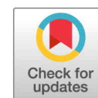
AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

RESEARCH ARTICLE



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

Ana P. Pereira,[a,b] Patrícia Antunes,[a,b,c] Rob Willems,[d] Jukka Corander,[e,f,g] Teresa M. Coque,[h,i] Luísa Peixe,[a,b] Ana R. Freitas,[a,b,j] Carla Novais[a,b]

[a]UCIBIO-Applied Molecular Biosciences Unit, Laboratory of Microbiology, Department of Biological Sciences, REQUIMTE Faculty of Pharmacy, University of Porto, Porto, Portugal

[b]Associate Laboratory i4HB, Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto, Porto, Portugal

[c]Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal

[d]Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

[e]Department of Biostatistics, Faculty of Medicine, University of Oslo, Oslo, Norway

[f]Parasites and Microbes, Wellcome Sanger Institute, Cambridge, United Kingdom

[g]Helsinki Institute of Information Technology, Department of Mathematics and Statistics, University of Helsinki, Helsinki, Finland

[h]Servicio de Microbiologia, Hospital Universitario Ramón y Cajal, Madrid, Spain

[i]Centro de Investigación Biomédica en Enfermedades Infecciosas (CIBER-EII), Madrid, Spain

[j]TOXRUN, Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal

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} ($\leq$1mg/L). Distinct ST40-*E. faecalis* subpopulations carrying truncated and nontruncated EfrE were detected, with the former being predominant in human isolates. This study provides a new insight about CHX susceptibility and ChlR-EfrEF variability within diverse *E. faecalis* populations. The MIC_{CHX}/MBC_{CHX} of more tolerant *E. faecalis* (MIC_{CHX} = 8 mg/L; MBC_{CHX} = 64 mg/L) remain lower than in-use concentrations of CHX ($\geq$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

Editor Cheryl P. Andam, University at Albany, State University of New York

Copyright © 2022 Pereira et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Carla Novais, casilva@ff.up.pt.

The authors declare no conflict of interest.

Received 11 April 2022
Accepted 17 June 2022
Published 7 July 2022

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 ChlR, a putative MerR family transcription regulator (23, 24).

Our aim was to evaluate CHX susceptibility, the variability of the *chlR-efrEF* genes and to correlate CHX phenotypes with *chlR-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 MIC_{50} of 4 mg/L and MIC_{90} 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)

