we really define them?Ana R. Freitas^{1,2}, Ana P. Pereira¹, Carla Novais^{1,2}, Luísa Peixe^{1,2,*}

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ABSTRACT

Enterococcus faecium is a significant opportunistic human pathogen with a broad host range, including humans, farm animals, pets and wildlife. Specialised subpopulations have globally evolved towards a powerful and convergent adaption to the healthcare environment by acquiring a cocktail of key antimicrobial resistance and virulence genes, enabling them to thrive in the disturbed microbiota of hospitalised patients. These populations can also be found in different community reservoirs, but the relevance of their dispersal in non-human hosts is greatly unknown and is here discussed. This review provides a brief historical overview of what we have been considering *E. faecium* high-risk clones worldwide alongside the advances in strain typing technologies that have revolutionised our understanding of the genetic evolution of this species over the last three decades.

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1. Introduction

Enterococci are recognised for their extraordinary adaptive behaviour to a wide range of environmental conditions and their ubiquitous distribution in nature [1]. The gastrointestinal tract (GIT) of nearly all terrestrial animals is the primary habitat for enterococci, which represent ~1% of the human microbiota, with *Enterococcus faecalis* (*Efs*) and *Enterococcus faecium* (*Efm*) corresponding to the main species colonising the human gut in similar abundance [2]. The functional role of enterococci is not fully understood, but in vivo experiments suggest that they may contribute to colonisation resistance against pathogen invasion [3]. The great dilemma is that enterococci, and especially *Efm*, are also intrinsically resistant to many antimicrobials and are particularly adept at acquiring new antimicrobial resistance (AMR) genetic elements, which are major drivers for *Efm* selection and propagation in environments with high selective pressure, such as hospitals [4,5].

Efm is a frequent cause of life-threatening infections (e.g. urinary tract infections, wound infections, septicaemia and endocarditis), which are especially problematic among patients

receiving long-course antimicrobial treatments, critically ill/ immunosuppressed patients and/or those with prolonged hospital stays, with an increase of the *Efm/Efs* infection ratio over the years (Fig. 1) [1]. *Efm* is now known to significantly dominate the gut microbiota in long-stay hospitalised patients, with multidrug-resistant (MDR) *Efm* quickly replacing susceptible populations before invasion of the bloodstream, a feat apparently not so prominent in *Efs* [11]. MDR-*Efm* human infections, and especially vancomycin-resistant *Efm* (VRE*fm*) infections, are indeed a major challenge to clinicians given the limited therapeutic options [12,13]. Rates of VRE*fm* are highly variable between different areas (<1–86.7%), with ongoing increasing trends in several countries [13–15]. Linezolid, daptomycin and tigecycline remain valid alternatives to treat severe infections caused by MDR-*Efm*, with general resistance rates <1% [16]. The great adaptation and resilience of *Efm* in the clinical setting owing to the intensive use of antimicrobials has made this species a current member of the 'ESKAPE' group, and VRE*fm* are included in the World Health Organization's (WHO) global priority list of antibiotic-resistant bacteria [17].

Efm historically emerged into distinct clonal lineages (ecotypes) more adapted to the hospital and hospitalised patient ecosystems, which are now epidemic and endemic in many hospitals worldwide [4,18]. Similar to other bacterial species, they have often been designated as high-risk clones owing to their worldwide distribution, colonisation and persistence in humans for long periods of time, and their involvement in threatening and/or recurrent in-

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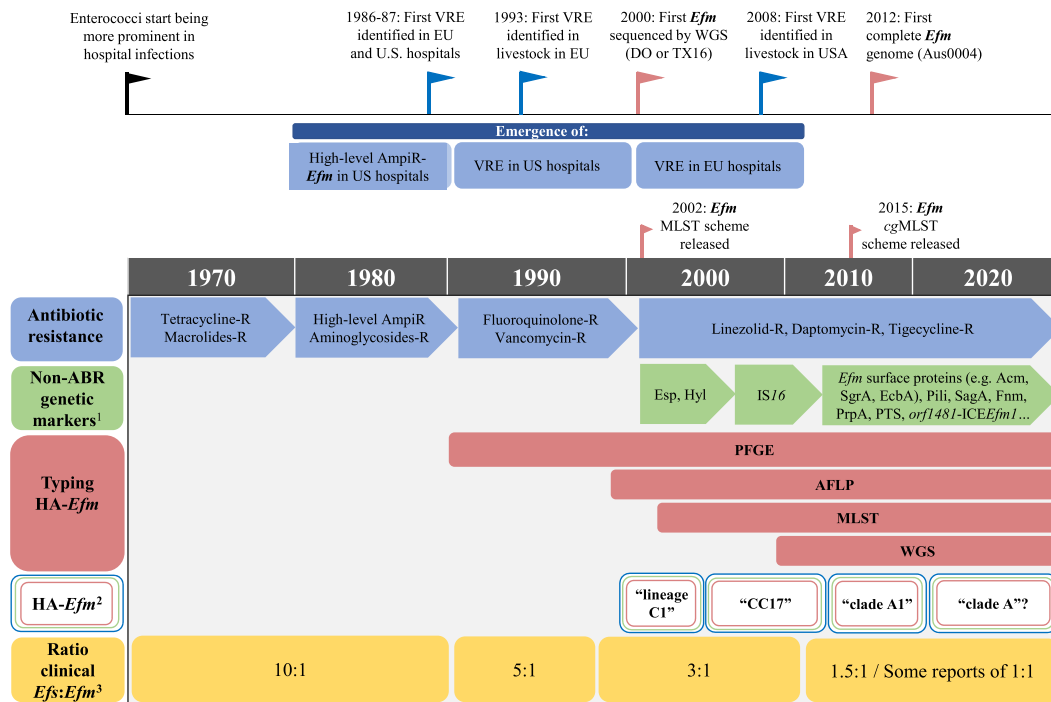


Fig. 1. Historical evolution of hospital-associated *Enterococcus faecium* (HA-Efm) clones and their features in parallel with the development of the main strain typing methods. ¹ Main genetic markers (mainly corresponding to virulence genes) greatly associated with global HA-Efm clones over time and putatively involved in adhesion/colonisation, biofilm formation and infection are represented according to the date of their description, but all remain current relevant markers. They are: *Efm* surface proteins or CWAPs (surface-exposed LPXTG cell wall-anchored proteins) including Esp (enterococcal surface protein located on a transferable pathogenicity island and enhancing initial cell adhesion, biofilm formation and pathogenesis in endocarditis), Acm (adhesin of collagen from *Efm* enhancing initial adhesion and with a role in endocarditis), Scm (second collagen adhesin from *Efm* that interacts with components of the extracellular matrix), SgrA (serine glutamate repeat-containing protein A, a nidogen-binding surface adhesin implicated in biofilm formation), EcbA (*Efm* collagen-binding protein A implicated in adhesion to components of the extracellular matrix), Hyl (glycosyl hydrolase that is likely to have β -N-acetylglucosaminidase activity but with an unelucidated role – the megaplasmids where it is located might be relevant in host colonisation or in metabolic processes), IS16 (conferring genomic plasticity), PrpA (peptidoglycan-anchored surface protein able to bind to fibrinogen, fibronectin and platelets), pili (filamentous protein surface structures contributing to primary cell attachment and infection), SagA (major secreted antigen A with broad-spectrum binding to extracellular matrix proteins, likely to play a critical role for cell growth, stress activation and cell wall metabolism – it has a specific repeat region only present in clade A1 strains; probably a good vaccine candidate), Fnm (fibronectin-binding protein of *Efm*), PTS (phosphotransferase systems, transmembrane proteins that phosphorylate specific carbohydrates – PtsD and BepA are PTS enriched in HA-Efm and possibly playing multiple roles during *Efm* adaption in hospitals and involved in stress responses, colonisation or biofilm formation) and *orf1481*, which is a good marker of ICE*Efm1*, an 8.5-kb genomic island involved in carbohydrate transport and metabolism [6,7]. ² HA-Efm classification has evolved throughout time according to the strain typing method used in each decade. We designated the current HA-Efm as interrogated 'clade A?' since the distinction between clade A1 and clade A2 is not confirmed in all genomic studies [3] and further works will better elucidate it. ³ The ratio of *Enterococcus faecalis* (*Efs*) to *E. faecium* (*Efm*) causing disease has greatly changed over the last decades, from 10:1 before 1990, to 5:1 during the 1990s, to 3:1 in the beginning 2000s and to 1.5:1 or even 1:1 in the actuality [8–10]. ABR, antibiotic resistance; AFLP, amplified fragment length polymorphism; AmpR, ampicillin-resistant; cgMLST, core genome multilocus sequence typing; EU, Europe; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; R, resistance; U.S., United States of America; VRE, vancomycin-resistant enterococci; WGS, whole-genome sequencing.

fections that are difficult to treat [19,20]. This review intends to gather the main features of *E. faecium* high-risk clones [from now on designated as hospital-associated *E. faecium* (HA-Efm) clones] that are continuously changing towards better survival, spreading and infecting patients at increasing rates. Their nomenclature and characteristics are revised according to the evolution of the different typing methods used over the last 30 years.

2. Historical perspective of hospital-associated *Enterococcus faecium* significance according to the growing knowledge of their genetic evolution and the development of high-resolution typing methods

Efm population structure studies were greatly motivated by the emergence of VRE*fm* in the early 1990s and continue to this day in parallel with the exponential rise of global MDR-VRE*fm* hospital

infections (Fig. 1) [8,10,15,19,20]. The development of genotyping methods to better cluster *Efm* populations, along with the increasing knowledge of *Efm* genetic evolution and sequentially recognition of specific MDR and virulence genes, led to the identification of specialised subpopulations whose structure is being continuously reshaped [4,19].

Pulsed-field gel electrophoresis (PFGE) was for a long time the gold-standard method for subtyping enterococci, with primary evidence of genetic identity between clinical isolates and isolates from farm animals and/or farmers occurring during the 1990–2000s [4]. The very first recognition of host-specific groups, including a subpopulation mainly associated with hospital outbreaks and infections worldwide ('lineage C1'), was obtained in 2000 by the systematic amplified fragment length polymorphism (AFLP) analysis of diverse VRE*fm* [21]. Five years later, this subpopulation was renamed 'clonal complex 17' (CC17) by multilocus sequence typ-

ing (MLST)/eBURST, originating from sequence type 17 (ST17) from which clinical isolates seemed to have evolved. It mostly included VREfm/vancomycin-susceptible Efm from hospitalised patients, expressing high levels of ampicillin and quinolone resistance and carrying *esp* and *hyl* genes [22]. At this time, the cumulative acquisition of different adaptive genes was considered pivotal in the specialisation of CC17. This occurred through a 'genetic capitalism' process (in which clones more fit in selective environments are more prone to acquire and accumulate novel adaptive traits) [23], favoured by the high recombination levels and horizontal gene transfer (HGT). In the following years, an incredible number of studies confirmed the worldwide dissemination of CC17-HA-Efm and added other virulence determinants (mostly surface proteins), mobile genetic elements (MGEs), phage genes and plasmid sequences to this specialised population [4]. However, placing all clinical Efm into a single CC was recognised as an oversimplification, with Bayesian Analysis of Population Structure (BAPS) delineating three major clonal lineages (ST17/ST18/ST78) from different ancestral strains that followed a convergent evolution in the acquisition of resistance and virulence traits [24].

The first genome ever sequenced was strain DO or TX16 (GenBank accession no. [GCA_000174395.2](https://www.ncbi.nlm.nih.gov/nuccore/GCA_000174395.2); https://www.ebi.ac.uk/ena/data/view/GCA_000174395.2) in 2000 [25], but the first analysis of the Efm genome content was only made available by van Schaik et al. 10 years later [26]. Subsequent robust whole-genome sequencing (WGS)-based studies proposed replacing 'CC17' by 'clade A1' based on genetic diversity between clades A (hospital-associated) and B (community-associated), and between clades A1 (mostly human epidemic/outbreaks scenarios) and A2 (mostly animal-associated) [20,27,28]. More recent studies also supported the phylogenetic distinction between clades A and B, although the ecological distinction between clades A1 and A2 was not always corroborated when larger collections were used [3,29,30]. Instead, HA-Efm appear to have emerged from an ancestor lineage linked to animals that diverged prior to clonal expansion of human clinical clades, with hospital-associated populations evolving faster than animal ones [29]. The high resolution, ease of use, and lower limitations of WGS compared with MLST (e.g. five MLST loci are in recombination hotspots; absence of the *ptsS* gene in several genomes) currently places WGS as the gold-standard technique to identify HA-Efm.

The first mention to a high-risk clone of Efm was likely made by Leavis et al. in 2006 [19] when referring to CC17 as a nosocomial clonal lineage that had spread globally and presented specific AMR and virulence traits [22]. Since then, numerous studies have used this terminology and variants of it with the same purpose, either applied to a clone or particular populations, such as 'high-risk populations', 'high-risk *E. faecium* genotypes', 'hospital adapted high-risk lineage', 'high-risk enterococcal clonal complexes (HiRECCs)', 'successful clones', 'hospital-adapted/acquired/associated clones' or 'hospital-adapted sub-population', but their use is decreasing since the universal attribution of specific features to such clones is difficult in this evolving topic and genetically dynamic species. Independently of its terminology, it is unquestionable that HA-Efm are enriched in a variety of adaptive traits enabling them to thrive in the harsh environment of hospitals and to better succeed during host colonisation and infection [3,29]. Notable examples of successful and emergent HA-Efm include the endemic Australian ST796 responsible for most VREfm bacteraemias in 2015 [15], a VREfm ST80 clone quickly spreading in Copenhagen hospitals during 2012–2015 [31] or the emergent ST117 that presently accounts for over one-third of VREfm from bloodstream infections in Germany and the Netherlands, for most infected/colonised patients in Spain [8,32], and previously associated with linezolid and tigecycline resistance [33–35].

3. Hospital-associated *Enterococcus faecium* special features promoting adaptation to the human host and hospital environment

3.1. Genomic diversification, colonisation ability and infection potential

Pan-genome analysis has shown that Efm from hospitalised patients harbour a very diverse accessory genome and have larger chromosomes than non-clinical isolates [3,27]. HA-Efm genome plasticity is sustained by the infrequent detection of genome defence mechanisms limiting HGT, such as CRISPR-Cas and a type IV restriction-modification system [26,27]. HGT among HA-Efm promotes the accumulation of diverse MGEs (e.g. plasmids, transposons, insertion sequences, phages or genomic islands) carrying multiple host/environment adaptive genes that are often involved in genomic inversions, recombination and fine-tuning gene expression [3,27]. HA-Efm genome diversification might occur during GIT colonisation and/or infection through a constant gain/loss of diverse genetic elements or mutations towards a strain optimisation to overcome challenges imposed by patients varying in illnesses, antimicrobial therapy and GIT microbiota composition [34,36–39]. Moreover, the emergence of multiple subpopulations might generate a heterogeneous response to antimicrobial treatment, especially if prolonged, making HA-Efm transmission tracking difficult [36–39]. The risk of infection, especially among immunocompromised hosts, and of recontamination of the patient's proximal environment and healthcare workers appears to be proportional to HA-VREfm or ampicillin-resistant (AmpR)-Efm domination of the GIT disturbed microbiota [5,36,40,41]. Although occasionally these HA-Efm can persist for long periods after elimination of the selective pressure, HA-Efm are replaced by commensal Efm when patients are no longer hospitalised [36]. Data suggest that HA-Efm struggle to efficiently compete in a normal human GIT compared with commensal strains (clade B), potentially by their particular adaptive evolution to the unusual ecosystem of the antimicrobial-exposed GIT [18]. For example, the differential use of carbohydrates has been pointed out as one of the main drivers for the divergent evolution of HA-Efm [42]. Commensal populations mostly contain genes involved in complex carbohydrate utilisation, contrasting with HA-Efm that are more enriched in genes for the utilisation of amino-sugars found at the epithelial surface and in specific carbohydrate-phosphotransferase systems (PtsD/BepA), improving their colonisation in the antimicrobial-treated GIT [6,27].

HA-Efm are also more enriched than community isolates in diverse putative virulence genes [the better known are cell surface (Esp/SgrA/EcbA/Acm/Scm/PrpA) proteins, pili and secreted virulence factors (SagA, Hyl-plasmids) (Fig. 1)] that contribute to initial cell adhesion, higher bacterial loads during colonisation, biofilm production, and pathogenesis in endocarditis [6,7,42]. Mutations in the putative capsular polysaccharide (*cps*) locus have been associated with an increase in biofilm formation and improved survival after exposure to lysozyme in diverse HA-VREfm strains [42].

3.2. Antimicrobial resistance and biocide tolerance

One main event boosting the emergence of HA-Efm in the 1980s was the acquisition of ampicillin resistance associated with mutated and/or overexpressed penicillin-binding protein 5 (PBP5), which preceded the emergence of VREfm in the 1990s in different geographical regions [5]. While resistance to vancomycin or other antibiotics is variable among HA-Efm, most of them are AmpR, with HGT of the PBP5 intrinsic gene already being demonstrated [4,16,43–45]. The evolution of PBP5 appears to reflect Efm phylogenomic diversification, as HA-Efm are greatly associated with

particular protein variants [43]. The low biological cost associated with the large transferred and recombinogenic *pbp5* chromosomal platform, and the co-transfer of genes encoding other adaptive features (e.g. carbohydrate metabolism, tolerance to acid and bile, adhesion, biofilm formation) as part of the same *pbp5* platform or in a co-transferred plasmid (pLRM23), have been reported [7,43,44]. Interestingly, a positive association between the presence of putative virulence markers and ampicillin minimum inhibitory concentrations (MICs) has been demonstrated in HA-*Efm*, with highly-resistant (MIC > 256 mg/L) strains enriched in specific virulence markers (*ptsD/sgrA/IS16/orf1481/pili*) being particularly linked to human infections [7]. It is tempting to speculate that all of these genetic events along with ampicillin local selective pressure might have contributed to the emergent and persistent high rates of HA-*Efm* worldwide [7,43,44].

The plasmidome is also a key component of the success and dynamics of HA-*Efm*, namely in the emergence and expansion of VRE*Efm* clones throughout the last decades. Detailed studies performed by Freitas et al. demonstrated that *Efm* possess a species-specific plasmid pool that is divergent from that of *Efs* and is highly consistent between different hosts (same plasmid families), but with a variable plasmid content among strains from different habitats [46,47]. Clinical strains present a plasmidome highly dissimilar to that of other hosts, with several plasmids per cell, higher size plasmids and unique plasmid chimeras [3,47]. Vancomycin resistance has been associated with expansion, diversification and persistence of new clouds of HA-*Efm*. Multiple acquisitions/losses of *vanA* plasmids or *vanB*-Tn1549/Tn5382, the creation of plasmid chimeras during HGT (potentially amplifying the host range or functional benefits) or de novo *vanB* acquisition from anaerobic microbiota have recently been described [31,47,48]. Such events have contributed to a complex scenario of VRE*Efm* spread, limiting the efficacy of infection control interventions. Besides AMR, plasmidomes of HA-*Efm* are enriched in mechanisms of plasmid and strain stabilisation (toxin-antitoxin systems, bacteriocin-like protein *bacA*) and exclusively carry genes conferring different adaptive traits (e.g. phosphotransferase systems, copper resistance, efflux permease ABC transporters), facilitating the persistence of different hierarchical units (e.g. AMR genes, transposons) related to niche adaption, colonisation and infection [3,27,47].

Despite the AmpR-VR-HA-*Efm* epidemic and endemic contexts worldwide and the need to use more recent antimicrobials to treat severe enterococcal infections, outbreaks or clonal spread of linezolid-, tigecycline- or daptomycin-resistant HA-*Efm* remain lesser described [16,33]. Linezolid resistance is mainly associated with chromosomal 23S rRNA mutations, often in HA-*Efm*-ST117/ST80, and less frequently with plasmid-borne *optrA*, *cfr* or *poxtA* genes that have been rarely linked to HA-*Efm* [16,49–51]. Non-susceptibility to daptomycin has been mainly associated with chromosomal mutations in the LiaFSR three-component regulatory cell envelope stress response pathway and the phospholipid biosynthesis enzyme cardiolipin synthase, mostly in the predisposed HA-*Efm*-ST736 [16,52].

Recent studies indicate an increasing tolerance of HA-*Efm* to antiseptics and disinfectants, critical antimicrobials to control MDR-*Efm* spread. Chlorhexidine tolerance conferred by a P102H mutation in a DNA-binding response regulator from the 2CS-CHXT operon was predominantly detected in HA-*Efm* compared with other *Efm* populations [53]. Also, a higher tolerance to isopropanol (23% v/v, lower than the 70% recommended for hand disinfection) through mutations involved in carbohydrate uptake and metabolism was mainly observed in the more recent Australian HA-*Efm* population [54]. However, follow-up studies using cell suspension and four-field standardised international tests to evaluate biocide efficacy as well as the same ST796 isopropanol-tolerant

Efm strains reassured that isopropanol used at 60–70% (v/v) with appropriate contact time and volume is effective against *Efm*. They also showed that 'isopropanol tolerant ST796-*Efm*' were no more tolerant than other *Efm* and non-*Efm* strains that were tested against isopropanol at 23% or 70% [55]. Studies using standardised methodologies involving a large number of strains from diverse genetic backgrounds in different contexts (e.g. in cell suspension, on different surface types including hands), as well as functional studies with biocides, are still needed to better understand the potential evolution of HA-*Efm* towards tolerance and to prevent biocide-ineffective scenarios. Despite concentrations used in marketed alcohol/chlorohexidine products remaining effective, prudent and correct use of biocides is advisable.

4. Dispersal of hospital-associated *Enterococcus faecium* (HA-*Efm*) in different One Health microbiospheres: are different players contributing to HA-*Efm* transmission to humans?

The occurrence of HA-*Efm* in non-clinical contexts is documented since the era of PFGE/MLST subtyping, yet the actual prevalence of such clones in the many community reservoirs may be undervalued since most studies focus on clinical isolates [56]. HA-*Efm* have been mainly identified in sewage from hospital effluents, but also in healthy humans with no recent hospitalisation history, dogs, farm animals, foodstuffs and wildlife [3,57–59]. The real phylogenetic relationship between human infection and community isolates is, however, only now starting to be explored with the recent WGS boom.

Although with the introduction of WGS our opportunities to compare genomes and to identify HA-*Efm* on a global scale hypothetically increased, the imbalance between the proportion of clinical versus non-clinical isolates prejudices such purposes. According to the PATRIC database (accessed 20 January 2020), the 2258 *Efm* genomes currently available include 1317 from humans (mostly clinical sources), 309 from animals and 632 from other/unknown sources. Most sequenced isolates belong to clade A [3] and the design of most studies does not capture the diversity of *Efm* populations (including the resistant HA-*Efm* minority) occurring in a bacterial community. This occurs because they only test one colony/sample, isolates are often recovered without antimicrobial selection, and only a few recent studies address a phylogenetic comparison between clinical and non-clinical (mostly wastewater) *Efm* genomes. Very recently, Arredondo-Alonso et al. showed that clinical and dogs' pan-genomes share a common evolutionary history positioning dogs as a possible source of HA-*Efm*, further evolving through acquisition of additional MGEs to succeed in hospitals [3]. Another recent study in the UK in 2014–2015 including farm animals, but not pets, found limited sharing of strains, resistance genes and *vanA* elements between livestock and humans in the UK [60]. Pig isolates were, however, more genetically related to hospital-associated strains (>50 single nucleotide polymorphisms (SNPs)), corroborating previous findings with older typing techniques [59]. We took this chance to phylogenetically compare the most common HA-*Efm* (ST16/ST17/ST18/ST78/ST117/ST132/ST192/ST203/ST262/ST412) from clinical and non-clinical sources that were selected from 1837 GenBank available genomes (at January 2020) plus 30 undeposited *Efm* from our collection. Selected genomes were analysed using QUAST: Quality Assessment Tool for Genome Assemblies (<http://bioinf.spbau.ru/quast>) to evaluate the quality of genome assembly, and genomes not fulfilling enough quality (based on the number of contigs, total length and N_{50}) were excluded. Of course among the few 'clade A1' clones identified outside the hospital setting, we really do not know whether they are real 'clade A1' strains as most cases only performed MLST [59,61,62] and even less non-clinical

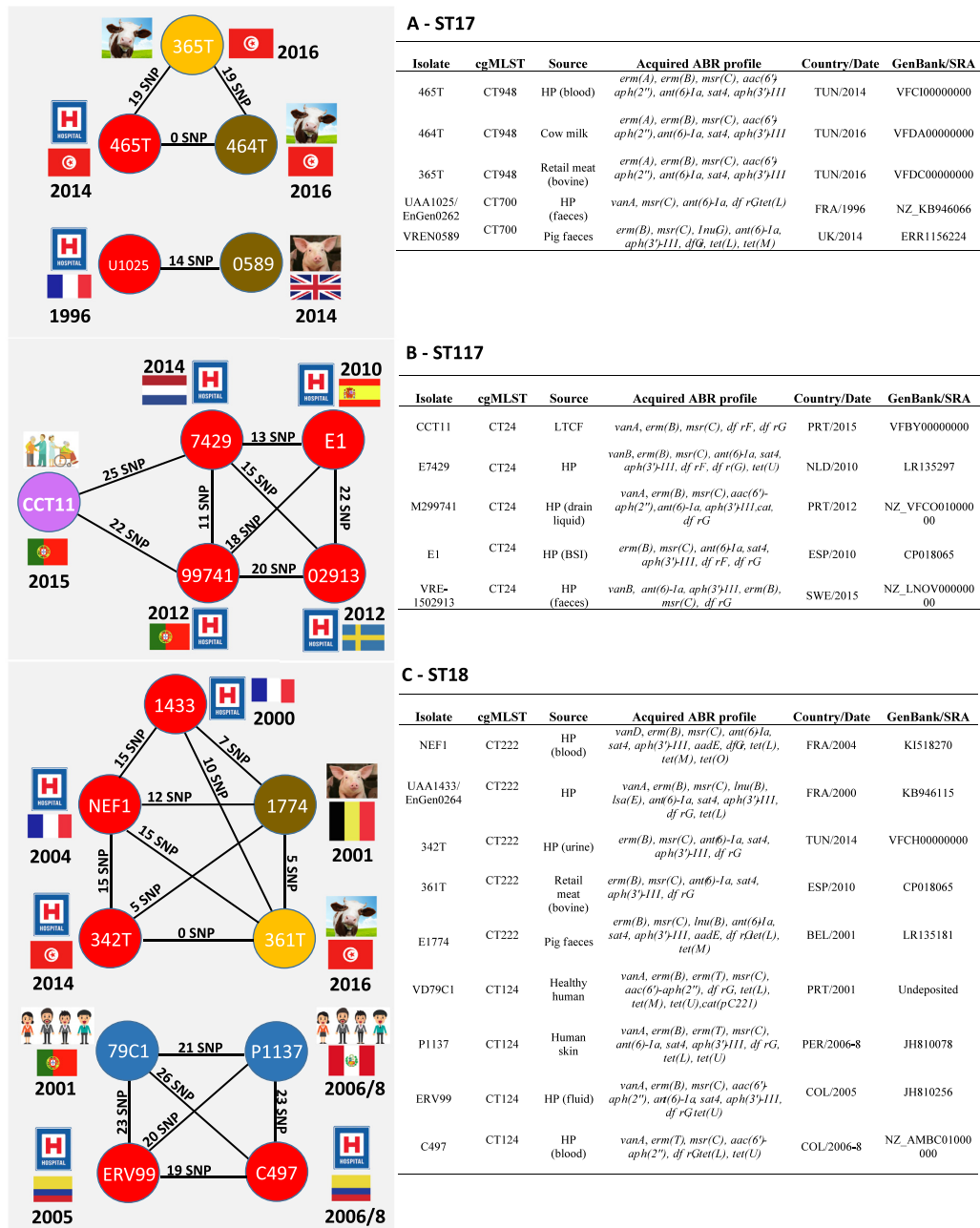


Fig. 2. Evidence of phylogenetically related hospital-associated *Enterococcus faecium* (HA-Efm) clones from different sources and hosts. From the 1837 available *E. faecium* genomes, only selected and high-quality genomes belonging to ST16 ($n = 6$), ST17 ($n = 117$), ST18 ($n = 81$), ST78 ($n = 55$), ST117 ($n = 46$), ST132 ($n = 27$), ST192 ($n = 42$), ST203 ($n = 93$), ST262 ($n = 23$) and ST412 ($n = 62$) were analysed. Genomes were mapped against three *E. faecium* reference strains (DO, GenBank accession no. **CP003583**; 64/3, GenBank accession no. **NZ_CP012522**; and Aus0004, GenBank accession no. **CP003351**) to infer a phylogeny based on the concatenated alignment of high-quality single nucleotide polymorphisms (SNPs) using CSI Phylogeny standard settings of the Center for Genomic Epidemiology (<https://cge.cbs.dtu.dk/services/CSIPhylogeny>). The SNP distances presented (only <26 SNPs) were obtained against *E. faecium* strain 64/3 since it generated the highest percentage of reference genome covered (SNP distances were generally similar between the three references). Input parameters were: $10 \times$ minimum depth and minimum relative depth at SNP positions; SNPs were filtered out if they were called within the vicinity of 300 bp of another SNP (pruning); minimum SNP quality of 30; minimum read mapping quality of 25; and minimum Z-score of 1.96. High-resolution genotyping was performed by using the *E. faecium* core genome multilocus sequence typing (cgMLST) scheme of the Ridom SeqSphere+ v. 7.2 software and complex types (CTs) were deduced. The assembled genomes (contigs) were screened for genes encoding antibiotic resistance (ABR) using the in silico genomic tools ResFinder 3.2 (<https://cge.cbs.dtu.dk/services/ResFinder/>) and the Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/home>). BEL, Belgium; BSI, bloodstream infection; COL, Colombia; ESP, Spain; FRA, France; LTCF, long-term care facility; HP, hospitalised patient; NLD, Netherlands; PER, Peru; PRT, Portugal; SRA, Sequence Read Archive; ST, sequence type; SWE, Sweden; TUN, Tunisia; UK, United Kingdom.

representatives have been sequenced. Despite that, we could identify phylogenetically related HA-*Efm* (<26 SNPs; whole genome used) obtained from human infection and non-clinical (farm animals, retail meat, healthy humans, long-term care facilities) isolates, also sharing the same complex types (CTs) as determined by core genome MLST (cgMLST) (Fig. 2). Among ST17 strains, we identified related CT948 strains (0–19 SNPs) in hospitalised patients, farm animals and retail meat from Tunisia as well as a CT700 strain pair (14 SNPs difference) including one pig isolate (2014) from the aforementioned UK study [59] and one isolate from the faeces of a patient in Toulouse hospital (France, 1996) (Fig. 2A). Although an epidemiological link between the Tunisian strains is probable, no obvious link is apparent between the UK and French ST17 strains recovered almost 20 years apart. Among ST117 (none from animals), only a relationship between CT24 strains from patients in hospitals and long-term care facilities was uncovered, including in different countries (Portugal and France; Fig. 2B). A phylogenetic link between diverse ST18/CT222 strains from patients, farm animals and retail meat (0–15 SNPs) in France, Belgium and Tunisia was identified (Fig. 2C). It is interesting to think that Tunisia has a close relationship with France, which in turn is a neighbour of and close to Belgium. Of course their content in MGEs and adaptive genes might differ substantially, but their core genetic makeup is undoubtedly related. We are aware of the limitations of the SNP-based approach, highly dependent on the reference used and precluding interstudy comparisons [35], but cgMLST analyses confirmed the same CTs being disseminated across decades in different countries. In any case, and given the low number of STs analysed, these data revealed the same MDR-HA-*Efm* in different hosts during long time frames, elucidating the persistence of common (successful) HA-*Efm* along time, the importance of the globalisation of goods and people, and the need for wider surveillance and genomic studies including broad animal collections. The acquired antibiotic resistance gene content was variable between phylogenetically related strains most probably due to their usual location on plasmids (e.g. *vanA* and *tet* genes) that are not present in the reference strain used, once again reinforcing the pivotal role of plasmids as possible good indicators of host specificity [3]. In fact, improved tracking of HGT events in different humans and hosts may reflect unique selective pressures and lifestyles [63], providing a better understanding of the intermix of genes transferred between different populations at a One Health scale. WGS typing has already proved the genetic intermixing between wastewater/pets and human-infection HA-*Efm* and between wastewater and livestock, whilst clonal relationships between HA-*Efm* and farm animals or foodstuffs seem scarce, with the main zoonotic risk being represented by HGT of AMR/virulence genes. The risk to humans associated with the presence of HA-*Efm* in non-clinical sources is arguable by the lack of evidence of direct food–human, animal–human or environment–human transmission.

Even with the potential problems related to research studies design to identify HA-*Efm* in non-clinical settings, the current data point to the specialisation of HA-*Efm* to the hospital setting and patient's selective gut and to a difficult competition of HA-*Efm* with commensal strains in contexts where antimicrobials are not used. These issues raise several questions about the ability of HA-*Efm* to survive and spread in diverse environments as well as their ability to be transmitted to humans from non-clinical sources. As we do not have studies including animals being subjected to conditions of immunosuppression, prolonged antibiotic therapies, etc. as we have in hospitals, we do not know whether HA-*Efm* could outcompete the gut microbiota of diverse mammals as happens in humans. We also do not know whether they would survive or expand under a variety of stresses co-occurring in nature (specificity of nutrients in particular environments, variety of chemicals associated with anthropogenic activities and pollution, diverse ranges

of pH, etc.), or what is the necessary bacterial load of HA-*Efm* to successfully colonise the intestinal tract through food/water consumption or animal contact under antibiotic therapy. These and other questions need to be answered by further genomic and longitudinal One Health studies to assess the true extent and human health risk of HA-*Efm* in the community.

5. Final remarks

The perception of HA-*Efm* has greatly changed over the past three decades, mostly due to the maturation of strain typing methods and cumulative knowledge regarding their genomic evolution. Nonetheless, regardless of the strain genotyping method used and the time and region, it is unquestionable that HA-*Efm* are genetically and evolutionarily distinct from non-clinical isolates and are greatly equipped to survive and expand in hospitalised human gut and healthcare environments, especially under antimicrobial selective pressure favouring their expansion, diversification and persistence. Defining MDR high-risk *Efm* clones and identifying particular genetic features supporting such a definition is, and possibly will remain, difficult. The *Efm* pan-genome seems unlimited and we expect that HA-*Efm* will not stop evolving and adapting to thrive in the perturbed microbiota of hospitalised patients by acquiring genes that improve their fitness, colonisation ability and virulence potential, and thus we should expect the emergence of novel genetic markers or novel key gene combinations to constantly redefine HA-*Efm* in future studies. The more we know about the HA-*Efm* unlimited/open pan-genome, the more we will learn about their ecological needs and pathways of gene sharing, which may be useful to redefine these specialised subpopulations but also to elaborate strategies to control them. Efficient hygiene measures and antimicrobial stewardship will always be critical in containing the spread of HA-*Efm* in hospitals, but future interventions might also include the use of next-generation probiotics derived from the gut microbiota, bacteriocins or the development of phage-derived mechanisms to control specific phosphotransferase system-positive isolates of HA-*Efm* [64], to reduce the density of MDR-*Efm* and, consequently, patient-to-patient-transmission of *Efm* with subsequent colonisation and, sometimes, infection. It is clear that this pathogen will remain a challenge in hospitals in years to come, requiring a multifaceted approach and (cross-border) collaboration to optimise diagnostics, infection prevention and treatment.

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3. *Enterococcus faecalis*: Insights into its Success and Adaptability

***Enterococcus faecalis*: Insights into its Success and Adaptability**

Enterococcus faecalis is a Gram-positive, facultative anaerobic bacterium, and one of the most prevalent species of the *Enterococcus* genus colonizing the human gastrointestinal tract (GIT) and causing human infections [1, 2]. Typically, it is implicated in urinary tract and wound infections, bacteremias, and endocarditis, as well as oral infections (e.g., caries, periodontitis) [1-4]. Of note, *E. faecalis* currently stands out as one of the predominant pathogens involved in healthcare-associated infections (HAI) worldwide [5, 6]. In hospitals in the United States, *E. faecalis* was the third most common organism recovered from adult HAI between 2018 and 2021, behind *Escherichia coli* and *Staphylococcus aureus* [6]. Similarly, it was one of the most reported bacterial species in 2021 in Europe, by the European Union Member States and two European Economic Area countries (Iceland and Norway), in fourth place following *E. coli*, *S. aureus* and *Klebsiella pneumoniae* [5]. Besides humans, *E. faecalis* is also a major colonizer and pathogen of pet animals such as dogs and cats as well as of food-producing animals (e.g., poultry) in which the high rates of mortality may be associated with economical losses [7, 8]. Its ability to thrive in different environmental conditions and hosts is further highlighted as a colonizer of insects and invertebrates, as well as a contaminant of food sources of animal or vegetable origin, drinking or recreational waters [1, 2, 9, 10]. Therefore, appropriate disinfection measures and protective barriers are critical to control these microorganisms in healthcare, food chain and environmental contexts [1].

The widespread nature of *E. faecalis* is associated with its ability to overcome diverse environmental stresses, as a broad range of temperatures (8-45°C) and pH values (4.8-9.6), up to 6.5% NaCl and 40% bile salts, starvation, desiccation, antimicrobials, among others [2, 3, 9, 11]. *E. faecalis* is intrinsically resistant to antibiotics of diverse families such as lincosamides, cephalosporins, monobactams, low concentrations of aminoglycosides, nitroimidazoles, antimetabolites, and streptogramins [3, 10, 12, 13]. As a result, hospitalized patients who are frequently under treatment with broad-spectrum antibiotics to which it is intrinsically resistant may have an overgrowth of *E. faecalis* in the GIT, facilitating potential infections, either by direct translocation into the bloodstream or leading to opportunistic urinary tract infections, among others [1, 11, 14]. Also, this increased GIT density may contribute to the spread of *E. faecalis* into the surrounding environment, where it can persist for long periods on environmental surfaces and medical equipment, and to other patients through the hands of the healthcare staff, patients and visitors [11, 12]. Besides intrinsic resistance, *E. faecalis* may acquire resistance to additional antibiotics, namely to high concentrations of aminoglycosides, important for the treatment of *E. faecalis* infections in synergy with cell wall-active agents, such as ampicillin or vancomycin [1, 12]. The occurrence of high-level resistance to aminoglycosides among *E. faecalis* is considerably common and has been

continually monitored in Europe, where it averaged around 30% in 2021, by the ECDC (European Centre for Disease Prevention and Control) and the WHO Regional Office for Europe [5]. Such acquired resistance has been found not only among clinical strains but also in isolates colonizing healthy humans, recovered from the aquatic environment or in the food chain, along with high rates of resistance to other antibiotics such as tetracyclines, macrolides or quinolones, often used in food-animal production [3, 9, 15, 16]. On the other hand, the rates of resistance to ampicillin and other antibiotics commonly used to treat severe infections, as vancomycin, linezolid, tigecycline, or daptomycin, remain low [1, 3, 9, 11, 17]. Still, the exponential number of non-clinical isolates (healthy humans, animal or environmental origin) carrying genes coding for linezolid resistance (*cfr*, *poxA*, *optrA*) described worldwide are of concern [9, 18-21]. The acquisition of antibiotic resistance traits in *E. faecalis* occurs primarily through horizontal gene transfer of plasmids and transposons [3, 13]. Accordingly, the presence of antibiotic resistance genes has been found inversely correlated with the presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-cas, a “defense system” against incoming foreign DNA, in *E. faecalis* [12, 22].

Along with antibiotic resistance, horizontal transfer of mobile genetic elements (MGEs) provides other accessory genes that enhance the adaptation and survival of populations in diverse environments (e.g., virulence factors, bacteriocins, metabolic pathways, metal and biocides tolerance genes), playing a key role in the ultimate success of *E. faecalis* [1, 12, 22-25]. Indeed, the high genome plasticity of this species is clearly indicated by the percentage of the genome, of up to 25%, attributed to MGEs, which may be shared between *E. faecalis* strains, as well as with different bacterial species [3, 9, 11, 13, 22, 26]. While virulence is not completely understood in *E. faecalis*, mainly because it depends on the complex interaction with the host, several genes have been identified as having a potential role in this species' pathogenesis and epidemiology [3, 10, 27]. Factors known or suspected of enhancing *E. faecalis* virulence in animal models are associated with enterococcal colonization, tissue invasion and translocation, lysis of eukaryotic cells, biofilm formation, modulation of the host immune response and immune evasion [3, 12, 28]. Examples include the enterococcal surface protein (Esp), endocarditis- and biofilm-associated pili (Ebp), cytolysin (Cyl), collagen adhesin (Ace), the co-transcribed gelatinase (GelE) and serine protease (SprE), aggregation substance proteins, and a bile salt hydrolase, among others [3, 12, 28]. Many known and putative virulence determinants (e.g., Cyl, Esp, aggregation substance proteins) cluster on a large pathogenicity island, a genetic element of variable content and size, widely distributed among clinical *E. faecalis*, although it has also been reported in commensal, both human and animal, and environmental strains [11, 25, 28, 29]. Cyl is also capable of lysing prokaryotic cells, showing bacteriocin activity against other Gram-positive organisms [3, 28]. Of note, *E. faecalis* is one of the greatest producers of bacteriocins (enterocins), with action against

microorganisms from different bacterial taxa, occurring in diverse epidemiological contexts [23]. Likewise, acquired metal and biocides tolerance genes have been reported in *E. faecalis* isolated across different environments, frequently co-located in MGEs with antibiotic resistance genes, genes coding for bacteriocins or amino acid or carbohydrates metabolism, revealing a potential for co-selection and co-transfer [24, 30-34].