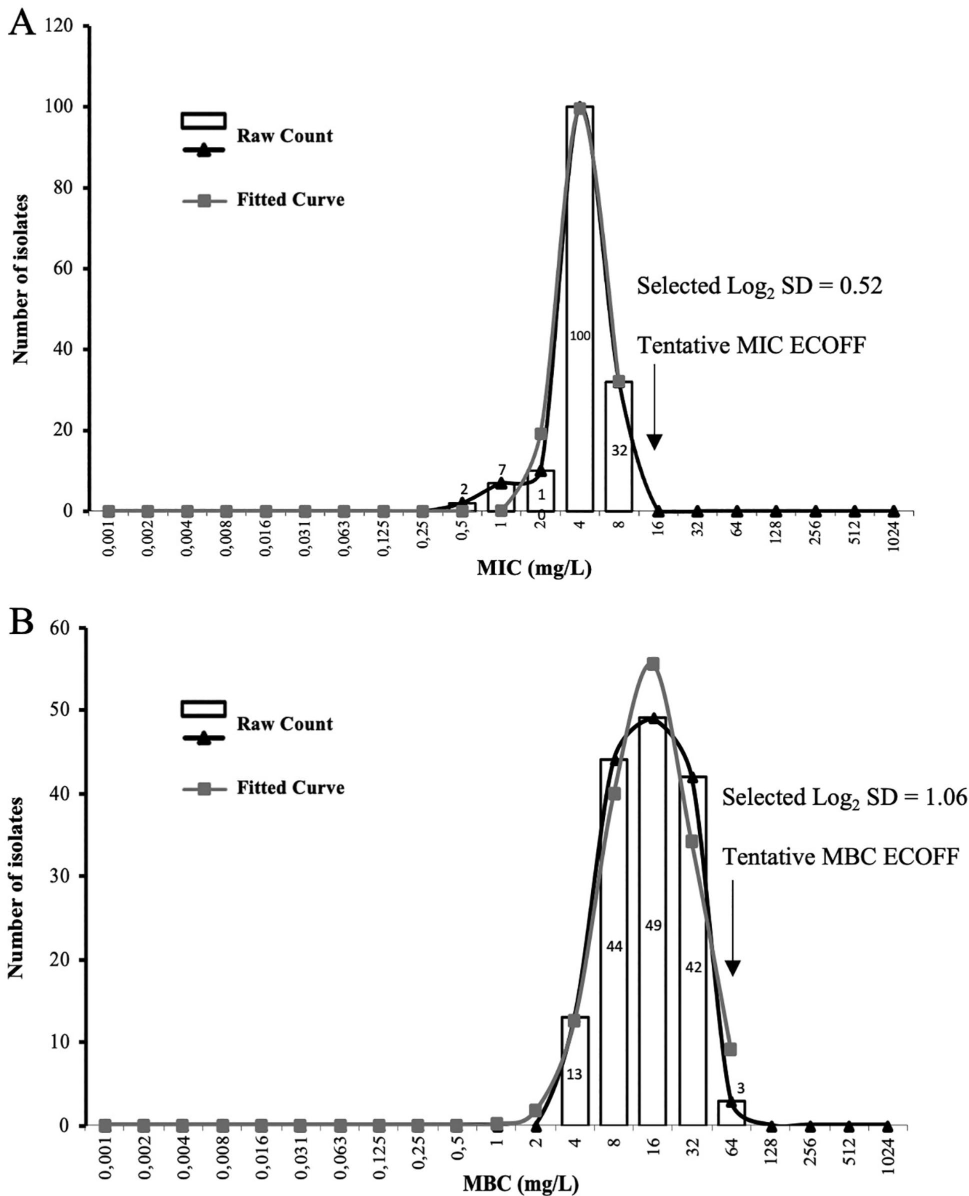


FIG 1 (A, B) Distribution of the *Enterococcus faecalis* studied by different chlorhexidine MICs (A) and minimum bactericidal concentrations (MBCs) (B). The graph-fitted curves were estimated using the ECOFFinder tool, which proposed 8 and 64 mg/L for MIC and MBC, respectively, as limits of 99% of wild-type 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 log_2 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 MBC_{50} of 16 mg/L and MBC_{90} 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 MBC $ECOFF_{CHX}$ 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 MBC_{CHX} = 64 mg/L and 0% probability of an MBC_{CHX} of >64 mg/L. Thus, both analyses point to a tentative MBC $ECOFF_{CHX}$ 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). MIC_{CHX}/MBC_{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 MIC_{CHX}/MBC_{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 MIC_{CHX}/MBC_{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 ChlR-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 ChlR (*n* = 2), EfrE (*n* = 25), or EfrF (*n* = 6) proteins (Fig. S1; Fig. 3; Table S2). To assess a potential association between the incomplete ChlR, 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 MIC_{CHX}/MBC_{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 ChlR-EfrEF proteins (Table S2; Fig. 3).

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

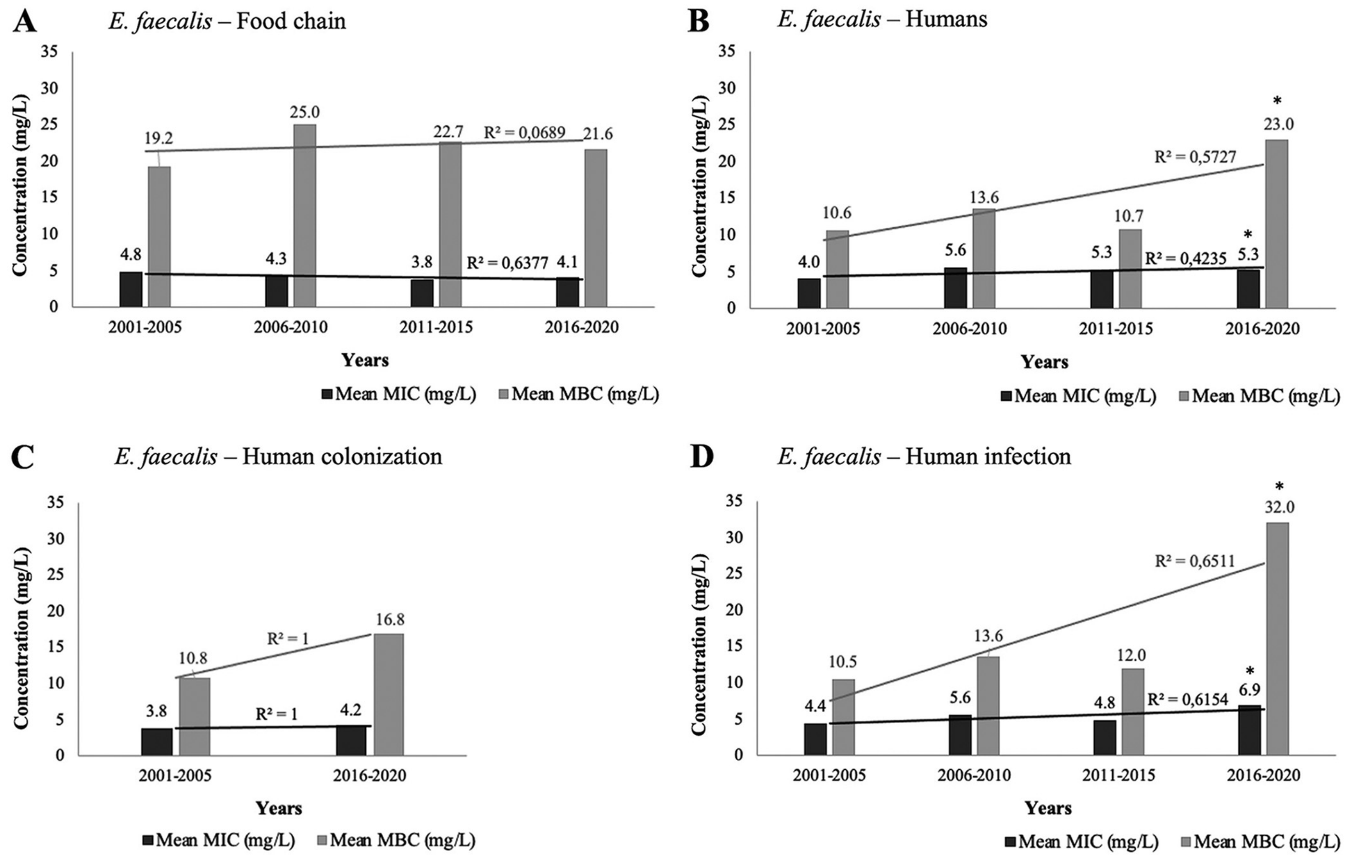


FIG 2 Chlorhexidine mean MIC and minimum bactericidal concentration (MBC) distribution over the years (5-year intervals, from 2001 to 2020) of *E. faecalis* from independently analyzed sources. (A) Distribution of food chain *E. faecalis* isolates ($n = 57$). (B) Distribution of *E. faecalis* isolates 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, BJTJ00000000, BJTS00000000, and BJTH00000000).

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)