Besides acquired biocide tolerance genes, whose occurrence and effects on phenotype still require extensive elucidation in this species, *E. faecalis* intrinsically encoded efflux pump activity (e.g. EfrEF) has also been shown to impact its susceptibility to chlorhexidine, a biocide with extensive applications in diverse sectors worldwide, namely in the reduction of colonization and infection by multidrug-resistant *Enterococcus* among hospitalized patients [35]. However, despite the key role of biocides in controlling the spread of *E. faecalis* in different settings and the substantial increase in their use over the years, facilitating the exposure of bacteria to variable concentration gradients occurring in diverse environments, *E. faecalis* response to these compounds remains largely unexplored [36-38].

The ability of *E. faecalis* to successfully circulate in overlapping ecosystems without the occurrence of clear niche-specific subpopulations suggests a generalist nature for this species [1, 25]. Accordingly, a comprehensive analysis of 2026 *E. faecalis* genomes from different sources and clonal lineages has shown that accessory gene contents did not reveal a prominent host specialization, although a few identified clusters mainly consisted of hospital isolates [25]. Further supporting the idea of the generalist nature of *E. faecalis*, this species seems to also lack a clear core-genome-based phylogenetic structure separating host-specific subpopulations. Still, some clonal lineages, ST6, ST9 and ST28, enriched in antibiotic resistance and virulence genes, are prevalent among clinical isolates worldwide, suggesting these are more adapted to persist in the healthcare environment [3, 25, 39]. The generalist lifestyle of *E. faecalis* might be the consequence of a high-level genetic connectivity of strains between different sources and hosts, facilitating the spread of adaptive traits between diverse contexts where these can be acquired and/or selected for [1].

Similar to *E. faecium*, the open pangenome of *E. faecalis* will continue to evolve and adapt to diverse stresses, enhancing their fitness, colonization ability and virulence potential. Consequently, we should anticipate the emergence of novel genetic features in contexts where high selective pressures associated with anthropogenic activities and pollutants occur. For instance, the role of subinhibitory concentrations of biocides present in diverse settings, resulting from the increasing use of antiseptics or disinfectants, needs to be elucidated within the *E. faecalis* epidemiological evolution. A deeper understanding of the promoters of *E. faecalis*' adaptation will enable the development of effective strategies for its control within a One Health framework.

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Chapter II

Objectives and Outline of the Study

“Science is not about being right or wrong, it's about being willing to ask the right questions and follow the evidence wherever it leads”

Neil deGrasse Tyson

1. Objectives

Cationic biocides (CBs) are critical to control the dissemination of bacterial pathogens across diverse environments and have significantly improved hygienic practices, thereby reducing infections and the need for antibiotic therapy. Nevertheless, the extensive use of CBs over the past decades in the healthcare, veterinary, food chain (e.g., farms, food industry), or human domestic contexts has led to widespread CBs subinhibitory concentrations, raising concerns about the potential selection of tolerant bacterial populations and cross-resistance to antibiotics. *Enterococcus* spp., particularly the species *Enterococcus faecium* and *Enterococcus faecalis*, are major agents of multidrug-resistant healthcare-associated infections worldwide with a remarkable ability to tolerate a broad range of environmental stresses and antimicrobials. They are also distributed in other non-human hosts and non-clinical environments, facilitating their frequent exposure to subinhibitory concentrations of CBs and possible adaptation. However, the evolution of genotypic and phenotypic susceptibility to diverse CBs in *E. faecium* and *E. faecalis* and their impact on antibiotic resistance remains underexplored, representing a current research gap that this thesis aims to address.

1.1 Study Hypothesis

Our **hypothesis** was that multidrug-resistant *Enterococcus* spp. from diverse epidemiological scenarios where CBs are traditionally used have evolved towards tolerance to these compounds over the past decades. Furthermore, exposure to subinhibitory concentrations of CBs contributed to the emergence and spread of antibiotic resistance.

1.2 General and specific objectives

The **general objective** of this study was to investigate whether biguanides and quaternary ammonium compounds (QACs) had an impact on the evolution of *E. faecium* and *E. faecalis* populations from diverse settings in the past decades, influencing their tolerance to these compounds and the emergence of resistance to clinically-relevant antibiotics.

To achieve this goal, we selected three widely used CBs, chlorhexidine, benzalkonium chloride, and didecyldimethylammonium chloride, representing a biguanide and two QACs, respectively. The response to these CBs was thoroughly analyzed in a comprehensive

collection of *E. faecium* and *E. faecalis* (786 isolates), representative of diverse sources, timeframes, and clonal backgrounds, recovered in previous studies, through a multi-layered phenotypic and genotypic approach, encompassing classical and high-throughput molecular microbiological methods.

Thus, our study focused on the following **specific objectives**:

1. To evaluate the phenotypic response of *Enterococcus* spp. to CBs in populations isolated from human infections or colonization, food chain and environmental sources over two decades, corresponding to a wide range of clonal lineages and antibiotic resistance profiles.
2. To assess the occurrence and variability of previously identified CBs tolerance genotypes among *Enterococcus* spp. from different epidemiological and clonal backgrounds and their correlation with CBs susceptibility.
3. To evaluate whether acquired CBs tolerance and antibiotic resistance genes of *Enterococcus* spp. share common genetic contexts, potentially contributing to co-selection events associated with the presence of different antimicrobials in various settings.
4. To assess the impact of variable environmental conditions (e.g., pH, temperature, anaerobiosis) on the *Enterococcus* spp. susceptibility to CBs.
5. To assess the effects of exposure to subinhibitory concentrations of CBs on the emergence of tolerance to these compounds and/or antibiotic resistance in *Enterococcus* spp., as well as their impact on the rate of horizontal transfer of genes coding for resistance to clinically-relevant antibiotics.

2. Outline of the thesis

The research carried out to meet the specific goals of this thesis produced results organized in three scientific papers. Two of these papers are already published in international scientific journals with peer-review and one paper is in preparation. The scientific data of this thesis were organized throughout the papers, according to the rationale proposed, as follows:

1. Susceptibility dynamics to CBs in *E. faecium* and *E. faecalis*: insights into phenotypes and genotypes in isolates from diverse epidemiological and clonal backgrounds, under variable environmental conditions.

1.1 **Pereira AP**, Antunes P, Willems R, Corander J, Coque TM, Peixe L, Freitas AR, Novais C. Evolution of Chlorhexidine Susceptibility and of the EfrEF Operon among *Enterococcus faecalis* from Diverse Environments, Clones, and Time Spans. *Microbiology Spectrum*. 2022; 10(4):e0117622. doi: 10.1128/spectrum.01176-22.

1.2 **Pereira AP**, Antunes P, Bierge P, Willems RJL, Corander J, Coque TM, Pich OQ, Peixe L, Freitas AR, Novais C. Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions. *Microbiology Spectrum*. 2023; 11(5):e0232423. doi: 10.1128/spectrum.02324-23.

2. Impact of subinhibitory concentrations of biguanides on the emergence and spread of antimicrobial resistance in *E. faecium*.

2.1 **Pereira AP**, Almeida-Santos AC, Duarte B, Antunes P, Peixe L, Freitas AR, Novais C. The Interplay of Chlorhexidine with Antimicrobial Resistance in *Enterococcus faecium*. (In preparation)



Chapter III

Results and Discussion

*“The greatest discoveries often lie not in finding new things,
but in seeing familiar things in new ways”*

Alexander Fleming

1. Susceptibility dynamics to CBs in *E. faecium* and *E. faecalis*: insights into phenotypes and genotypes in isolates from diverse epidemiological and clonal backgrounds, under variable environmental conditions

1.1. Evolution of Chlorhexidine Susceptibility and of the EfrEF Operon among *Enterococcus faecalis* from Diverse Environments, Clones, and Time Spans

1.2. Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions

Evolution of chlorhexidine susceptibility and of the EfrEF operon among *Enterococcus faecalis* from diverse environments, clones and time spans

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Evolution of Chlorhexidine Susceptibility and of the EfrEF Operon among *Enterococcus faecalis* from Diverse Environments, Clones, and Time Spans

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ABSTRACT Chlorhexidine (CHX) is widely used to control the spread of pathogens (e.g., human/animal clinical settings, ambulatory care, food industry). *Enterococcus faecalis*, a major nosocomial pathogen, is broadly distributed in diverse hosts and environments facilitating its exposure to CHX over the years. Nevertheless, CHX activity against *E. faecalis* is understudied. Our goal was to assess CHX activity and the variability of ChlR-EfrEF proteins (associated with CHX tolerance) among 673 field isolates and 1,784 *E. faecalis* genomes from the PATRIC database from different sources, time spans, clonal lineages, and antibiotic-resistance profiles. The CHX MIC (MIC_{CHX}) and minimum bactericidal concentration (MBC_{CHX}) against *E. faecalis* presented normal distributions (0.5 to 64 mg/L). However, more CHX-tolerant isolates were detected in the food chain and recent human infections, suggesting an adaptability of *E. faecalis* populations in settings where CHX is heavily used. Heterogeneity in ChlR-EfrEF sequences was identified, with isolates harboring incomplete ChlR-EfrEF proteins, particularly the EfrE identified in the ST40 clonal lineage, showing low MIC_{CHX} (≤ 1 mg/L). Distinct ST40-*E. faecalis* subpopulations carrying truncated and nontruncated EfrE were detected, with the former being predominant in human isolates. This study provides a new insight about CHX susceptibility and ChlR-EfrEF variability within diverse *E. faecalis* populations. The MIC_{CHX}/MBC_{CHX} of more tolerant *E. faecalis* (MIC_{CHX} = 8 mg/L; MBC_{CHX} = 64 mg/L) remain lower than in-use concentrations of CHX (≥ 500 mg/L). However, increased CHX use, combined with concentration gradients occurring in diverse environments, potentially selecting multidrug-resistant strains with different CHX susceptibilities, signals the importance of monitoring the trends of *E. faecalis* CHX tolerance within a One Health approach.

IMPORTANCE Chlorhexidine (CHX) is a disinfectant and antiseptic used since the 1950s and included in the World Health Organization's list of essential medicines. It has been widely applied in hospitals, the community, the food industry, animal husbandry and pets. CHX tolerance in *Enterococcus faecalis*, a ubiquitous bacterium and one of the leading causes of human hospital-acquired infections, remains underexplored. Our study provides novel and comprehensive insights about CHX susceptibility within the *E. faecalis* population structure context, revealing more CHX-tolerant subpopulations from the food

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chain and recent human infections. We further show a detailed analysis of the genetic diversity of the *efrEF* operon (previously associated with *E. faecalis* CHX tolerance) and its correlation with CHX phenotypes. The recent strains with a higher tolerance to CHX and the multiple sources where bacteria are exposed to this biocide alert us to the need for the continuous monitoring of *E. faecalis* adaptation toward CHX tolerance within a One Health approach.

KEYWORDS Bacillota (former Firmicutes), biocide, minimum inhibitory concentration, minimum bactericidal concentration, One Health

Chlorhexidine (CHX) is a broad-spectrum disinfectant and antiseptic used since the 1950s and included in the World Health Organization's list of essential medicines (1, 2). It has been widely used for different purposes (e.g., surface disinfectants, antiseptics, mouthwashes, personal care products) in hospitals, the community, the food industry, animal husbandry, and pets (3). Currently, CHX is recommended in the prevention of health care-associated infections by multidrug-resistant (MDR) bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) (4–9). As a bisbiguanide, CHX interacts with the cell wall and membrane anionic sites affecting the osmotic equilibrium of the cell, resulting in a bacteriostatic or bactericidal action depending on the concentration applied (2, 3, 10). Recommended CHX concentrations in disinfectants and antiseptics are usually high (0.05% and 4%; 500 to 40,000 mg/L) (2). However, CHX's wide use has also negative effects, including ecotoxicity to aquatic life, horizontal transfer promotion of genetic elements carrying antimicrobial resistance genes, and changes in bacterial communities (e.g., in the oral microbiota toward a greater abundance of Firmicutes, now designated Bacillota) (11–14).

Within Bacillota, *Enterococcus* spp. is one of the most frequently found taxa in both humans and animals (15). They are members of the oral and gut microbiota of mammals, birds, and reptiles; are able to cause infections in animals; and are one of the leading causes of human hospital-acquired infections globally (15). Their ability to tolerate different stresses facilitates their survival in the environment, being frequently recovered from plants and vegetables, water bodies, and soil (15, 16). Also, this ability to survive and persist in abiotic surfaces is of particular concern in hospitals, increasing the risk of their transmission to patients followed by potential colonization or infection (17).

Enterococcus faecium populations of clade A1, a cluster overrepresented by clinical isolates, have shown a trend toward CHX tolerance (18). Strains belonging to this clade carry a single amino acid change (P102H) in a conserved DNA-binding response regulator (ChtR) from the 2CS-CHX^T operon (18, 19). CHX tolerance in *Enterococcus faecalis* remains, however, scarcely explored. Most available studies are restricted to clinical isolates, especially causing oral infections, and do not analyze the clonal diversity of the studied isolates (20–22). Recently, the *efrEF* operon, coding for the heterodimeric ATP-binding cassette (ABC) transporter EfrEF, was shown to be involved in the tolerance of the *E. faecalis* V583 strain to CHX by deletion and complementation experiments (23). The EfrEF transporter is composed by the EfrE and EfrF proteins, and their upregulation under CHX exposure is mediated by ChIR, a putative MerR family transcription regulator (23, 24).

Our aim was to evaluate CHX susceptibility, the variability of the *chIR-efrEF* genes and to correlate CHX phenotypes with *chIR-efrEF* genotypes among a large collection of *E. faecalis* isolates from human, animal, food, and environmental sources and available genomes from the last century. CHX activity results will be also discussed within the *E. faecalis* population structure context.

RESULTS

Chlorhexidine susceptibility of *E. faecalis* from diverse sources and clonal lineages. The MIC(s) of CHX digluconate (MIC_{CHX}) of the 151 *E. faecalis* ranged from 0.5 to 8 mg/L, with an MIC₅₀ of 4 mg/L and MIC₉₀ of 8 mg/L (Fig. 1A). The highest MIC_{CHX} of 8 mg/L was observed in 21% ($n = 32/151$) of the population studied, while 6% ($n = 9/151$)

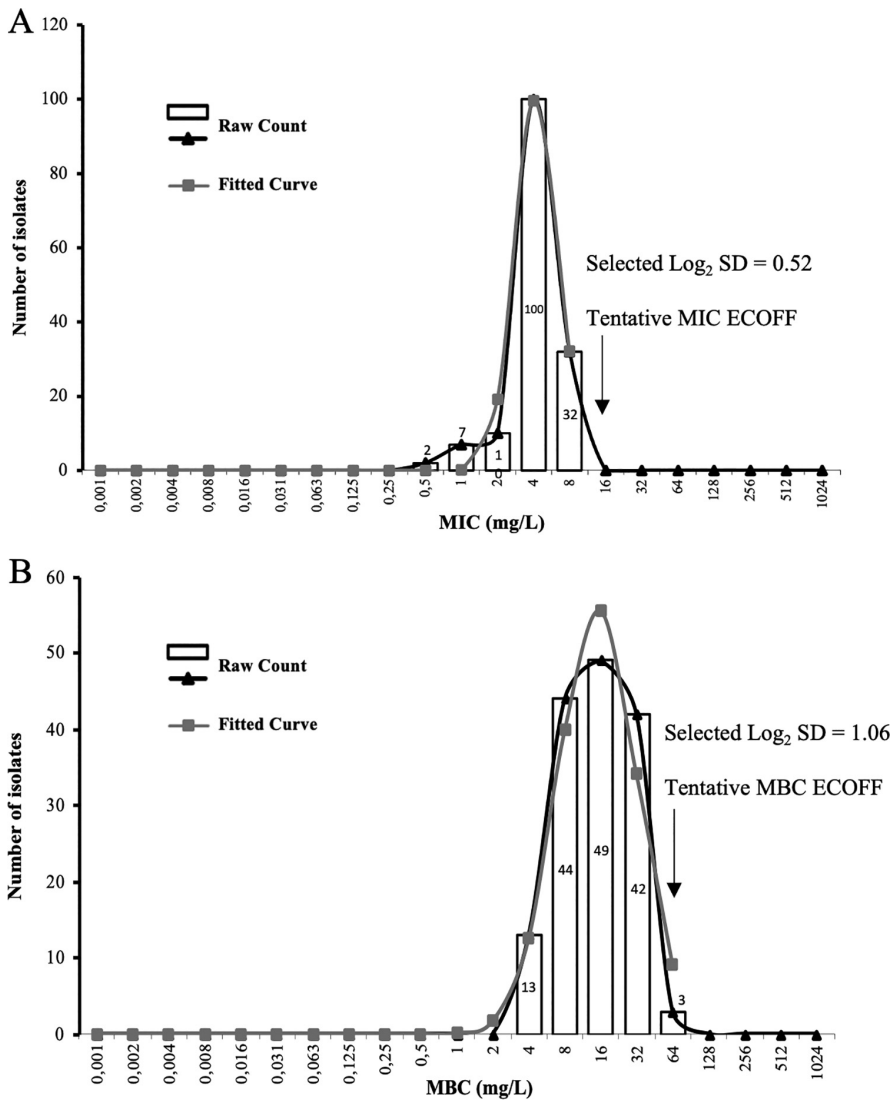


FIG 1 (A, B) Distribution of the *Enterococcus faecalis* studied by different chlorhexidine MICs (A) and minimum bactericidal concentrations (MBCs) (B). The graph-fitted curves were estimated using the ECOFFinder tool, which proposed 8 and 64 mg/L for MIC and MBC, respectively, as limits of 99% of wild-type population. The NORM.DIST Excel 16.44 function indicates that the probability of occurrence of an isolate with an MIC > 8 and ≤ 16 mg/L is 4% and 0% > 16 mg/L and with an MBC ≤ 64 mg/L is 100% and 0% > 64 mg/L. The tentative ECOFFs for MIC and MBC suggested are therefore 16 and 64 mg/L, respectively. ECOFF, epidemiologic cutoff value; SD, standard deviation.

of isolates showed an MIC_{CHX} of 0.5 to 1 mg/L, corresponding in both cases to *E. faecalis* recovered from different sources and clonal lineages. MIC_{CHX} values presented a normal distribution, with a selected log₂ standard deviation (SD) of 0.52 and a fitted curve overlapping the raw count distribution (Fig. 1A). The MIC epidemiologic cutoff value of CHX (ECOFF_{CHX}) proposed for 99% of the population by the ECOFFinder tool was 8 mg/L. However, the MIC_{CHX} distribution analysis using the NORM.DIST Excel function showed a 4% probability of a wild-type isolate having an MIC_{CHX} of > 8 and ≤ 16 mg/L and 0% probability of an MIC_{CHX} of > 16 mg/L. Therefore, based on the normal distribution data, a tentative MIC ECOFF_{CHX} of ≤ 16 mg/L is suggested for *E. faecalis*.

CHX digluconate minimum bactericidal concentration(s) (MBC_{CHX}) ranged from 4 to 64 mg/L, with an MBC₅₀ of 16 mg/L and MBC₉₀ of 32 mg/L. A normal MBC_{CHX} distribution

was also observed, being the selected $\log_2$ SD of 1.06 (Fig. 1B). The highest MBC_{CHX} of 32 to 64 mg/L (30%; $n = 45/151$) and the lowest MBC_{CHX} of 4–8 mg/L (38%; $n = 57/151$) comprised in both cases isolates from different sources and clonal lineages. The $\text{MBC ECOFF}_{\text{CHX}}$ proposed for 99% of the population by the ECOFFinder tool was 64 mg/L, and the NORM.DIST Excel function estimated a 12% probability of a wild-type isolate having an $\text{MBC}_{\text{CHX}} = 64$ mg/L and 0% probability of an MBC_{CHX} of >64 mg/L. Thus, both analyses point to a tentative $\text{MBC ECOFF}_{\text{CHX}}$ of ≤ 64 mg/L for *E. faecalis*.

The analysis of CHX activity regarding isolates' antibiotic-resistance profiles showed that MDR *E. faecalis* had higher mean MIC_{CHX} but similar mean MBC_{CHX} comparing to non-MDR ones (5.0 versus 4.2 [$P \leq 0.05$] and 16.1 versus 19.4 mg/L [$P \geq 0.05$], respectively). The MIC_{CHX} and MBC_{CHX} among VRE was variable and ranged, respectively, between 4 and 8 mg/L and between 4 and 32 mg/L ($n = 14$; human infection, hospital sewage, human fecal samples at hospital admission, and dog feces; from 1996 to 2016). $\text{MIC}_{\text{CHX}}/\text{MBC}_{\text{CHX}}$ of linezolid-resistant isolates varied between 1 and 8 mg/L and between 16 and 64 mg/L ($n = 6$; raw frozen pet food in 2019 to 2020), respectively.

***E. faecalis* isolates from the food chain and recent human samples express higher tolerance to chlorhexidine.** The MIC_{CHX} and MBC_{CHX} distribution of the 151 *E. faecalis* isolates tested were analyzed separately by source and time span (5-year intervals). The MIC_{CHX} distribution of the 151 *E. faecalis* revealed that the mean MIC_{CHX} of isolates from humans (4.8 mg/L; 44 sequence type (STs) among 77 isolates) was higher than the associated with isolates from the food chain (4.1 mg/L; 47 STs among 59 isolates) ($P \leq 0.05$) but similar to those from the environment (4.8 mg/L; 11 STs among 12 isolates) ($P \geq 0.05$). Within the group of *E. faecalis* from humans, the mean MIC_{CHX} was significantly higher among those associated with infection (5.4 mg/L; 27 STs among 41 isolates) than colonization (4.2 mg/L; 29 STs among 36 isolates) ($P \leq 0.05$). In contrast, the mean MBC_{CHX} values were significantly higher among isolates from the food chain (22.6 mg/L) than isolates from humans or the environment (15.3 and 13.0 mg/L, respectively) ($P \leq 0.001$). MBC_{CHX} of *E. faecalis* from human infection or colonization isolates were similar (17.1 mg/L versus 13.2 mg/L, respectively; $P \geq 0.05$).

Food chain *E. faecalis* from different time spans showed variable MIC_{CHX} and MBC_{CHX} , with no apparent increasing trend over time (Fig. 2A). However, a significant increasing trend in the mean MIC_{CHX} and MBC_{CHX} over the years was detected in isolates from human sources (Fig. 2B) ($P \leq 0.05$). We also analyzed the $\text{MIC}_{\text{CHX}}/\text{MBC}_{\text{CHX}}$ trends separately for strains associated with human infection or colonization (including isolates mostly from feces or the urinary tract of healthy humans but also feces from long-term-care facility patients and individuals at hospital admission) (Table S1). The mean MIC_{CHX} and MBC_{CHX} of isolates obtained from human colonization in 2001 to 2005 (3.8 and 10.8 mg/L, respectively; 15 STs among 16 isolates) was statistically similar to that of more recent ones (2016 to 2020: 4.2 and 16.8 mg/L; 13 STs among 16 isolates) ($P \geq 0.05$), although an increase was observed (Fig. 2C). In isolates from human infections, the mean $\text{MIC}_{\text{CHX}}/\text{MBC}_{\text{CHX}}$ significantly increased, with the mean MBC_{CHX} tripling between 2001 and 2005 (10.5 mg/L; 12 STs among 13 isolates) and between 2016 and 2020 (32.0 mg/L; 10 STs among 11 isolates) ($P \leq 0.05$) (Fig. 2D).

Diversity of ChIR-EfrEF sequences and association of incomplete proteins with *E. faecalis* low MIC_{CHX} values. The *efrEF* operon was identified in all but one of the 666 *E. faecalis* genomes analyzed, with 5% ($n = 33/666$) carrying genes coding for incomplete ChIR ($n = 2$), EfrE ($n = 25$), or EfrF ($n = 6$) proteins (Fig. S1; Fig. 3; Table S2). To assess a potential association between the incomplete ChIR, EfrE, and EfrF proteins and susceptibility to CHX, the MIC_{CHX} and MBC_{CHX} were also determined for all isolates with incomplete proteins that were not included in the group of 151 isolates formerly tested in the $\text{MIC}_{\text{CHX}}/\text{MBC}_{\text{CHX}}$ assays. Whereas the MIC_{CHX} values of most of these strains were consistently low (0.5 to 1 mg/L for 91% of the strains, $n = 30/33$), the MBC_{CHX} values ranged from 1 to 64 mg/L, similar to the values observed for other isolates without frameshift, nonframeshift, or nonsense mutations in the ChIR-EfrEF proteins (Table S2; Fig. 3).

Among the 33 *E. faecalis* with incomplete ChIR-EfrEF, 25 isolates carrying a truncated EfrE and recovered from different sources belonged to ST40 (Table S2; Fig. 3). All of

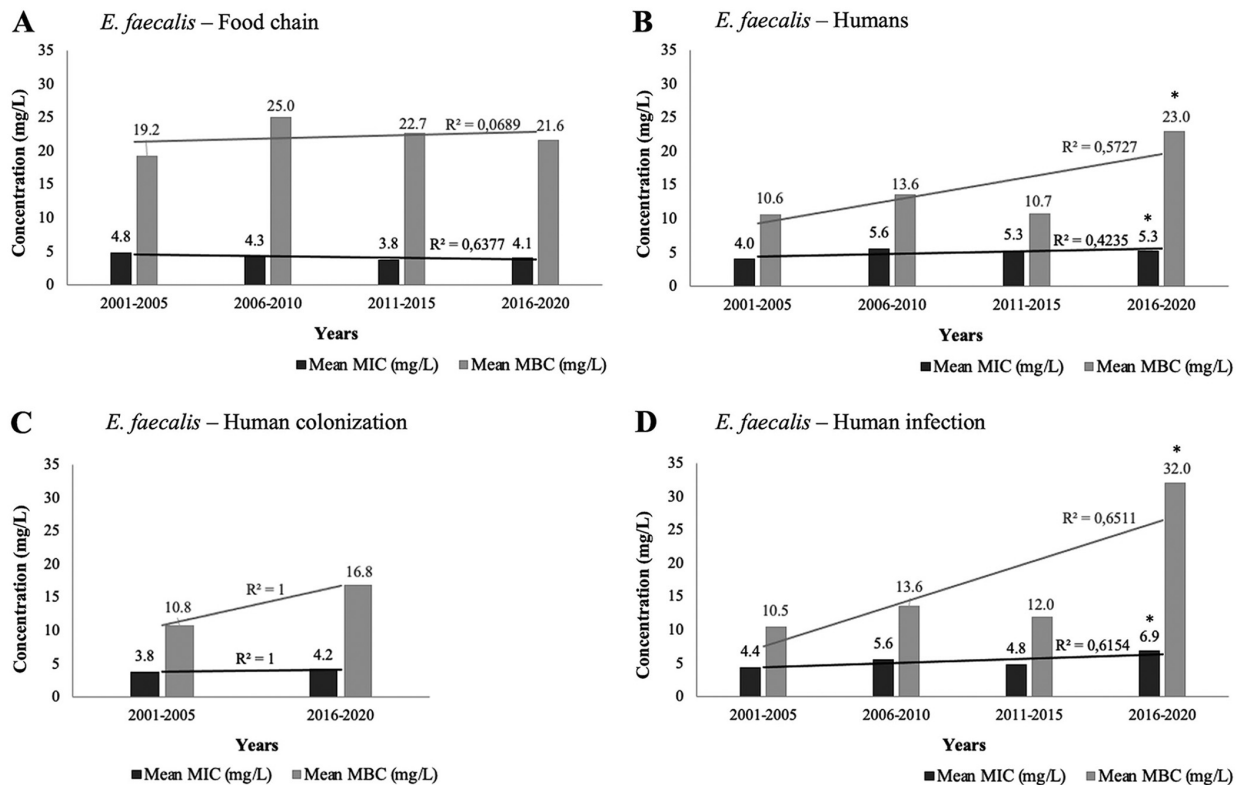


FIG 2 Chlorhexidine mean MIC and minimum bactericidal concentration (MBC) distribution over the years (5-year intervals, from 2001 to 2020) of *E. faecalis* from independently analyzed sources. (A) Distribution of food chain *E. faecalis* isolates ($n = 57$). (B) Distribution of *E. faecalis* isolates recovered from all human sources ($n = 75$). (C) Distribution of *E. faecalis* isolated from human colonization (including isolates from healthy humans, long-term-care patients, and human fecal samples at hospital admission) between 2001 and 2005 and between 2016 and 2020 ($n = 32$). (D) Distribution of *E. faecalis* from human infection ($n = 39$). *, $P \leq 0.05$; two-tailed unpaired Student's t test. *E. faecalis* from earlier years, between 1996 and 2000 ($n = 4$), human colonization isolates from 2006 to 2015 ($n = 4$), and those with other origins ($n = 15$) were not included in the analysis due to the low number of isolates. A linear trendline and the R^2 value were added to each distribution using Excel 16.44.

them showed a missing guanine in the nucleotide position 186 of the *efrE* gene associated with a frameshift mutation resulting in a stop codon at amino acid 79 of EfrE (Fig. S1; Tables S2 and S3). The search for common mutations in the PATRIC database available genomes showed that 85% ($n = 76/89$) of the published ST40 *E. faecalis* also carried this *efrE* mutation (Fig. 4; Table S3). Proteins 100% identical to the truncated EfrE of ST40 *E. faecalis* were also found in five ST268 *E. faecalis* human fecal isolates (GenBank accession numbers [NZ_CABGJG000000000](#), [CABGJA000000000](#), [BJTJ000000000](#), [BJTS000000000](#), and [BJTH000000000](#)).

E. faecalis with incomplete ChlR or EfrF proteins were less represented in our collection (Table S2), as well as in the *E. faecalis* genomes searched in the PATRIC database. Concerning ChlR mutations, two human isolates from our collection (ST59 and ST319) showed the deletion of an adenine in *chlR* nucleotide 5 associated with frameshift mutations resulting in an early truncated protein at amino acid 7 (Fig. S1; Table S2). One published ST40 *E. faecalis* (food chain), with the previously described truncated EfrE, also showed an incomplete ChlR protein due to the insertion of an adenine in *chlR* nucleotide 530, resulting in an early truncated protein at amino acid 181 (Table S3).

Concerning EfrF mutations, an isolate from our collection presented a nonsense mutation in *efrF* (C1567T), resulting in an early stop codon at amino acid 523 in a single ST179 fecal isolate. This mutation was not found in other 30 ST179 human isolates analyzed (15 from our collection and 15 from PATRIC database) (Fig. S1; Tables S2 and S3). In addition, a deletion of 39 nt (696 to 734 nt) resulting in a shortened EfrF protein without amino acids from positions 233 to 245 was found in all ST200 analyzed (5 from our collection and 1 available at PATRIC database; 3 human and 3 food chain isolates).

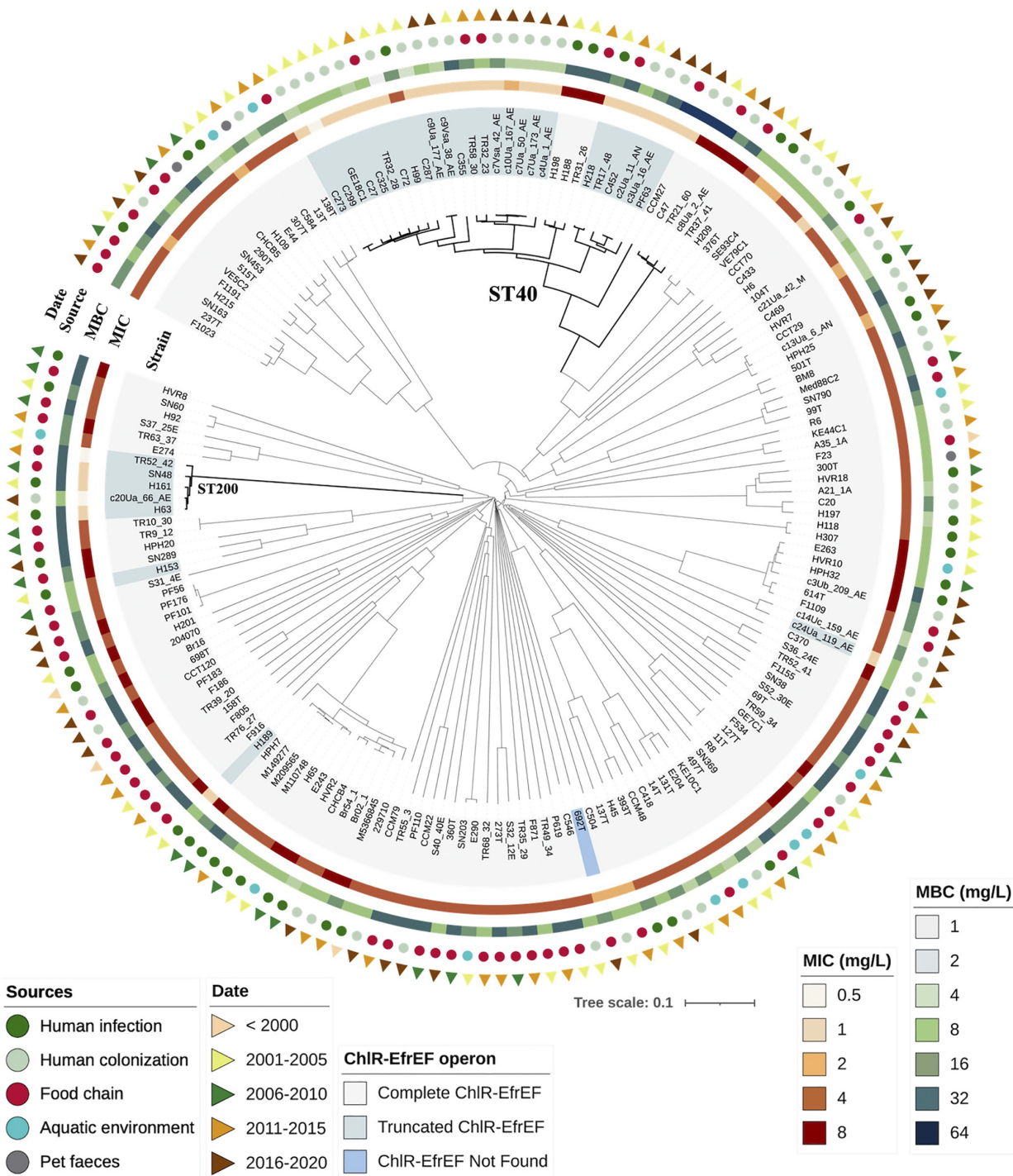


FIG 3 Phylogenetic tree based on the core genome MLST (cgMLST) allelic profiles of all sequenced *Enterococcus faecalis* studied with phenotypic assays ($n = 174$). The clonal relationship of the strains was established from the sequence analysis of 1,972 gene targets according to the *E. faecalis* cgMLST scheme (47), using Ridom SeqSphere+ software version 7.2. The features of the *E. faecalis* isolates, marked with different colors and shapes using iTol software (<https://itol.embl.de>), from the inner to the external part of the phylogenetic tree are complete, incomplete, or not found ChlR-EfrEF proteins marked in the “strain” line, chlorhexidine MICs, chlorhexidine MBCs, source, and date of isolation. For more isolate details, see Table S2.

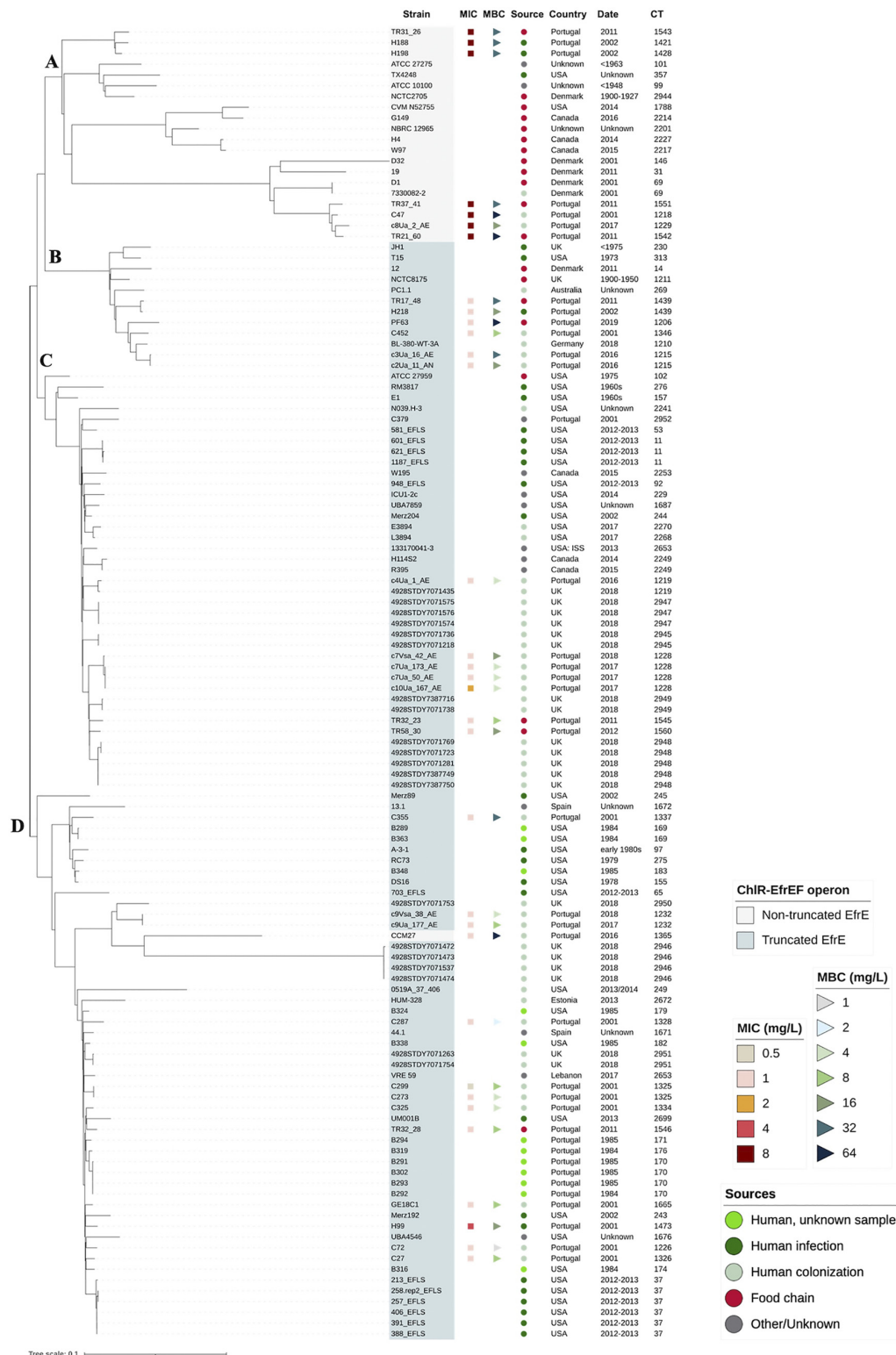


FIG 4 Phylogenetic tree based on the core genome MLST (cgMLST) allelic profiles of *Enterococcus faecalis* identified as ST40 from our collection and available at the PATRIC database (until 18 December 2020) ($n = 122$). The clonal relationship of the (Continued on next page)

(Fig. S1). Finally, one public ST40 *E. faecalis* from human origin with the truncated EfrE protein had an EfrF with a frameshift mutation, caused by the insertion of an adenine in *efrF* nucleotide 1138, associated with an early stop codon at amino acid position 392 (Table S3).

Among the 632 isolates with complete ChlR-EfrEF proteins, a broad range of missense mutations was identified in each of the proteins studied, but no correlation between specific mutations and MIC_{CHX} and/or MBC_{CHX} was noted (Table S2).

EfrE-truncated ST40 *E. faecalis* clustered separately from nontruncated ST40 ones in the phylogenetic tree and were mostly recovered from humans. To assess a potential association between clonal lineages and CHX phenotypes, we performed the core genome multilocus sequence typing (cgMLST) based phylogeny of all sequenced *E. faecalis* isolates with available phenotypic information ($n = 174$). We identified 77 STs and 160 complex types (CTs) with variable MIC_{CHX} and MBC_{CHX} values for isolates of each ST or CT (Fig. 3; Table S2). Nonetheless, it is of note that ST40 *E. faecalis* (18 CTs) and ST200 *E. faecalis* (5 CTs) isolates expressing lower MIC_{CHX} (0.5 to 1 mg/L) clustered separately, while the few ST179, ST308, and ST319 with low MIC_{CHX} were dispersed throughout the phylogenetic tree (Fig. 3).