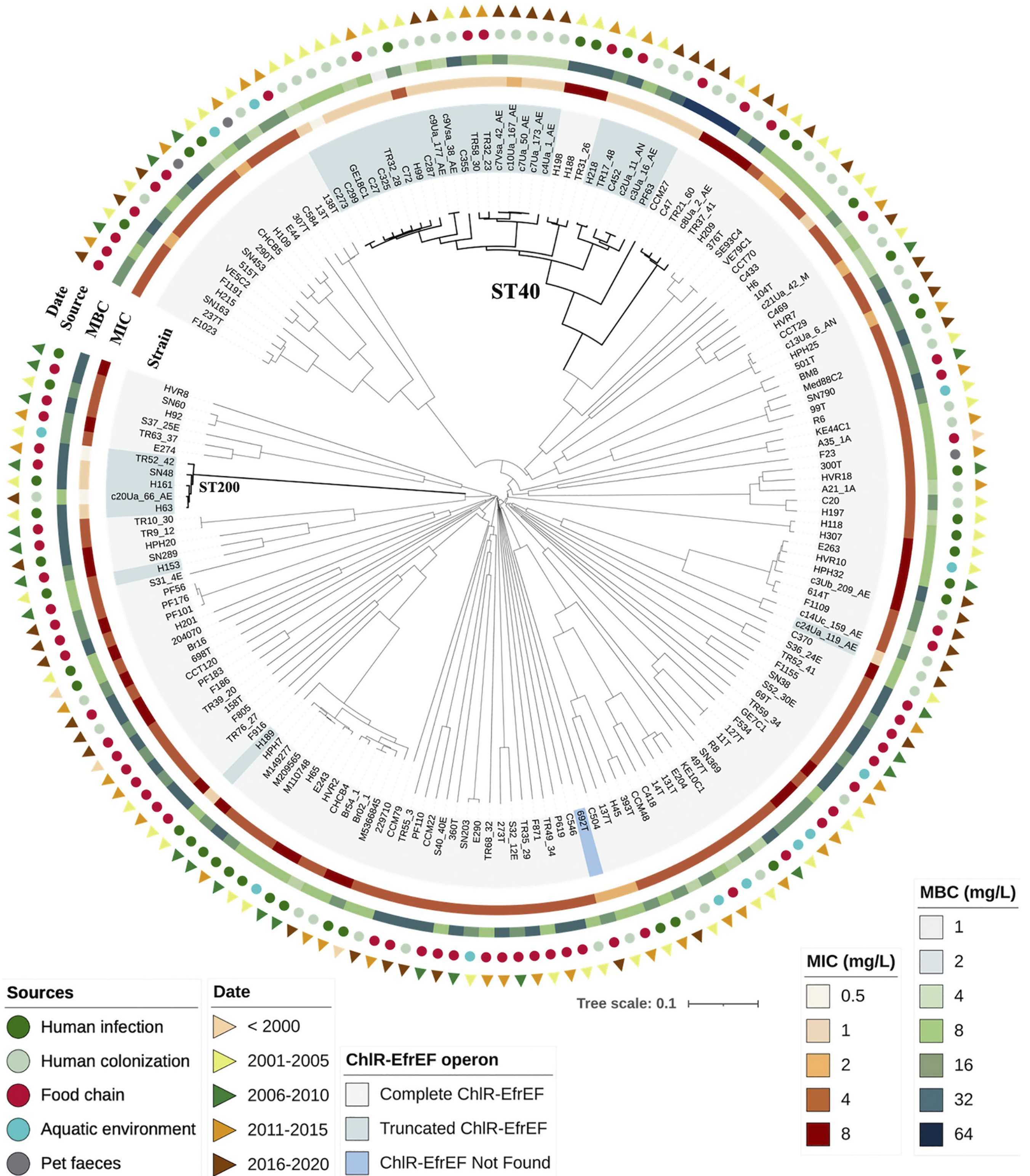


FIG 3 Phylogenetic tree based on the core genome MLST (cgMLST) allelic profiles of all sequenced *Enterococcus faecalis* studied with phenotypic assays (n = 174). The clonal relationship of the strains was established from the sequence analysis of 1,972 gene targets according to the *E. faecalis* cgMLST 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.