To further analyze the ST40 *E. faecalis*, all 33 ST40 genomes from our collection and the 89 available at the PATRIC database ($n = 122$) were separately analyzed in a new cgMLST-based phylogenetic tree (Fig. 4). Isolates with operons encoding a truncated EfrE protein clustered separately from those with operons encoding a complete EfrE protein. Cluster A grouped 20 of the 21 strains with a nontruncated EfrE (Fig. 4; Table S3), whereas ST40 *E. faecalis* with a truncated EfrE grouped in clusters B ($n = 12$ isolates), C ($n = 39$ isolates), or D ($n = 50$ isolates), with the latter comprising also one isolate with nontruncated EfrE. The oldest *E. faecalis* with a truncated EfrE was recovered from the food chain in 1900 to 1950. Overall, ST40 *E. faecalis* with a truncated EfrE included in clusters C and D were isolated predominantly from humans (81%; $n = 82/101$; $P < 0.0001$) of different geographical regions.

ST40 *E. faecalis* isolates from cluster A had an MIC_{CHX} of 8 mg/L, while most ST40 isolates of clusters B, C, and D ($n = 24/26$) had an MIC_{CHX} of 1 mg/L. The only ST40 *E. faecalis* with nontruncated EfrE included in cluster D presented the same ChlR-EfrEF mutations as a ST308 *E. faecalis* from a healthy human, which also had an MIC_{CHX} of 1 mg/L without possessing an incomplete ChlR-EfrEF (Table S2; Fig. 3). Additionally, most of our isolates of clusters A and B had an MBC_{CHX} of ≥ 16 mg/L (92.3%, $n = 12/13$; $P < 0.0001$), while strains in clusters C and D mostly had an MBC_{CHX} of < 16 mg/L (75%, $n = 15/20$; $P \leq 0.05$).

DISCUSSION

The increasing challenge to control the growth and transmission of human and animal pathogens in clinical settings, in ambulatory care, or in the food industry explains the rising use of biocides in different sectors, namely, of CHX. However, the scarcity of available data concerning both wild-type bacterial phenotypes and subpopulations' adaptation to biocides over the years limits the perception and the restraint of a potential biocide resistance threat.

In this study, we showed that the MIC_{CHX} and MBC_{CHX} normal distributions for the *E. faecalis* isolates analyzed were in accordance with the ranges previously reported for this species (20, 25). However, the higher mean MBC_{CHX} values found in isolates from the food chain as well as the increasing mean MIC_{CHX}/MBC_{CHX} values of recent isolates from human infections may suggest the adaptability of *E. faecalis* populations in settings where CHX is heavily used. Tentative MIC ECOFF_{CHX} and MBC ECOFF_{CHX} values of 16 and 64 mg/L, respectively, proposed by the ECOFFinder tool and the NORM.DIST Excel function analysis based on *E. faecalis* normal

FIG 4 Legend (Continued)

strains was established from the sequence analysis of 1,972 gene targets accordingly to the *E. faecalis* cgMLST scheme (47), using Ridom SeqSphere+ software version 7.2. Four clusters (A, B, C, and D) were identified. Strains with a truncated EfrE (marked in the "strain" column), chlorhexidine MICs, and minimum bactericidal concentrations (MBC), source, date of isolation, and complex type (CT) of each isolate were marked with different colors, with different shapes, or by text, using iTol software (<https://itol.embl.de>). For more isolate details, see Table S3.

distribution, therefore seem limited because they comprise isolates with heterogeneous phenotypes and genotypes. Although further molecular analyses are needed to understand the significance of such diversity in bacterial populations classified as “wild-type” for CHX, the MIC/MBC_{CHX} values found are considerably below the in-use concentrations of CHX (500 to 40,000 mg/L) (2, 3). Nevertheless, they are within or higher than the levels that have been detected in the skin of patients subjected to CHX bathing (<4.69 to 600 mg/L), in cow milk (4 to 78 mg/L), or in sewage (28 to 1,300 ng/L) (5, 26, 27). As CHX tends to persist in water, sediment, and soils (28), diverse *E. faecalis* populations showing different CHX susceptibilities could hypothetically be selected and adapt within gradients of subinhibitory concentrations occurring not only in patients’ skin but also in diverse environments (5, 26–30).

The detection of *E. faecalis* isolates falling into the upper borderline of the MBC_{CHX} distribution (32 to 64 mg/L), with many of them recently recovered from human infections or the food chain and some showing resistance to vancomycin or linezolid, warns of the possibility of MDR strain selection by CHX, as well as an adaptation toward CHX tolerance in the following years. Such an increase in CHX tolerance over time has been described for other relevant bacterial species, such as *S. aureus*, *Klebsiella pneumoniae*, or *Acinetobacter baumannii* (31–34), suggesting that the increasing use of CHX since the 2000s in community, veterinary, and hospital contexts (27, 32, 35) might have been contributing to selection or ecological adaptation of different bacteria genera. Moreover, other bacterial stresses, such as those with impact in membrane fluidity (e.g., temperature, acids, other biocides), should also be considered in future studies to assess cross-tolerance with CHX (36, 37) and to help explain the higher MBC_{CHX} found in isolates from the food chain throughout the study, when comparing to isolates from humans sources, more tolerant to CHX in recent years.

The few articles addressing the genetic mechanisms involved in CHX tolerance among *E. faecalis* described the upregulation of different genes, especially the conserved *chlR-efrEF* genes (23). We observed that *chlR-efrEF* diversity does not seem to have a direct impact in the MBC_{CHX} values, but variants with incomplete proteins encoded by *chlR-efrEF* correlated with an *E. faecalis* growth perturbation at low CHX concentrations (corresponding to MIC_{CHX}), particularly in ST40 *E. faecalis* from humans. ST40 *E. faecalis* are known to be widely distributed in different environments and hosts (38), but a divergent evolution among strains with truncated and nontruncated EfrE was detected, being both selected across different time spans and geographical regions. Most *E. faecalis* with truncated EfrE, presenting the same mutation, were of human origin, being isolated from this source at least since the 1960s. However, whether this truncated EfrE subpopulation reflects multiple evolved genomic regions of ST40 *E. faecalis* with a better human host adaptation, namely, to colonization, remains to be clarified. More studies are also needed to better understand the role of the EfrEF operon in the metabolism of *E. faecalis* and specifically in the tolerance to CHX and other stresses, as this operon was described to be involved in the transport of ethoxylated fatty amines, fluoroquinolones, and fluorescent dyes (23, 24, 39). Although changes in the *chlR-efrEF* genes were associated with strains’ growth inhibition by CHX in most cases, a few isolates (ST40, ST59, and ST860) with incomplete/deleted ChlR-EfrEF exhibited MIC_{CHX} levels of >1 mg/L, suggesting the occurrence of other cellular mechanisms implicated in bacteria growth under CHX exposure.

In conclusion, our study provides novel and comprehensive insights about CHX susceptibility within the *E. faecalis* population structure context, revealing more CHX-tolerant subpopulations recovered from the food chain and recent human infections. Although the presence of the *efrEF* operon was previously shown to be important in *E. faecalis* V583 response to CHX (23), we further show a detailed analysis of the genetic diversity of the operon and the correlation with CHX phenotypes, namely, the apparent impact of incomplete ChlR-EfrE proteins on isolates’ growth (MIC_{CHX}). The recent strains with a higher tolerance to CHX and the known multiple sources where bacteria are exposed to CHX (e.g., hospital anti-sepsis and disinfection, diffuse pollution by down-the-drain of CHX containing products used in diverse society sectors) (28) alert us to the potential consequences of the growing CHX use and to the need for continuous monitoring of *E. faecalis* adaptation toward CHX tolerance within a One Health approach.

MATERIALS AND METHODS

Epidemiological background of field isolates included in the different assays. A collection of 673 *E. faecalis* isolates (666 sequenced), representative of different geographical regions, sources, time spans, and genomic backgrounds (BioProjects [PRJEB28327](#), [PRJEB40976](#), and [PRJNA663240](#)) (38, 40) was selected for this study. They were recovered in previous studies from human infection ($n = 174$), human colonization ($n = 163$), food chain (animal production settings, animal meat and other food products) ($n = 275$), pets ($n = 9$), and aquatic environment ($n = 45$) samples, in diverse regions (Portugal, Tunisia, Angola, and Brazil) and time spans (1996 to 2020) (40–42). Among them, 181 isolates were included in the CHX susceptibility assays (details in Table S1), with 41% ($n = 75/181$) classified as MDR (resistance to three or more antibiotics from different families), 8% ($n = 14/181$) as resistant to vancomycin and 3% ($n = 6/181$) to linezolid, in previous studies (40–42). Of these, 151 *E. faecalis*, representative of the different sources, geographical regions, time frames, clonal lineages, and antibiotic-resistance profiles (Table S1), were initially considered to evaluate *E. faecalis* MIC_{CHX}/MBC_{CHX} distributions. Subsequently, 30 additional *E. faecalis* with ChlR-EfrEF incomplete proteins and/or belonging to ST40 were considered for phenotypic-genotypic comparative studies along with the former 151 isolates. These 30 additional strains were not included in the first set of phenotypic assays so as not to introduce an overrepresentation of *E. faecalis* with ChlR-EfrEF incomplete proteins and/or belonging to ST40 in MIC_{CHX}/MBC_{CHX} distributions.

Chlorhexidine susceptibility. The MIC_{CHX} (CAS: 18472-51-0, Sigma-Aldrich) of the 181 *E. faecalis* was established by broth microdilution, using the methodological approach proposed by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing (Muller-Hinton broth; pH 7.4; 37°C/20 h) (43). Using a 96-well microtiter plate containing serial 2-fold dilutions of CHX (concentration range, 0.125 to 128 mg/L), bacterial suspensions in log-phase growth, adjusted to reach a final inoculum of 5×10^5 CFU/mL in each well, were incubated for 20 h at 37°C. Microdilution panels were prepared before each assay. The first concentration of CHX without visible growth was considered the MIC_{CHX}. Pinpoint growth was often observed and disregarded as recommended (43).

To determine the MBC_{CHX}, 10 μ L of each well without visible growth from the 96-well MIC_{CHX} plate were incubated onto brain heart infusion (BHI) agar plates at 37°C for 24 h, as defined by the CLSI (44). The MBC_{CHX} was defined as the lowest CHX concentration for which the number of colonies was equal or less than the rejection value defined by CLSI guidelines, based on the final inoculum of each well confirmed by actual count (44). Each experiment was repeated three to six times, and the MIC_{CHX}/MBC_{CHX} values corresponded to the mean of the determinations. *E. faecalis* ATCC 29212 and *E. faecalis* V583 strains were used as controls.

The assessment of MIC_{CHX} and MBC_{CHX} wild-type distribution was performed using the ECOFFinder tool (ECOFFinder_XL_2010_V2.1; available at http://www.eucast.org/mic_distributions_and_ecoffs/), which attempts to fit a log-normal distribution to the presumptive wild-type counts by the so-called iterative statistical method (45). In order to increase specificity to identify wild-type strains, the percentage selected to set the ECOFF was 99%, as suggested by the guidelines of the ECOFFinder tool. The NORM.DIST Excel version 16.44 function was used to calculate the probability of occurrence of isolates at higher concentrations and, consequently, evaluate the potential presence of an acquired tolerance mechanism if such probability was too low, using the mean and the standard deviation and with the cumulative normal distribution function option set to TRUE (45).

Finally, the statistical significance of the differences between MIC_{CHX} and MBC_{CHX} of isolates from the diverse sources, time spans and with disparate antibiotic-resistance profiles was assessed using the two-tailed unpaired Student's *t* test (Excel version 16.44), and the differences associated with the source and MBC_{CHX} distribution among *E. faecalis* ST40 populations were analyzed by the Fisher exact test using GraphPad Prism software, version 9.0., with *P* values of ≤ 0.05 considered significant.

Whole-genome sequence analysis. The genomic search of *chlR*, *efrE*, and *efrF* genes (reference strain *E. faecalis* V583; GenBank accession no. [AE016830.1](#); locus-tag EF_2225 to EF_2227) was performed in the 666 *E. faecalis* sequenced genomes by using the MyDBfinder tool available at the Center for Genomic Epidemiology (www.genomicepidemiology.org). The *chlR*-*efrEF* genes identified in each genome were translated into the corresponding amino acid sequences by the DNA translate tool of ExPASy SIB Bioinformatics Resource Portal (<https://web.expasy.org/translate/>), and the occurrence of incomplete ChlR-EfrEF proteins was evaluated.

For the sequenced *E. faecalis* included in the phenotypic assays, a comparison of the amino acid sequences with the reference strain *E. faecalis* V583 was performed using Clustal Omega software (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) to identify specific mutations. Their clonal relationship was also established by MLST and cgMLST (46, 47) (<http://pubmlst.org>; Ridom SeqSphere+, version 7.2). A phylogenetic tree based on their cgMLST allelic profiles was constructed using Ridom SeqSphere+ software, and isolate information was added to the tree using iTol software (<https://itol.embl.de>).

Comparative genomics. In order to evaluate the frequency of strains with genes coding for incomplete ChlR, EfrE, or EfrF proteins in other collections, ChlR, EfrE, and EfrF sequences with 100% identity until the stop codon with those found in our isolates with incomplete ChlR-EfrEF were searched in 1,784 *E. faecalis* genomes of the PATRIC database, representing a timespan between 1900 and 2020 (last update on 18 December 2020). In addition, to assess whether *E. faecalis* isolates containing genes encoding incomplete ChlR, EfrE, or EfrF proteins had a similar genomic evolution, a cgMLST-based phylogenetic tree was constructed with all *E. faecalis* genomes identified as ST40 ($n = 122$), both from our collection and available at the PATRIC database (last update on 18 December 2020), using Ridom SeqSphere+ software. Isolate information was added to the tree using iTol software (<https://itol.embl.de>).

Data availability. The genome sequences have been deposited in GenBank under BioProjects [PRJEB28327](#), [PRJEB40976](#), and [PRJNA663240](#).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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Supplementary material

Table S1 – Epidemiological background of the 181 *E. faecalis* included in the CHX susceptibility phenotypic assays.

Origin (n isolates)	Source (n isolates)	Date	Country (n isolates)	ST (n isolates)	CT (n isolates)	MDR
HUMANS n=101	Human infection: diverse biological products (47)	1996-2019	Portugal (43), Tunisia (4)	2 (1), 6 (10), 9 (2), 16 (3), 21 (2), 22 (1), 25 (1), 30 (1), 40 (4), 41 (1), 55 (2), 59 (1), 64 (1), 97 (1), 116 (1), 126 (1), 133 (1), 159 (1), 191 (1), 200 (2), 275 (1), 286 (1), 319 (1), 397 (1), 631 (1), 679 (1), 1105 (1), 1164 (1), 1165 (1)	1248 (3), 1252 (1), 1255 (1), 1265 (1), 1266 (1), 1267 (1), 1271 (1), 1376 (1), 1387 (1), 1393 (1), 1408 (1), 1412 (1), 1421 (1), 1422 (1), 1427 (1), 1428 (1), 1431 (1), 1435 (1), 1438 (1), 1439 (1), 1442 (1), 1448 (1), 1460 (1), 1463 (1), 1464 (1), 1471 (1), 1473 (1), 1474 (1), 1475 (1), 1479 (1), 1481 (1), 1486 (1), 1487 (1), 1647 (1), 2904 (1), 2905 (1), 2906 (1), 2907 (1), NA (7)	47%
	Healthy humans colonization: faeces (27), urinary tract (14), vagina (2), breast milk (1)	2001-2018	Portugal (42), Angola (2)	16 (1), 21 (1), 23 (1), 30 (1), 40 (20), 55 (2), 56 (1), 63 (1), 64 (1), 81 (2), 96 (1), 116 (1), 117 (1), 168 (1), 179 (2), 191 (1), 200 (1), 206 (1), 209 (1), 275 (1), 308 (1), 394 (1)	1215 (2), 1216 (1), 1218 (1), 1219 (1), 1228 (4), 1229 (1), 1232 (2), 1293 (1), 1294 (1), 1295 (1), 1309 (1), 1311 (1), 1319 (1), 1320 (1), 1321 (1), 1323 (1), 1325 (2), 1326 (2), 1328 (1), 1334 (1), 1337 (1), 1340 (1), 1343 (1), 1344 (1), 1346 (1), 1348 (1), 1350 (1), 1352 (1), 1356 (1), 1380 (1), 1489 (1), 1491 (1), 1493 (1), 1503 (2), 1567 (1), 1665 (1)	43%
	Long-term care patients: faeces (7)	2015-2016	Portugal (7)	25 (1), 26 (1), 40(1), 143 (1), 191 (1), 398 (1), 679 (1)	1359 (1), 1364 (1), 1365 (1), 1366 (1), 1368 (1), 1372 (1), 1645 (1)	14%
	Patients at hospital admission: faeces (3)	2015-2016	Brasil (3)	6 (2), 525 (1)	1296 (2), 1298 (1)	100%
FOOD CHAIN n=65	Animal production settings: aquaculture (11), piggery (9), poultry and ovine faeces (3)	2006-2015	Portugal (20), Tunisia (3)	21 (1), 22 (2), 35 (1), 40 (6), 59 (1), 65 (1), 100 (1), 139 (1), 200 (1), 209 (1), 330 (2), 386 (1), 445 (1), 631 (1), 749 (1), 872 (1)	1236 (1), 1246 (1), 1262 (1), 1439 (1), 1505 (1), 1506 (1), 1511 (1), 1513 (1), 1519 (1), 1520 (1), 1524 (1), 1528 (1), 1534 (1), 1537 (2), 1542 (1), 1543 (1), 1545 (1), 1546 (1), 1548 (1), 1549 (1), 1551 (1), 1556 (1)	39%
	Food of animal origin: poultry carcass (13), trout (8), bovine and goat milk (6), bovine meat (3), raw meat frozen pet food (6)	1999-2020	Portugal (25), Tunisia (11)	4 (1), 16 (2), 21 (1), 22 (1), 25 (1), 27 (1), 30 (1), 32 (1), 34 (1), 40 (2), 49 (1), 68 (1), 82 (1), 117 (1), 141 (1), 200 (1), 202 (1), 206 (1), 209 (1), 227 (1), 249 (1), 288 (1), 436 (1), 648 (1), 674 (1), 721 (1), 843 (1), 860 (1), 1106 (1), 1107 (1), 1008 (3), 1009 (1)	1205 (2), 1206 (1), 1207 (1), 1208 (1), 1209 (1), 1235 (1), 1238 (1), 1240 (1), 1242 (1), 1243 (1), 1269 (1), 1275 (1), 1285 (1), 1288 (1), 1290 (1), 1292 (1), 1494 (1), 1557 (1), 1558 (1), 1559 (1), 1560 (1), 1561 (1), 1563 (1), 1564 (1), 1565 (1), 1569 (1), 1571 (1), 1576 (1), 1583 (1), 1588 (1), 1589 (1), 1600 (1), 1625 (1), 1629 (1), 1640 (1)	39%
	Ready-to-eat salads (6)	2010	Portugal (6)	100 (1), 141 (1), 165 (1), 309 (1), 579 (1), 594 (1)	1496 (1), 1497 (1), 1498 (1), 1499 (1), 1500 (1), 1501 (1)	0%
PET FAECES n=3	Pet faeces: dog (1), cat (1), bird (1)	2014-2015	Tunisia (3)	21 (2), 116 (1)	1258 (1), 1260 (1), 1264 (1)	33%
AQUATIC ENVIRONMENT n=12	River (2)	2003	Portugal (2)	1 (1), 4 (1)	1 (1), 1467 (1)	0%
	Hospital sewage (6)	2001-2002	Portugal (6)	6 (1), 16 (1), 21 (1), 35 (1), 49 (1), 206 (1)	1651 (1), 1653 (1), 1656 (1), 1658 (1), 1659 (1), 1661 (1)	67%
	Urban wastewater treatment plant (4)	2014-2015	Tunisia (4)	4 (1), 23 (1), 86 (1), 117 (1)	20 (1), 1238 (1), 1241 (1), 1244 (1)	25%

Abbreviations: ST, sequence type; CT, complex type; MDR, multidrug resistance; NA, not available.

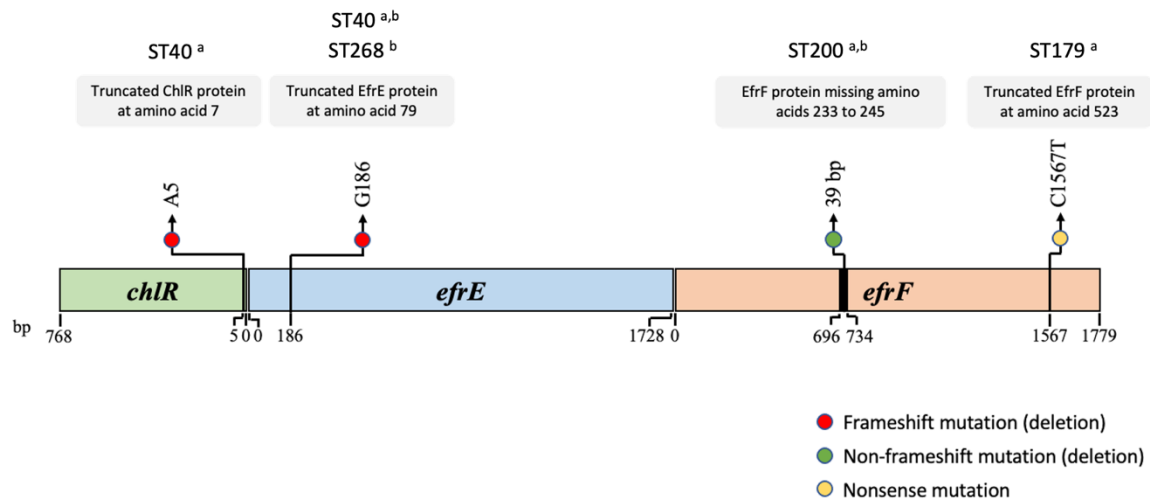


Figure S1. Gene mutations in *chlR* (green), *efrE* (blue) and *efrF* (orange) resulting in incomplete proteins detected among the 666 sequenced *E. faecalis* genomes from our collection^a or published at the PATRIC database^b (when protein sequences had 100% identity until the stop codon with those found in our isolates with incomplete ChlR-EfrEF). The sequence type (ST) of isolates in which the mutations were found are indicated. Frameshift, non-frameshift or nonsense mutations are marked with different colours. Detailed information on the epidemiological background and chlorhexidine phenotypes of isolates from our collection carrying incomplete ChlR-EfrEF proteins is available on Fig. 3 and Table S2.

TABLE S2 – Distribution of the ChIR-EfrEF variants among *Enterococcus faecalis* isolates showing different chlorhexidine phenotypes and with diverse epidemiological backgrounds.

Strain	MIC (mg/L)	MBC (mg/L)	ChIR-EfrEF mutations 1			Source	Geographical Region	Date	ST	CT
			ChIR	EfrE	EfrF					
C299	0.5	8	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1325
c20Ua_66_AE	0.5	8	S188P	Q234R; K435E	I ^c	HH	Portugal	2017	200	1320
TR 52-42	0.5	32	S188P	Q234R; K435E	I ^c	T	Portugal	2012	200	1558
H189	1	8	Tr ^a	-	-	HI	Portugal	2002	319	1422
C72	1	1	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1226
C287	1	2	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1328
C273	1	4	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1325
C325	1	4	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1334
c4Ua_1_AE	1	4	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2016	40	1219
c7Ua_50_AE	1	4	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2017	40	1228
c7ua_173_AE	1	4	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2017	40	1228
c9Vsa_38_AE	1	4	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2017	40	1232
GE18C1	1	8	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1665
C27	1	8	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1326
C452	1	8	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1346
TR 32-23	1	8	L185V	Tr ^b	R58H; P67S; E365K	T	Portugal	2011	40	1545
TR 32-28	1	8	L185V	Tr ^b	R58H; P67S; E365K	T	Portugal	2011	40	1546
c9Ua_177_AE	1	8	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2017	40	1232
H218	1	16	L185V	Tr ^b	R58H; P67S; E365K	HI	Portugal	2002	40	1439
TR 58-30	1	16	L185V	Tr ^b	R58H; P67S; E365K	T	Portugal	2012	40	1560
c2Ua_11_AN	1	16	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2016	40	1215
c7Vsa_42_AE	1	16	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2018	40	1228
C355	1	32	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	40	1337
TR 17_48	1	32	L185V	Tr ^b	R58H; P67S; E365K	T	Portugal	2011	40	1439
c3Ua_16_AE	1	32	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2016	40	1215
PF63	1	64	L185V	Tr ^b	R58H; P67S; E365K	PF	Portugal	2019	40	1206
H63	1	32	S188P	Q234R; K435E	I ^c	HI	Portugal	2001	200	1463
H161	1	32	S188P	Q234R; K435E	I ^c	HI	Portugal	2002	200	1412
SN48	1	32	S188P	Q234R; K435E	I ^c	P	Portugal	2007	200	1524
c24ua_119_AE	1	8	D138N	V264I; A369S	Tr ^d	HH	Portugal	2018	179	1323
C433	1	16	S188P	Q234R; T324I; S470A; S519A	D240A; V372I; G493R	HH	Portugal	2001	308	1343
CCM27	1	64	S188P	Q234R; T324I; S470A; S519A	D240A; V372I; G493R	LTCP	Portugal	2016	40	1365
c10Ua_167_AE	2	4	L185V	Tr ^b	R58H; P67S; E365K	HH	Portugal	2001	30	1348
692T	2	16	NF	NF	NF	PC	Tunisia	2017	860	1288
SE93C4	2	8	-	-	-	HH	Portugal	2001	55	1503
376T	2	8	-	-	-	HI	Portugal	2016	55	1271
F1191	2	32	-	A63T; Q234R; A369S	-	PC	Portugal	2018	202	1583
E44	2	8	-	Q234R; A369S	-	HS	Portugal	2001	21	1661
HPH25	2	16	-	Q234R; E386D	L297S; V329F	HI	Portugal	2019	191	2905
C504	2	8	-	A369S	S137L; A207S	HH	Portugal	2001	168	1350
C469	2	4	-	A369S	A207S; E567K	HH	Portugal	2017	40	1228
137T	2	8	S188P	Q234R; T324I; S470A; A501T; S519A	D240A	BGM	Portugal	2015	25	1242
H153	4	8	Tr ^a	N11S	L7I; R58H; P67S; D240A	HI	Portugal	2001	59	1408
H99	4	16	L185V	Tr ^b	R58H; P67S; E365K	HI	Portugal	2001	40	1473
CHCB4	4	4	-	-	-	HI	Portugal	2007	6	1376
229710	4	8	-	-	-	HI	Portugal	1996	6	1255
H65	4	8	-	-	-	HI	Portugal	2001	6	1464
H209	4	8	-	-	-	HI	Portugal	2002	55	1435
VE79C1	4	8	-	-	-	HH	Portugal	2001	55	1503
M110748	4	8	-	-	-	HI	Portugal	2010	6	1248
Br54_1	4	8	-	-	-	HH	Brasil	2016	6	1296
M209565	4	16	-	-	-	HI	Portugal	2011	6	1248
TR39_20	4	16	-	-	-	AQ	Portugal	2011	139	1549
M149277	4	32	-	-	-	HI	Portugal	2010	6	1248
158T	4	32	-	-	-	AF	Tunisia	2015	65	1246
698T	4	32	-	-	-	BM	Tunisia	2017	1107	1290
F1155	4	16	-	-	L7I; R58H; P67S	PC	Portugal	2018	843	1571
TR55-3	4	32	-	-	D240A; K419E	T	Portugal	2012	227	1559
F805	4	16	-	-	A577T	PC	Portugal	2001	648	1625
SN453	4	16	-	T21A; Q234R; A369S	-	P	Portugal	2007	21	1520
515T	4	8	-	T21A; Q234R; E386D	-	BM	Tunisia	2016	21	1275
VE5C2	4	16	-	T21A; A369S	-	HH	Portugal	2001	21	1567
SN163	4	8	-	A63T; Q234R; A369S	-	P	Portugal	2007	22	1505
H215	4	16	-	A63T; Q234R; A369S	-	HH	Portugal	2002	22	1438
F186	4	32	-	A63T; Q234R; A369S	-	PC	Portugal	1999	82	1588
237T	4	16	-	A63T; Q234R; E386D	-	AF	Tunisia	2015	22	1236
F1023	4	16	-	A63T; Q234R; E386D	-	PC	Portugal	2018	22	1569
E290	4	16	-	Q234R; I254L; D520E	L7I; R58H; D240A; I277T; L278F	HS	Portugal	2002	35	1659
SN203	4	32	-	Q234R; I254L; D520E	L7I; R58H; D240A; I277T; L278F	P	Portugal	2008	35	1506

TABLE S2 (continued) - Distribution of the ChlR-EfrEF variants among *Enterococcus faecalis* isolates showing different chlorhexidine phenotypes and with diverse epidemiological backgrounds.

Strain	MIC (mg/L)	MBC (mg/L)	ChlR-EfrEF mutations ¹			Source	Geographical Region	Date	ST	CT
			ChlR	EfrE	EfrF					
Med88C2	4	16	-	Q234R; V357F; Q409K	D240A	HH	Portugal	2001	209	1493
SN790	4	16	-	Q234R; V357F; Q409K	D240A	P	Portugal	2007	209	1534
99T	4	32	-	Q234R; V357F; Q409K	D240A	BGM	Tunisia	2014	209	1292
CHCB5	4	16	-	Q234R; A369S	-	HI	Portugal	2008	21	1647
290T	4	32	-	Q234R; A369S	-	F	Tunisia	2015	21	1258
CCT29	4	16	-	Q234R; A369S	L297S; V329F	LTCP	Portugal	2016	191	1359
c13Ua_6_AN	4	32	-	Q234R; A369S	L297S; V329F	HH	Portugal	2017	191	1309
TR9_12	4	32	-	Q234R; A369S; V410I; S519A	L74F	AQ	Portugal	2010	330	1537
TR10_30	4	32	-	Q234R; A369S; V410I; S519A	L74F	AQ	Portugal	2010	330	1537
H109	4	4	-	Q234R; E386D	-	HI	Portugal	2001	21	1387
307T	4	16	-	Q234R; E386D	-	F	Tunisia	2014	21	1260
F1109	4	4	-	Q234R; Q409K	-	PC	Portugal	2018	16	1576
R8	4	16	-	Q234R; Q409K	-	R	Portugal	2003	4	1463
614T	4	16	-	Q234R; Q409K	-	PC	Tunisia	2017	16	1285
TR76-27	4	32	-	Q234R; Q409K	D240A; A577T	T	Portugal	2012	436	1565
F534	4	8	-	Q234R; Q409K	M400I	PC	Portugal	2001	32	1600
E274	4	16	-	V264I; A369S	-	HS	Portugal	2002	49	1658
S37_25E	4	32	-	V264I; E386D	-	RS	Portugal	2010	309	1499
H92	4	16	-	E330K; Q409K	P67S	HI	Portugal	2001	631	1471
SN60	4	32	-	E330K; Q409K	P67S	P	Portugal	2007	631	1528
S36_24E	4	32	-	N353S; A369S; K435E	-	RS	Portugal	2010	141	1498
TR52_41	4	32	-	N353S; E386D; K435E	-	T	Portugal	2012	141	1557
HVR7	4	4	-	A369S	A207S; E567K	HI	Portugal	2007	30	1486
c21Ua_42_M	4	8	-	A369S	A207S; E567K	HH	Portugal	2017	56	1321
SN38	4	32	-	A369S; K435E; S519A	P67S	P	Portugal	2007	100	1519
104T	4	4	-	E386D	A207S; E567K	BGM	Tunisia	2014	30	1235
S52_30E	4	32	-	E386D; K435E; S519A	P67S	RS	Portugal	2010	100	1501
F23	4	8	-	K435E	-	PC	Portugal	1999	27	1589
CCT70	4	8	-	K435E	L278F	LTCP	Portugal	2016	26	1645
204070	4	8	-	S519A	P67S	HI	Portugal	1999	159	1252
H197	4	4	-	A554D	L7I; R58H; P67S	HI	Portugal	2002	275	1427
C20	4	16	-	A554D	L7I; R58H; P67S	HH	Portugal	2001	275	1319
SN369	4	32	E86K; D138N	Q234R; A369T; Q409K	-	P	Portugal	2008	445	1511
KE10C1	4	16	H98Q	Q234R; V354A; K435E	-	HH	Portugal	2001	206	1489
131T	4	8	H98Q	Q234R; K435E	-	BGM	Tunisia	2015	206	1240
C418	4	4	H98Q; E222K	Q234R; K435E	-	HH	Portugal	2001	23	1344
14T	4	16	H98Q; E222K	Q234R; K435E	-	UWTP	Tunisia	2014	23	1241
E204	4	4	H98Q; E254K	Q234R; K435E	-	HS	Portugal	2001	206	1651
TR59_34	4	32	A112V	E386D	-	T	Portugal	2012	68	1561
A21_1A	4	4	V120F	Q268E; A369S	D240A	HH	Angola	2015	116	1293
HVR18	4	8	V120F	Q268E; A369S	D240A	HI	Portugal	2008	116	1481
300T	4	8	V120F	Q268E; A369S	D240A	F	Tunisia	2015	116	1264
501T	4	16	D138N	P33S; V264I; A369S	-	HI	Tunisia	2014	64	1265
BM8	4	32	D138N	P33S; V264I; A369S	-	HH	Portugal	2017	64	1295
P619	4	32	D138N	Q234R; S519A	-	PC	Portugal	2001	249	1494
C584	4	4	D138N	V264I; A369S	-	HH	Portugal	2001	117	1356
CCM79	4	8	D138N	V264I; A369S	-	LTCP	Portugal	2016	679	1368
13T	4	16	D138N	V264I; A369S	-	UWTP	Tunisia	2014	117	1244
138T	4	16	D138N	V264I; A369S	-	BGM	Tunisia	2015	117	1243
c14Uc_159_AE	4	16	D138N	V264I; A369S; A554D	-	HH	Portugal	2018	179	1311
TR49_34	4	32	L185F	F96I; A369S	P67S; E365K	AQ	Portugal	2012	749	1556
R6	4	16	L185V	Q234R	A207S	R	Portugal	2003	1	1
TR35-29	4	32	L185V	Q234R; K435E; S519A	G60E	AQ	Portugal	2011	386	1548
C546	4	8	L185V	K435E; A516T; A554D	L7I; R58H; P67S; H246Q; E365K	HH	Portugal	2001	96	1352
360T	4	8	S188P	-	L278F	BM	Tunisia	2016	34	1269
S40_40E	4	32	S188P	-	L278F	RS	Portugal	2010	579	1500
497T	4	4	S188P	Q234R; T324I; S470A; S519A	D240A	HI	Tunisia	2011	133	1267
393T	4	8	S188P	Q234R; T324I; S470A; S519A	D240A	HI	Tunisia	2015	25	1266
H6	4	16	S188P	Q234R; T324I; S470A; S519A	D240A	HI	Portugal	2001	41	1460
H45	4	16	S188P	Q234R; T324I; S470A; S519A	D240A	HI	Portugal	2001	97	1448
CCM48	4	16	S188P	Q234R; T324I; S470A; S519A	D240A	LTCP	Portugal	2016	25	1366
273T	4	32	S188P	Q234R; T324I; S470A; S519A	D240A	AF	Tunisia	2015	872	1262
S32_12E	4	16	S188P	Q234R; M431T; K435E; S470A; S519A; L524F	D240A	RS	Portugal	2010	594	1497
PF56	4	16	S188P	Q234R; S470A; S519A	D240A; A577T	PF	Portugal	2019	1008	1205
PF101	4	32	S188P	Q234R; S470A; S519A	D240A; A577T	PF	Portugal	2019	1008	1209
S31_4E	4	8	S188P	Q234R; S519A	D240A	RS	Portugal	2010	165	1496
TR68_32	4	8	S188P	H320N	D240A; L278F	T	Portugal	2012	1106	1564
KE44C1	4	8	S188P; E211K	K231I; E386D; K435E; S519A	N51K; P67S	HH	Portugal	2001	81	1491
A35_1A	4	8	S188P; E211K	A369S; K435E; S519A	N51K; P67S; P578Q	HH	Angola	2013	81	1294
F871	4	8	S188P; I242V	-	D240A	PC	Portugal	2001	288	1629
H118	4	8	E211G	Q234R; K435E	R58H; P67S; E365K	HI	Portugal	2001	9	1393
69T	4	16	V226I	Q234R; Q409K; L557I	-	UWTP	Tunisia	2015	86	20
CCM22	4	32	I249V	-	L278F	LTCP	Portugal	2016	143	1364
PF110	4	32	I249V	M116I; V300I	L278F	PF	Portugal	2019	674	1207

TABLE S2 (continued) - Distribution of the ChlR-EfrEF variants among *Enterococcus faecalis* isolates showing different chlorhexidine phenotypes and with diverse epidemiological backgrounds.

Strain	MIC (mg/L)	MBC (mg/L)	ChlR-EfrEF mutations ¹			Source	Geographical Region	Date	ST	CT
			ChlR	EfrE	EfrF					
E243	8	8	-	-	-	HS	Portugal	2002	6	1653
HVR2	8	8	-	-	-	HI	Portugal	2007	6	1479
Br02_1	8	8	-	-	-	HS	Portugal	2015	6	1296
HPH7	8	16	-	-	-	HI	Portugal	2007	6	1474
M5366845	8	16	-	-	-	HI	Portugal	2013	6	2907
CCT120	8	32	-	-	Q233P; E236K	LTCP	Portugal	2016	398	1372
PF183	8	16	-	-	D240A	PF	Portugal	2020	1009	1208
SN289	8	16	-	N11S	L7I; R58H; P67S; D240A	P	Portugal	2007	59	1513
HPH20	8	32	-	N11S	L7I; R58H; P67S; D240A	HI	Portugal	2019	1105	2904
C370	8	8	-	Q234R	D240A	HH	Portugal	2001	63	1340
E263	8	8	-	Q234R; Q409K	-	HS	Portugal	2002	16	1653
HVR10	8	8	-	Q234R; Q409K	-	HI	Portugal	2008	16	1475
c3Ub_209_AE	8	16	-	Q234R; Q409K	-	HH	Portugal	2017	16	1216
HPH32	8	32	-	Q234R; Q409K	-	HI	Portugal	2019	16	2906
F916	8	32	-	Q234R; K435E; S519A; T552S	P67S	PC	Portugal	2001	721	1640
TR63_37	8	16	-	V264I; A369S	-	T	Portugal	2012	49	1563
11T	8	16	-	N353S; Q409K	-	UWTP	Tunisia	2014	4	1238
127T	8	16	-	N353S; Q409K	-	BGM	Tunisia	2015	4	1238
GE7C1	8	32	-	A369S	-	HH	Portugal	2001	394	1380
Br16	8	16	-	S519A	P67S	HH	Portugal	2015	525	1298
HVR8	8	32	D138N	V264I; A369S	S347F	HI	Portugal	2007	397	1486
H201	8	8	Y145C	-	T188N	HI	Portugal	2002	2	1431
c8Ua_2_AE	8	16	L185V	Q234R; K435E	R58H; P67S; E365K	HH	Portugal	2017	40	1229
H188	8	32	L185V	Q234R; K435E	R58H; P67S; E365K	HI	Portugal	2002	40	1421
H198	8	32	L185V	Q234R; K435E	R58H; P67S; E365K	HI	Portugal	2002	40	1428
TR31_26	8	32	L185V	Q234R; K435E	R58H; P67S; E365K	AQ	Portugal	2011	40	1543
TR37_41	8	32	L185V	Q234R; K435E	R58H; P67S; E365K	AQ	Portugal	2011	40	1551
C47	8	64	L185V	Q234R; K435E	R58H; P67S; E365K	HH	Portugal	2001	40	1218
TR21_60	8	64	L185V	Q234R; K435E	R58H; P67S; E365K	AQ	Portugal	2011	40	1542
PF176	8	16	S188P	Q234R; S470A; S519A	D240A; A577T	PF	Portugal	2020	1008	1205
H307	8	8	E211G	S46G; Q234R; K435E	R58H; P67S; E365K	HI	Portugal	2002	9	1442

¹ Amino acid variation.

^a Truncated ChlR at amino acid 7. ^b Truncated EfrE at amino acid 79. ^c Incomplete EfrF missing amino acids 233 to 245. ^d Truncated EfrF at amino acid 523. The reference strain used was *E. faecalis* V583 [GenBank accession no. AE016830.1; locus-tag EF_2225 (*chlR*), EF_2226 (*efrE*) and EF_2227 (*efrF*)].

Abbreviations: -, no mutations detected; AF, animal faeces; AQ, aquaculture; BGM, bovine and goat milk; BM, bovine meat; F, pet faeces; HA, human faecal samples in hospital admission; HH, healthy-humans; HI, human infection; HS, hospital sewage; I, incomplete; LTCP, long-term care patients; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; NA, not available; NF, not found; P, piggy; PC, poultry carcass; PF, raw frozen pet food; T, trout; Tr, truncated ChlR, EfrE or EfrF protein; R, river water; RS, ready-to-eat salads; ST, sequence type; UWTP, urban wastewater treatment plant.

TABLE S3 – ChlR-EfrEF variability and epidemiological background of *E. faecalis* genomes identified as ST40 from our collection and available at the PATRIC database (until the 18th of December 2020) (n=122).

ChlR-EfrEF proteins			<i>E. faecalis</i> strain	Date	Isolation Source	Geographic Location	Genome ID PATRIC	GenBank Acc. / Bioproject Number
ChlR	EfrE	EfrF						
+	Tr ^a	+	NCTC8175	1900-1950	Milk	UK	1351.1221	UGIM01000000
			E1	1960s	Human infection	USA	1158673.3	AJEU00000000
			T15	1973	Human infection	USA	1158672.3	AJET00000000
			JH1	<1975	Human infection	UK	565648.4	ACAP00000000
			DS16	1978	Human infection	USA	1158677.3	AJEY00000000
			RC73	1979	Human infection	USA	1158678.3	AJEZ00000000
			A-3-1	early 1980s	Human infection	USA	1169246.3	ASEF00000000
			B289	1984	Human	USA	1391472.3	JAHH00000000
			B292	1984	Human	USA	1391474.3	JAHO00000000
			B316	1984	Human	USA	1391480.3	JAHP00000000
			B319	1984	Human	USA	1391482.3	JAHR00000000
			B363	1984	Human	USA	1391493.3	JAIC00000000
			B291	1985	Human	USA	1391473.3	JAHI00000000
			B293	1985	Human	USA	1391475.3	JAHK00000000
			B294	1985	Human	USA	1391476.3	JAHL00000000
			B302	1985	Human	USA	1391478.3	JAHN00000000
			B324	1985	Human	USA	1391485.3	JAHU00000000
			B338	1985	Human	USA	1391488.3	JAHX00000000
			B348	1985	Human	USA	1391491.3	JAIA00000000
			GE18C1	2001	Human colonization	Portugal		PRJEB28327
			C27	2001	Human colonization	Portugal		PRJEB28327
			C72	2001	Human colonization	Portugal		PRJEB28327
			C273	2001	Human colonization	Portugal		PRJEB28327
			C287	2001	Human colonization	Portugal		PRJEB28327
			C299	2001	Human colonization	Portugal		PRJEB28327
			C325	2001	Human colonization	Portugal		GCA_905123595.1
			C355	2001	Human colonization	Portugal		PRJEB28327
			C452	2001	Human colonization	Portugal		PRJEB28327
			H99	2001	Human infection	Portugal		PRJEB28327
			H218	2002	Human infection	Portugal		PRJEB28327
			Merz192	2002	Human infection	USA	1169248.3	ASEH00000000
			Merz204	2002	Human infection	USA	1169249.3	ASEI00000000
			Merz89	2002	Human infection	USA	1169250.3	ASEG00000000
			12	2011	Copper fed pig	Denmark	1351.116	JTKT00000000
			TR17_48	2011	Aquaculture	Portugal		PRJEB28327
			TR32_23	2011	Aquaculture	Portugal		PRJEB28327
			TR32_28	2011	Aquaculture	Portugal		PRJEB28327
			TR58_30	2012	Trout	Portugal		PRJEB28327
			1187_EFLS	2012-2013	Human infection	USA	1351.233	JVZS01000000
			258.rep2_EFLS	2012-2013	Human infection	USA	1351.242	JVOA01000000
			391_EFLS	2012-2013	Human infection	USA	1351.244	JVIY01000000
			406_EFLS	2012-2013	Human infection	USA	1351.245	JVIK01000000
			581_EFLS	2012-2013	Human infection	USA	1351.248	JVBG01000000
			213_EFLS	2012-2013	Human infection	USA	1351.255	JVPS01000000
			257_EFLS	2012-2013	Human infection	USA	1351.259	JVOC01000000
			388_EFLS	2012-2013	Human infection	USA	1351.262	JVJB01000000
			601_EFLS	2012-2013	Human infection	USA	1351.270	JVAM01000000
			621_EFLS	2012-2013	Human infection	USA	1351.271	JUZT01000000
			703_EFLS	2012-2013	Human infection	USA	1351.276	JUWK01000000
			948_EFLS	2012-2013	Human infection	USA	1351.289	JUMK01000000
			133170041-3	2013	Air	USA: International Space Station	1351.3790	GCA_013344605.1
			HUM-328	2013	Human colonization	Estonia	1351.4120	JACZBA00000000
			UM001B	2013	Human infection	USA	1351.4157	CP053026.1
			0519A_37_406	2013/2014	Human colonization	USA	1351.440	MKXV00000000
			ICU1-2c	2014	NA	USA	1351.328	LQAM00000000
			H114S2	2014	Wastewater (primary effluent)	Canada	1351.3401	WMGT01000000
			W195	2015	Wastewater (final effluent)	Canada	1351.3409	WMGS01000000
			R395	2015	Wastewater (primary effluent)	Canada	1351.3411	WMGP01000000
			C379	2016	Wastewater (final effluent)	Canada	1351.3404	WMGU01000000
			c2Ua_11_AN	2016	Human colonization	Portugal		PRJEB28327
			c3Ua_16_AE	2016	Human colonization	Portugal		PRJEB28327
			c4Ua_1_AE	2016	Human colonization	Portugal		PRJEB28327
			L3894	2017	Human colonization	USA	1351.3473	WVTT01000000
			E3894	2017	Human colonization	USA	1351.3475	WVTL01000001
			VRE 59	2017	NA	Lebanon	1351.4086	JAAIGK01000000

TABLE S3 (continued) – ChlR-EfrEF variability and epidemiological background of *E. faecalis* genomes identified as ST40 from our collection and available at the PATRIC database (until the 18th of December 2020) (n=122).

ChlR-EfrEF proteins			<i>E. faecalis</i> strain	Date	Isolation Source	Geographic Location	Genome ID PATRIC	GenBank Acc. / Bioproject Number
ChlR	EfrE	EfrF						
+	Tr ^a	+	c7Ua_50_AE	2017	Human colonization	Portugal		GCA_905123655.1
			c7ua_173_AE	2017	Human colonization	Portugal		PRJEB28327
			c9Ua_177_AE	2017	Human colonization	Portugal		PRJEB28327
			c10Ua_167_AE	2017	Human colonization	Portugal		PRJEB28327
			c7Vsa_42_AE	2018	Human colonization	Portugal		PRJEB28327
			c9Vsa_38_AE	2018	Human colonization	Portugal		PRJEB28327
			4928STDY7071218	2018	Human colonization	UK	1351.2101	CABGHW000000000
			4928STDY7071474	2018	Human colonization	UK	1351.2120	CABGJI000000000
			4928STDY7071574	2018	Human colonization	UK	1351.2124	CABGJT000000000
			4928STDY7071435	2018	Human colonization	UK	1351.2169	CABGOB000000000
			4928STDY7071473	2018	Human colonization	UK	1351.2180	CABGPI000000000
			4928STDY7071472	2018	Human colonization	UK	1351.2185	CABGPH000000000
			4928STDY7071537	2018	Human colonization	UK	1351.2198	CABGRJ000000000
			4928STDY7071576	2018	Human colonization	UK	1351.2207	CABGSN000000000
			4928STDY7071575	2018	Human colonization	UK	1351.2210	CABGSG000000000
			4928STDY7071281	2018	Human colonization	UK	1351.2272	CABGYG000000000
			4928STDY7071723	2018	Human colonization	UK	1351.2284	CABGZY000000000
			4928STDY7071736	2018	Human colonization	UK	1351.2298	CABHAI000000000
			4928STDY7071738	2018	Human colonization	UK	1351.2306	CABHAQ000000000
			4928STDY7071753	2018	Human colonization	UK	1351.2308	CABHBB000000000
			4928STDY7071754	2018	Human colonization	UK	1351.2317	CABHAW000000000
			4928STDY7071769	2018	Human colonization	UK	1351.2326	CABHBQ000000000
			4928STDY7387716	2018	Human colonization	UK	1351.2350	CABHDL000000000
			4928STDY7387750	2018	Human colonization	UK	1351.2364	CABHEG000000000
			4928STDY7387749	2018	Human colonization	UK	1351.2365	CABHEI000000000
			4928STDY7071263	2018	Human colonization	UK	1351.2431	LR607346
			BL-380-WT-3A	2018	Human colonization	Germany	1351.3672	JABAFU000000000
			PF63	2019	Raw frozen pet food	Portugal		PRJEB28327
			N039.H-3	NA	Human colonization (infant)	USA	1351.2073	QYNC01000000
			44.1	NA	NA	Spain	1351.591	NSJR000000000
			13.1	NA	NA	Spain	1351.592	NSJS000000000
			UBA7859	NA	Metal/plastic	USA	1351.655	DLSPO1000000
			PC1.1	NA	Human colonization	Australia	791166.14	ADKN01000000
NA ^b	Tr ^a	+	UBA4546	NA	New York City MTA subway	USA	1351.634	DGLK01000000
Tr ^c	Tr ^a	+	ATCC 27959	1975	Bovine mastitis	USA	1158676.3	AJEX000000000
+	Tr ^a	Tr ^d	RM3817	1960s	Human infection	USA	1158674.3	AJEV000000000
+	+	Tr ^e	ATCC 10100	<1949	NA	NA	1169312.3	ASEA000000000
+	+	+	NCTC2705	1900-1927	Udder of cows	Denmark	1351.1229	UGIW010000000
			ATCC 27275	<1963	NA	NA	1158675.3	AJEW000000000
			7330082-2	2001	Human colonization	Denmark	1151198.3	AIIW000000000
			D1	2001	Pig	Denmark	1169247.3	ASEJ000000000
			D32	2001	Pig faeces	Denmark	1206105.3	CP003726
			C47	2001	Human colonization	Portugal		PRJEB28327
			H188	2002	Human infection	Portugal		PRJEB28327
			H198	2002	Human infection	Portugal		PRJEB28327
			19	2011	Copper fed pig	Denmark	1351.119	JTKW000000000
			TR21_60	2011	Aquaculture	Portugal		PRJEB28327
			TR31_26	2011	Aquaculture	Portugal		GCA_905123745.1
			TR37_41	2011	Aquaculture	Portugal		PRJEB28327
			H4	2014	Beef packing plant and retail meat	Canada	1351.2050	VHRN01000000
			CVM N52755	2014	Ground turkey	USA	1351.873	PTYC01000000
			W97	2015	Beef packing plant and retail meat	Canada	1351.2036	VHQO01000000
			G149	2016	Beef packing plant and retail meat	Canada	1351.2032	VHRX01000000
			CCM27	2016	Human colonization	Portugal		PRJEB28327
			c8Ua_2_AE	2017	Human colonization	Portugal		PRJEB28327
			NBRC 12965	NA	Citrus juice	NA	1351.2018	BJMP010000000
			TX4248	NA	Human infection	USA	749495.4	AEBR000000000

^a Truncated EfrE at amino acid 79. ^b ChlR mutations were not assessed due to sequencing error. ^c Truncated ChlR at amino acid 181. ^d Truncated EfrF at amino acid 392. ^e Truncated EfrF at amino acid 146. The reference strain used was *E. faecalis* V583 [GenBank accession no. AE016830.1; locus-tag EF_2225 (*chlR*), EF_2226 (*efrE*) and EF_2227 (*efrF*)]. Abbreviations: +, complete protein; Tr, truncated protein; NA, not available; UK, United Kingdom; USA, United States of America.

Unraveling *Enterococcus* Susceptibility to Quaternary Ammonium Compounds: genes, phenotypes, and the impact of environmental conditions

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Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions

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ABSTRACT Quaternary ammonium compounds (QACs) have been extensively used in the community, healthcare facilities, and food chain, in concentrations between 20 and 30,000 mg/L. *Enterococcus faecalis* and *Enterococcus faecium* are ubiquitous in these settings and are recognized as nosocomial pathogens worldwide, but QACs' activity against strains from diverse epidemiological and genomic backgrounds remained largely unexplored. We evaluated the role of *Enterococcus* isolates from different sources, years, and clonal lineages as hosts of QACs tolerance genes and their susceptibility to QACs in optimal, single-stress and cross-stress growth conditions. Only 1% of the *Enterococcus* isolates included in this study and 0.5% of publicly available *Enterococcus* genomes carried *qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*, *qrg*, *bcrABC* or *oqxAB* genes, shared with >60 species of Bacillota, Pseudomonadota, Actinomycetota, or Spirochaetota. These genes were generally found within close proximity of antibiotics and/or metals resistance genes. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of benzalkonium chloride (BC) and didecyldimethylammonium chloride ranged between 0.5 and 4 mg/L (microdilution: 37°C/20 h/pH = 7/aerobiosis) for 210 *E. faecalis* and *E. faecium* isolates (two isolates carrying *qacZ*). Modified growth conditions (e.g., 22°C/pH = 5) increased MIC_{BC}/MBC_{BC} (maximum of eightfold and MBC_{BC} = 16 mg/L) and changed bacterial growth kinetics under BC toward later stationary phases in both species, including in isolates without QACs tolerance genes. In conclusion, *Enterococcus* are susceptible to in-use QACs concentrations and rarely carry QACs tolerance genes. However, their potential gene exchange with different microbiota, the decreased susceptibility to QACs under specific environmental conditions, and the presence of subinhibitory QACs concentrations in various settings may contribute to the selection of particular strains and, thus, require a One Health strategy to maintain QACs effectiveness.

IMPORTANCE Despite the increasing use of quaternary ammonium compounds (QACs), the susceptibility of pathogens to these antimicrobials remains largely unknown. *Enterococcus faecium* and *Enterococcus faecalis* are susceptible to in-use QACs concentrations and are not main hosts of QACs tolerance genes but participate in gene transfer pathways with diverse bacterial taxa exposed to these biocides. Moreover, QACs tolerance genes often share the same genetic contexts with antibiotics and/or metals resistance genes, raising concerns about potential co-selection events. *E. faecium* and *E. faecalis* showed increased tolerance to benzalkonium chloride under specific environmental conditions (22°C, pH = 5), suggesting that strains might be selected in settings where they occur along with subinhibitory QACs concentrations. Transcriptomic studies investigating the cellular mechanisms of *Enterococcus* adaptation to QACs tolerance, along with longitudinal metadata analysis of tolerant populations dynamics under the

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influence of diverse environmental factors, are essential and should be prioritized within a One Health strategy.

KEYWORDS Bacillota, biocide, disinfection, One Health

Enterococcus spp. are major opportunistic pathogens of humans and animals that easily spread through contaminated abiotic surfaces, enhancing the risk of transmission between different hosts (1, 2). They are widely distributed in various environments, including foods, water systems, and soil and serve as indicators of fecal contamination due to their ability to withstand harsh conditions and different stresses. Additionally, they are known to engage in horizontal genetic transfer with local microbiota (1–3). Despite the significant increase in the usage of biocides, such as quaternary ammonium compounds (QACs), in recent years, and their key role in controlling the spread of pathogens, the susceptibility of major opportunistic pathogens, including *Enterococcus* spp., to these antimicrobials remains largely unknown (4, 5).

QACs are cationic biocides used extensively as disinfectants, antiseptics, and preservatives in human and veterinary healthcare facilities, food production settings, and consumer products (e.g., hygiene products, eye drops, and mouthwashes) (5–7). Among the most used are benzalkonium chloride (BC) and didecyldimethylammonium chloride (DDAC) (5, 8). Their action may be bacteriostatic or bactericidal depending on the concentration used, and it involves the loss of the membrane's physical and ionic integrity, with leakage of cytoplasmic components, osmotic dysregulation, inhibition of respiratory enzymes and transport, and oxidative stress (6, 8, 9). In-use concentrations of QACs range from 20 to 30,000 mg/L (7, 10). Diverse concentration gradients (less than 0.001 to 6 mg/L) have also been found in sewage and surface water and soil/sediments, after domestic, hospital, and industrial (including food processing) discharges, either directly or via wastewater treatment systems, followed by dilution in the environment and biodegradation (6, 7, 11). Insufficient cleaning prior to disinfection or inadequate application of the biocide (e.g., dosage error, excess of organic matter, or the use of cotton and microfiber cloths that greatly reduce QACs concentration) in the clinical or food processing settings may also contribute to QACs use in subinhibitory concentrations in these particular contexts (8, 12). Thus, diverse microbiomes are often exposed to a wide range of QACs concentrations that may result in changes in their taxonomic composition and promote the selection of populations with decreased susceptibility to QACs and other antimicrobial compounds (biocides or antibiotics) by a co- or cross-selection process (7, 13–16). When the in-use concentrations of a biocide remain higher than the minimum inhibitory concentrations (MIC) or minimum bactericidal concentrations (MBC) observed *in vitro*, a decreased susceptibility to biocides is often described as “tolerance” (the term used in this manuscript) (17–19). On the contrary, the term “resistance” implies that the microorganisms are not inactivated by the in-use concentrations of a biocide (17–19). Tolerance to QACs is mainly associated with the acquisition or overexpression of genes encoding efflux pumps, although adaptive tolerance mechanisms such as changes in the cell membrane composition have also been reported (6, 19).

QACs efflux determinants belonging to the MFS (major facilitator superfamily; e.g., *qacA/B*), SMR (small multidrug resistance; e.g., *qacC*, *qacZ*, *qrg*, *bcrABC*), and RND (resistance nodulation division; e.g., *oqxAB*) families have been detected in *Enterococcus* spp. (20–26). However, few studies have addressed their susceptibility and genomic adaptation to QACs (10, 20, 21, 26, 27), especially within a comprehensive context of *Enterococcus* population structure, sources, and time spans. In fact, the role of QACs tolerance genes on *Enterococcus* adaptation to these compounds remains elusive due to contradictory data regarding the increase of QACs tolerance in strains carrying such genes (20–22, 24). This discrepancy can be attributed, in part, to the absence of standardized methodologies and universally accepted epidemiological cutoffs (28). Of note, most studies assessing the susceptibility of *Enterococcus* and other bacteria

to QACs typically conduct tests under ideal standardized growth conditions. Thus, the impact of various physicochemical challenges present in diverse natural ecosystems, such as pH, temperature, and other pollutants, remains largely unknown (29).

In this study, we aimed to assess the occurrence of known transferable QACs tolerance genes in *Enterococcus* spp. isolates and public genomes and their flow between *Enterococcus* and other bacterial taxa. Also, we evaluated the activity of BC and DDAC in different growth conditions, among a collection of phylogenetically diverse *E. faecalis* and *E. faecium* from different sources, time spans, and antibiotic resistance profiles.

RESULTS

Enterococcus carry broad-host QACs tolerance genes widely spread across different bacterial taxa

The presence of QACs tolerance genes was investigated in *Enterococcus* from diverse epidemiological backgrounds, including 210 *Enterococcus* isolates from human and non-human sources (105 *E. faecium* and 105 *E. faecalis*) and 22,428 genomes publicly available at the NCBI database.

Only 1% of the isolates, which included one ST17 *E. faecium* and one ST6 *E. faecalis* carrying the *qacZ* gene, were found to carry QACs tolerance genes. Additionally, among the public genomes analyzed, the presence of QACs tolerance genes (*qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*, *qrg*, *bcrABC*, and *oqxAB*) was observed in only 0.5% of the genomes ($n = 117$), which encompassed 93 *E. faecalis*, 23 *E. faecium*, and 1 *Enterococcus lactis* genomes (Fig. 1; Table S1). The isolates ST17 *E. faecium*-E241 and ST6 *E. faecalis*-V583 were collected from a hospital sewage (2002) and a hospitalized patient (1987), respectively (21, 30). The 117 *Enterococcus* isolates from public genomes were collected between 1987 and 2020 and belonged to diverse clonal lineages (30 STs among 93 *E. faecalis*; 13 STs among 23 *E. faecium*) and sources (Table S1). Among these, the *qac* and *qrg* genes were mostly detected in humans, including clinical and surveillance isolates (97%, $n = 69/71$, among isolates with identifiable sources) ($P \leq 0.01$), whereas *bcrABC* and *oqxAB* were more prevalent in *Enterococcus* strains from the food chain environment (68%, $n = 28/41$) ($P \leq 0.01$) (Fig. 1; Table S1).

QACs tolerance genes identical to the ones found in *Enterococcus* strains were shared with bacterial species from Bacillota, Pseudomonadota, Actinomycetota, and Spirochaetota (former Firmicutes, Proteobacteria, Actinobacteria, and Spirochaetes, respectively) phyla (Fig. 1) (32). The *qacA/B*, *qacC*, and *qacJ* were detected in 20 genera (20, 38, and 4 species, respectively), mainly in *Staphylococcus* spp. (97%, $n = 2,691/2,777$, among bacteria carrying *qac* genes) ($P \leq 0.01$) and particularly in *S. aureus* (58%, $n = 1,565/2,691$ of the *Staphylococcus* genomes carrying *qac* genes) (Fig. 1). The predominant genera sharing an identical *qrg* with *Enterococcus* strains was *Streptococcus* sp. (98%, $n = 183/187$) ($P \leq 0.01$), among the five genera (14 species) in which the gene was found. The *qacG* and *qacZ* were only found in *Enterococcus*, according to our sequence selection criteria. As observed for *Enterococcus* isolates, most strains carrying *qac* and *qrg* genes had a human origin (88%, $n = 2,387/2,699$, among isolates with identifiable sources) ($P \leq 0.01$) (Fig. 1). The *bcrABC* gene cluster was identified in six genera (10 species), and *oqxAB* gene cluster was identified in four genera (five species), mainly in *Listeria monocytogenes* (97%, $n = 2,975/3,060$, among those carrying *bcrABC*) ($P \leq 0.01$) and *Escherichia coli* (78%, $n = 80/102$, among those carrying *oqxAB*) ($P \leq 0.01$), respectively (Fig. 1). Of note, *bcrABC* and *oqxAB* were, as mentioned above for enterococci, more prevalent in food chain isolates (84%, $n = 2,645/2,942$) ($P \leq 0.01$) (Fig. 1).

Genetic contexts of QACs tolerance genes are diverse and enriched in antimicrobial resistance genes

The co-occurrence of QACs tolerance genes with other antimicrobial resistance genes (e.g., antibiotics, metals) in the same genetic contexts and the similarity of these QACs

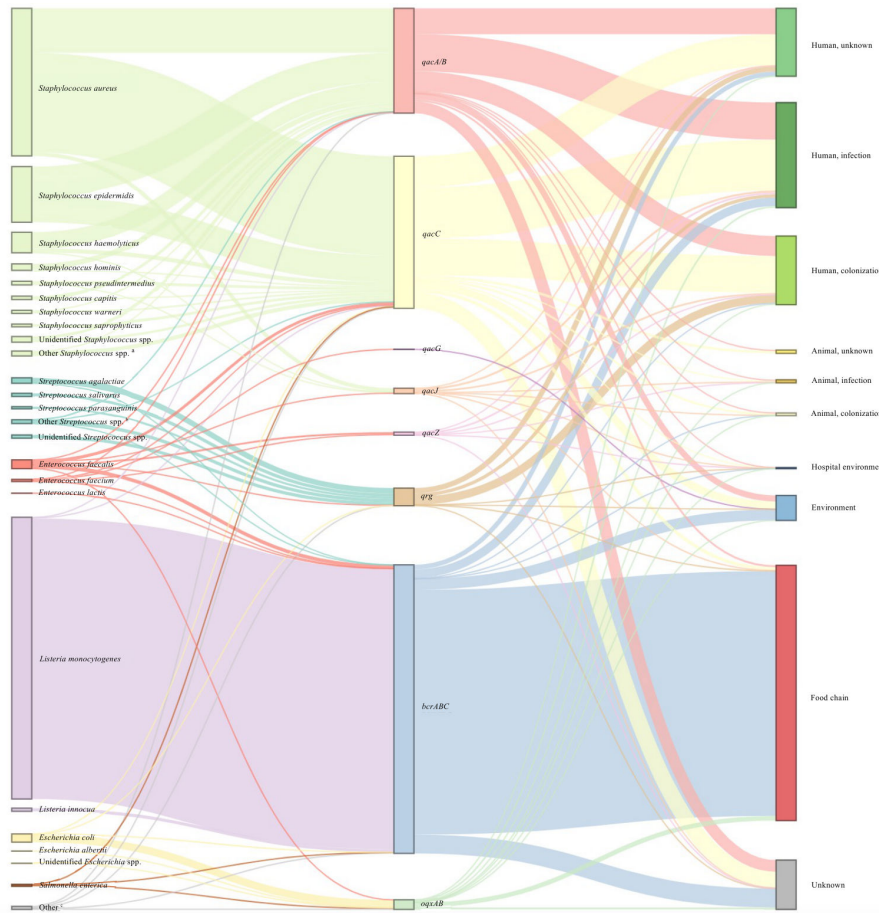


FIG 1 Sankey diagram showing the distribution of QACs tolerance genes found among *Enterococcus* spp. and shared with other bacterial taxa. Using the blastp tool, the following genes were searched in *Enterococcus* spp. genomes and proteins available at the NCBI database: *qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*, *qrg*, *bcrABC*, *oxqAB* (GenBank accession no. [HE579074.1](#), [GQ900434.1](#), [Y16944.1](#), [NG_048046.1](#), [KM083808.1](#), [HQ663849.2](#), [NZ_CP011399.1](#), [KT716391.1](#), respectively). Sequences with $\geq 90\%$ of query-cover and $\geq 97\%$ identity were considered. Identical proteins (100% coverage and identity) to the ones identified were then searched in other NCBI published bacterial taxa genomes and proteins in order to infer about the genetic flow of these genes. The diagram was constructed in R using the package networkD3 v0.4 (31). ^aOther *Staphylococcus* spp.: *Staphylococcus argenteus*, *Staphylococcus arlettae*, *Staphylococcus borealis*, *Staphylococcus caprae*, *Staphylococcus cohnii*, *Staphylococcus croceilyticus*, *Staphylococcus equorum*, *Staphylococcus lugdunensis*, *Staphylococcus massiliensis*, *Staphylococcus pasteurii*, *Staphylococcus petrasii*, *Staphylococcus pettenkoferi*, *Staphylococcus schleiferi*, *Staphylococcus simulans*, *Staphylococcus singaporensis*. ^bOther *Streptococcus* spp.: *Streptococcus anginosus*, *Streptococcus australis*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus pyogenes*, *Streptococcus vestibularis*. ^cOther: *Acinetobacter baumannii*, *Burkholderia cepacia*, *Campylobacter jejuni*, *Carnobacterium divergens*, *Clostridioides difficile*, *Cutibacterium acnes*, *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, *Leptospira noguchii*, *Mammaliococcus sciuri*, *Mycobacteroides abscessus*, *Oenococcus oeni*, *Providencia alcalifaciens*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Shinella curvata*, *Streptomyces* sp., *Vibrio cholerae*.

tolerance genetic contexts between *Enterococcus* and other bacterial taxa were evaluated.

Considering the few complete genomes included in the analysis for the different taxa, namely of *Enterococcus* spp., most QACs tolerance genes were found on plasmids of different sizes ($n = 18/19$; 2–129 kb) rather than in the chromosome ($n = 1/19$) (Fig. S1). For the most part, the genetic contexts of QACs tolerance genes studied

presented high variability among the different bacterial taxa (Fig. S1-A-G), with exception of some isolates sharing few genes. This was the case for *E. faecium* C132 and *Staphylococcus aureus* CC1-1 or *Staphylococcus warneri* Ani-LG-025, sharing the *qacA/B* and beta-lactam resistance genes, or *E. faecalis* C32 and *Staphylococcus capitis* 15–101 or *Staphylococcus massiliensis* P3, sharing the *qacA/B* and copper tolerance genes (Fig. S1-A), among others. *Enterococcus* spp. from the clinical and environmental settings shared *qacZ* genetic contexts carrying *aac(6)-Ie-aph(2)-Ia*, coding for aminoglycoside resistance, insertion sequences, and recombinases (Fig. S1-D). Also, *qrg* genetic contexts were shared between *E. faecalis* and *Streptococcus* spp., mostly presenting insertion sequences, recombinases, and hypothetical proteins (Fig. S1-E). Finally, *E. faecalis* from different epidemiological backgrounds shared *bcrABC* genes, insertion sequences, genes coding for recombinases, hypothetical proteins, or bacteriocin associated (Fig. S1-F).

Regardless of the strains' source, geographical region, or date of isolation, we observed that different genetic determinants for antibiotic resistance and metal tolerance were located adjacent to QACs tolerance genes. Of note, several of the genetic contexts analyzed ($n = 32$) in diverse bacteria, including *Enterococcus* spp., harbored genes conferring resistance to aminoglycosides, beta-lactams, macrolides, lincosamides, streptogramin B, mupirocin, bleomycin, tetracycline, chloramphenicol/florfenicol, trimethoprim, fosfomycin, and/or sulfonamide (Fig. S1-A,B,C,D,F,G). Metal tolerance genes, namely to copper, cadmium, zinc, or arsenic, were also detected within the vicinity of *qacA/B*, *qacC*, *qacJ*, *qrg*, and *bcrABC* genes (Fig. S1-A,B,C,E,F). Additionally, the genetic contexts analyzed were highly enriched in insertion sequences, recombinases, and replication associated proteins.

QACs susceptibility assays of *E. faecalis* and *E. faecium*

The susceptibility of 105 *E. faecalis* and 105 *E. faecium* isolates (including two isolates carrying *qacZ*), with diverse epidemiological and clonal backgrounds (Table S2), to BC and DDAC was determined.

The MIC_{BC} in standard conditions were similar for the *E. faecalis* and *E. faecium* studied (MIC₅₀ = 2 mg/L and MIC₉₀ = 2 mg/L for both) (Fig. 2). The highest MIC_{BC} of 4 mg/L was observed in 1 *E. faecalis* and 9 *E. faecium* recovered from different sources, years, and clonal lineages. Of these, most were MDR ($n = 8/10$; resistant to three or more antibiotics from different families), and two of them harbored the gene *qacZ* (ST17 *E. faecium*-E241 and ST6 *E. faecalis*-V583). MBC_{BC} distributions for both species also showed similar MBC₅₀ = 2 mg/L and MBC₉₀ = 4 mg/L (Fig. 2). Likewise, the 43% ($n = 45/105$) of *E. faecalis* and 19% ($n = 20/105$) of *E. faecium* isolates showing the highest MBC_{BC} of 4 mg/L comprised, in both cases, isolates from different epidemiological and genetic backgrounds. On the contrary, *E. faecium* ($n = 8$) with the lowest MBC_{BC} of 1 mg/L were mostly from the food chain ($n = 7/8$), belonging to different STs and years. The value of MIC_{BC} and MBC_{BC} of the control strain *E. faecalis* ATCC 29212 varied between 1–2 mg/L and 2–4 mg/L, respectively.

Both species were susceptible to lower concentrations of DDAC compared to BC ($P \leq 0.01$) (Fig. 2). For DDAC, the MIC₅₀ was 1 mg/L for both species, and the MIC₉₀ was 1 mg/L or 2 mg/L for *E. faecalis* and *E. faecium*, respectively (Fig. 2). Strains with an MIC_{DDAC} of 2 mg/L ($n = 8$ *E. faecalis* and $n = 29$ *E. faecium*) were diverse and comprised most of the *Enterococcus* with the highest MIC_{BC} ($n = 7/10$, including the two isolates carrying *qacZ*). Finally, the MBC₅₀ was 2 mg/L for both species, and the MBC₉₀ was 4 mg/L and 2 mg/L for *E. faecalis* and *E. faecium*, respectively. Again, isolates showing the highest MBC_{DDAC} of 4 mg/L or the lowest MBC_{DDAC} of 1 mg/L (18%, $n = 19/105$, and 4%, $n = 4/105$, of the *E. faecalis*; 4%, $n = 4/105$, and 23%, $n = 24/105$, of the *E. faecium* populations tested, respectively) were associated with different sources, years, and STs. The MIC_{DDAC} and MBC_{DDAC} of the control strain *E. faecalis* ATCC 29212 varied between 0.5–1 mg/L and 1–2 mg/L, respectively.

Considering all sources, *E. faecalis* seem to be more tolerant to bactericidal concentrations of the QACs tested (higher MBC) than *E. faecium* ($P \leq 0.01$), although the latter

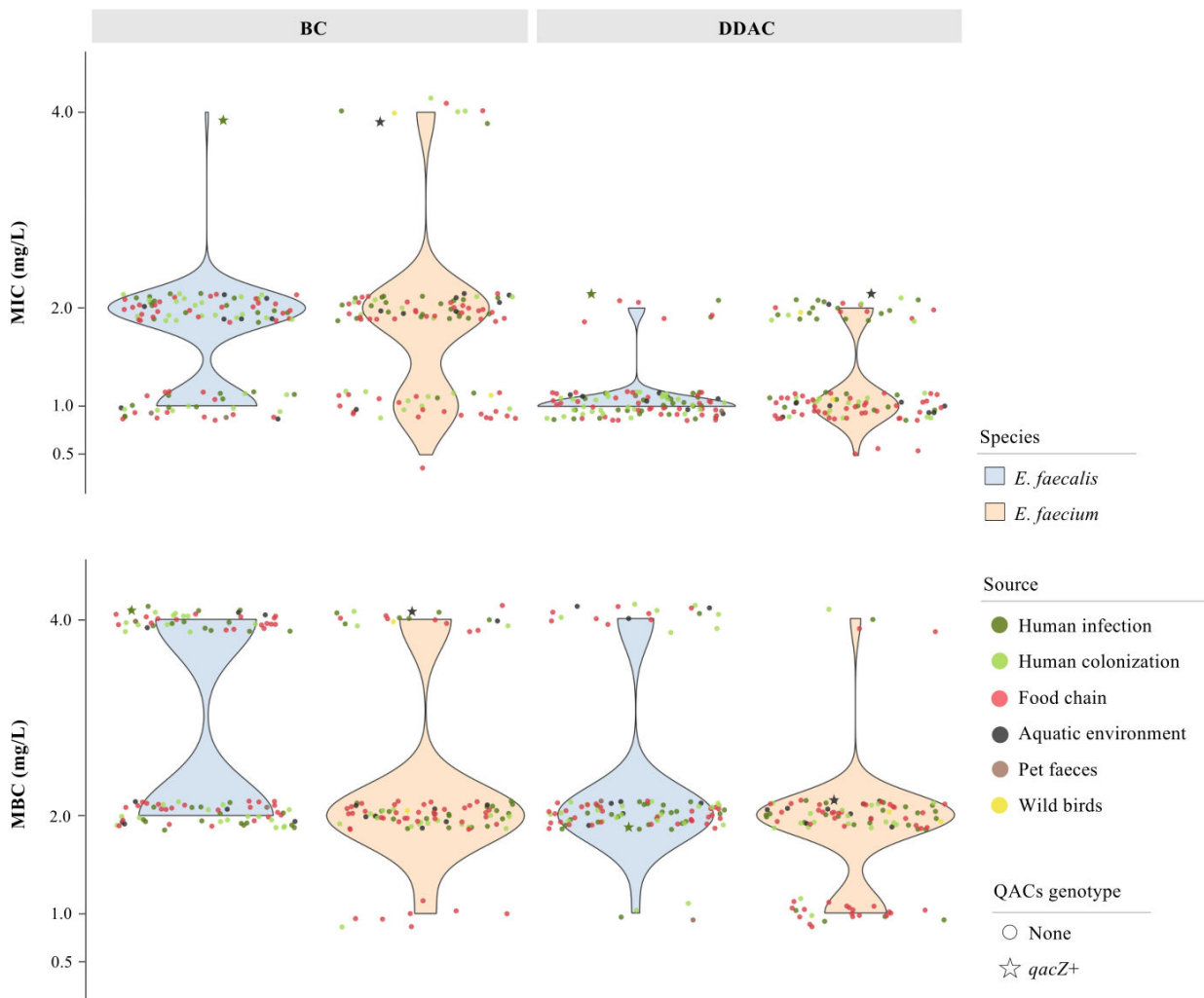


FIG 2 Benzalkonium chloride (BC) and didecyltrimethylammonium chloride (DDAC) minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) distribution of *Enterococcus faecalis* (blue) and *Enterococcus faecium* (orange) isolates from diverse epidemiological backgrounds. MIC were established by broth microdilution in standard growth conditions [Mueller-Hinton broth (MHB); pH = 7.2; 37°C/20 h], followed by MBC determination, using the methodological approach proposed by the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing (33, 34). The source and QACs genotype of each isolate are indicated with different colors and shapes, respectively. For more isolates' details see Table S2. Graphics were done in R, using the ggplot2 package v3.4.0 (35).

presented a significantly higher MIC_{DDAC} ($P \leq 0.01$) (Fig. 2). The MIC and MBC distributions of the isolates tested were analyzed separately by source and time span (5-year intervals) (Fig. S2), with the following significant differences among them: the MIC_{DDAC} and MIC_{BC} were higher for *E. faecium* recovered from human infections compared to those from the food chain ($P \leq 0.01$), but a significant increasing trend in the MIC_{BC} over the years was detected in *E. faecium* isolates from the food chain ($P \leq 0.01$) (Fig. S2). QACs susceptibility among MDR and non-MDR *E. faecalis* and *E. faecium* was similar ($P > 0.01$) (Fig. S3). BC and DDAC MIC and MBC values for vancomycin- or linezolid-resistant isolates varied within the ranges described for the whole population (Fig. S3).

Different growth conditions occurring in the environment affect *Enterococcus* spp. susceptibility to QACs

To assess the activity of QACs on *Enterococcus* growth or survival under diverse environmental conditions that mimic real contexts, MIC_{BC} and MBC_{BC} were determined in anaerobic conditions (e.g., occurring in sewage) (37, 38), room temperature (22°C; representing abiotic surfaces in healthcare or agri-food sectors) (39), and mildly acidic pH (pH = 5; resembling human skin and fresh fruits/vegetables contaminated with QACs residues) (40–42). BC was the QAC chosen for the susceptibility assays with modified conditions, as both species demonstrated higher tolerance and phenotype variability (wider MIC and MBC ranges) to this compound compared to DDAC under standard conditions. MIC_{BC} and MBC_{BC} distributions for the modified conditions studied are shown in Fig. 3.

The analysis revealed similar results between aerobic (standard) and anaerobic conditions ($P > 0.01$), but increased tolerance to BC at room temperature (22°C) and/or mildly acidic pH (pH = 5) conditions for *E. faecalis* and *E. faecium* strains ($P \leq 0.01$) (Fig. 3). In anaerobic conditions, MIC_{BC} or MBC_{BC} increased by no more than twofold in a few strains (six *E. faecalis* and six *E. faecium*) belonging to diverse sources, dates, and STs. The MBC_{BC} of *qacZ* + ST17 *E. faecium* E241 increased from 4 to 8 mg/L, outside the range obtained in standard conditions. Also, the MIC_{BC} and MBC_{BC} increased from 4 to 8 mg/L in a human *E. faecium* strain (ST412), recovered from a clinical infection in 2011, that did not contain any of the known QACs tolerance genes.

Contrastingly, four to eightfold MIC_{BC} and MBC_{BC} increases were observed at 22°C and/or pH = 5 conditions in diverse strains of both species. When isolates were tested at pH = 5, the MIC_{BC} increased significantly for *E. faecalis* ($P \leq 0.01$) but not for *E. faecium* ($P > 0.01$), although MBC_{BC} were higher for both ($P \leq 0.01$) (Fig. 3). The MIC_{BC} of one *E. faecalis* increased fourfold (from 1 to 4 mg/L; urban wastewater treatment plant from Tunisia, ST23, 2014) and the MIC_{BC} of the *qacZ* + *E. faecalis* V583 increased from 4 to 8 mg/L. The highest MBC_{BC} obtained among the investigated *E. faecalis* strains was also 8 mg/L, observed in nine strains exhibiting a twofold ($n = 8$; from several sources and STs) or fourfold rise ($n = 1$; poultry carcass, ST843, 2018). In the case of *E. faecium* isolates, the highest MBC_{BC} at pH = 5 was 16 mg/L, corresponding to *qacZ* + *E. faecium* E241 and one strain isolated from the faeces of a long-term care facility patient in 2016 (ST262). It is noteworthy that these strains exhibited an MBC_{BC} of 4 mg/L under standard conditions.

An increased tolerance to BC was more pronounced at 22°C than at pH = 5 for *E. faecium*, reflected both in MIC_{BC} ($n = 3$ isolates with fourfold and $n = 2$ isolates with eightfold increases at 22°C, compared to standard conditions) and MBC_{BC} ($n = 6$ isolates with fourfold and $n = 2$ isolates with eightfold increases at 22°C) values. The highest MBC_{BC} of 16 mg/L was detected in two food chain isolates (ready-to-eat salad and poultry carcass, ST12 and ST352, 2010–2019), while the remaining strains had an MBC_{BC} of 8 mg/L ($n = 18$). Among *E. faecalis*, in all but one of the isolates, the MBC_{BC} increased to 8 mg/L [following twofold ($n = 17$) or fourfold ($n = 4$) MBC_{BC} increases] at 22°C, whereas the MIC_{BC} did not change ($P > 0.01$) for most strains ($n = 19$).