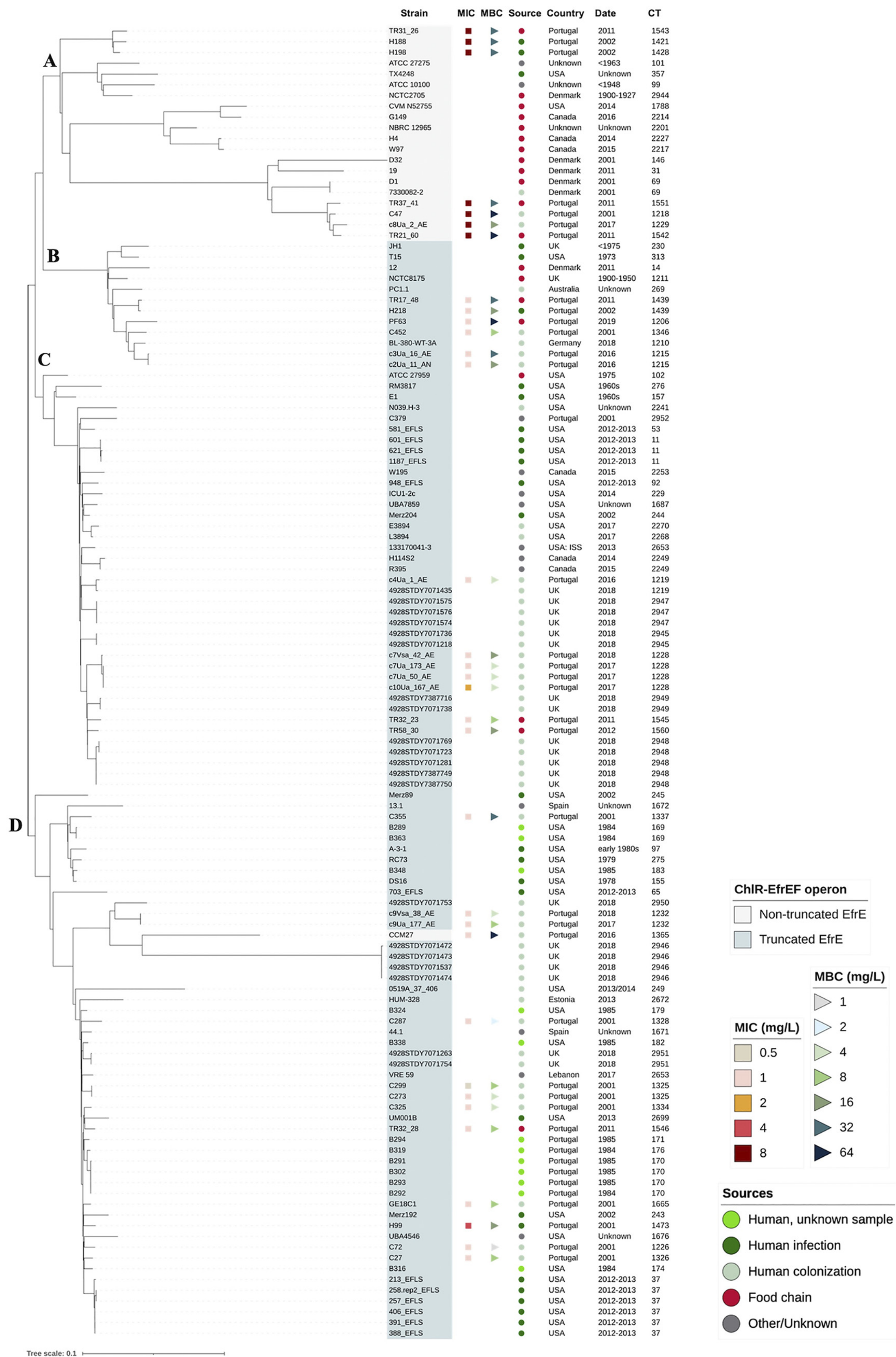


FIG 4 Phylogenetic tree based on the core genome MLST (cgMLST) allelic profiles of *Enterococcus faecalis* identified as ST40 from our collection and available at the PATRIC database (until 18 December 2020) (n = 122). The clonal relationship of the

(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 $\geq$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 baumanni* (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 antisepsis 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 μL 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 $\leq$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.

ACKNOWLEDGMENTS

This work was financed by national funds from Fundação para a Ciência e a Tecnologia (FCT), I.P., in the scope of projects UIDP/04378/2020 and UIDB/04378/2020 of the Research Unit on Applied Molecular Biosciences–UCIBIO and project LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy–i4HB, by the AgriFood XXI I&D&I project (NORTE-01-0145-FEDER-000041) cofinanced by the European Regional Development Fund (ERDF) and through the Programa Operacional Regional do Norte 2014/2020. Ana Paula Pereira was supported by Ph.D. fellowship SFRH/BD/144401/2019 from FCT, cofinanced by European Social Fund through Norte Portugal Regional Operational Program (NORTE 2020), and Ana R. Freitas was supported by the Junior Research Position (CEECIND/02268/2017 – Individual Call to Scientific Employment Stimulus 2017) granted by FCT/MCTES through national funds.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Patrícia Antunes, Teresa M. Coque, Luísa Peixe, Ana R. Freitas, and Carla Novaisa are active members of the European Congress of Clinical Microbiology & Infectious Diseases Study Group on Food- and Water-borne Infections.

Ana Paula Pereira: methodology, software, formal analysis, investigation, writing – original draft, writing – review & editing. Patrícia Antunes: formal analysis, investigation, writing – review & editing, funding acquisition. Rob Willems: formal analysis, writing – review & editing; Jukka Corander: formal analysis, writing – review & editing; Teresa M. Coque: formal analysis, writing – review & editing. Luísa Peixe: supervision, funding acquisition, formal analysis, writing – review & editing. Ana R. Freitas: conceptualization, methodology, formal analysis, supervision, writing – review & editing, funding acquisition. Carla Novais: conceptualization, methodology, software, formal analysis, investigation, supervision, writing – original draft, writing – review & editing, funding acquisition, project administration.

We declare no conflict of interest.