The effect of the combination of the test conditions (growth at 22°C and pH = 5) was also studied, excluding anaerobiosis since it did not significantly influence QACs susceptibility. Indeed, higher MIC_{BC} or MBC_{BC} were observed for both species under 22°C and pH = 5 stress than when each condition was tested separately ($P \leq 0.01$). MBC_{BC} increased from 1 to 4 mg/L (standard conditions) to 8 mg/L at 22°C (93%; $n = 21/22$ *E. faecalis* and $n = 18/20$ *E. faecium*) and 16 mg/L (100%; $n = 7/7$ *E. faecalis* and $n = 7/7$ *E. faecium*) for the combination of 22°C and pH = 5 for most isolates, regardless of the species, epidemiological or genetic background. MIC_{BC} also reached the highest values of 16 mg/L in four *E. faecium* strains from human infection and colonization, hospital sewage (*E. faecium* ST17 carrying *qacZ*), and food chain (2002–2020), and of 8 mg/L in four *E. faecalis* from human infection ($n = 2$, including an ST6 strain carrying *qacZ*), food chain, and environmental origins (1987–2019).

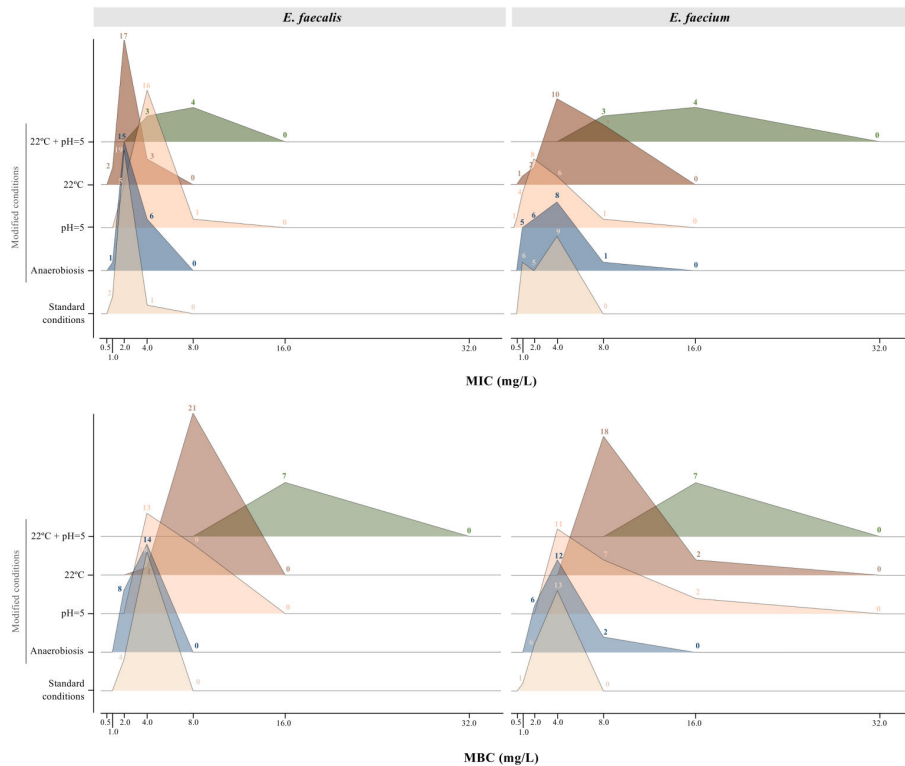


FIG 3 Ridgeline chart depicting the distribution of benzalkonium chloride minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) determined in standard or modified conditions. The non-standard conditions tested were anaerobiosis, room temperature (22°C), or mildly acidic pH (pH = 5) for 22 *Enterococcus faecalis* and 20 *Enterococcus faecium* isolates and a combination of 22°C and pH = 5 for 7 strains of each species. For more isolates' details see Table S3. Graphics were built using the ggridges R package v0.5.4 (36).

Two *E. faecalis* (V583 with *qacZ*; S37-25 without *qacZ*) and two *E. faecium* (E241 with *qacZ*; F1651 without *qacZ*) were included in kinetic assays, showing changes in their growth dynamics under the modified conditions studied compared with the standard ones (Fig. S4 and S5). Such modifications in the growth curves were strain specific, with *E. faecalis* isolates showing more similarities among each other compared to the *E. faecium* isolates. This was observed in the kinetic assays conducted both with subinhibitory concentrations of BC (0.5 mg/L for *E. faecium* F1651 and 1 mg/L for *E. faecium* E241, *E. faecalis* V583, and S37-25) as well as without BC (Fig. 4; Fig. S4 and S5). Comparing the growth kinetics of each strain under BC plus modified conditions with the standard ones (Fig. 4), different bacterial adaptations were observed, including extended lag phases and, in several cases, slower exponential growth, resulting in a delayed entry into the stationary phase. Also, for the *E. faecium* F1651 and the two *E. faecalis*, their growth with BC plus 22°C or 22°C + pH = 5 surpassed that occurring in standard conditions during the time of the assay, which may suggest that these modified conditions are better for bacterial multiplication under BC stress.

Specifically, at pH = 5 or 22°C, we described earlier that both *E. faecium* and *E. faecalis* increased their MBC_{BC} (two to eightfold) compared to standard conditions, which may be explained by the longer exponential growth phase in such modified conditions (Fig. 4). Additionally, the highest MBC_{BC} increase observed for *E. faecium* (eightfold) may be related to their exponential growth for even longer periods compared to *E. faecalis*, which enter much earlier into the stationary phase (<20 hours) (Fig. 4). Of note, for the combination of 22°C + pH = 5 conditions, growth kinetics for all *E. faecium* and *E. faecalis*

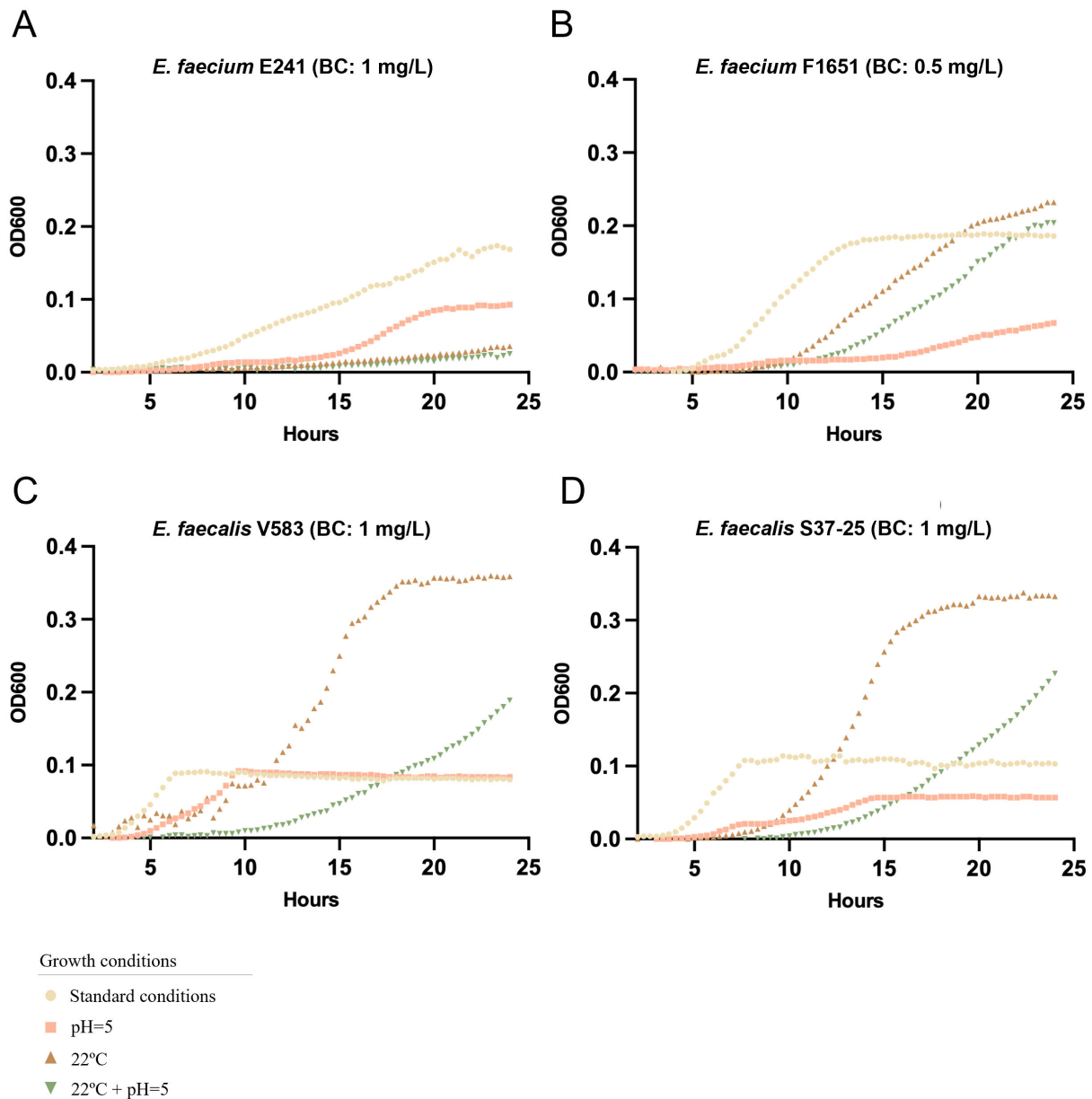


FIG 4 Growth kinetics of two *Enterococcus faecium* and two *Enterococcus faecalis* in the presence of subinhibitory concentrations of benzalkonium chloride [1 mg/L for *E. faecium* E241 with *qacZ* (A), *E. faecalis* V583 with *qacZ* (C), and *E. faecalis* S37-25 without *qacZ* (D) and 0.5 mg/L for *E. faecium* F1651 without *qacZ* (B)] in standard (light-yellow circles) or modified conditions. The non-standard conditions tested were 22°C (light pink squares), mildly acidic pH = 5 (brown triangles) and a combination of 22°C and pH = 5 (inverted green triangles). For more isolates' details see Table S3. Growth curves were determined using a Biotek Synergy HT plate reader (Marshall Scientific), with absorbance at 600 nm (A600) recorded every 20 minutes for 24 hours. Graphics were built using the Prism software v9.0 (GraphPad Software; www.graphpad.com).

strains included in the analysis have shown that they were still in the exponential multiplication phase after 24 hours. This may explain the higher MBC_{BC} observed for both species under the combination of 22°C and pH = 5 stress than for any other growth condition tested, with all isolates reaching the highest MBC_{BC} of 16 mg/L. No clear

differences were detected in isolates carrying the *qacZ* gene (*E. faecium* E241; *E. faecalis* V583) or not (*E. faecium* F1651; *E. faecalis* S37-25) in any condition tested.

Pre-exposure to BC does not increase *E. faecalis* and *E. faecium* tolerance to the biocide

In order to assess if a pre-exposure to QACs would increase QACs-tolerant phenotypes, we exposed seven *E. faecalis* and seven *E. faecium* strains to subinhibitory concentrations of BC ($0.5 \times \text{MIC}_{\text{BC}}$) at different contact times (3, 5, and 10 minutes), but no change in their susceptibility to BC was observed ($P > 0.01$). This was also the case for the *E. faecium* and *E. faecalis* isolates harboring *qacZ*.

DISCUSSION

This study highlights the increased tolerance of *Enterococcus* spp. to QACs under different environmental stresses, although QACs typical in-use concentrations remain effective in all conditions tested. Furthermore, it shows that *Enterococcus* spp. share known QACs tolerance genes with diverse bacterial taxa within the same ecosystems, despite the low content of QACs tolerance genes among enterococci. These findings show the importance of understanding the complex dynamics of QACs tolerance in microbial communities and provide new opportunities for further exploration in this field.

Despite the increased use of QACs in recent years (4, 5), *E. faecalis* and *E. faecium* from various epidemiological backgrounds remain susceptible to QACs concentrations lower than those recommended for disinfection or preservation, with MIC and MBC distributions for BC and DDAC being consistent with previous reports (10, 20, 22, 27, 43). The observed similar QACs phenotypes in isolates with and without *qacZ* gene, along with supporting published data (20, 22), suggest that QACs tolerance genes may not have a significant impact on the survival of *Enterococcus* spp. under QACs exposure. It is also possible that the standard susceptibility determination methods did not accurately replicate the necessary conditions for the expression of QACs tolerance genotypes. The apparent lack of selective advantage conferred by QACs tolerance genes in *Enterococcus* seems to be also corroborated by their low prevalence among *Enterococcus* isolates, which could be explained by rare horizontal transfer events, lack of stability of the acquired genes or genetic contexts due to, for example, high fitness costs (11). Accordingly, literature mostly supports that *Enterococcus* from diverse sources do not seem to be relevant reservoirs of known QACs tolerance genes (20–22, 43), with few exceptions (24, 25). In addition, we found that pre-exposure to BC did not induce a higher tolerance to this biocide in isolates carrying *qacZ*, which may indicate that the BC concentration and exposure times tested were not able to induce the gene expression of the efflux pump encoded by *qacZ*, that increased expression levels of *qacZ* did not lead to a detectable increase in *Enterococcus* tolerance to BC, or that other gene expression regulatory systems or environmental inducers are needed. In a few studies, bacterial adaptation and higher tolerance to QACs have been observed following repeated exposure to sublethal/increasing concentrations of QACs (44, 45), but QACs tolerance genes were either not considered or detected, making it difficult to draw conclusions about their role on the increased phenotypes.

The main source and bacterial species harboring identical QACs proteins that were found in *Enterococcus* spp. might reflect the respective microbial communities and settings in which specific QACs tolerance genes circulate. For example, the genes *qac* and *qrg*, identified mostly in human *Enterococcus* were, respectively, predominant in *Staphylococcus* and *Streptococcus* isolates mainly associated with human colonization and infection (46–48), whereas *bcrABC* and *oqxAB*, mostly found in *Enterococcus* spp. recovered from the food chain, were largely detected in bacterial species often occurring as food chain pathogens or hygiene indicators, such as *Listeria* spp. and *E. coli* (49–51). The genetic contexts of QACs tolerance genes studied were very diverse in *Enterococcus* and other taxa, probably as a result of a high number of recombination events,

as suggested by the abundance of insertion sequences and recombinases detected. They were often co-located with genes coding for resistance or tolerance to other antimicrobial agents, such as antibiotics or metals, coexisting as selective agents in many ecosystems (52–54), and highlighting the possibility of co- and cross-selection events.

However, phenotypes of reduced susceptibility to QACs have not only been attributed to the acquisition of particular genes. It has been suggested that environmental conditions may contribute to bacterial cellular adaptations protective against the action of biocides (29). Here, when testing bacterial susceptibility to QACs under conditions such as lower temperatures than ideal for growth (e.g., room temperature, as found on many abiotic surfaces) (39) and/or mildly acidic pH (e.g., on human skin or on fruits and vegetables) (40–42), *Enterococcus* tolerance to BC significantly increased and growth kinetics changed, regardless of whether they contained or not known QACs tolerance genes. Environmental stresses can cause cellular modifications in both Gram-positive and Gram-negative bacteria (e.g., changes in membrane composition and fluidity, cellular metabolism), with implications on the activity of cationic compounds, such as QACs (29, 55–57). Although the effects of these stresses on *Enterococcus* spp. susceptibility to QACs are still rather unexplored, in other Bacillota, such as *Listeria* spp. or *S. aureus*, studies have shown that adaptation to acid pH, lower temperature, and anaerobiosis resulted in strains more tolerant to different QACs (58–61). Also, similar findings have been reported for Gram-negative bacteria (55, 62).

While the more tolerant phenotypes detected in this study at pH = 5 and/or 22°C were found to be below the in-use QACs concentrations, the potential implications of environmental stress on biocide efficacy emphasize the importance of conducting susceptibility tests under conditions mimicking those occurring in settings where *Enterococcus* are exposed to QACs. Also, QACs concentrations within or below the MIC and MBC ranges obtained in this study have been detected in wastewater and surface waters (less than 0.001 to 6 mg/L) as well as in various types of food, including fruits and nuts, meat, or dairy products (up to 14.4 mg/kg) (6, 7, 42), and might promote the selection and persistence of particular strains, possibly increased by environmental factors. Moreover, exposure to sublethal concentrations of disinfectants, including QACs, has been associated with an increased horizontal gene transfer via conjugation by upregulating the SOS response, enhancing the membrane permeability and production of reactive oxygen species (63–65).

In conclusion, this study provides a comprehensive phenotypic and genomic analysis of the *E. faecalis* and *E. faecium* tolerance to QACs. Although *Enterococcus* do not seem to be significant hosts of QACs tolerance genes, they can adapt to modified growth conditions occurring in the environment toward more tolerant phenotypes. Further studies, including whole transcriptome analyses, are needed to understand the expression of known QACs tolerance genes and the cellular mechanisms influencing *Enterococcus* adaptation to QACs. Continuous surveillance, namely through longitudinal metadata studies considering the influence of environmental interventions and local physicochemical factors on the dynamics of the microbiota composition in different settings, will also be crucial to assess the selection of tolerant *Enterococcus* populations exposed to varying concentrations of QACs. Such research can offer valuable insights for One Health intervention strategies aimed at preventing future biocide inefficacy, with significant implications for Public Health.

MATERIALS AND METHODS

Epidemiological background of the bacterial isolates studied

A collection of 105 *E. faecium* and 105 *E. faecalis* isolates, representative of different geographical regions, sources, time spans, and genomic backgrounds, was selected for this study (Table S2). They were recovered in previous studies from human infections ($n = 53$), human colonization ($n = 47$), food chain (food-animal production settings, meat of animal origin, and other food products) ($n = 89$), pets ($n = 2$), wild birds ($n = 2$),

and aquatic environment ($n = 17$) samples, in diverse regions (Portugal, Tunisia, Angola, Brazil, Spain, Germany, Canada, United States) and time spans (1996–2020) (Table S2) (66–71). Among them, 73% ($n = 77/105$) of *E. faecium* and 43% ($n = 45/105$) of *E. faecalis* were classified as MDR (resistance to three or more antibiotics from different families), 34% ($n = 36/105$) and 10% ($n = 11/105$) as resistant to vancomycin and 5% ($n = 5/105$) and 4% ($n = 4/105$) to linezolid, respectively (Table S2) (66–71). Clonal relationship was established by multilocus sequence typing (MLST; sequence-type-ST) (72–74) and core genome MLST (cgMLST; Complex Type-CT; Ridom SeqSphere+, version 8.2.0; <https://www.ridom.de/seqsphere>) (75, 76).

Screening of QACs tolerance genes in *Enterococcus* spp. isolates and public genomes of diverse bacterial taxa

Several *qac* genes (*qacA/B*, *qacC*, *qacG*, *qacJ*, *qacZ*), the *qrg*, *bcrABC*, and the *oqxAB* genes (GenBank accession no. [HE579074.1](#), [GQ900434.1](#), [Y16944.1](#), [NG_048046.1](#), [KM083808.1](#), [HQ663849.2](#), [NZ_CP011399.1](#), [KT716391.1](#), respectively), coding for efflux pumps, were searched among the isolates included in the phenotypic assays. For the *E. faecium* ($n = 61$) and *E. faecalis* ($n = 101$) isolates sequenced in previous studies (67, 70, 77, 78), the genetic screening was performed using the MyDBfinder tool available at the Center for Genomic Epidemiology (www.genomicepidemiology.org). In the remaining isolates, the presence of genes associated with tolerance to QACs was identified by PCR, using primers and conditions described previously (21, 23, 79, 80).

Additionally, the frequency and distribution of *Enterococcus* strains with QACs tolerance genes were also assessed among *Enterococcus* genomes ($n = 22,428$, until 28 April 2022; <https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=1350>) and proteins from other collections available at the NCBI database. Amino acid sequences with $\geq 90\%$ of query-cover and $\geq 97\%$ identity were considered. The source of isolates carrying QACs tolerance genes was retrieved from the NCBI and BV-BRC (Bacterial and Viral Bioinformatics Resource Center) databases and their clonality determined at the PubMLST website (72–74).

Also, QACs tolerance proteins variants identified in *Enterococcus* were searched in other NCBI published bacterial taxa genomes ($n = 1,162,672$, until 28 April 2022) and proteins, in order to infer about the genetic flow of QACs tolerance genes. This was performed using the blastp tool (81), by looking at the 100% identical amino acid sequences (coverage and identity) of each of the proteins found in *Enterococcus* strains. The source of the isolates of all taxa carrying such QACs tolerance proteins variants was retrieved from the NCBI Biosample database (<http://www.ncbi.nlm.nih.gov/biosample/>), when available.

Comparative analysis of the genetic contexts of QACs tolerance genes

The synteny and nucleotide identity of the genetic contexts of QACs tolerance genes among *Enterococcus* and other bacterial taxa genomes were analyzed using the BLASTN option of Easyfig v2.2.2 (82) and based on NCBI genome annotations. To assess the genetic context of each QACs tolerance gene, *Enterococcus* strains and one representative species from other bacterial taxa from several sources and time spans were selected for comparison. The criteria used to select the boundaries of the analyzed sequences were the limit of the contigs in which the QACs tolerance genes were located or the presence of genetic elements of interest (e.g., antibiotic resistance, biocides or virulence genes, mobile genetic elements).

QACs susceptibility assays in standard conditions

The MIC_{BC} (BC: CAS 68391–01–5, VWR) and MIC_{DDAC} (DDAC: CAS 7173–51–5, Sigma Aldrich) of the 210 *Enterococcus* isolates were established by broth microdilution, using the methodological approach proposed by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing [Mueller-Hinton broth (MHB); pH

= 7.2; 37°C/20 hours] (33). A 96-well microtiter plate containing serial twofold dilutions of the disinfectant (concentration range of 0.125 to 128 mg/L) was used to assess the susceptibility of bacterial suspensions in log-phase growth (adjusted to reach a final inoculum of 5×10^5 CFU/mL in each well) at 37°C for 20 hours. Microdilution panels were freshly prepared before each assay. The first concentration of QAC without visible growth was considered the MIC (33).

To determine the MBC_{BC} and MBC_{DDAC} , 10 μ L of each well without visible growth from the 96-well MIC plate was incubated onto brain heart infusion (BHI) agar plates at 37°C for 24 hours, as defined by the CLSI (34). The MBC was defined as the lowest QAC concentration for which the number of colonies was equal or less than the rejection value defined by CLSI guidelines, based on the final inoculum of each well confirmed by actual count (34). Each experiment was repeated three to six times, and the MIC/MBC values corresponded to the mean of the determinations. The *E. faecalis* ATCC 29212 strain (without any known QACs tolerance genes) was included as control to guarantee the reproducibility of all assays. The MIC_{50} and the MIC_{90} (minimum concentration of an antimicrobial agent that inhibits the growth of 50% or 90% of the bacterial population, respectively) as well as the MBC_{50} and the MBC_{90} (lowest concentration of an antimicrobial agent that is required to kill 50% or 90% of the bacterial population, respectively) were determined.

QACs susceptibility in modified environmental conditions

QACs assays performed in modified environmental conditions (anaerobiosis, pH = 5, 22°C, or pH = 5 + 22°C) were performed for 20 *E. faecium* and 22 *E. faecalis* (Table S3). The bacterial strains selected were representative of the MIC and MBC distribution ranges in standard conditions, different geographical regions, sources, time spans, antimicrobial resistance profiles, and genomic backgrounds of the initial collections. Modified conditions were initially tested separately (anaerobiosis, pH = 5, 22°C), and subsequently, MIC_{BC} and MBC_{BC} were determined with cross-environmental conditions (pH = 5 + 22°C) for seven *E. faecium* and seven *E. faecalis* (Table S3), based on the results obtained from individual assays. From these 14 isolates, two *E. faecium* isolates (E241 with *qacZ* from hospital sewage and F1651 without *qacZ* from poultry meat, belonging to ST17 and ST12, respectively) and two *E. faecalis* isolates (V583 with *qacZ* from clinical origin and S37-25 without *qacZ* from a ready-to-eat salad, identified as ST6 and ST309) were selected for kinetic assays. These assays, which provide more quantitative information compared to the endpoint measurements used in MIC and MBC determinations, were performed under different growth conditions including standard conditions (37°C; pH = 7.2), pH = 5, 22°C, and pH = 5 + 22°C. BC concentrations tested corresponded to the $0.5 \times MIC_{BC}$ of the lowest MIC_{BC} obtained in the three replicates in standard conditions for each strain. Growth curves were determined using a Biotek Synergy HT plate reader (Marshall Scientific), with absorbance at 600 nm (A600) recorded every 20 minutes for 24 hours. Graphics were built using the Prism software v9.0 (GraphPad Software; www.graphpad.com).

QACs susceptibility after bacteria pre-exposure to BC

The seven *E. faecalis* and seven *E. faecium* strains previously selected for cross-stress testing (including *qacZ* + ST17 *E. faecium* E241 and ST6 *E. faecalis* V583) (Table S3) were pre-exposed to BC. The protocol was designed based on the European standards EN 1276 (83) and EN 13727 (84) and a methodology previously described by Skive et al. (85). Briefly, MHB bacterial suspensions in log-phase of growth were split into two glass tubes and exposed to either $0.5 \times MIC_{BC}$ or to only MHB as a control. The cultures were left in the presence of BC for 3, 5, or 10 minutes at room temperature to determine the effect of usually recommended disinfectant contact times (17, 86, 87). Following exposure, 1 mL of the suspension was added to 9 mL of neutralizer solution prepared according to EN 13727 (84) containing Tween 80 (30 g/L; CAS: 9005-65-6, Sigma Aldrich), sodium dodecyl sulfate (4 g/L; CAS: 151-21-3, VWR), and lecithin (3 g/L; MP Biomedicals).

Cells were centrifuged at $2,500 \times g$ for 10 minutes. Supernatant was removed and cells were resuspended in 3 mL of PBS (phosphate-buffered saline). Cells were centrifuged at $2,500 \times g$ for 10 minutes, and supernatant was removed from the pelleted cells. The washed cells were resuspended in 2 mL of NaCl 0.9% and volume adjusted to reach a final inoculum of 5×10^5 CFU/mL in each well. MIC_{BC} and MBC_{BC} were then determined as described above, in standard conditions, following the CLSI guidelines (33, 34).

The effectiveness and toxicity of the neutralizer were evaluated according to the protocol described by Vali et al. (88). First, neutralizer toxicity was tested by adding 1 mL of neutralizer to a bacterial suspension and left in contact for 5 minutes. Cells were serially diluted to reach a final inoculum of 5×10^5 CFU/mL and incubated in Mueller-Hinton agar plates at 37°C for 24 hours. The number of CFU/mL was compared to a control with NaCl 0.9% replacing the neutralizer. To confirm BC was being quenched effectively by the neutralizer, a bacterial suspension was split into three glass tubes and, in each, was added a mixture containing 1 mL of biocide (at bactericidal concentration) and 9 mL of neutralizer, a mixture of 1 mL biocide (at bactericidal concentration) and 9 mL NaCl 0.9%, or 10 mL NaCl 0.9%. After 5 minutes of contact time, cells were diluted and incubated as previously, and the number of CFU/mL was compared. The results for the effectiveness and toxicity test of the neutralizer are shown in Table S4.

Statistical analysis

The statistical significance of the differences between MIC and MBC distributions of isolates from the diverse sources, time spans, and with disparate antibiotic resistance profiles in standard conditions, as well as differences in MIC_{BC} and MBC_{BC} between standard and modified conditions, were assessed using the Wilcoxon Signed Rank and Mann-Whitney Wilcoxon tests (R Statistical Software, v4.1.2) (89). The statistical analysis of potential associations between QACs tolerance genes found in *Enterococcus* and particular bacterial taxa or sources was performed by Fisher's exact test (Prism software, v9.0, GraphPad Software; www.graphpad.com). *P* values ≤ 0.01 were considered significant.

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Ana P. Pereira, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review and editing | Patrícia Antunes, Formal analysis, Funding acquisition, Writing – review and editing | Paula Bierge, Investigation, Writing – review and editing | Rob J. L. Willems, Formal analysis, Writing – review and editing | Jukka Corander, Formal analysis, Writing – review and editing | Teresa M. Coque, Formal analysis, Writing – review and editing | Oscar Q. Pich, Formal analysis, Writing – review and editing | Luisa Peixe, Formal analysis, Funding acquisition, Supervision, Writing – review and editing | Ana R. Freitas, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review and editing | Carla Novais, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Writing – original draft, Writing – review and editing

DATA AVAILABILITY

Genome sequences used in this study have been deposited in GenBank in previous studies under the BioProject numbers [PRJEB40976](#), [PRJNA663240](#), [PRJNA546230](#), and [PRJNA800622](#).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (supplemental_material.pdf). Tables S1, S2, S3, and S4 and Figures S1, S2, S3, S4, and S5.

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Supplementary material

TABLE S1 – Epidemiological background of all *Enterococcus* spp. strains harboring *qac*, *qrg*, *bcrABC* and *oqxAB* genes published at the NCBI database (until the 28th of April 2021).

Gene(s)	Species	Isolate	ST	Date	Isolation Source	Country	GenBank Acc. Number
<i>qacA/B</i>	<i>E. faecalis</i>	C27	81	2019	Human infection	South Africa	NZ_JADBP000000000
		C32	81	2019	Human infection	South Africa	NZ_JADBP000000000
	<i>E. faecium</i>	C132	761	2019	Human infection	South Africa	NZ_JADBP000000000
<i>qacC</i>	<i>E. faecalis</i>	SF28073 (EnGen0243)	2	2003	Human infection	USA	NZ_AJBC000000000
		4	2	2010	Human colonization	USA	DACBBL000000000
		19	2	2010	Human colonization	USA	DACAXD000000000
		352	2	2010	Human colonization	USA	DACBCW000000000
		1238	2	2011	Human colonization	USA	DACAYD000000000
		1750	2	2011	Human colonization	USA	DACBAN000000000
		31	2	2012	Human colonization	USA	DACAZT000000000
		3448	2	2012	Human colonization	USA	DACBDB000000000
		3447	2	2012	Human colonization	USA	DACBDD000000000
		3366	2	2012	Human colonization	USA	DACAYK000000000
		3367	2	2012	Human colonization	USA	DACAYL000000000
		ERV31	2	NA	Human	NA	NZ_ALZN000000000
		ERV37	2	NA	Human	NA	NZ_ALZO000000000
		ERV129	2	NA	Human colonization	NA	NZ_ALZL000000000
		TX0104	2	NA	Human infection	NA	NZ_ACGL000000000
		BCW_3742	2	NA	NA	NA	DACKBI000000000
		C39	81	2019	Human infection	South Africa	NZ_JADBP000000000
		Merz151 (EnGen0358)	104	2002	Human infection	USA	NZ_ASDS000000000
		VRE33500	254	2018	Human colonization	USA	NZ_JAEIZP000000000
		VRE33417	891	2018	Human colonization	USA	NZ_JAEIUF000000000
		VRE33393	891	2018	Human colonization	USA	NZ_JAEIVA000000000
		VRE33217	891	2018	Human colonization	USA	NZ_JAEIVU000000000
		VRE34953	891	2019	Human infection	USA	NZ_JAEJIO000000000
		VRE32555	891	2017	Human infection	USA	NZ_JAEJYH000000000
		VRE32929	891	2017	Human colonization	USA	DACBVV000000000
		VRE33136	891	2018	Human colonization	USA	NZ_JAEKAL000000000
		VRE33093	891	2018	Human colonization	USA	DACBWK000000000
		72	778	2014	Human colonization	USA	DACAXQ000000000
		ERV81	New	NA	Human	NA	NZ_ALZW000000000
	<i>E. faecium</i>	V138	80	2013	Human	Denmark	DACJDL000000000
		V37	80	2013	Human	Denmark	DACJFD000000000
		V63	117	2013	Human	Denmark	DADXYD000000000
		V1666	203	2016	Human colonization	Denmark	DAFCJN000000000
		V1668	203	2016	Human colonization	Denmark	DAFCJS000000000
		V1418	1297	2016	Human colonization	Denmark	DADQWF000000000
		E41209_2017	NA	2017	NA	Australia	DACECH000000000
<i>qacG</i>	<i>E. faecium</i>	8D1-48	1753	2018	Soil	South Africa	NZ_JAAMS000000000
<i>qacJ</i>	<i>E. faecalis</i>	C30	81	2019	Human infection	South Africa	NZ_JADBP000000000
<i>qacZ</i>	<i>E. faecalis</i>	V583	6	1987	Human infection	USA	NC_004668
		V587 (EnGen0242)	6	1987	Human infection	USA	NZ_AJBB000000000
		NCTC 12203	6	1987	Human infection	UK	NZ_UGIK000000000
		ATCC 51299	6	1993	Human infection	USA	NZ_JSES000000000
		ARL04-446	6	2004	Human infection	New Zealand	NZ_QPTF000000000
		UW4687	6	2004	Human colonization	Germany	DACBDW000000000
		UW7354	6	2008	Human	Portugal	DACBIG000000000
		BSAC-ec1762	6	2008	Human infection	UK	DACBPU000000000
		BSEF-36	6	2014	Human infection	USA	NZ_QAWN000000000
		BSEF-39	6	2014	Human infection	USA	NZ_QAWM000000000
		BSEF-42	6	2014	Human infection	USA	NZ_QAWL000000000
		UAA946 (EnGen0253)	26	1996	NA	USA	NZ_AIZQ000000000
		UAA948 (EnGen0232)	26	1996	NA	USA	NZ_AIZS000000000
		NCTC 12201	28	1986	Human infection	UK	NZ_UGIZ000000000
		BSAC-ec292	28	2002	Human infection	UK	DACBPJ000000000
		BSAC-ec1263	28	2006	Human infection	UK	DACBSK000000000
		CLB21560	28	2017	Human infection	USA	NZ_CP019512
		HC_NS0057	525	2015	Human	Canada	JABRHT000000000
		HC_NS0822	525	2015	Human	Canada	JABRLU000000000
		BSEF-44	New	2014	Human infection	USA	NZ_QAWK000000000

Chapter III – Results and Discussion

TABLE S1 (continued) – Epidemiological background of all *Enterococcus* spp. strains harboring *qac*, *qrg*, *bcrABC* and *oqxAB* genes published at the NCBI database (until the 28th of April 2021).

Gene(s)	Species	Isolate	ST	Date	Isolation Source	Country	GenBank Acc. Number
<i>qacZ</i>	<i>E. faecium</i>	E241*	17	2002	Hospital sewage	Portugal	KM083808
		E7	18	2009	Human infection	USA	DACEKJ000000000
		32	18	2010	Human infection	Russia	NZ_PDFP000000000
		E290	18	2013	Human infection	USA	DACEMY000000000
		E301	18	2013	Human infection	USA	DACEKV000000000
		VRE33148	18	2018	Human colonization	USA	JADUPV000000000
		E178	282	2009	Human infection	USA	DACEIS000000000
		E438	282	2009	Human infection	USA	DACEMD000000000
		E440	282	2009	Human infection	USA	DACEOU000000000
		VRE34923	282	2019	Human colonization	USA	NZ_JADRDG000000000
		E180	736	2010	Human infection	USA	DACZFN000000000
		E0369	872	2015	Human	India	DADMXL000000000
		ISMMS_VRE_1	2272	2011	Human infection	USA	NZ_CP012430
<i>qrg</i>	<i>E. faecalis</i>	DVT_1043	6	2020	Human infection	USA	DAEEVS000000000
<i>bcrABC</i>	<i>E. faecalis</i>	3983	6	2013	Human colonization	USA	DACAYW000000000
		VRE32801	6	2017	Human colonization	USA	JA EKAV000000000
		G138E	21	2016	Beef plant	Canada	VHRY000000000
		FSIS11816003	22	2018	Swine	USA	AAXEIS000000000
		EF72PII	25	NA	Human infection	USA	DACAME000000000
		703-EFLS	40	2012-2013	Human infection	USA	JUWK000000000
		G149	40	2016	Beef plant	Canada	VHRX000000000
		FSIS12029501	40	2020	Catfish	USA	AAXDJB000000000
		BSAC-ec2240	73	2009	Human infection	UK	DACBSF000000000
		H44	76	2015	Beef carcass	Canada	VHRM000000000
		G119	76	2015	Beef processing facility	Canada	JAGLAU000000000
		G90	84	2015	Beef processing facility	Canada	JAGLAW000000000
		FSIS12142132	84	2020	Catfish	USA	ABCEYE000000000
		4928STDY7071666	97	2018	Human colonization	UK	CABGVE000000000
		263EA1	126	NA	Chicken meat	USA	LEPN000000000
		4928STDY7071628	133	2018	Human colonization	UK	CABGUD000000000
		1726	158	2011	Human colonization	USA	DACBAR000000000
		2015	158	2011	Human colonization	USA	DACBCG000000000
		3016	158	2011	Human colonization	USA	DACAZS000000000
		CVM N54595	192	2014	Pork chop	USA	PUAC000000000
		R49	192	2015	Retail beef	Canada	VHRC000000000
		CVM N53420	228	2014	Pork chop	USA	PTYL000000000
		R48	228	2015	Beef processing facility	Canada	JAGLBN000000000
		W100	260	2015	Beef carcass	Canada	VHQU000000000
		R37	260	2015	Retail beef	Canada	VHRE000000000
		R52	260	2015	Retail beef	Canada	VHQY000000000
		R53	260	2016	Retail beef	Canada	VHQX000000000
		CVM N55185	273	2014	Pork chop	USA	PUAM000000000
		CVM N52729	282	2014	Pork chop	USA	PTXY000000000
		4928STDY7071622	290	2018	Human colonization	UK	CABGTR000000000
		G81	624	2015	Beef plant	Canada	VHRU000000000
		EC1251	648	2009	Human infection	UK	DACBSY000000000
		W84	949	2015	Beef carcass	Canada	VHQP000000000
		R5E	New	2014	Retail beef	Canada	VHQW000000000
		G127E	New	2015	Beef plant	Canada	VHRZ000000000
		DVT_872	New	2019	Human infection	USA	DAEEVX000000000
		UBA9870	NA	NA	NA	NA	DQAR000000000
	<i>E. faecium</i>	42EA1	1950	NA	Chicken meat	USA	NZ_LERK000000000
	<i>E. lactis</i>	CVM N54519		2014	Ground beef	USA	NZ_PTZP000000000
<i>oqxAB</i> ¹	<i>E. faecalis</i>	3EF	NA	2010-2012	Pig faeces	China	KT716391
		4EF	NA	2010-2012	Pig faeces	China	KT716392
		5EF	NA	2010-2012	Pig faeces	China	KT727030

¹ - Genes found among published *Enterococcus* spp. proteins and not complete genomes.

* - Strain from our collection.

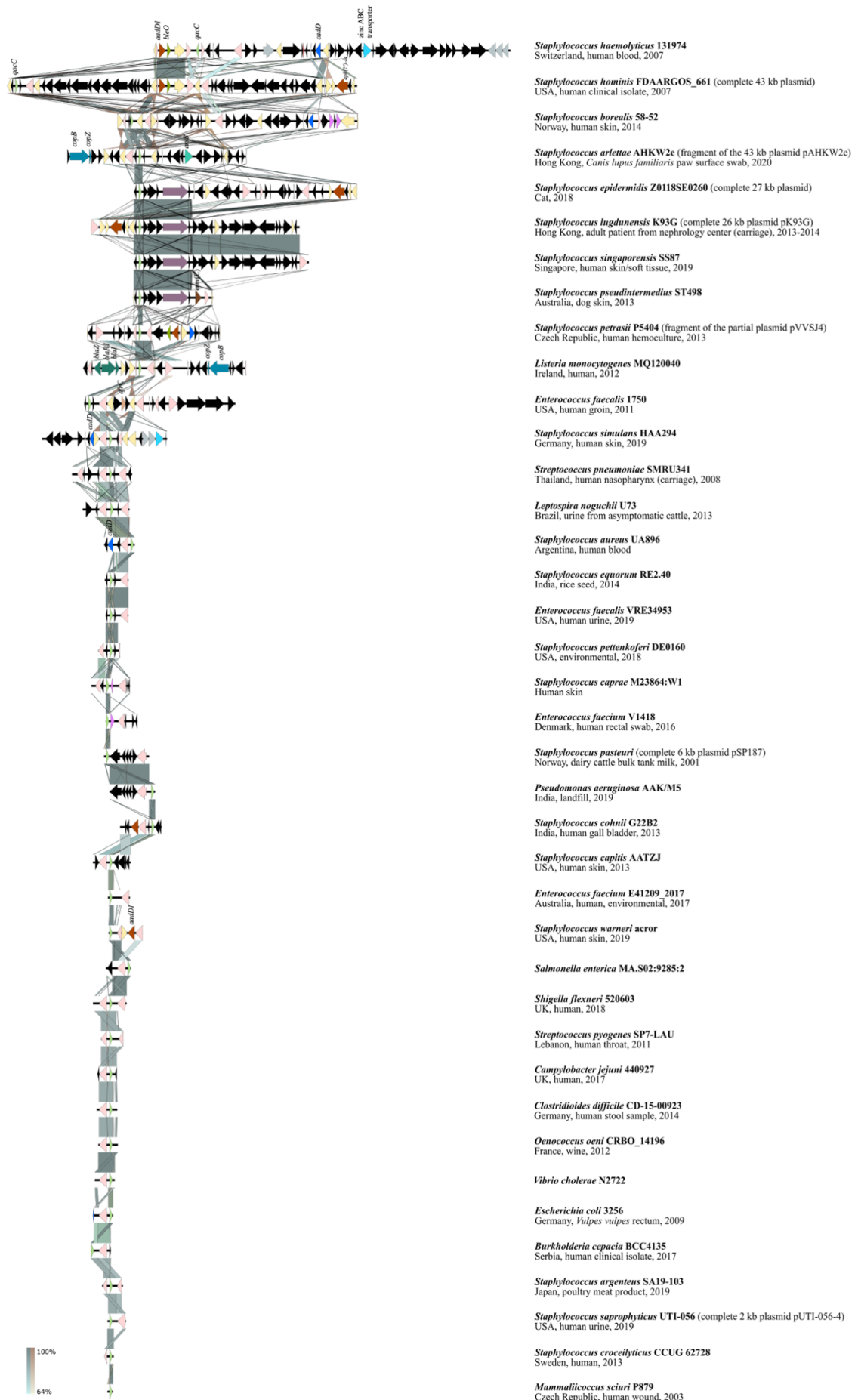
Abbreviations: NA, not available; UK, United Kingdom; USA, United States of America.

A

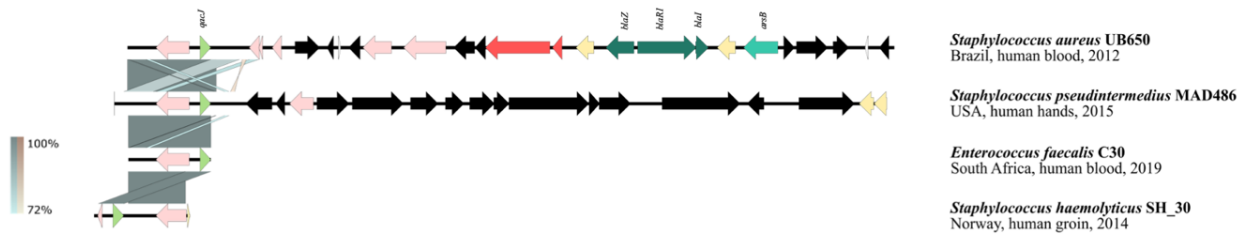


Shigella flexneri 126020
UK, human, 2012

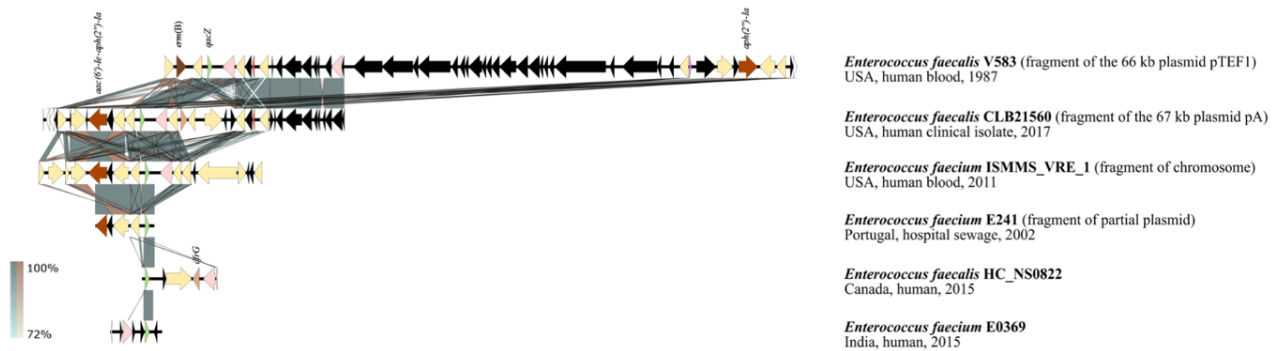
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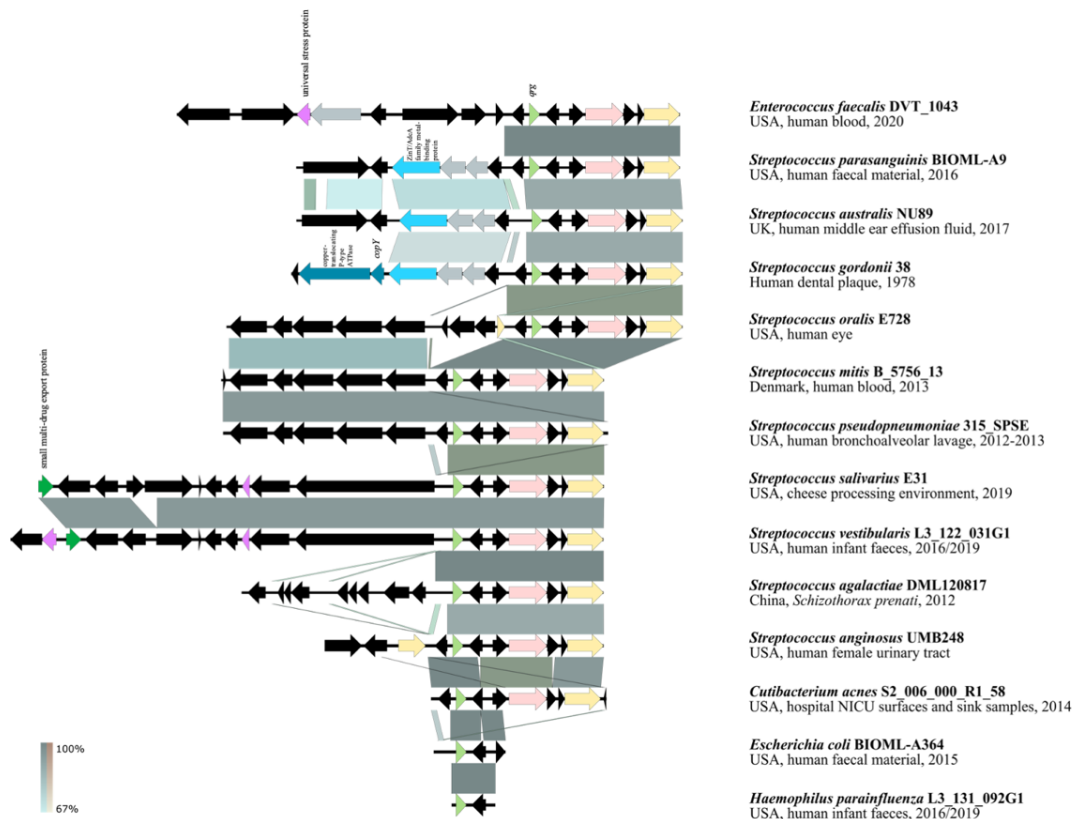
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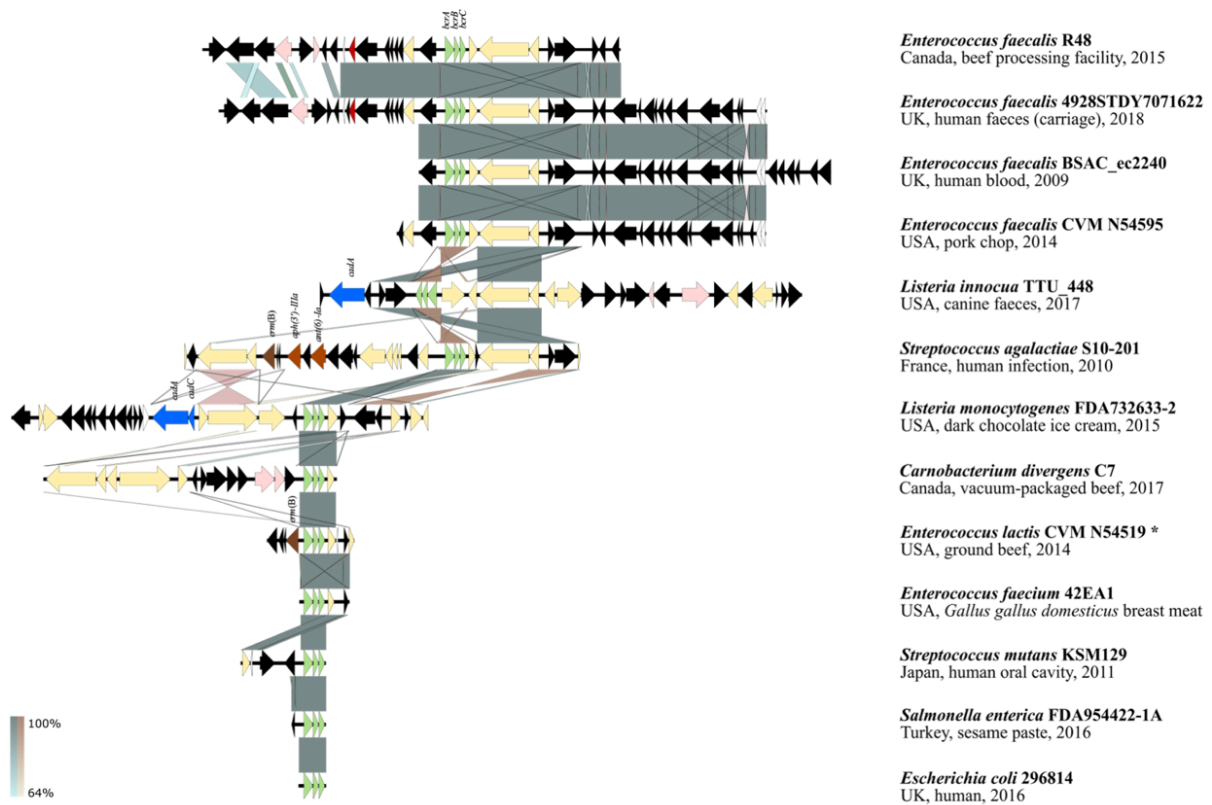
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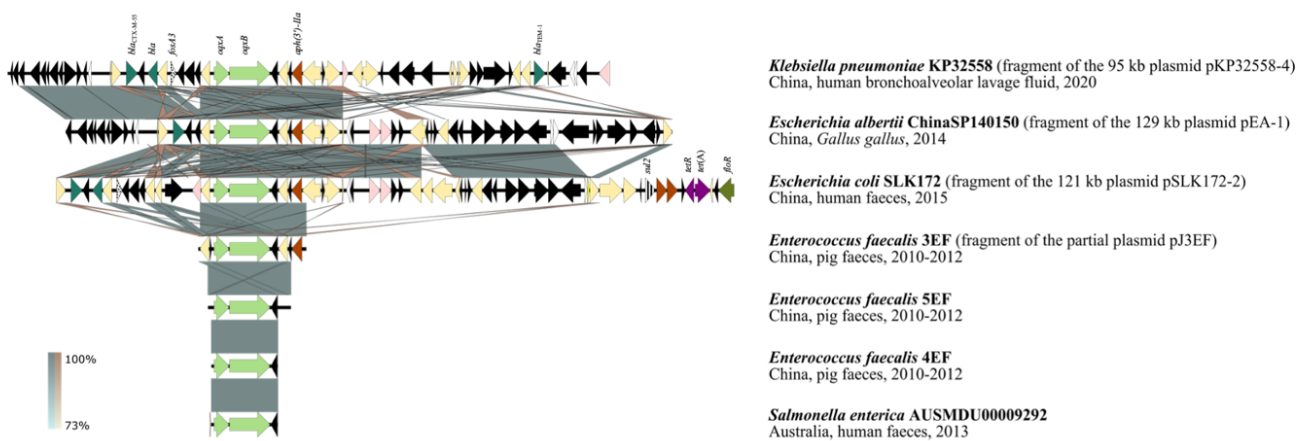
E



F



G



Antimicrobials

Quaternary ammonium compounds tolerance associated genes 

Antibiotic resistance: Aminoglycosides  Beta-lactams  Bleomycin  Chloramphenicol/Florfenicol  Fosfomycin 

Macrolides, Lincosamides and/or Streptogramin B  Mupirocin  Sulfonamides  Tetracyclines 

Trimethoprim 

Metal tolerance: Arsenic  Cadmium  Copper  Zinc  Metal transporter 

Bacteriocin associated 

Multiple functions

UV resistance  Toxin-Antitoxin system  Stress response  Insertion sequence/Recombinase/Integrase/Transposase 

Replication associated 

Figure S1. Diversity of genetic contexts among *Enterococcus* and other bacterial taxa genomes of QACs tolerance genes: A) *qacA/B*; B) *qacC*; C) *qacJ*; D) *qacZ*; E) *qrg*; F) *bcrABC*; G) *oqxAB*. For each gene, *Enterococcus* strains representative of the different sources available were selected as well as one genome corresponding to each of the remaining species, from several sources and timespans. Images were generated using the BLASTN option of Easyfig 2.2.2 (33) and based on NCBI genome annotations.

* For *Enterococcus* strain CVM N54519 the best-matching type-strain, *Enterococcus lactis*, was considered.

Chapter III – Results and Discussion

Table S2 - Epidemiological background of the 105 *E. faecium* and 105 *E. faecalis* included in the QACs susceptibility phenotypic assays.

Species	Origin (n isolates)	Source (n isolates)	Date	Country (n isolates)	ST (n isolates)	CT (n isolates)	MDR
<i>Enterococcus faecium</i> n=105	Humans n=45	Human infection: diverse biological products (26)	1996-2020	Portugal (20), Spain (3), Tunisia (3)	17 (2), 18 (3), 22 (2), 78 (1), 80 (2), 117 (3), 190 (1), 280 (2), 391 (1), 412 (2), 419 (1), 494 (1), 515 (1), 780 (1), NA (3)	24 (1), 222 (1), 258 (1), 730 (1), 948 (1), 962 (2), 1764 (1), 4639 (1), 4643 (1), 4651 (1), 4652 (1), 4658 (1), 4659 (1), 4660 (1), 4719 (1), 5005 (1), NA (9)	92%
		Healthy humans colonization: faeces (14), vagina (1)	2001-2017	Portugal (10), Angola (2), Unknown (3)	6 (2), 8 (1), 18 (1), 22 (1), 32 (1), 89 (1), 147 (1), 845 (2), 1051 (1), 2095 (1), NA (3)	124 (1), 128 (1), 4641 (1), 4647 (1), 4672 (1), NA (10)	73%
		Long-term care patients: faeces (4)	2015-2016	Portugal (4)	80 (2), 117 (1), 262 (1)	24 (1), NA (3)	100%
	Food chain. n=49	Animal production settings: aquaculture (5), piggery (10), poultry faeces (3)	2001-2020	Portugal (11), Angola (3), Unknown (4)	5 (1), 6 (2), 29(1), 30 (1), 94 (1), 101 (1), 132 (1), 185 (1), 264 (1), 393 (1), 430 (1), 434 (1), 891 (1), 970 (1), 971 (1), NA (2)	128 (2), 374 (1), 549 (1), 749 (1), 967 (1), 4644 (1), 4645 (1), 4664 (1), 4665 (1), 4666 (1), 4667 (1), NA (6)	61%
		Food of animal origin: poultry carcass (10), trout (5), bovine milk (4), bovine meat (3), raw meat frozen pet food (5)	1999-2020	Portugal (18), Tunisia (9)	9 (2), 12 (1), 17 (2), 18 (2), 25 (1), 27 (1), 32 (1), 80 (1), 157 (1), 203 (1), 245 (1), 264 (1), 451 (1), 683 (1), 1058 (2), 1059 (1), 1091 (1), 1263 (1), 2092 (1), NA (4)	106 (1), 184 (1), 222 (1), 272 (1), 284 (1), 374 (1), 948 (2), 2661 (1), 3399 (1), 4638 (2), 4653 (1), 4654 (1), 4655 (1), 4656 (1), 4657 (1), 4668 (1), 4669 (1), 4670 (1), NA (7)	39%
		Ready-to-eat salads (4)	2010	Portugal (4)	352 (1), 640 (2), 666 (1)	4662 (1), 4663 (1), NA (2)	0%
	Wild birds n=2	Wild birds (2)	2011-2013	Germany (1), Canada (1)	78 (1), 448 (1)	63 (1), 4642 (1)	100%
	Aquatic environment n=9	River (3)	2003	Portugal (3)	22 (1), 132 (1), 2093 (1)	5140 (1), NA (2)	67%
		Hospital sewage (3)	2001-2002	Portugal (3)	17 (1), 368 (1), 2091 (1)	4649 (1), NA (2)	67%
		Urban wastewater treatment plant (3)	2014-2015	Tunisia (3)	666 (1), NA (2)	NA (3)	67%
<i>Enterococcus faecalis</i> n=105	Humans n=55	Human infection: diverse biological products (27)	1987-2020	Portugal (22), Tunisia (4), USA (1)	2 (1), 6 (6), 9 (1), 16 (3), 22 (1), 25 (1), 30 (1), 55 (1), 59 (1), 64 (1), 116 (1), 133 (1), 159 (1), 191 (1), 200 (1), 631 (1), 1105 (1), 1165 (1), NA (2)	1248 (3), 1252 (1), 1265 (1), 1266 (1), 1267 (1), 1271 (1), 1376 (1), 1408 (1), 1431 (1), 1438 (1), 1442 (1), 1463 (1), 1471 (1), 1475 (1), 1481 (1), 1486 (1), 2904 (1), 2905 (1), 2906 (1), 2907 (1), NA (5)	59%
		Healthy humans colonization: faeces (14), urinary tract (6), breast milk (1)	2001-2018	Portugal (19), Angola (2)	21 (1), 30 (1), 40 (6), 63 (1), 64 (1), 81 (1), 116 (1), 179 (2), 191 (1), 200 (1), 206 (1), 209 (1), 275 (1), 308 (1), 394 (1)	1219 (1), 1226 (1), 1232 (1), 1293 (1), 1294 (1), 1295 (1), 1309 (1), 1311 (1), 1319 (1), 1320 (1), 1323 (1), 1325 (1), 1328 (1), 1340 (1), 1343 (1), 1348 (1), 1380 (1), 1489 (1), 1493 (1), 1567 (1), 1665 (1)	48%
		Long-term care patients: faeces (4)	2015-2016	Portugal (4)	25 (1), 143 (1), 398 (1), 679 (1)	1364 (1), 1366 (1), 1368 (1), 1372 (1)	25%
	Food chain n=40	Patients at hospital admission: faeces (3)	2015-2016	Brasil (3)	6 (2), 525 (1)	1296 (2), 1298 (1)	100%
		Animal production settings: aquaculture (5), piggery (7), poultry and ovine faeces (2)	2006-2015	Portugal (12), Tunisia (3)	21 (1), 22 (1), 35 (1), 40 (1), 59 (1), 65 (1), 139 (1), 200 (1), 209 (1), 330 (1), 386 (1), 631 (1), 749 (1), 872 (1)	1246 (1), 1262 (1), 1439 (1), 1505 (1), 1506 (1), 1513 (1), 1520 (1), 1524 (1), 1537 (1), 1548 (1), 1549 (1), 1556 (1), 1528 (1), 1534 (1)	36%
		Food of animal origin: poultry carcass (7), trout (6), bovine and goat milk (3), bovine meat (2), raw meat frozen pet food (4)	1999-2020	Portugal (17), Tunisia (5)	21 (1), 27 (1), 32 (1), 34 (1), 40 (1), 49 (1), 117 (1), 141 (1), 200 (1), 202 (1), 206 (1), 209 (1), 227 (1), 288 (1), 436 (1), 674 (1), 721 (1), 843 (1), 860 (1), 1008 (2), 1106 (1)	1205 (2), 1206 (1), 1207 (1), 1240 (1), 1243 (1), 1269 (1), 1275 (1), 1288 (1), 1292 (1), 1557 (1), 1558 (1), 1559 (1), 1563 (1), 1564 (1), 1565 (1), 1571 (1), 1583 (1), 1589 (1), 1600 (1), 1629 (1), 1640 (1)	36%
		Ready-to-eat salads (4)	2010	Portugal (4)	141 (1), 165 (1), 309 (1), 594 (1)	1496 (1), 1497 (1), 1498 (1), 1499 (1)	0%
		Pet faeces: cat (1), bird (1)	2014-2015	Tunisia (2)	21 (1), 116 (1)	1260 (1), 1264 (1)	0%
		River (2)	2003	Portugal (2)	1 (1), 4 (1)	1 (1), 1467 (1)	0%
	Aquatic environment. n=8	Hospital sewage (3)	2002	Portugal (3)	16 (1), 35 (1), 49 (1)	1656 (1), 1658 (1), 1659 (1)	100%
		Urban wastewater treatment plant (3)	2014-2015	Tunisia (3)	23 (1), 86 (1), 117 (1)	20 (1), 1241 (1), 1244 (1)	33%

Abbreviations: CT, complex type; MDR, multidrug resistance; n, number; NA, not available; QACs, quaternary ammonium compounds; ST, sequence type; USA, United States of America.

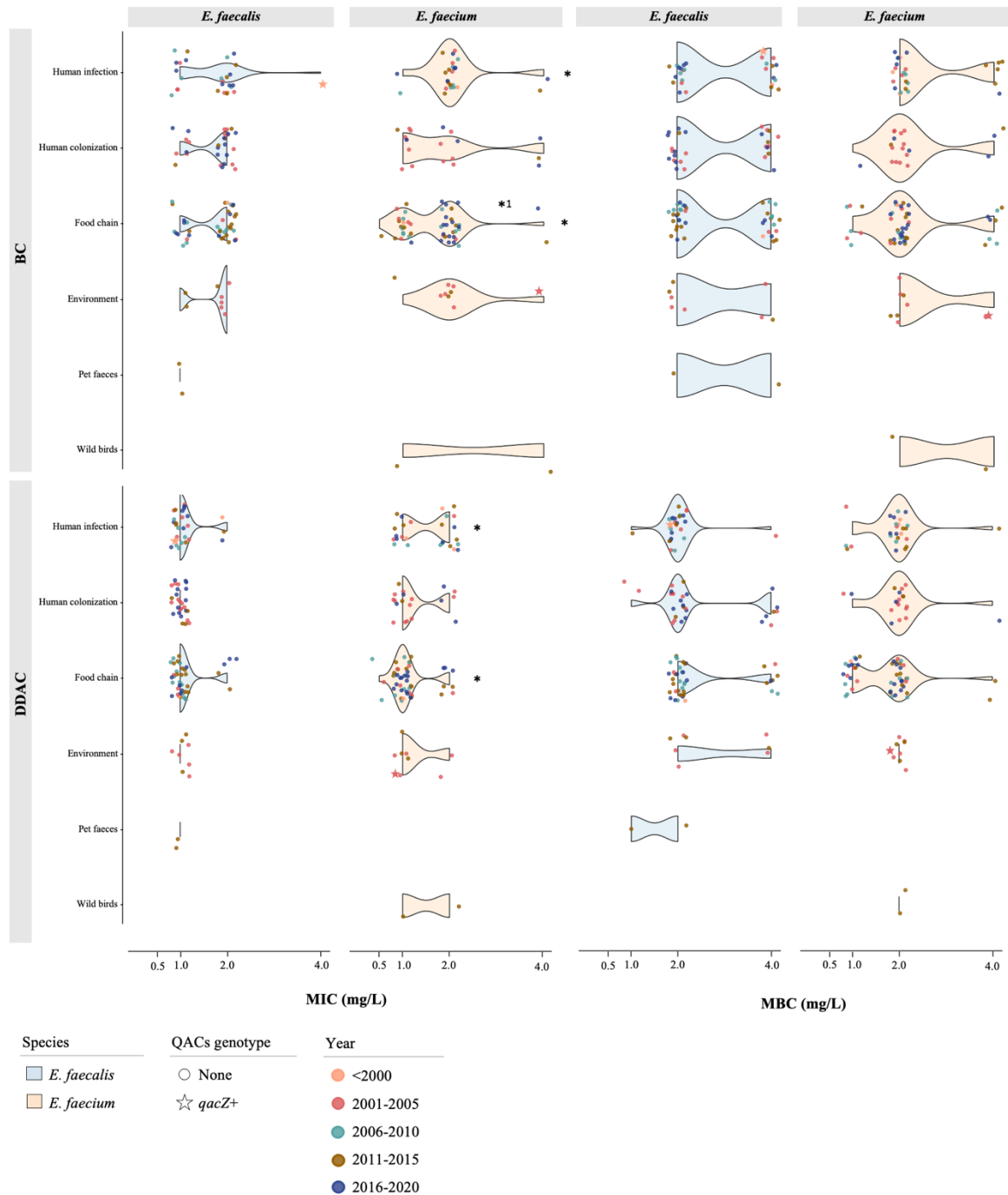


Figure S2. Benzalkonium chloride (BC) and didecylmethylammonium chloride (DDAC) minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) distribution of *Enterococcus faecalis* (blue) and *Enterococcus faecium* (orange) isolates from independently analysed sources. The isolation date and QACs genotype of each isolate is indicated with different colours and shapes, respectively. For more isolates' details see Table S2. * MIC_{DDAC} and MIC_{BC} were higher for *E. faecium* recovered from human infections compared to those from the food chain ($P \leq 0.01$). *¹ MIC_{BC} significantly increased over the years in isolates from the food chain ($P \leq 0.01$). Graphics were done in R, using the ggplot2 package v3.4.0 (34).

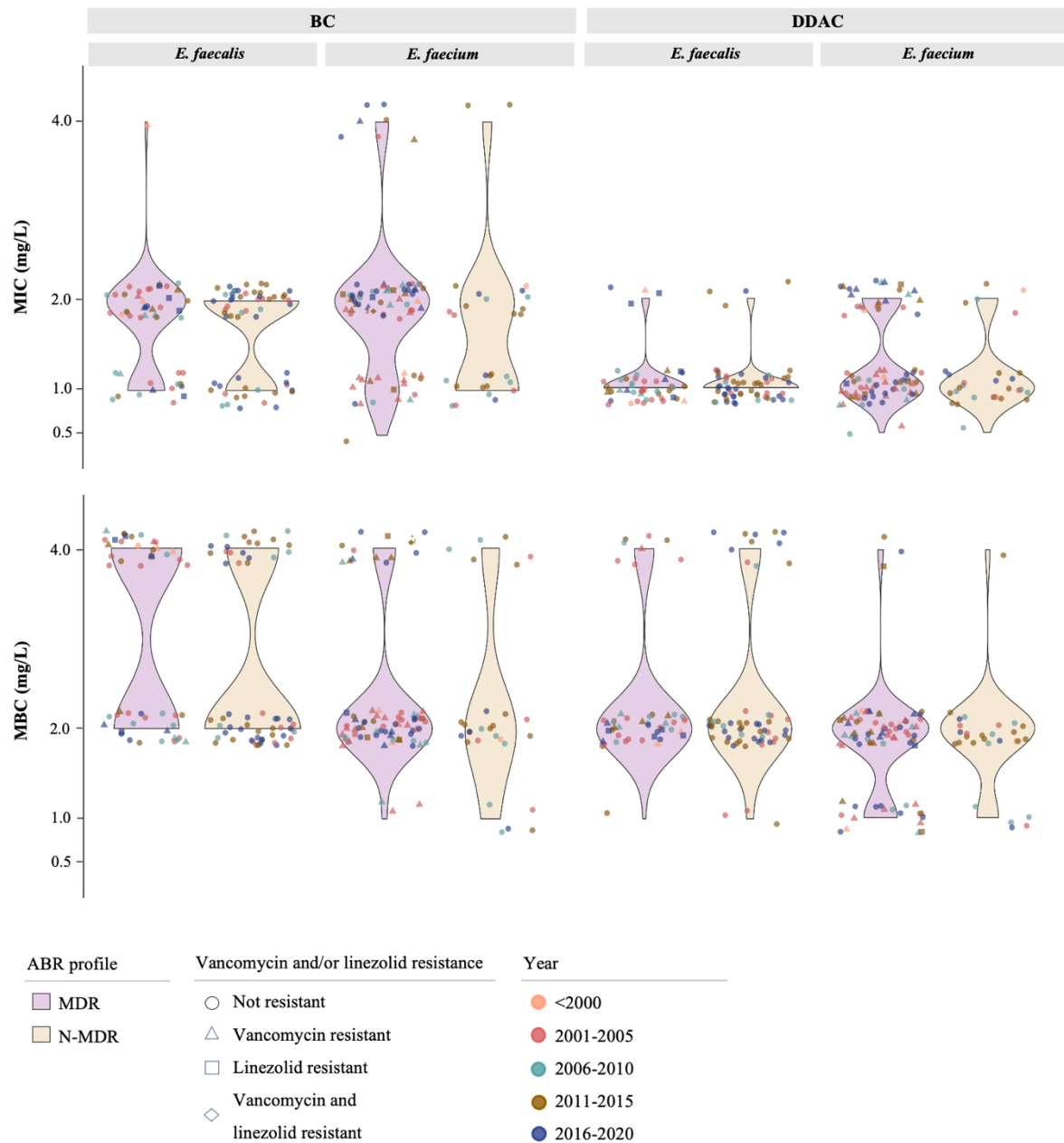


Figure S3. Benzalkonium chloride (BC) and Didecyltrimethylammonium chloride (DDAC) minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) distribution of *Enterococcus faecalis* and *Enterococcus faecium* multidrug resistant (MDR; pink) and non-multidrug resistant (N-MDR; beige) isolates. The isolation date of each isolate and its resistance to vancomycin and/or linezolid is indicated with different colours and shapes, respectively. For more isolates' details see Table S2. Graphics were done in R, using the ggplot2 package v3.4.0 (34).

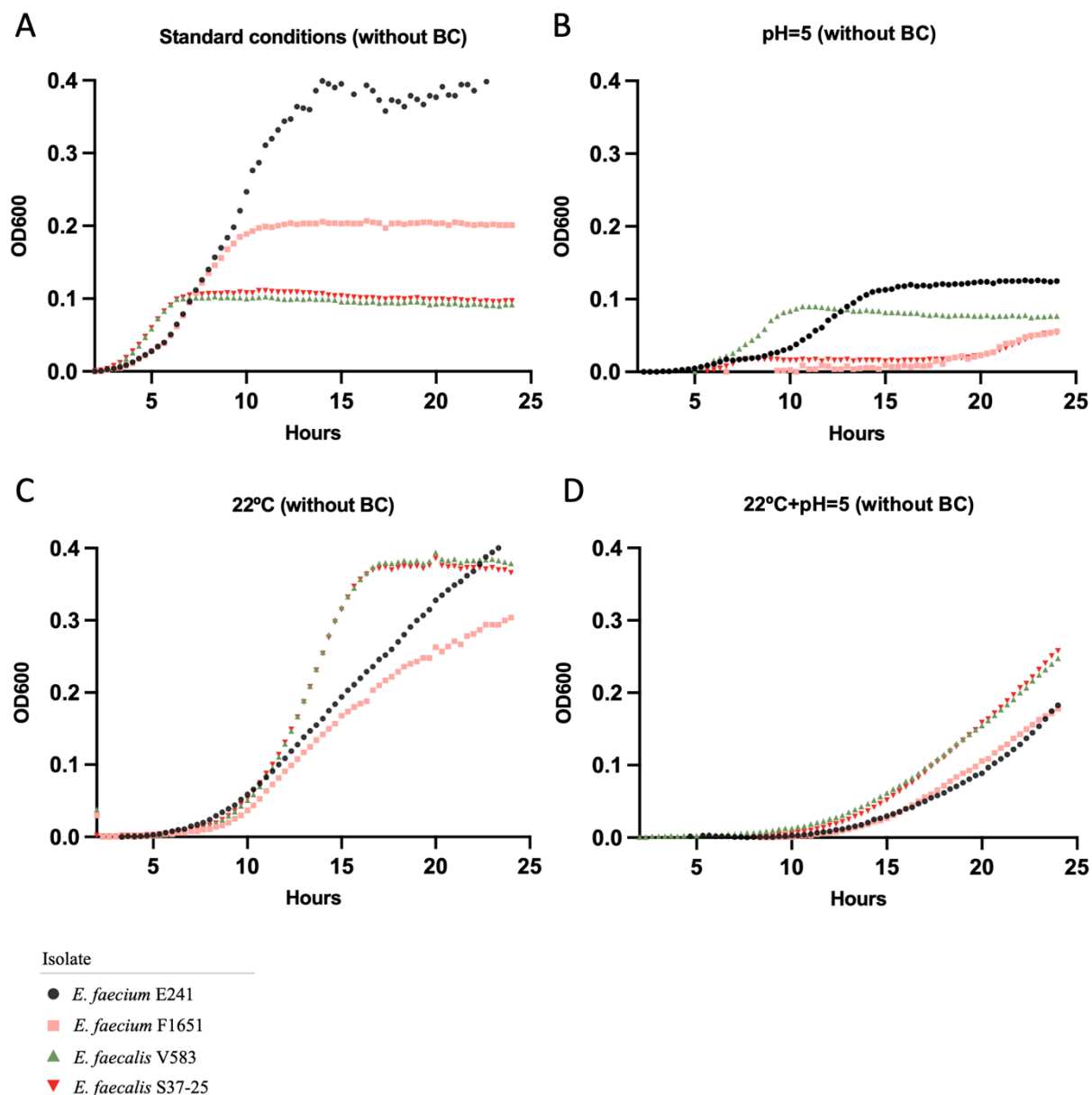


Figure S4. Growth curves without benzalkonium chloride (BC) for *E. faecium* E241 (black circles), *E. faecium* F1651 (pink squares), *E. faecalis* V583 (green triangles) and *E. faecalis* S37-25 (inverted red triangles) in standard (A) or modified conditions (B: pH=5; C: 22°C; D: 22°C+pH=5). For more isolates' details see Table S3. Growth curves were determined using a Biotek Synergy HT plate reader (Marshall Scientific), with absorbance at 600 nm (A600) recorded every 20 minutes for 24 hours. Graphics were built using the Prism software v8.1.1 (GraphPad Software; www.graphpad.com).

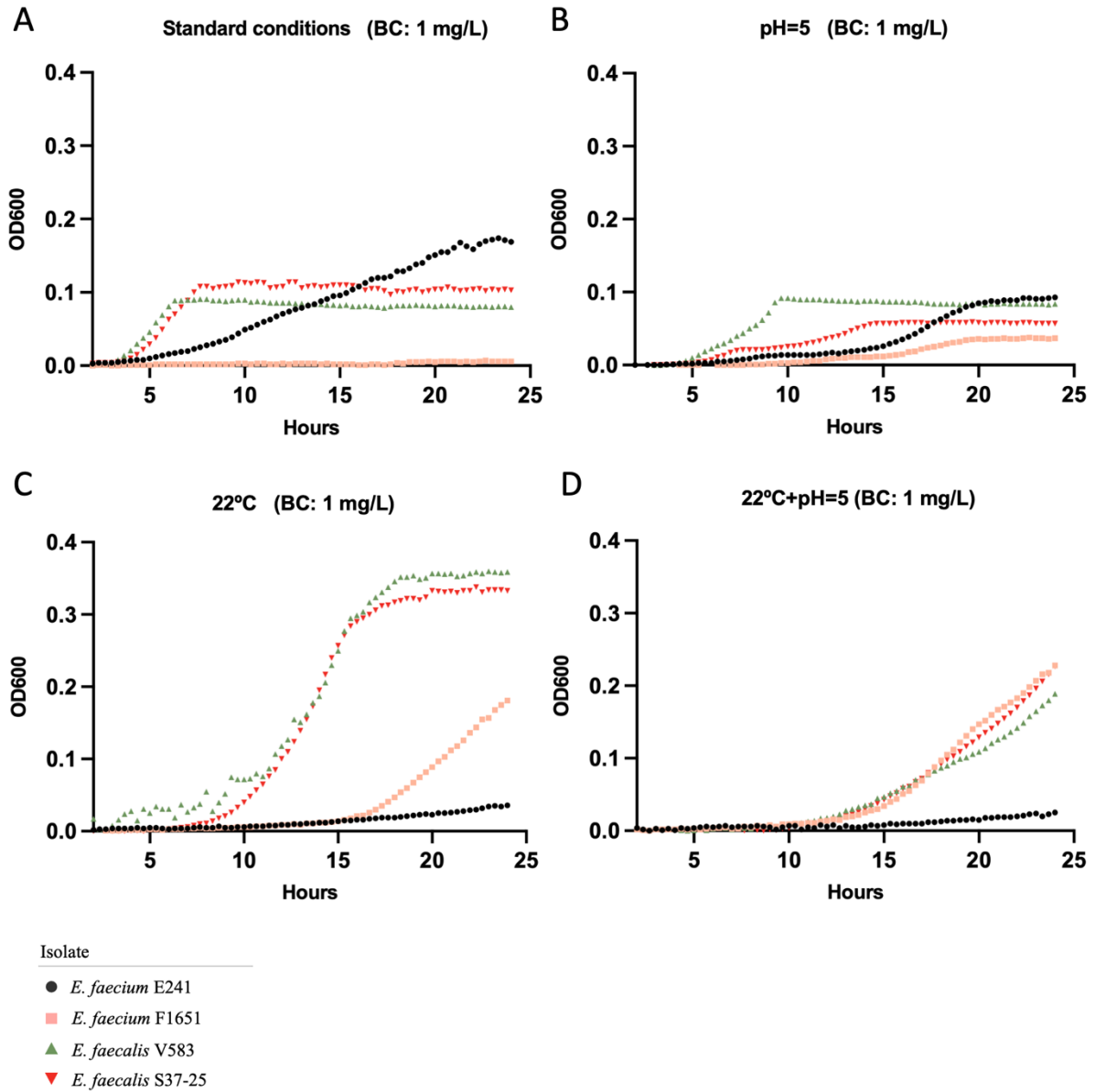


Figure S5. Growth curves in the presence of 1 mg/L of benzalkonium chloride (BC) for *E. faecium* E241 (black circles), *E. faecium* F1651 (pink squares), *E. faecalis* V583 (green triangles) and *E. faecalis* S37-25 (inverted red triangles) in standard (A) or modified conditions (B: pH=5; C: 22°C; D: 22°C+pH=5). For more isolates' details see Table S3. Growth curves were determined using a Biotek Synergy HT plate reader (Marshall Scientific), with absorbance at 600 nm (A600) recorded every 20 minutes for 24 hours. Graphics were built using the Prism software v8.1.1 (GraphPad Software; www.graphpad.com).

Chapter III – Results and Discussion

TABLE S3 – Epidemiological background of the 22 *E. faecalis* and 20 *E. faecium* included in the modified QACs susceptibility phenotypic assays.