REFERENCES

1. WHO. 2021. World Health Organization Model List of Essential Medicines − 22nd List, 2021. World Health Organization, Geneva, Switzerland. WHO/MHP/HPS/EML/2021.02. Licence CC BY-NC-SA 3.0 IGO.
2. Williamson DA, Carter GP, Howden BP. 2017. Current and emerging topical antibacterials and antiseptics: agents, action, and resistance patterns. Clin Microbiol Rev 30:827–860. https://doi.org/10.1128/CMR.00112-16.
3. Kampf G. 2018. Chlorhexidine digluconate, p 429–534. *In* Antiseptic stewardship. Springer International Publishing, Cham, Switzerland.
4. Climo MW, Sepkowitz KA, Zuccotti G, Fraser VJ, Warren DK, Perl TM, Speck K, Jernigan JA, Robles JR, Wong ES. 2009. The effect of daily bathing with chlorhexidine on the acquisition of methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and healthcare-associated bloodstream infections: results of a quasi-experimental multicenter trial. Crit Care Med 37:1858–1865. https://doi.org/10.1097/CCM.0b013e31819ffe6d.
5. Popovich KJ, Lyles R, Hayes R, Hota B, Trick W, Weinstein RA, Hayden MK. 2012. Relationship between chlorhexidine gluconate skin concentration and microbial density on the skin of critically ill patients bathed daily with chlorhexidine gluconate. Infect Control Hosp Epidemiol 33:889–896. https://doi.org/10.1086/667371.
6. Yokoe DS, Anderson DJ, Berenholtz SM, Calfee DP, Dubberke ER, Ellingson KD, Gerding DN, Haas JP, Kaye KS, Klompas M, Lo E, Marschall J, Mermel LA, Nicolle LE, Salgado CD, Bryant K, Classen D, Crist K, Deloney VM, Fishman NO, Foster N, Goldmann DA, Humphreys E, Jernigan JA, Padberg J, Perl TM, Podgorny K, Septimus EJ, VanAmringe M, Weaver T, Weinstein RA, Wise R, Maragakis LL. 2014. A compendium of strategies to prevent healthcare-associated infections in acute care hospitals: 2014 updates. Infect Control Hosp Epidemiol 35:S21–S31. https://doi.org/10.1017/s0899823x00193833.
7. Marschall J, Mermel LA, Fakih M, Hadaway L, Kallen A, O'Grady NP, Pettis AM, Rupp ME, Sandora T, Maragakis LL, Yokoe DS, Society for Healthcare Epidemiology of America. 2014. Strategies to prevent central line-associated bloodstream infections in acute care hospitals: 2014 update. Infect Control Hosp Epidemiol 35:753–771. https://doi.org/10.1086/676533.
8. Klompas M, Branson R, Eichenwald EC, Greene LR, Howell MD, Lee G, Magill SS, Maragakis LL, Priebe GP, Speck K, Yokoe DS, Berenholtz SM, Society for Healthcare Epidemiology of America (SHEA). 2014. Strategies to prevent ventilator-associated pneumonia in acute care hospitals: 2014 update. Infect Control Hosp Epidemiol 35:915–936. https://doi.org/10.1086/677144.
9. NHMRC. 2019. Australian guidelines for the prevention and control of infection in healthcare. National Health and Medical Research Council, Canberra, Australia.
10. Gilbert P, Moore LE. 2005. Cationic antiseptics: diversity of action under a common epithet. J Appl Microbiol 99:703–715. https://doi.org/10.1111/j.1365-2672.2005.02664.x.
11. ECHA. Substance infocard: chlorhexidine. European Chemicals Agency, Helsinki, Finland. https://echa.europa.eu/pt/substance-information/-/substanceinfo/100.000.217.
12. Jutkina J, Marathe NP, Flach CF, Larsson DGJ. 2018. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Sci Total Environ 616-617:172–178. https://doi.org/10.1016/j.scitotenv.2017.10.312.
13. Bescos R, Ashworth A, Cutler C, Brookes ZL, Belfield L, Rodiles A, Casas-Agustench P, Farnham G, Liddle L, Burleigh M, White D, Easton C, Hickson M. 2020. Effects of chlorhexidine mouthwash on the oral microbiome. Sci Rep 10:5254. https://doi.org/10.1038/s41598-020-61912-4.
14. Oren A, Garrity GM. 2021. Valid publication of the names of forty-two phyla of prokaryotes. Int J Syst Evol Microbiol 71. https://doi.org/10.1099/ijsem.0.005056.