Species	Isolate	ST	CT	Date	Isolation Source	Country	Antibiotic Resistance Profile	Modified conditions tested			
								Anaerobiosis	22°C	pH5	22°C + pH5
<i>E. faecium</i>	M239495	412	258	2011	Human infection	Portugal	AMP, CIP, ERY, STR, TET	•	•	•	
	342T	18	222	2014	Human infection	Tunisia	AMP, CIP, ERY, STR	•	•	•	
	HPH109	494	NA	2020	Human infection	Portugal	AMP, CIP, ERY, GEN, STR, TET, TGC	•	•	•	•
	TR 58_34	683	4669	2012	Trout	Portugal	TET	•	•	•	
	R7	22	NA	2003	River	Portugal	CIP	•	•	•	
	E241 *	17	NA	2002	Hospital sewage	Portugal	AMP, CIP, ERY, GEN, Q-D, STR, TET	•	•	•	•
	66T	666	NA	2015	Urban wastewater treatment plant	Tunisia	CIP, ERY, STR, TET	•	•	•	
	S4_1E	640	4663	2010	Ready-to-eat salad	Portugal	-	•	•	•	
	S53_34E	352	NA	2010	Ready-to-eat salad	Portugal	-	•	•	•	•
	D1	147	NA	2003	Human colonization (faeces)	Unknown	ERY, STR, TEC, TET, VAN	•	•	•	
	A29.1A	1051	NA	2015	Human colonization (faeces)	Angola	Q-D	•	•	•	
	C75	89	NA	2001	Human colonization (faeces)	Portugal	ERY, Q-D, STR, TEC, VAN	•	•	•	•
	c29VSb_20_AE	8	NA	2017	Human colonization (vagina)	Portugal	ERY, TET	•	•	•	
	CCT11	117	24	2015-2016	Long-term care patient (faeces)	Portugal	AMP, CIP, ERY, TEC, VAN	•	•	•	
	CCT96	262	NA	2015-2016	Long-term care patient (faeces)	Portugal	AMP, CIP, ERY	•	•	•	•
	D2	6	128	2001	Piggery	Unknown	AMP, ERY, TEC, TET, VAN	•	•	•	
	F1651	12	NA	2019	Poultry carcass	Portugal	ERY, Q-D, STR, TET	•	•	•	•
	145T	1058	4638	2015	Bovine milk	Tunisia	CIP, ERY, LZD, Q-D, TET	•	•	•	
	4275	448	4642	2011	Wild bird	Germany	AMP, CIP, ERY, STR, TEC, TET, VAN	•	•	•	
	PF97	264	374	2019	Raw meat frozen pet food	Portugal	AMP, CIP, Q-D, STR, TET	•	•	•	
<i>E. faecalis</i>	V583 *	6	371	1987	Human infection	USA	ERY, GEN, VAN	•	•	•	•
	204070	159	1252	1999	Human infection	Portugal	CIP, ERY, GEN, TEC, TET, VAN	•	•	•	•
	H307	9	1442	2002	Human infection	Portugal	CHL, CIP, ERY, GEN, STR, TEC, VAN	•	•	•	
	HPH25	191	2905	2019	Human infection	Portugal	TET	•	•	•	
	M5366845	6	2907	2013	Human infection	Portugal	CIP, ERY, GEN, STR, TET	•	•	•	
	TR 39-20	139	1549	2011	Aquaculture	Portugal	ERY, TET	•	•	•	
	R8	4	1467	2003	River	Portugal	-	•	•	•	•
	E263	16	1656	2002	Hospital sewage	Portugal	ERY, GEN, TET, STR	•	•	•	
	14T	23	1241	2014	Urban wastewater treatment plant	Tunisia	TET	•	•	•	
	S37-25E	309	1499	2010	Ready-to-eat salad	Portugal	-	•	•	•	•
	C72	40	1226	2001	Human colonization (faeces)	Portugal	CHL, ERY, STR, TET	•	•	•	
	C370	63	1340	2001	Human colonization (faeces)	Portugal	CHL, ERY, STR, TET	•	•	•	
	A35_1A	81	1294	2013	Human colonization (faeces)	Angola	ERY, TET	•	•	•	
	Br16	525	1298	2015	Human colonization (faeces)	Brazil	GEN, TET	•	•	•	•
	c20Ua_66_AE	200	1320	2017	Human colonization (urine)	Portugal	CIP, ERY, GEN, TEC, TET, VAN	•	•	•	
	CCM48	25	1366	2015-2016	Long-term care patient (faeces)	Portugal	STR	•	•	•	
	SN163	22	1505	2006-2007	Piggery	Portugal	CHL, ERY, MIN, STR, TET	•	•	•	
	F1155	843	1571	2018	Poultry carcass	Portugal	ERY, TET	•	•	•	•
	138T	117	1243	2015	Bovine milk	Tunisia	-	•	•	•	
	273T	872	1262	2015	Ovine faeces	Tunisia	-	•	•	•	
	PF63	40	1206	2019	Raw meat frozen pet food	Portugal	CHL, ERY, LZD, TET	•	•	•	
	PF110	674	1207	2019	Raw meat frozen pet food	Portugal	CHL, CIP, ERY, LZD, STR, TET	•	•	•	•

* *qacZ*+

- not resistant to the antibiotics tested

Abbreviations: AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CT, complex type; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; MIN, minocycline; QACs, quaternary ammonium compounds; Q-D, quinupristin-dalfopristin; ST, sequence type; STR, streptomycin; TEC, teicoplanin; TET, tetracycline; TGC, tigecycline; USA, United States of America.

Table S4 - Benzalkonium chloride neutralizer* effectiveness and toxicity test.

Strain	Test solution	Bacterial cell count (CFU/mL) ¹
<i>Enterococcus faecalis</i> ATCC29212	NaCl 0.9 % (Control)	3.2×10^5
		1.8×10^5
		2.1×10^5
	Neutralizer* + 2 mg/L of Benzalkonium chloride ²	2.8×10^5
		3.3×10^5
		2.4×10^5
	NaCl 0.9 % + 2 mg/L of Benzalkonium chloride ²	0.4×10^5
		0.1×10^5
		0.1×10^5
<i>Enterococcus faecalis</i> V583	NaCl 0.9 % (Control)	2.3×10^5
		3.2×10^5
		1.7×10^5
	Neutralizer* + 4 mg/L of Benzalkonium chloride ²	2.7×10^5
		4.5×10^5
		3.0×10^5
	NaCl 0.9 % + 4 mg/L of Benzalkonium chloride ²	Absence of colonies
		Absence of colonies
		Absence of colonies

* The neutralizer solution was prepared according to EN 13727 (ref) and contained Tween 80 (30 g/L; CAS: 9005-65-6, Sigma Aldrich), sodium dodecyl sulfate (4 g/L; CAS: 151-21-3, VWR) and lecithin (3 g/L; MP Biomedicals).

¹ The three values presented for each test solution correspond to different replicates.

² Benzalkonium chloride concentrations used in these tests corresponded to the minimum bactericidal concentration (MBC) of each strain in standard conditions.

2. Impact of subinhibitory concentrations of biguanides on the emergence and spread of antimicrobial resistance in *E. faecium*

2.1. The Interplay of Chlorhexidine with Antimicrobial Resistance in *Enterococcus faecium*

The Interplay of Chlorhexidine with Antimicrobial Resistance in *Enterococcus faecium*

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Research article in preparation

Abstract

Enterococcus faecium, a human and animal commensal broadly distributed in the environment, is currently one of the most challenging multidrug-resistant (MDR) healthcare-associated pathogens worldwide. Chlorhexidine (CHX), a broad-spectrum antiseptic, is extensively used in healthcare, domestic, and food production settings. This leads to widespread environmental gradients of CHX concentrations, including at subinhibitory levels, whose impact on *E. faecium* adaptation to various antimicrobials remains unclear. Our study aimed to explore the effects of subinhibitory CHX concentrations on biocides and antibiotics susceptibility as well as in the transfer of antibiotic resistance genes among *E. faecium* from different epidemiological and clonal backgrounds. Serial CHX exposure resulted in strain-specific MIC_{CHX} and MBC_{CHX} trends, limited to twofold, in six *E. faecium* over ten CHX-passages. A potential link to known CHX genotypes was observed, with the P102H mutation in DNA-binding response regulator ChtR present in two of three strains showing increased MIC_{CHX} and/or MBC_{CHX}, while the EfrEF transporter was absent in the sole strain exhibiting increased CHX susceptibility after exposure. Whole-genome comparison between parental and CHX-adapted strains found no alterations in recognized CHX tolerance genes. Subinhibitory CHX exposure did not induce antibiotic resistance but notably enhanced plasmid-mediated gene transfer, up to 12.5-fold, among five clinical and non-clinical *E. faecium* strains, carrying vancomycin or linezolid resistance genes, except in a strain lacking a functional EfrEF. This study sheds light on the effects of exposure to subinhibitory CHX concentrations in diverse *E. faecium* populations. Further investigation is warranted to elucidate the underlying mechanisms driving these phenomena in *E. faecium*, ensuring the continued effectiveness of both CHX and antibiotics and safeguarding Public Health.

1. Introduction

Enterococcus faecium is one of the most challenging human healthcare-associated pathogens worldwide [1-3]. Over recent decades, this opportunistic Gram-positive bacterium, part of the human and animal gut microbiota, has developed resistance to all clinically-relevant antibiotics, particularly ampicillin, vancomycin, linezolid, daptomycin, or tigecycline, with varying global occurrence rates [1, 2]. *E. faecium*'s infections mostly target immunocompromised hospital patients, who are often under multiple antibiotic therapy and show, as a result, an increased abundance of multidrug-resistant (MDR) *E. faecium* in the gastrointestinal tract, facilitating the spread of these microorganisms in the hospital environment through the close contact between healthcare staff and patients [4-6]. Their remarkable ability to survive harsh conditions, such as heat, nutrient scarcity, and desiccation, for extended periods on contaminated medical equipment and other abiotic surfaces also explains its successful spread in healthcare environments [5, 7-9]. Effective infection control practices are, thus, crucial to prevent colonization and potential life-threatening infections by MDR *E. faecium*.

Chlorhexidine (CHX), a broad-spectrum cationic biocide, plays a key role in this context as one of the most used antiseptics or disinfectants (typical in-use concentrations: 25 – 40,000 mg/L) [10, 11]. Moreover, it has been increasingly used in recent years, not only in hospitals but also in domestic, veterinary, food production, and industrial settings, where *Enterococcus* are also present as human and animal colonizers or pathogens, as well as food contaminants [10, 11]. As a result, *E. faecium* are often exposed to various subinhibitory concentrations of CHX, namely on the skin of patients undergoing daily CHX bathing, or in sewage (0.00028-600 mg/L) [12-15].

Previous studies have shown an evolution towards CHX tolerance among various pathogenic bacterial species from environments where CHX is known to be extensively used in recent years [16-19]. In *Enterococcus*, few reports have identified an increased tolerance to CHX in clinical strains [20-23], namely in patients undergoing daily CHX bathing compared to those not exposed [24], or among food chain isolates [21, 25]. Additionally, *in vitro* repeated exposure of clinical and food-associated *E. faecium* to subinhibitory CHX concentrations lead to adapted strains with increased CHX tolerance as well as other alterations directly implicated in antibiotic resistance [9, 26-30], such as the upregulation of genes involved in vancomycin (*vanHAX*) and daptomycin (*liaXYZ*) resistance [9].

Genes linked to CHX susceptibility in *E. faecium* have been identified, including the heterodimeric ATP-binding cassette (ABC) multidrug efflux-pump *efrEF* and its transcription regulator *chlR*, and a putative two-component regulatory system (2CS) composed by a histidine kinase sensor and a DNA-binding response regulator, encoded by the genes *chtS*

and *chtR*, respectively [9, 20, 23]. Deletion of the 2CS genes in *E. faecium* strains resulted in an increased susceptibility to CHX [9, 23]. Furthermore, a single amino acid mutation (P102H) in ChtR, predominantly detected in clonal lineages of clade A1 (mostly associated with human infections and outbreaks), was linked to a CHX tolerant phenotype [20, 23]. The role of other efflux-pumps found in *E. faecium*, with a demonstrated impact on CHX tolerance in other species, such as the *qacA/B* or *oqxAB* genes, remains, however, more questionable [31-35]. Recently, subinhibitory concentrations of different biocides [tens to hundreds of times below the minimum inhibitory concentrations (MICs)], including CHX, have been shown to increase horizontal gene transfer (HGT) via conjugation [36-40]. Nevertheless, most of these recent studies included Gram-negative *Escherichia coli* as the conjugative donor and/or recipient strain [36-40], leaving the extent of this phenomenon in Gram-positive bacteria, particularly *E. faecium*, largely unexplored. Conjugation plays a pivotal role in the evolution and success of *E. faecium* populations, allowing them to acquire and share adaptive traits, most notably antimicrobial resistance genes [2, 41, 42]. Case in point, the Tn1546 transposon carrying *vanA*, the most prevalent genetic platform associated with resistance to vancomycin in *E. faecium*, or the *cfp*, *optrA*, and *poxA* genes, which code for resistance to linezolid and chloramphenicol, are frequently located on transferable plasmids [1, 2].

Despite the large use of CHX in different sectors and of the potential biological implications for *Enterococcus* suggested by a few studies, the impact of *E. faecium*'s wide exposure to CHX on the populations' epidemiology and antimicrobial resistance selection and spread remains to be elucidated. In this study, we explored the phenotypic and genomic outcomes of exposure to CHX subinhibitory concentrations on biocides and antibiotics susceptibility, as well as in the transfer of genes coding for resistance to clinically-relevant antibiotics, among *E. faecium* isolates with different epidemiological and clonal backgrounds.

2. Methods

2.1. Epidemiological background of the bacterial isolates

A collection of 11 *E. faecium* isolates, representative of different geographical regions, sources, time spans, genomic backgrounds and antimicrobial resistance profiles, was selected for this study. They included the laboratory strain *E. faecium* GE1 [43, 44] and ten isolates recovered from human infections (n=3), human colonization (n=3), and food chain (n=4; food-animal production settings, meat) samples, in diverse regions of Portugal and Tunisia, from 1999 to 2022. Among them, 6 were classified as MDR (resistance to 3 or more antibiotics from different families) [45]. The three or two isolates resistant to vancomycin or linezolid were selected as donor strains of *vanA* or *poxA* and/or *optrA* genes, respectively, for

the conjugation assays. Contrastingly, *E. faecium* isolates susceptible to these and most of the other antibiotics tested were included in the CHX serial exposure experiment, to test the hypothesis that subinhibitory concentrations of CHX could promote antibiotic resistance.

2.2. Whole-genome sequencing and analysis of strains genomic background

The genomes of most of selected *E. faecium* (n=9/11) had been sequenced in previous studies (BioProjects PRJNA546230, PRJNA580369, PRJNA1055432) [20, 46]. The remaining two isolates (SN134 and SN558) were sequenced in this study to include fully characterized isolates in the subsequent experiments with CHX, as briefly described next.

Genomic DNA was extracted using a Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Whole-genome sequencing (WGS) was accomplished by an Illumina NovaSeq 6000 platform, with average median depth of coverage 100% to obtain 5 million read pairs (2×150 bp), according to standard Illumina protocols performed at Eurofins Scientific (Italy). The quality of the raw and preprocessed sequencing data was analyzed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The SPAdes genome assembler v3.15.2 was used for de novo assembly of the paired end reads and the quality of genome assembly was evaluated by QUAST (<http://cab.cc.spbu.ru/quast/>).

Clonal relationship was established for all isolates by multilocus sequence typing (MLST; Sequence Type-ST) [47, 48] and core genome MLST (cgMLST; Complex Type-CT; Ridom SeqSphere+, version 8.2.0; <https://www.ridom.de/seqsphere>) [49].

The screening for antibiotic resistance genes was performed using ResFinder v4.4.2, available at the Center for Genomic Epidemiology (CGE; <https://www.genomicepidemiology.org>) [50]. Specifically for isolates included in the conjugation assays, plasmids carrying vancomycin and linezolid resistance genes were predicted. Initially, Unicycler v0.5.0 was used to generate an assembly graph for each donor strain [51]. Plasmid-derived contigs from the assembly graph were predicted through PlasmidEC v1.3, a tool that combines three binary classification tools and implements a majority vote system [52]. The binning of plasmid-nodes into individual plasmid predictions was achieved using gplas2 v2.0, a tool based on sequence composition, coverage, and assembly graph information, with a modularity threshold of 0.1 [52]. Finally, typing of plasmid replicons was performed with PlasmidFinder v2.1, available at CGE [53].

To characterize CHX tolerance genotypes in all isolates, the presence and nucleotide sequence of genes with confirmed or predicted roles in tolerance to CHX in *E. faecium* were assessed through BLAST [54]. The search included the *Enterococcus* genes *chtS* and *chtR* (reference strain: *E. faecium* E1162; GenBank accession no. NZ_ABQJ01000097.1; locus-tag EfmE1162_2202 and EfmE1162_2203, respectively), and the *efrE*, *efrF* and *chlR* genes

(reference strain: *E. faecium* 1,231,410; GenBank accession no. NZ_GG692477.1; locus-tag EFTG_02286 to EFTG_02288) [20, 21, 23, 55, 56]. Also, acquired *qacA/B* (GenBank accession no. HE579074.1; locus-tag SAI8T7_20080) and *oqxAB* (GenBank accession no. KT716391.1) genes were searched [25, 34, 57]. Genes identified in each genome were translated into the corresponding amino acid sequences by the DNA translate tool of ExPASy SIB Bioinformatics Resource Portal (<https://web.expasy.org/translate/>) and the occurrence of truncated proteins was evaluated. Comparisons of the amino acid sequences obtained with the reference strains used in the search, detailed above, were performed with the Clustal Omega software (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) for the identification of specific mutations.

2.3. Chlorhexidine susceptibility testing

The MIC and minimum bactericidal concentrations of CHX, MIC_{CHX} and MBC_{CHX}, respectively, were established by broth microdilution for all isolates, using the methodological approach proposed by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing (Mueller-Hinton (MH) broth; pH 7.3; 37°C/20 h) as previously described [21, 58, 59]. Briefly, microdilution panels were freshly prepared before each assay. The first concentration of CHX without visible growth was considered the MIC_{CHX} [21, 58]. The MBC_{CHX} was defined as the lowest concentration for which the number of colonies was equal or less than the rejection value defined by CLSI guidelines, based on the final inoculum of each well confirmed by actual count [21, 59]. Each experiment was repeated three times, and the MIC_{CHX} or MBC_{CHX} values corresponded to the mean of the determinations. *E. faecalis* ATCC 29212 was used as control to guarantee the reproducibility of all assays.

2.4. Serial exposure of *E. faecium* to subinhibitory concentrations of chlorhexidine

From the initial *E. faecium* collection, a subset of 6 isolates, with different CHX phenotypes, were exposed to subinhibitory concentrations of CHX (CAS: 18472-51-0, Sigma-Aldrich). CHX agar plates were prepared the previous day in glass petri dishes and refrigerated until use (maximum of 24h). Briefly, on the first day (P0), 30 µL of 0.5 McFarland bacterial suspensions in log-phase growth were added to MH agar plates containing CHX concentrations ranging from 0.25 mg/L (8-16-fold below the MIC_{CHX}) to 64 mg/L (16-32-fold above the MIC_{CHX}), using twofold concentration intervals, and incubated for 24h at 37°C. On the subsequent days, the bacterial populations from the two highest CHX concentrations still exhibiting visible growth were mixed in saline solution and 30 µL were used for the inoculation of the next passage. This procedure was executed for 10 passages (P1 to P10), a number described in previous studies to result in CHX phenotype changes in *Enterococcus* spp. [27, 29, 30]. Serial passages in CHX-free MH agar plates were carried out in parallel for each strain as the control. To

determine the stability of any CHX susceptibility changes, bacteria passaged in CHX, as well as controls, were then repeatedly subcultured for 5 days in the absence of CHX (X1 to X5). Susceptibility to CHX and benzalkonium chloride (BC; CAS: 68391-01-5, VWR), another cationic biocide which belongs to the quaternary ammonium compounds (QACs) class, and to several antibiotics was assessed at P0, P5, P10, X1 and X5. The MIC and MBC of BC (MIC_{BC} and MBC_{BC}, respectively) were established by broth microdilution, using the methodology described above for CHX, following the CLSI guidelines (MH broth; pH 7.3; 37°C/20 h), as previously described [58-60]. Antibiotic susceptibility to ampicillin (AMP2), vancomycin (VA5), teicoplanin (TEC30), ciprofloxacin (CIP5), tetracycline (TET30), erythromycin (E15), gentamicin (CN30), streptomycin (S300), linezolid (LZD10), chloramphenicol (C30), and quinupristin-dalfopristin (QD15) was tested by disk diffusion (Thermo Scientific™ Oxoid™ Antimicrobial Susceptibility Disks) following the EUCAST (European Committee on Antimicrobial Susceptibility Testing; <https://www.eucast.org/>) guidelines. In addition, the MIC of vancomycin and linezolid was established by standard broth microdilution and of ampicillin by E-test (AMP 0.016-256 mg/L, Liofilchem) [58]. Results were interpreted according to the EUCAST [61] or, when not available (tetracycline, chloramphenicol, erythromycin), by the CLSI [62] clinical breakpoints. *E. faecalis* ATCC 29212 was used as the control in all susceptibility assays.

The genomes of the two CHX-adapted isolates at passage X1, in which the increased MIC_{CHX} and/or MBC_{CHX} were still not reversed at X5, were sequenced as previously described. Polymorphisms occurring in the strains' whole-genome sequences compared to the parental strains were detected using Snippy v4.6.0 [63] and Breseq v0.36.1 [64]. Additionally, the occurrence of identical mutations among other *E. faecium* genomes (n= 22,466; until 20/01/2023) available at the NCBI (National Center for Biotechnology Information) database was assessed using the blastp tool [54].

From the 6 isolates included in the passages, three *E. faecium* (TR68_31, C1177 and SN134), representative of different CHX-passages phenotypic outcomes and CHX genotypes, were selected for kinetic growth assays. Growth curves were determined for the CHX-exposed strain at X1 and the parental strain (P0), for each of the three *E. faecium*. For both CHX-exposed and parental strains, assays were performed at standard conditions (37°C; pH=7.3) under CHX concentrations 0-4 mg/L. Growth curves were determined using a Biotek Synergy HT plate reader (Marshall Scientific), with absorbance at 600 nm (A600) recorded every 20 minutes for 24 hours.

2.5. Conjugation assays

For the conjugation assays, a different subset of 6 *E. faecium* isolates was selected from the study collection. Among these, 5 strains, representative of the different CHX phenotypes and harboring either vancomycin (H323, M1241285, SN558: *vanA*) or linezolid (C1299: *optrA* and *poxA*; 149T: *poxA*) resistance genes, were used as the donors of mobile resistance genes. *E. faecium* GE1, resistant to fusidic acid, rifampicin and tetracycline, was included as the recipient strain.

Conjugation was tested through a broth mating assay, in which donor and recipient bacterial suspensions in log-phase of growth, adjusted at 0.5 McFarland in MH broth, were mixed at a ratio of 1:1. CHX was added to the mating paired systems to reach final concentrations, for each donor/recipient pair, 200 times lower than the MIC_{CHX} of the donor strain (SN558: 0,0025 mg/L; M1241285, 149T: 0.01 mg/L; H323, C1299: 0.02 mg/L) [65]. CHX-free conjugation mixtures were also tested in the same experimental conditions for each conjugal pair as control of conjugation in standard conditions. After conjugation at 37°C for 22h, the mixtures were decimally diluted and plated onto BHI agar supplemented with 30 mg/L rifampicin (CAS: 13292-46-1, Sigma-Aldrich) and 20 mg/L fusidic acid (CAS: 751-94-0, Sigma-Aldrich) plus either 6 mg/L vancomycin (CAS: 1404-93-9, Sigma-Aldrich) or 10 mg/L chloramphenicol (CAS: 56-75-7, Sigma-Aldrich). Transconjugants were recovered after incubation at 37 °C for 24-72 h and confirmed by antibiotic susceptibility testing and PCR detection of the *vanA*, *poxA* and/or *optrA* genes, using primers and conditions previously described [66]. All experiments were conducted in triplicate. The conjugation frequency was calculated as the ratio between the number of transconjugants per mL and the number of recipients per mL, determined prior the mating step [67].

2.6. Statistical analysis

The statistical analysis of MIC_{CHX} and MBC_{CHX} comparison between CHX-passaged strains and controls was performed using the Related-Samples Friedman's Two-Way Analysis of Variance by Ranks and the Related-Samples Wilcoxon Signed Rank test on IBM SPSS Statistics v28.0. *P* values ≤0.05 were considered significant.

3. Results

3.1. Chlorhexidine phenotypic and genotypic characterization of the *E. faecium* strains

The MIC_{CHX} of the 11 *E. faecium* ranged from 0.5 to 4 mg/L, whereas the MBC_{CHX} varied between 2 and 4 mg/L (**Table 1**). The highest MIC_{CHX} and MBC_{CHX} of 4 mg/L were obtained for seven and eight isolates, respectively, corresponding to *E. faecium* recovered from diverse

Chapter III – Results and Discussion

Table 1 - Chlorhexidine (CHX) phenotypic and genotypic characterization of the *E. faecium* strains from different epidemiological and clonal backgrounds included in this study.

Isolate	Methodologies		ST	CT	Date	Isolation Source	Country	Antibiotic Resistance Profile	Antibiotic Resistance Genes ²	CHX susceptibility		CHX genotypes ³								
	CHX Passages	Conjugation								MIC (mg/L)	MBC (mg/L)	2CS		chlR-efrEF			qac		oqxAB	
												chtS	chtR	chlR	efrE	efrF	qacA/B	oxxA	oxxB	
M918981	●		494	6690	2019	Human infection	Portugal	AMP, CIP, ERY, GEN, STR, TET	aac(6')-aph(2''), aac(6')-Ii, ant(6)-Ia, aph(3')-III, msr(C), erm(B), tet(M), tet(L)	4	4	-	-	-	Q25E, H185Q	F260C	NF	NF	NF	
C1177	●		269	7695	2022	Human colonization (faeces)	Portugal	ERY, Q-D, TET	aac(6')-Ii, catA8, erm(B), lnu(G), msr(C), narAB, tet(L), tet(M)	2	2	G189R	H102P	NF	NF	NF	NF	NF	NF	
C1304	●		2323	7708	2022	Human colonization (faeces)	Portugal	TET	aac(6')-Ii, lnu(A), msr(C), tet(L), tet(M)	4	4	-	H102P	E105A, Q106R	H185Q, E326K, I347T	T159A, K177R, T572A	NF	NF	NF	
SN134	●		848	8178	2006	Piggery	Portugal	ERY, Q-D, STR, TET	aac(6')-Ii, ant(6)-Ia, aph(3')-III, erm(B), lnu(B), lsa(E), tet(L), tet(M)	4	4	-	H102P	E105A, Q106R	H185Q, E326K, I347T	T159A, A456T, T572A	NF	NF	NF	
TR68_31	●		1059	4670	2012	Trout	Portugal	-	aac(6')-Ii, msc(C)	4	4	-	-	Q106R	H185Q	-	NF	NF	NF	
GE1 (ATCC 51558)	●	●	515	978	*	*	*	FUS, RIF, TET	aac(6')-Ii, msr(C), tet(M)	4	4	G189R, N233K	H102P, I151V	Q106R	H185Q, I193V	D455N	NF	NF	NF	
H323		●	17	4658	2002	Human infection	Portugal	AMP, CIP, ERY, GEN, TEC, VAN	aac(6')-aph(2''), aac(6')-Ii, aph(2'')-Ia, erm(B), msr(C), vanA	4	4	-	-	Q106R, I183S	H185Q	-	NF	NF	NF	
M1241285		●	494	6704	2021	Human infection	Portugal	AMP, CIP, ERY, GEN, TEC, TET, VAN	aac(6')-aph(2''), aac(6')-Ii, ant(6)-Ia, aph(3')-III, erm(B), msr(C), tet(L), tet(M), vanA	2	4	-	-	-	Q25E, H185Q	F260C	NF	NF	NF	
C1299		●	128	7706	2022	Human colonization (faeces)	Portugal	CHL, LZD	aac(6')-Ii, fexA, fexB, msr(C), optrA, poxtA	4	4	-	-	Q106R	H185Q	-	NF	NF	NF	
SN558		●	185	351	2007	Piggery	Portugal	TEC, TET, VAN	aac(6')-Ii, msr(C), tet(M), vanA	0.5	2	-	H102P, L164W	Trunc.	Trunc.	F35L, E57Q, T92A, A240S, M283I, D327E, D344E, R346K, Q350E, K352E, A356V, L417R, D479E, A556V, S579A, I593V	NF	NF	NF	
149T		●	1058	4638	2015	Bovine milk	Tunisia	CHL, CIP, ERY, LZD, TET	aac(6')-Ii, erm(B), dfpK, fexB, msr(C), narAB, poxtA, tet(L), tet(M)	2	2	-	-	P63S, Q106R	H185Q	-	NF	NF	NF	

* : GE-1 is laboratory mutant of a penicillin-hypersusceptible strain designated *E. faecium* 4379-S, derived from the clinical isolate *E. faecium* 4379 (United States; <1982) through serial exposure to gradients of novobiocin concentrations [43, 44].

1 : Antibiotics tested in previous studies (ref) included: AMP, CHL, CIP, ERY, GEN, LZD, Q-D, STR, TEC, TET, TGC, VAN. For strains included in the conjugation assays, FUS and RIF were also tested. Antibiotic susceptibility was tested by disk diffusion following the EUCAST (European Committee on Antimicrobial Susceptibility Testing; <https://www.eucast.org/>) guidelines. In addition, the MIC of LZD and VAN was established by standard broth microdilution and of AMP by E-test. Results were interpreted according to the EUCAST [61] or, when not available (CHL, ERY, TET), by the CLSI [62] clinical breakpoints.

2 : The screening for antibiotic resistance genes was performed using ResFinder v4.4.2, available at the Center for Genomic Epidemiology (CGE; <https://www.genomic epidemiology.org>) [50]. The *aac(6')-li* as an intrinsic gene of *E. faecium* was not included in the table.

3 : Amino acidic mutations. The genes used as reference were: *chtS* and *chtR* (reference strain: *E. faecium* E1162; GenBank accession no. NZ_ABQJ01000097.1; locus-tag EfmE1162_2202 and EfmE1162_2203, respectively); *efrE*, *efrF* and *chlR* (reference strain: *E. faecium* 1,231,410; GenBank accession no. NZ_GG692477.1; locus-tag EFTG_02286 to EFTG_02288); *qacA/B* (GenBank accession no. HE579074.1; locus-tag SA18T7_20080); *oqxAB* (GenBank accession no. KT716391.1).

- : No antibiotic resistance or mutations were detected.

Abbreviations: AMP, ampicillin; ATCC, American Type Culture Collection; CHL, chloramphenicol; CHX, chlorhexidine; CIP, ciprofloxacin; CT, complex type; ERY, erythromycin; FUS, fusidic acid; GEN, gentamicin; LZD, linezolid; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; NF, not found; Q-D, quinupristin-dalfopristin; RIF, rifampicin; ST, sequence type; STR, streptomycin; Trunc., truncated; TEC, teicoplanin; TET, tetracycline; TGC, tigecycline; VAN, vancomycin.

epidemiological and clonal backgrounds. On the other hand, the most CHX susceptible strain, with an MIC_{CHX} of 0.5 mg/L, was only observed in a vancomycin-resistant strain from the food chain (piggery, ST185, Portugal, 2017) (**Table 1**). CHX susceptibility values of *E. faecium* from different sources and with different antibiotic resistance profiles were within the same ranges (**Table 1**).

Enterococcus genes implicated in CHX tolerance were found among isolates from diverse sources and clonal lineages (**Table 1**). All strains harbored the genes *chtS* and *chtR* (**Table 1**), and 6 out of the 11 isolates studied harbored the mutated P102H-ChtR (**Table 1**). These isolates had MIC_{CHX} and MBC_{CHX} varying from 2 to 4 mg/L and were recovered from human infections (n=3), human colonization (n=1), and the food chain (n=2), from Portugal and Tunisia, between 2002-2022 (**Table 1**).

The *chlR-efrEF* operon encoding a complete multidrug efflux-pump was present in all but two of the *E. faecium* analyzed, encoding proteins with amino acid sequence variability among them (**Table 1**). The two other isolates either without *chlR-efrEF* or with a truncated operon were from human colonization (ST269, Portugal, 2022; MIC_{CHX} and MBC_{CHX} = 2 mg/L) or the food chain environment (ST185, Portugal, 2017; MIC_{CHX} = 0.5 mg/L and MBC_{CHX} = 2 mg/L), respectively. The latter carried a truncated operon missing part of the *chlR* and *efrE* genes (**Table 1**).

Considering the remaining genotypes searched, neither the *qacA/B* or *oqxAB* genes were found among the *E. faecium* strains studied (**Table 1**).

3.2. Impact of serial exposure to subinhibitory concentrations of chlorhexidine on tolerance to biocides and resistance to antibiotics

Serial exposure of six *E. faecium* from diverse epidemiological and genetic backgrounds to subinhibitory concentrations of CHX resulted in increased MIC_{CHX} and MBC_{CHX} ($P < 0.05$) when comparing to the value distribution of the same strains passaged in CHX-free medium (**Fig. 1-A**). However, MIC_{CHX} and MBC_{CHX} trends along CHX passages differed between the strains studied (**Fig. 1-B**). Among the six CHX-exposed *E. faecium*, two and three strains showed a twofold increase in their MIC_{CHX} or MBC_{CHX}, respectively, compared to the control, from 4 to 8 mg/L, after ten passages (P10) (**Fig. 1-B**). The two strains with changes in both MIC_{CHX} and MBC_{CHX}, *E. faecium* C1304 and *E. faecium* TR68_31, were isolated from different sources in Portugal (human colonization and the food chain, respectively), ten years apart, and belonged to distinct clonal lineages (ST2323 and ST1059, respectively) (**Fig. 1-B; Table 1**). While the higher MIC_{CHX} detected for CHX-adapted *E. faecium* C1304 reverted to the one of the parental strain (P0) after 5 CHX-free passages (X5), the increased MBC_{CHX} was stable, similarly to the altered MIC_{CHX} or MBC_{CHX} in *E. faecium* TR68_31, until X5 (**Fig. 1-B**). Additionally, human infection *E. faecium* M918981 strain (ST494, Portugal, 2019) passages in CHX subinhibitory

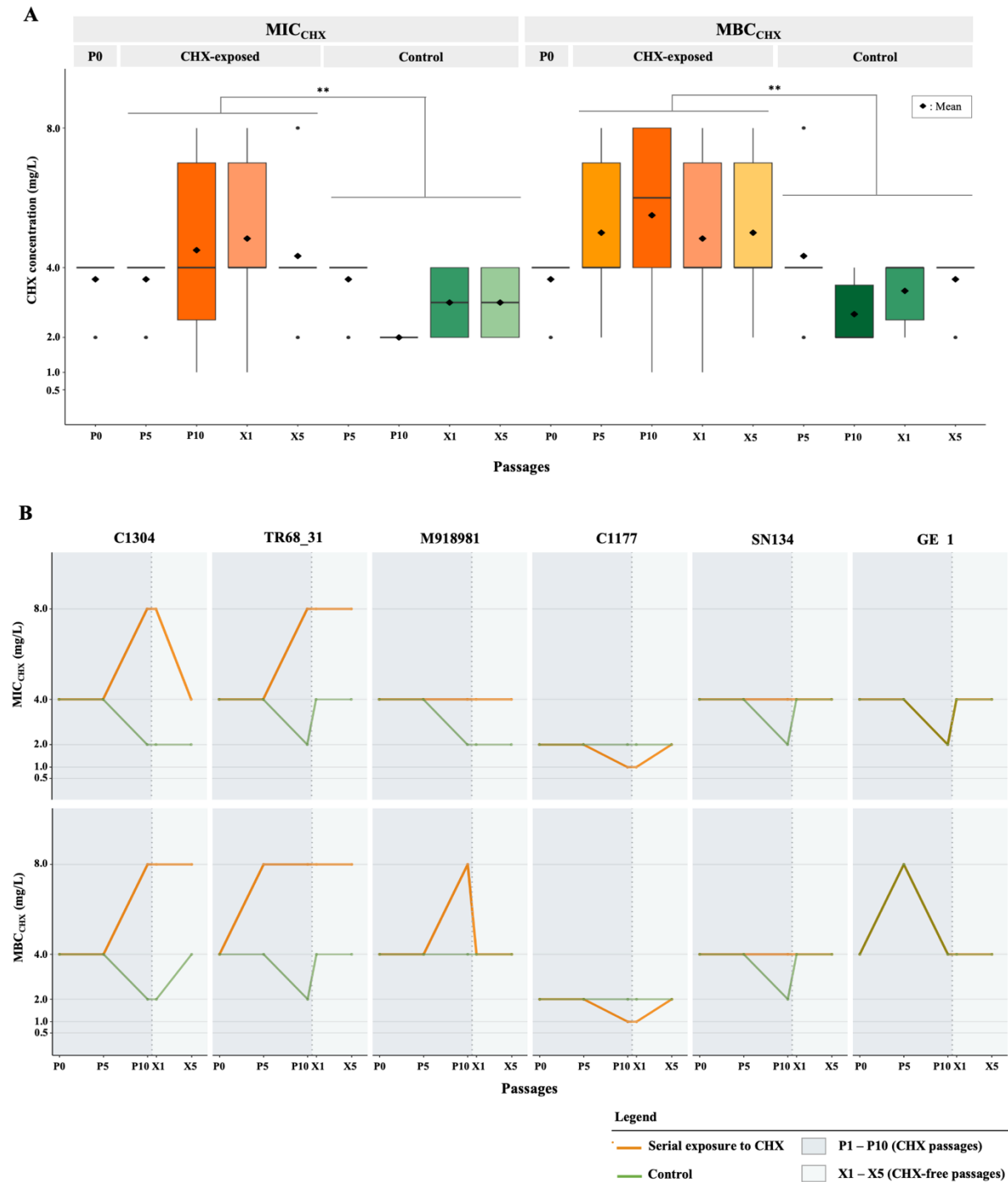


Figure 1. A) Chlorhexidine (CHX) minimum inhibitory concentrations (MIC_{CHX}) and minimum bactericidal concentrations (MBC_{CHX}) distribution of the 6 *E. faecium* strains serially exposed to CHX (CHX-exposed; shades of orange) or passaged in CHX-free medium (control; shades of green). **B)** MIC_{CHX} and MBC_{CHX} evolution of each of the 6 *E. faecium* strains serially exposed to CHX (orange) or passaged in CHX-free medium (control; green). P0 corresponds to the initial MIC or MBC. Exposure to CHX was performed for ten passages (P1 to P10), followed by five passages in CHX-free medium (X1 to X5), for the CHX-exposed strains. Serial passages in CHX-free medium (P1 to X5) were carried out in parallel for each strain as the control. **: $P \leq 0.01$, Related-Samples Wilcoxon Signed Rank test (IBM SPSS Statistics v28.0)

concentrations resulted in a twofold increase in the MBC_{CHX} , which was reversed after one CHX-free passage, but no changes in the MIC_{CHX} (**Fig. 1-B; Table 1**). Regarding their CHX genotypes, the three CHX-adapted *E. faecium* with reduced susceptibility at P10 (MIC and/or MBC) compared to the control carried genes encoding complete ChtRS and ChlR-EfrEF proteins, similarly to other CHX-exposed isolates with no phenotype changes (**Fig. 1-B; Table 1**). Of note, two of the three strains with reduced susceptibility, *E. faecium* M918981 and *E. faecium* TR68_31, were the only ones harboring the P102H mutated ChtR included in this experiment (**Table 1**). For the remaining three isolates (C1177, SN134 and GE1), MIC_{CHX} or MBC_{CHX} of CHX passaged strains were either stable or varied twofold compared to the initial value (P0) without ever being higher than the values obtained for the control at the same experimental point (**Fig. 1-B**). Notably, the human colonization isolate *E. faecium* C1177, lacking the *chlR-efrEF* operon, showed a twofold increased susceptibility for the CHX-exposed strain at P10 and X1 compared to the control (**Fig. 1-B; Table 1**).

Changes in the growth dynamics under CHX for CHX-exposed strains, compared to the parental strains, confirm that CHX adaptation is strain specific among the three *E. faecium* included in the kinetic growth assays. **Figure 2** shows the data related to the highest CHX concentration in which growth was still observed for either the parental and/or CHX-exposed strains. For the CHX-adapted TR68_31, harboring the P102H mutated ChtR, the ability to grow at higher CHX concentrations than the parental strain was evidenced by their growth kinetics under 4 mg/L of CHX, supporting the altered MIC_{CHX} and MBC_{CHX} detected after passages (**Fig. 1 and 2**). Contrastingly, in *chlR-efrEF*-lacking *E. faecium* C1177, which showed increased susceptibility to CHX after passages, compared to the control, the parental strain displayed a greater level of growth during the logarithmic phase than the CHX-exposed strain (**Fig. 1 and 2**). Additionally, whereas the parental strain was still multiplying exponentially after 24h, the latter reached stationary phase before the 20h of growth (**Fig. 2**). Finally, no remarkable difference was observed between the growth curves of the CHX-exposed or parental strain for *E. faecium* SN134, which is also in line with an absence of adaptation as suggested by the MIC_{CHX} and MBC_{CHX} determined after CHX passages (**Fig. 1 and 2**).

Concerning the susceptibility to QACs, MIC_{BC} and MBC_{BC} for the six *E. faecium* ranged between 2 and 4 mg/L at P0 (**Table S1**). The highest MIC_{BC} or MBC_{BC} of 4 mg/L were obtained for *E. faecium* M918981 and *E. faecium* C1304, both isolated from humans (**Table 1 and S1**). Values were mostly stable between passages or fluctuated within a twofold range, which was observed both in passages with and without CHX (**Table S1**). No reduced susceptibility to BC of CHX-adapted bacteria compared to the controls was detected.

The susceptibility or resistance to several antibiotics, accordingly to clinical breakpoints, did not change, both in CHX-adapted and control bacterial populations, from those initially

determined (**Table 1**). However, heteropopulations were frequently observed after passages with and without CHX for the different strains, particularly for antibiotics ampicillin, gentamycin, streptomycin, or erythromycin. Once more, the MIC for ampicillin, vancomycin and linezolid were mostly stable or fluctuated within a twofold range between CHX or CHX-free passages (data not shown).

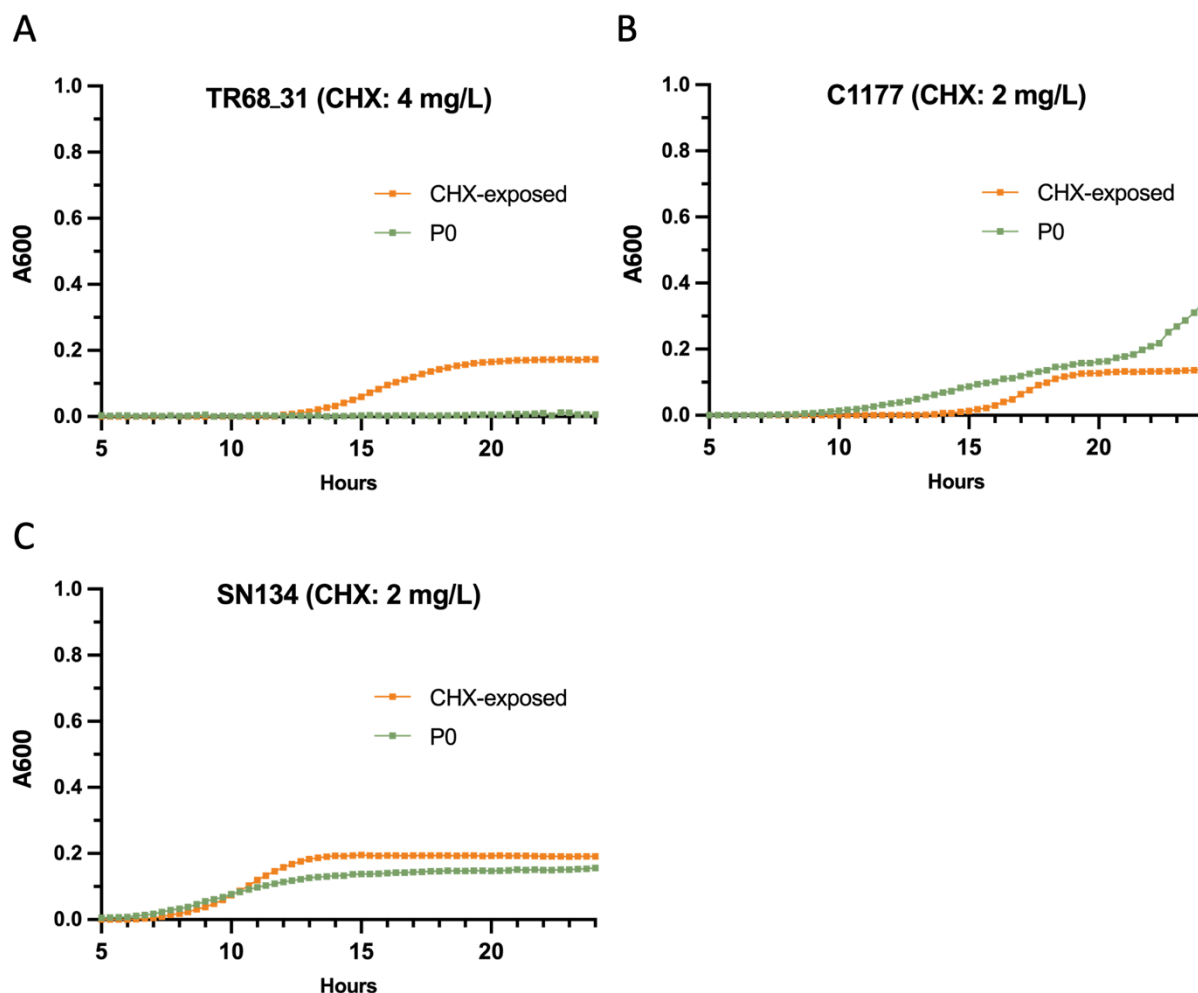


Figure 2. Growth curves obtained for *Enterococcus faecium* TR68_31 (**A**), C1177 (**B**), or SN134 (**C**) strains serially exposed to chlorhexidine (CHX-exposed; orange) or parental strains before passages (P0; green), in the presence of CHX. Isolates were representative of different phenotypic outcomes after CHX-passages (**Fig. 1**) as well as diverse CHX genotypes (**Table 1**). The CHX concentrations represented in the figure correspond to the highest in which growth was observed for the parental and/or CHX-exposed strains, for each of the three isolates. This corresponds to the CHX MIC of the parental TR68_31 and C1177 strains and 1/2 MIC for SN134. Assays were performed at standard conditions (37°C; pH=7.3). Growth curves were determined using a Biotek Synergy HT plate reader (Marshall Scientific), with absorbance at 600 nm (A600) recorded every 20 minutes for 24 hours. Graphics were built using the Prism software v8.1.1 (GraphPad Software; www.graphpad.com).

3.3. Occurrence of mutations in chlorhexidine-adapted strains

The genomic mutations occurring in the coding sequences of two CHX-adapted strains, *E. faecium* C1304 and *E. faecium* TR68-31, with reduced susceptibility to CHX revealed by both higher MIC_{CHX} and MBC_{CHX}, relative to the parental *E. faecium* were identified through WGS (**Table 2**). Various mutations (single nucleotide polymorphisms, deletions and insertions) were detected in different genes, namely in different insertions sequences of the CHX-adapted *E. faecium* C1304 as well as in a LysM peptidoglycan-binding domain-containing protein and a pentapeptide repeat-containing protein for CHX-adapted *E. faecium* TR68_31 (**Table 2**). LysM peptidoglycan-binding domain-containing proteins 100% identical to those identified in the adapted TR68_31 were found across *E. faecium* genomes from diverse sources available at the NCBI database (until 20/01/2023).

Table 2 - Genome mutations in the CHX-adapted *E. faecium* with increased chlorhexidine minimum inhibitory concentrations and minimum bactericidal concentrations, compared to the parental strain.

Isolate	Gene description	Genbank accession number	Nucleotide variation ¹	Amino acid change ¹
C1304	IS30 family transposase	WP_002301399.1	SNP: A609C SNP: A621T SNP: T710C	K204N Q208H V237A
	IS630 family transposase ISBs2	WP_002325012.1	INS: 509A	IAE170-172NC
	IS630 family transposase IS642	WP_226069527.1	SNP: G337A SNP: A364G	V113M K122E
	ISEfm1, transposase		DEL: T390 SNP: T595A	130-144-Trunc. F199I
	IS3 family transposase ISEfa10	WP_322743831.1	SNP: G61T	Trunc. at aa 21
TR68_31	Pentapeptide repeat-containing protein	WP_139910015.1	SNP: A590C	E197A
	LysM peptidoglycan-binding domain-containing protein	WP_002326685.1	INS: 302CAAGCTGAACCTGTT SNP: A335C	101QAEPV K112T

1: Polymorphisms occurring in the strains' whole-genome sequences compared to the parental strains were detected using Snippy v4.6.0 [63] and Breseq v0.36.1 [64].

Abbreviations: aa, amino acid; DEL, deletion; INS, insertion; SNP, single nucleotide polymorphism; Trunc., truncated.

3.4. Effect of subinhibitory concentrations of chlorhexidine on antibiotic resistance gene transfer

The conjugation frequency of 1:1 mating systems in MH broth without the presence of CHX (controls) varied between 8.71×10^{-6} - 5.19×10^{-5} for *vanA* (3 strains), 2.33×10^{-6} - 2.44×10^{-5} for *poxA* (2 strains) and 2.09×10^{-5} for *optrA* (1 strain) (**Fig. 3, Table S2**). When CHX was added to the mating paired systems, at concentrations 200x lower than each donor's strain MIC_{CHX}, the conjugation frequency ranged between 4.80×10^{-5} - 1.09×10^{-4} , 6.07×10^{-6} - 3.07×10^{-5} , and 6.96×10^{-5} for *vanA*, *poxA* and *optrA*, respectively (**Fig. 3, Table S2**). The selected CHX concentrations did not visually affect the isolates' growth rates compared to the controls.

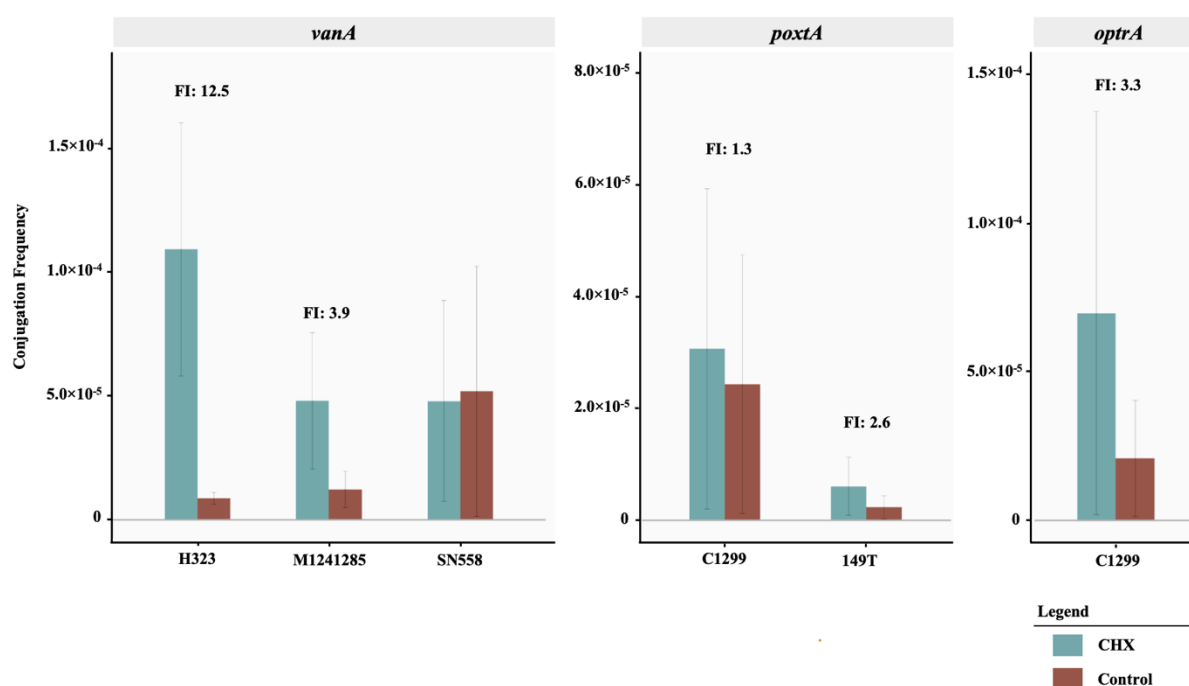


Figure 3. Conjugation frequency for the genes *vanA*, *pox1A* and *optrA* of diverse *E. faecium* strains, in the presence of subinhibitory concentrations of chlorhexidine (CHX; blue) or in the absence of CHX (control; brown). The fold increase (FI) in the conjugation frequency for each mating pair in the presence of CHX compared to the control is indicated. Donor and recipient strains' characteristics are detailed in **Table 1**. Conjugation was tested through a 1:1 broth mating assay. CHX was added to the mating paired systems to reach final concentrations, for each donor/recipient pair, 200 times lower than the CHX minimum inhibitory concentration of the donor strain (SN558: 0,0025 mg/L; M1241285, 149T: 0.01 mg/L; H323, C1299: 0.02 mg/L). CHX-free conjugation mixtures (controls) were tested in the same experimental conditions for each conjugal pair.

The effect of CHX subinhibitory concentrations on the HGT of vancomycin and linezolid resistance genes was variable among the *E. faecium* strains tested (**Fig. 3, Table S2**). Also, for each mating pair tested, the absolute conjugation frequency obtained varied between replicas, both for CHX and CHX-free conjugation assays (**Table S2**). Regarding *vanA*, the conjugation frequency was increased in the presence of CHX for two of the three donor strains tested (**Fig. 3, Table S2**). Increases of 12.5 and 3.9-fold were obtained for *E. faecium* H323 and *E. faecium* M1241285, respectively, two MDR human infection strains, identified as ST17 and ST494, respectively (**Fig. 3, Tables 1 and S2**). In these isolates, the *vanA* was located on the Inc18 plasmid rep_{2_pRE25} (34 kb) and the mosaic Inc18-RepA_N plasmid rep_{2_pRE25}+rep_{17_pRUM} (65 kb), respectively (**Table S3**). For *E. faecium* SN558, a food chain isolate belonging to ST185, the transfer rate was similar in the presence of subinhibitory concentrations of CHX compared to the control (**Fig. 3, Table S2**). Of note, this was the only

strain harboring a truncated *chlR-efrEF* included in the conjugation assays. In *E. faecium* SN558, the *vanA* was located in a plasmid rep_{US15_D03} (RepA_N) of 117 kb (**Table S3**). Similarly, the rate of transfer of genes conferring resistance to linezolid was higher in the presence of CHX, compared to controls, although in lower levels than observed for *vanA* (**Fig. 3, Table S2**). The increase in conjugation frequency varied between 1.3 to 2.6-fold for *poxxA*, among the donor strains *E. faecium* C1299 (ST128, human colonization, 2022) and *E. faecium* 149T (ST1058, food chain, 2015), respectively (**Fig. 3, Tables 1 and S2**). The gene *poxxA* was predictably carried on a rep_{29_Aus0085p4} (Rep_3) plasmid of 15-25 kb in the two isolates (**Table S3**). In the *E. faecium* that also harbored *optrA*, strain C1299, this gene was not located on the same plasmid as *poxxA*. Instead, it was predicted to be on a RepA_N plasmid of 56 kb, whose conjugation frequency was 3.3-fold higher in the presence of CHX (**Fig. 3, Tables S2 and S3**).

4. Discussion

This study shows that exposure to subinhibitory concentrations of CHX can increase the tolerance to this biocide as well as facilitate HGT of genes conferring resistance to clinically-relevant antibiotics among *E. faecium* strains with different epidemiological and clonal backgrounds.

Our data suggest that CHX adaptation seems to be a strain-specific process in clinical and non-clinical *E. faecium*, with variable outcomes on MIC_{CHX} and/or MBC_{CHX} changes and their stability. While both strains carrying the P102H-ChtR mutation (TR68_31 and M918981) adapted to CHX after serial exposure, the increased MIC_{CHX} and MBC_{CHX} in a mutation-lacking *E. faecium* (C1304) suggests that other genotypes or differential gene expression are involved in CHX phenotypic adaptation. Additionally, it is striking that for *E. faecium* C1177, a strain without a functional EfrEF, known to be associated with a lower MIC_{CHX} in *Enterococcus* spp. [9, 20, 21, 55], sustaining growth and/or even survival seems to become increasingly challenging over consecutive exposure to subinhibitory CHX concentrations.

The observed increments in MIC_{CHX} and/or MBC_{CHX} were limited to twofold in this study, which constitutes the lower limit of the previously reported range of 2 to 16-fold for MIC_{CHX} increases following adaptation in *E. faecium* [56, 68]. Higher differences were described in studies with adaptation protocols incorporating a higher number of CHX passages and/or longer incubation periods [56, 68], possibly allowing for an increased density of adapted populations with a higher fitness cost and, consequently, slower growth [11, 27, 56]. Additionally, employing narrower concentration intervals than those used in this study may be advantageous as it can

potentially facilitate the selection of low fitness cost variants and subsequent steps in phenotype evolution [69].

While mutations were detected in the genomes of CHX-adapted strains, the genes affected do not have a known role in CHX susceptibility. The LysM (Lysin Motif) peptidoglycan-binding domain-containing proteins are mostly bacterial extracellular proteins, including peptidoglycan hydrolases, adhesins and virulence factors, but also outer-membrane proteins, lipoproteins or proteins bound to the cell wall [70, 71]. Functional studies on the altered LysM peptidoglycan-binding domain-containing protein are required to assess its role in *E. faecium* and potential connections to CHX response. It is also possible that the changes in CHX susceptibility for these strains resulted from transitory phenotypic adaptations which were still not reversed after 5 CHX-free subcultures, such as alterations in membrane composition, structure and permeability, upregulation of efflux pump activity, or induction of stress response mechanisms [27, 29, 68, 72]. Comprehensive transcriptomic analyses coupled with WGS before and after serial CHX exposure in *E. faecium* from diverse epidemiological and clonal backgrounds should be incorporated in future studies to better understand the transitory and permanent cellular modifications involved in *E. faecium* adaptation to CHX.

Although subinhibitory concentrations of CHX did not induce resistance to several clinically-relevant antibiotics in this study, as has been previously described [68], they were shown to promote the conjugal transfer of plasmids of diverse families and sizes carrying vancomycin or linezolid resistance genes, among clinical and non-clinical *E. faecium* strains. The increases in the conjugation frequency observed here, up to 12.5-fold, are similar to those observed in the transfer of different antibiotic resistance genes between other bacterial taxa by the presence of subinhibitory concentrations of various biocides [65, 73-76]. These studies have suggested the implication of multiple cellular processes, including increased production of reactive oxygen species (ROS), upregulated stress and SOS responses, enhanced cell membrane permeability, and changes in the expression of conjugative transfer genes, among others [65, 73-76]. In *E. faecium*, the mechanisms behind the CHX stimulation of HGT remain to be clarified. Of note, the inability of CHX exposure to increase the conjugation frequency in the only strain lacking a functional EfrEF included in these assays, *E. faecium* SN558, demands more studies to fully understand the role of EfrEF in mitigating CHX stress, which may have potential ramifications for horizontal gene transfer events.

In conclusion, this is a unique study showing the impact of exposure to the widespread subinhibitory CHX concentrations and their potential implications on *E. faecium* from diverse epidemiological and clonal backgrounds. The CHX concentrations leading to strains with reduced CHX susceptibility (0.5-8 mg/L) or enhanced conjugation frequency (0.0025-0.02 mg/L) in our study align with gradients found in various healthcare, food chain, and environmental contexts (0.000028-600 mg/L) [12-15, 77], suggesting that these may promote

the evolution of strains towards CHX tolerance or the spread of antibiotic resistance in diverse scenarios [56, 73, 75]. Simultaneous exposure to other chemicals or physicochemical factors in real environments may additionally enhance or counteract these events and further research in this direction is imperative to comprehensively understand the complex interplay of factors influencing such evolution fostered by CHX exposure. Addressing these challenges will allow the elaboration of One Health strategies to ensure the continued efficacy of critical antimicrobial agents and safeguarding Public Health.

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Supplementary material

Table S1 - Chlorhexidine (CHX) and benzalkonium chloride (BC) minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) distribution of the 6 *E. faecium* strains serially exposed to CHX (CHX-exposed) or passaged in CHX-free medium (control).

Isolates		CHX-exposed ¹					Control ¹				
		P0	P5	P10	X1	X5	P0	P5	P10	X1	X5
CHX susceptibility	MIC (mg/L)	M918981	4	4	4	4	4	4	2	2	2
		C1177	2	2	1	1	2	2	2	2	2
		C1304	4	4	8	8	4	4	2	2	2
		SN134	4	4	4	4	4	4	2	4	4
		TR68-31	4	4	8	8	4	4	2	4	4
		GE1	4	4	2	4	4	4	2	4	4
	MBC (mg/L)	M918981	4	4	8	4	4	4	4	4	4
		C1177	2	2	1	1	2	2	2	2	2
		C1304	4	4	8	8	8	4	4	2	4
		SN134	4	4	4	4	4	4	2	4	4
		TR68-31	4	8	8	8	8	4	4	2	4
		GE1	4	8	4	4	4	8	4	4	4
BC susceptibility	MIC (mg/L)	M918981	2	2	2	4	2	2	2	2	2
		C1177	1	2	1	1	1	1	2	2	2
		C1304	2	2	2	2	2	2	2	2	2
		SN134	2	2	2	2	2	2	2	2	2
		TR68-31	2	2	2	2	2	2	2	2	2
		GE1	2	2	2	2	2	2	2	2	2
	MBC (mg/L)	M918981	4	4	2	4	4	2	2	2	4
		C1177	1	2	1	2	2	1	2	2	2
		C1304	2	2	2	2	4	2	2	4	2
		SN134	2	2	2	2	2	2	2	2	2
		TR68-31	2	2	2	2	2	2	2	2	2
		GE1	2	2	2	2	2	2	2	2	2

1: P0 corresponds to the initial MIC or MBC. Exposure to CHX was performed for ten passages (P1 to P10), followed by five passages in CHX-free medium (X1 to X5), for the CHX-exposed strains. Serial passages in CHX-free medium (P1 to X5) were carried out in parallel for each strain as the control.

Table S2 - Conjugation frequency for the genes *vanA*, *poxA* and *optrA* of diverse *E. faecium* strains, in the presence of subinhibitory concentrations of chlorhexidine (CHX) or in the absence of CHX (control).

Gene	Donor strain ¹	Recipient strain ¹	Condition	Replica	Conjugation frequency ²	Mean conjugation frequency	Fold increase
<i>vanA</i>	H323	GE1	CHX	1	1,69E-04	1,09E-04	12,5
				2	7,40E-06		
				3	1,51E-04		
			Control	1	1,03E-05	8,71E-06	
				2	1,18E-05		
				3	4,02E-06		
	M1241285	GE1	CHX	1	2,78E-06	4,80E-05	3,9
				2	4,33E-05		
				3	9,79E-05		
			Control	1	4,17E-06	1,23E-05	
				2	5,79E-06		
				3	2,68E-05		
	SN558	GE1	CHX	1	1,29E-04	4,80E-05	-
				2	7,95E-06		
				3	7,00E-06		
			Control	1	1,53E-04	5,19E-05	
				2	1,48E-06		
				3	1,23E-06		
<i>poxA</i>	C1299	GE1	CHX	1	8,80E-05	3,07E-05	1,3
				2	1,85E-06		
				3	2,22E-06		
			Control	1	7,06E-05	2,44E-05	
				2	1,11E-06		
				3	1,34E-06		
	149T	GE1	CHX	1	1,64E-05	6,07E-06	2,6
				2	4,26E-07		
				3	1,39E-06		
			Control	1	6,55E-06	2,33E-06	
				2	1,17E-07		
				3	3,35E-07		
<i>optrA</i>	C1299	GE1	CHX	1	2,05E-04	6,96E-05	3,3
				2	1,65E-06		
				3	2,22E-06		
			Control	1	6,00E-05	2,09E-05	
				2	1,48E-06		
				3	1,07E-06		

1: Donor and recipient strains' characteristics are detailed in **Table 1**.

2: Conjugation was tested through a 1:1 broth mating assay. CHX was added to the mating paired systems to reach final concentrations, for each donor/recipient pair, 200 times lower than the CHX minimum inhibitory concentration of the donor strain (SN558: 0,0025 mg/L; M1241285, 149T: 0.01 mg/L; H323, C1299: 0.02 mg/L). CHX-free conjugation mixtures (controls) were tested in the same experimental conditions for each conjugal pair.

Table S3 - Predicted plasmid replicon and size carrying the antibiotic resistance genes studied in the conjugation assays.

Isolate	Transferable gene	Predicted plasmid replicon(s) ¹	Predicted size (base pairs) ¹
H323	<i>vanA</i>	rep2_pRE25	33766
M1241285	<i>vanA</i>	rep17_pRUM rep2_pRE25	64850
SN558	<i>vanA</i>	repUS15_DO3	117380
C1299	<i>poxA</i>	rep29_Aus0085p4	24681
	<i>optrA</i>	RepA_N	55596
149T	<i>poxA</i>	rep29_Aus0085p4	15157

1: Plasmid-derived contigs from the assembly graph were predicted through PlasmidEC v1.3 and the binning of plasmid-nodes into individual plasmid predictions was achieved using gplas2 v2.0 [52]. The screening for resistance genes was performed with ABRicate using the ResFinder database v4.4.2 and identification and typing of plasmid replicons was performed with PlasmidFinder v2.1, available at CGE [50, 53].



Chapter IV

Conclusions

“-To be confused is really a very exciting thing.

-Science is the desire to remain confused”

Robin Ince & Brian Cox

Summarizing the research carried out in this thesis, the overall results point to the following general conclusion:

Although cationic biocides retain a good activity against *E. faecium* and *E. faecalis* from diverse epidemiological and clonal backgrounds, an evolution towards higher tolerance has been observed over the years in bacterial populations from the healthcare or food chain environments. Exposure of *Enterococcus* spp. to widespread subinhibitory concentrations of cationic biocides in diverse settings with variable physicochemical factors may be contributing to such evolution as well as to the spread of antibiotic resistance.

The detailed conclusions, supporting each previously defined specific objective, are summarized as follows:

1) *E. faecium* and *E. faecalis* populations from diverse sources, timeframes, clonal lineages, and antibiotic resistance profiles are susceptible to cationic biocides concentrations below those typically used for antiseptis or disinfection.

Susceptibility to chlorhexidine (CHX), benzalkonium chloride (BC) and didecyldimethylammonium chloride (DDAC) was observed in a comprehensive collection of *E. faecium* and *E. faecalis* isolates, representative of diverse sources, timeframes, clonal lineages, and antibiotic resistance profiles. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of these cationic biocides (CBs) against both species were, similarly to the ranges reported in previous literature, below the typical in-use concentrations, both when standard or modified growth conditions were used. Nevertheless, they fall within the CBs concentrations gradients that have been detected in the skin of patients, food products, sewage or surface water. Thus, populations of *E. faecium* and *E. faecalis* showing different susceptibilities to CBs could hypothetically be selected and adapt to gradients of subinhibitory concentrations occurring in various environments. Such data justifies a careful monitoring of CBs tolerance evolution in settings where these compounds are extensively used or discharged into.

More details in the following references:

1.1 Pereira AP, Antunes P, Willems R, Corander J, Coque TM, Peixe L, Freitas AR, Novais C. Evolution of Chlorhexidine Susceptibility and of the EfrEF Operon among *Enterococcus*

faecalis from Diverse Environments, Clones, and Time Spans. *Microbiology Spectrum*. 2022; 10(4):e0117622. doi: 10.1128/spectrum.01176-22.

1.2 Pereira AP, Antunes P, Bierge P, Willems RJL, Corander J, Coque TM, Pich OQ, Peixe L, Freitas AR, Novais C. Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions. *Microbiology Spectrum*. 2023; 11(5):e0232423. doi: 10.1128/spectrum.02324-23.

2) Tolerance to CBs has evolved among *E. faecium* and *E. faecalis* populations from human infections and the food chain, across diverse clonal lineages.

By analyzing the MICs and MBCs distributions of isolates separately by source or time span, significant differences or increasing trends were detected across the diverse populations, revealing an adaptation possibly driven by the widespread growing use of CBs and the resulting concentration gradients in multiple contexts. *E. faecium* or *E. faecalis* populations showing an increased tolerance to CHX, BC or DDAC were, in all cases, recovered either from the healthcare setting or the food chain, two environments where CBs are extensively used. These data are aligned with few published reports of *Enterococcus* spp. and other Gram-positive and Gram-negative species, although obtained from smaller collections less representative of various sources.

A significant correlation between multidrug resistance (MDR) and higher CBs tolerance was not observed among the *Enterococcus* spp. collection tested. Nevertheless, the detection of MDR *E. faecium* or *E. faecalis* isolates in the upper limit of the MICs or MBCs distributions, namely vancomycin or linezolid resistant human infection or food chain strains, alerts for the possibility of a greater selection of these features, particularly by CHX.

Similarly, our results did not suggest an association between *E. faecium* or *E. faecalis* clonal lineages and CHX, BC or DDAC phenotypes, as variable MIC and MBC values were obtained for isolates of each Sequence Type (ST; MLST: multilocus sequence typing) or Complex Type (CT; core genome MLST).

Although the CBs MIC or MBC values of evolved populations were still way below the typical in-use CBs concentrations, the potential continuation of the *Enterococcus* spp. evolution towards CBs tolerance must be assessed in the next years, namely for human infection *E. faecalis* in which the mean CHX MBCs increase was the most remarkable. Future studies will determine the need to develop appropriate interventions in specific sectors highly exposed to CBs in order to preserve their efficacy.

More details in the following references:

1.1 Pereira AP, Antunes P, Willems R, Corander J, Coque TM, Peixe L, Freitas AR, Novais C. Evolution of Chlorhexidine Susceptibility and of the EfrEF Operon among *Enterococcus faecalis* from Diverse Environments, Clones, and Time Spans. *Microbiology Spectrum*. 2022; 10(4):e0117622. doi: 10.1128/spectrum.01176-22.

1.2 Pereira AP, Antunes P, Bierge P, Willems RJL, Corander J, Coque TM, Pich OQ, Peixe L, Freitas AR, Novais C. Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions. *Microbiology Spectrum*. 2023; 11(5):e0232423. doi: 10.1128/spectrum.02324-23.

3) *Enterococcus* spp. from different epidemiological and clonal backgrounds carry intrinsic and broad-host acquired genes with a known or suspected role in CBs tolerance.

Currently, CBs tolerance genotypes in *Enterococcus* spp. primarily associate with intrinsic or acquired efflux activity. The intrinsic heterodimeric multidrug ATP-binding cassette (ABC) transporter EfrEF and the respective transcription regulator ChIR, with a confirmed role in CHX tolerance in both *E. faecium* and *E. faecalis*, were present in all but two of the isolates tested (one of each species). Isolates carrying genes coding for incomplete ChIR, EfrE or EfrF proteins exhibited low CHX MICs but variable MBCs, suggesting that the EfrEF transporter has a protective action against CHX stress only at CHX concentrations affecting growth (corresponding to MIC) but not their survival. A truncated EfrE protein was associated with ST40 *E. faecalis* strains with a human origin, from different time spans and geographical regions, revealing a divergent evolution within this particular clonal lineage for which the cause is not known. In 5 of the 36 isolates with incomplete or absent ChIR-EfrEF, MIC values were similar to *E. faecalis* and *E. faecium* with complete proteins (MIC>1 mg/L), suggesting the implication of other cellular mechanisms in their response to CHX.

On the other hand, *Enterococcus* spp. do not seem to act as relevant hosts of acquired genes encoding efflux pumps that have been associated with tolerance to both CHX and quaternary ammonium compounds (QACs) (*qacA/B* and *oqxAB*) or only to QACs (*qacC*, *qacG*, *qacJ*, *qacZ*, *qrg* and *bcrABC*) in different bacterial species. Nevertheless, the occurrence of identical acquired CBs tolerance protein variants among *Enterococcus* spp. and a broad range of bacterial species from diverse phyla significantly correlated with their predominant isolation source, reflecting the respective microbial communities and settings in which specific CBs

tolerance genes circulate. Although these genes have been found in 0.5% (n=117) of *Enterococcus* spp. published genomes from diverse sources, time spans and STs, their CBs susceptibility phenotypes were not assessed. Among our collection, the single *E. faecium* and *E. faecalis* strains harboring *qacZ* had similar QACs phenotypes to those observed in isolates without the gene, both with or without pre-exposure to subinhibitory concentrations of BC, suggesting that QACs tolerance genes may not have a significant impact on the survival of *Enterococcus* spp. under QACs exposure, as also suggested by few other studies. While it is also possible that the susceptibility assessment methods used did not accurately replicate the necessary conditions for the expression of tolerance, the apparent lack of selective advantage conferred by acquired CBs tolerance genes in *Enterococcus* seems to corroborate their low prevalence.

Future investigations should be designed to assess the true occurrence of acquired CBs genotypes in settings where these compounds are heavily used. This can be achieved by incorporating culture media supplemented with subinhibitory concentrations of CBs, facilitating the selection of more tolerant isolates, which may exist in small populations or require induced phenotypic changes to distinguish themselves from the general population. Additionally, functional studies are needed to ascertain the phenotypic effects of genes with a predicted role in CBs tolerance in *Enterococcus* spp.

More details in the following references:

1.1 Pereira AP, Antunes P, Willems R, Corander J, Coque TM, Peixe L, Freitas AR, Novais C. Evolution of Chlorhexidine Susceptibility and of the EfrEF Operon among *Enterococcus faecalis* from Diverse Environments, Clones, and Time Spans. *Microbiology Spectrum*. 2022; 10(4):e0117622. doi: 10.1128/spectrum.01176-22.

1.2 Pereira AP, Antunes P, Bierge P, Willems RJL, Corander J, Coque TM, Pich OQ, Peixe L, Freitas AR, Novais C. Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions. *Microbiology Spectrum*. 2023; 11(5):e0232423. doi: 10.1128/spectrum.02324-23.

2.1 Pereira AP, Almeida-Santos AC, Duarte B, Antunes P, Peixe L, Freitas AR, Novais C. The Interplay of Chlorhexidine with Antimicrobial Resistance in *Enterococcus faecium*. (In preparation)

4) Genetic contexts of acquired CBs tolerance genes are diverse and enriched in metal and antibiotic resistance genes

Acquired CBs tolerance genes in *Enterococcus* seem to be, like for other bacterial species, mainly located on plasmids, potentially facilitating their transmission via horizontal gene transfer within the microbial communities and settings where *Enterococcus* spp. are found. Furthermore, several CBs tolerance genes (*qacA/B*, *qacC*, *qacZ*, *bcrABC*, *oqxAB*) were co-located with genes conferring resistance to diverse antibiotics (aminoglycosides, beta-lactams, macrolides, lincosamides, streptogramin of group B, or trimethoprim) or metal tolerance genes, namely to copper and cadmium, in *Enterococcus* spp. from different sources, geographical regions, or dates of isolation. These genetic contexts that carry genes responding to different stresses may be co-selected, not only in clinical settings but also in the community, food production or wastewater, where CBs, antibiotics, and other antimicrobials such as metals may occur as selective agents, facilitating their maintenance and spread among bacterial populations.

Additionally, the alignment of CBs tolerance genetic contexts found in *Enterococcus* spp. and other Gram-positive and Gram-negative bacterial species revealed a high variability among them, potentially lead by a high number of recombination events, as suggested by the abundance of insertion sequences and recombinases detected.

More details in the following references:

1.2 Pereira AP, Antunes P, Bierge P, Willems RJL, Corander J, Coque TM, Pich OQ, Peixe L, Freitas AR, Novais C. Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions. *Microbiology Spectrum*. 2023; 11(5):e0232423. doi: 10.1128/spectrum.02324-23.

5) Different growth conditions occurring in the environment affect *Enterococcus* spp. susceptibility to BC regardless of the presence of the *qacZ* gene

The *E. faecium* and *E. faecalis* isolates from different epidemiological and clonal backgrounds exhibiting higher MICs and MBCs to BC at 22°C and/or pH = 5 confirm the impact of variable growth conditions on BC susceptibility, regardless of whether they harbored a known QACs tolerance gene (*qacZ*) or not. While biocide efficacy was, once more, not compromised, considering in-use BC concentrations, these and additional external factors at play in different dynamic environments (e.g., physicochemical, wide range of CBs concentrations, presence

of additional compounds with antimicrobial properties, presence of organic matter) may have important implications in diverse CBs susceptibility and should, thus, be considered in the establishment of standardized biocidal susceptibility assessment tests. Also, environmental stresses may impact the selection of more tolerant populations since the conditions tested, corresponding to room temperature and a mildly acidic pH, are found in diverse contexts on abiotic surfaces or on human skin and fruits/vegetables, respectively, where CBs have also been reported.

Although the mechanisms justifying such changes in BC phenotype in *Enterococcus* spp. need to be elucidated in future analyses, similar effects observed for other Gram-positive or Gram-negative bacteria exposed to CBs at lower temperature or acidic pH have been associated with multiple cellular processes, such as changes in membrane composition and fluidity or reduced cellular metabolism. Indeed, growth kinetics of two *E. faecium* and two *E. faecalis* were altered at 22°C and/or pH = 5, exhibiting extended lag phases and slower exponential growth, resulting in a delayed entry into the stationary phase, compared to standard conditions (37°C, pH = 7.2) under BC exposure. The longer exponential multiplication phases might explain the higher MBCs observed in such conditions.

More details in the following references:

1.2 Pereira AP, Antunes P, Bierge P, Willems RJL, Corander J, Coque TM, Pich OQ, Peixe L, Freitas AR, Novais C. Unraveling *Enterococcus* susceptibility to quaternary ammonium compounds: genes, phenotypes, and the impact of environmental conditions. *Microbiology Spectrum*. 2023; 11(5):e0232423. doi: 10.1128/spectrum.02324-23.

6) CHX adaptation is strain-specific among *E. faecium* from diverse epidemiological and clonal backgrounds

The serial *in vitro* exposure to subinhibitory concentrations of CHX in *E. faecium* allowed valuable insights into their adaptation to this biocide. It produced different outcomes among the strains tested, from diverse epidemiological and clonal backgrounds, although the observed increases in CHX MICs or MBCs were limited to twofold. In previous studies, similar or higher MIC increases from 2 to 16-fold were described following adaptation with a higher number of CHX passages and/or longer incubation periods, possibly allowing for an increased density of adapted populations with a higher fitness cost and, consequently, slower growth. Employing passages with narrower concentration intervals in adaptation protocols may also

be advantageous as it can potentially facilitate the selection of low fitness cost variants and subsequent steps in phenotype evolution.

Of note, previously known CHX genotypes might be connected to the strain-specific CHX adaptations observed. Specifically, the single amino acid mutation P102H in the conserved DNA-binding response regulator ChtR, part of an intrinsic putative two-component regulatory system, appears to confer a potential advantage in the evolution towards higher CHX tolerance. Still, the involvement of other cellular mechanisms, such as additional unknown genotypes or differential gene expression, was indicated by the higher tolerance of a CHX-adapted *E. faecium* without this mutation. Contrastingly, in the sole strain lacking a functional EfrEF, susceptibility to CHX increased with successive CHX passages. This implies that the absence of EfrEF not only hinders the selection of a more tolerant phenotype but also poses challenges for bacteria in overcoming the stress induced by CHX, even at subinhibitory concentrations, following consecutive exposure.

While mutations were detected in the genomes of CHX-adapted strains, the role of the affected genes in CHX susceptibility is unknown and needs to be clarified. Nevertheless, increased tolerance may also result from phenotypic adaptations, possibly alterations in membrane structure and permeability, upregulation of efflux pump activity or induction of stress response mechanisms. Thus, future studies using comprehensive transcriptomic analyses coupled with whole-genome sequencing before and after serial CHX exposure are needed to better understand the reversible and irreversible cellular changes involved in *E. faecium* adaptation to CHX.

The subinhibitory concentrations of CHX leading to *E. faecium* adapted strains with reduced susceptibility are similar to those found in healthcare, food chain, and environmental contexts, suggesting that these may promote the evolution of strains towards CHX tolerance in diverse settings with potential implications to Public Health, from a One Health perspective.

More details in the following references:

2.1 Pereira AP, Almeida-Santos AC, Duarte B, Antunes P, Peixe L, Freitas AR, Novais C. The Interplay of Chlorhexidine with Antimicrobial Resistance in *Enterococcus faecium*. (In preparation)

7) Subinhibitory concentrations of CHX promote horizontal transfer of antibiotic resistance genes among clinical and non-clinical *E. faecium*

Subinhibitory concentrations of CHX were also shown to promote the conjugal transfer of plasmids of different families and sizes carrying vancomycin or linezolid resistance genes, in all but one of the *E. faecium* strains tested, from diverse epidemiological and clonal backgrounds. The increases in the conjugation efficiency observed here, up to 12.5-fold, are similar to the effects observed for the transfer of different antibiotic resistance genes between other bacterial taxa by the presence of subinhibitory concentrations of various biocides. Of note, CHX exposure did not increase the conjugation frequency in a strain lacking a functional EfrEF, with more studies required to understand the full extent of EfrEF's impact on overcoming CHX stress, and consequently, horizontal gene transfer.

Although the mechanisms behind the CHX stimulation of horizontal gene transfer in *E. faecium* have not yet been investigated, studies in other species suggest the implication of multiple cellular processes, including increased production of reactive oxygen species (ROS), upregulated stress and SOS responses, enhanced cell membrane permeability, and changes in the expression of conjugative transfer genes, among others.

Thus, besides implications directly associated with the promotion of CHX tolerance, residual CHX concentrations in diverse environments might enhance the spread of antibiotic resistance in *E. faecium* populations, ultimately contributing to the global antibiotic resistance threat and constituting a challenge for Public Health. These results support an in-depth evaluation of the extent to which these phenomena may be occurring in real contexts, where simultaneous exposure to other chemicals or physicochemical factors may additionally impact these events.

More details in the following references:

2.1 Pereira AP, Almeida-Santos AC, Duarte B, Antunes P, Peixe L, Freitas AR, Novais C. The Interplay of Chlorhexidine with Antimicrobial Resistance in *Enterococcus faecium*. (In preparation)

CLOSING REMARKS

This thesis provides key insights into understanding the susceptibility and adaptation dynamics of *E. faecium* and *E. faecalis* populations to CBs. Although their efficacy against *Enterococcus* spp. was not called into question, potential consequences associated with

widespread subinhibitory concentrations of these compounds were here identified and require the utmost attention in future research.

Critically, immediate attention should be directed at understanding the long-term consequences of exposure to subinhibitory concentrations of CBs, namely the mechanisms involved in the adaptation to these compounds or in the co-selection or cross-resistance to antibiotics under diverse environmental conditions, through comprehensive transcriptomic and whole-genome sequencing analyses. Concomitantly, it is crucial to determine the range of CBs concentrations able to impact the evolution of *Enterococcus* spp. populations and promote the horizontal gene transfer of relevant adaptive traits, such as antibiotic resistance genes, among these. Furthermore, standardizing biocide susceptibility assessment methodologies that are of relevance to real environments where CBs concentrations are present would allow for the design of longitudinal surveillance studies evaluating their effect on the local microbial communities, namely on *Enterococcus* spp.

Fulfilling these research gaps will allow a more complete picture of the response of *Enterococcus* spp. to CBs across dynamic environments and the elaboration of One Health strategies to halt their evolution towards antimicrobial resistance, ensuring the continued efficacy of these critical agents and safeguarding Public Health.

"I am among those who think that science has great beauty"

Marie Curie

Response of *Enterococcus* spp. to Cationic Biocides Across Dynamic Environments: Phenotypic and Genotypic Insights

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