15. Garcia-Solache M, Rice LB. 2019. The *Enterococcus*: a model of adaptability to its environment. Clin Microbiol Rev 32:e00058-18. https://doi.org/10.1128/CMR.00058-18.
16. Gaca AO, Lemos JA. 2019. Adaptation to adversity: the intermingling of stress tolerance and pathogenesis in enterococci. Microbiol Mol Biol Rev 83:e00008-19. https://doi.org/10.1128/MMBR.00008-19.
17. Correa-Martinez CL, Tonnies H, Frobose NJ, Mellmann A, Kampmeier S. 2020. Transmission of vancomycin-resistant enterococci in the hospital setting: uncovering the patient-environment interplay. Microorganisms 8:203. https://doi.org/10.3390/microorganisms8020203.
18. Duarte B, Pereira AP, Freitas AR, Coque TM, Hammerum AM, Hasman H, Antunes P, Peixe L, Novais C. 2019. 2CS-CHX(T) operon signature of chlorhexidine tolerance among *Enterococcus faecium* isolates. Appl Environ Microbiol 85:e01589-19. https://doi.org/10.1128/AEM.01589-19.
19. Guzman Prieto AM, Wijngaarden J, Braat JC, Rogers MRC, Majoor E, Brouwer EC, Zhang X, Bayjanov JR, Bonten MJM, Willems RJL, van Schaik W. 2017. The two-component system ChtRS contributes to chlorhexidine tolerance in *Enterococcus faecium*. Antimicrob Agents Chemother 61:e02122-16. https://doi.org/10.1128/AAC.02122-16.
20. Suwantarat N, Carroll KC, Tekle T, Ross T, Maragakis LL, Cosgrove SE, Milstone AM. 2014. High prevalence of reduced chlorhexidine susceptibility in organisms causing central line– associated bloodstream infections. Infect Control Hosp Epidemiol 35:1183–1186. https://doi.org/10.1086/677628.
21. Ekizoglu M, Sagiroglu M, Kilic E, Hascelik AG. 2016. An investigation of the bactericidal activity of chlorhexidine digluconateagainst multidrug-resistant hospital isolates. Turk J Med Sci 46:903–909. https://doi.org/10.3906/sag-1503-140.
22. Komiyama EY, Lepesqueur LS, Yassuda CG, Samaranayake LP, Parahitiyawa NB, Balducci I, Koga-Ito CY. 2016. *Enterococcus* species in the oral cavity: prevalence, virulence factors and antimicrobial susceptibility. PLoS One 11:e0163001. https://doi.org/10.1371/journal.pone.0163001.
23. Li FJ, Palmer KL. 2018. EfrEF and the transcription regulator ChlR are required for chlorhexidine stress response in *Enterococcus faecalis* V583. Antimicrob Agents Chemother 62:e00267-18. https://doi.org/10.1128/AAC.00267-18.
24. Hurlimann LM, Corradi V, Hohl M, Bloemberg GV, Tieleman DP, Seeger MA. 2016. The heterodimeric ABC transporter EfrCD mediates multidrug efflux in *Enterococcus faecalis*. Antimicrob Agents Chemother 60:5400–5411. https://doi.org/10.1128/AAC.00661-16.
25. Bhardwaj P, Ziegler E, Palmer KL. 2016. Chlorhexidine induces VanA-type vancomycin resistance genes in enterococci. Antimicrob Agents Chemother 60:2209–2221. https://doi.org/10.1128/AAC.02595-15.
26. Ostman M, Lindberg RH, Fick J, Bjorn E, Tysklind M. 2017. Screening of biocides, metals and antibiotics in Swedish sewage sludge and wastewater. Water Res 115:318–328. https://doi.org/10.1016/j.watres.2017.03.011.
27. EMEA. 1996. Chlorhexidine: Summary report. Committee for Veterinary Medicinal Products, EMEA/MRL/107/96. European Agency for the Evaluation of Medicinal Products - Veterinary Medicines Evaluation Unit, London, UK.
28. ECCC. 2019. Screening assessment – chlorhexidine and its salts. Canada Gazette 153:26. Environment and Climate Change Canada, Vancouver, Canada. https://www.canada.ca/en/environment-climate-change/services/evaluating-existing-substances/screening-assessment-chlorhexidine-salts.html.
29. Forbes S, Dobson CB, Humphreys GJ, McBain AJ. 2014. Transient and sustained bacterial adaptation following repeated sublethal exposure to microbicides and a novel human antimicrobial peptide. Antimicrob Agents Chemother 58:5809–5817. https://doi.org/10.1128/AAC.03364-14.
30. Baquero F, Coque TM. 2014. Widening the spaces of selection: evolution along sublethal antimicrobial gradients. mBio 5:e02270. https://doi.org/10.1128/mBio.02270-14.
31. Bock LJ, Wand ME, Sutton JM. 2016. Varying activity of chlorhexidine-based disinfectants against *Klebsiella pneumoniae* clinical isolates and adapted strains. J Hosp Infect 93:42–48. https://doi.org/10.1016/j.jhin.2015.12.019.
32. Hardy K, Sunnucks K, Gil H, Shabir S, Trampari E, Hawkey P, Webber M. 2018. Increased usage of antiseptics is associated with reduced susceptibility in clinical isolates of *Staphylococcus aureus*. mBio 9:e00894-18. https://doi.org/10.1128/mBio.00894-18.
33. Buxser S. 2021. Has resistance to chlorhexidine increased among clinically-relevant bacteria? A systematic review of time course and subpopulation data. PLoS One 16:e0256336. https://doi.org/10.1371/journal.pone.0256336.
34. Sethi DK, Felgate H, Diaz M, Faust K, Kiy C, Clarke P, Hartel C, Rupp J, Webber MA. 2021. Chlorhexidine gluconate usage is associated with antiseptic tolerance in staphylococci from the neonatal intensive care unit. JAC Antimicrob Resist 3:dlab173. https://doi.org/10.1093/jacamr/dlab173.
35. Van den Poel B, Saegeman V, Schuermans A. 2022. Increasing usage of chlorhexidine in health care settings: blessing or curse? A narrative review of the risk of chlorhexidine resistance and the implications for infection prevention and control. Eur J Clin Microbiol Infect Dis 41:349–362. https://doi.org/10.1007/s10096-022-04403-w.
36. Gadea R, Glibota N, Perez Pulido R, Galvez A, Ortega E. 2017. Adaptation to biocides cetrimide and chlorhexidine in bacteria from organic foods: association with tolerance to other antimicrobials and physical stresses. J Agric Food Chem 65:1758–1770. https://doi.org/10.1021/acs.jafc.6b04650.
37. Kaspar JR, Godwin MJ, Velsko IM, Richards VP, Burne RA. 2019. Spontaneously arising *Streptococcus mutans* variants with reduced susceptibility to chlorhexidine display genetic defects and diminished fitness. Antimicrob Agents Chemother 63:e00161-19. https://doi.org/10.1128/AAC.00161-19.
38. Pontinen AK, Top J, Arredondo-Alonso S, Tonkin-Hill G, Freitas AR, Novais C, Gladstone RA, Pesonen M, Meneses R, Pesonen H, Lees JA, Jamrozy D, Bentley SD, Lanza VF, Torres C, Peixe L, Coque TM, Parkhill J, Schurch AC, Willems RJL, Corander J. 2021. Apparent nosocomial adaptation of *Enterococcus faecalis* predates the modern hospital era. Nat Commun 12:1523. https://doi.org/10.1038/s41467-021-21749-5.
39. Bhardwaj P, Hans A, Ruikar K, Guan Z, Palmer KL. 2018. Reduced chlorhexidine and daptomycin susceptibility in vancomycin-resistant *Enterococcus faecium* after serial chlorhexidine exposure. Antimicrob Agents Chemother 62:e01235-17. https://doi.org/10.1128/AAC.01235-17.
40. Freitas A, Finisterra L, Tedim A, Duarte B, Novais C, Peixe L, from the ESCMID Study Group on Food- and Water-borne Infections (EFWISG). 2021. Linezolid- and multidrug-resistant enterococci in raw commercial dog food, Europe, 2019–2020. Emerg Infect Dis 27:2221–2224. https://doi.org/10.3201/eid2708.204933.
41. Freitas AR, Elghaieb H, Leon-Sampedro R, Abbassi MS, Novais C, Coque TM, Hassen A, Peixe L. 2017. Detection of optrA in the African continent (Tunisia) within a mosaic *Enterococcus faecalis* plasmid from urban wastewaters. J Antimicrob Chemother 72:3245–3251. https://doi.org/10.1093/jac/dkx321.
42. Silveira E, Freitas AR, Antunes P, Barros M, Campos J, Coque TM, Peixe L, Novais C. 2014. Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages. J Antimicrob Chemother 69:899–906. https://doi.org/10.1093/jac/dkt479.
43. CLSI. 2018. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, CLSI standard M07, 11th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
44. CLSI. 1999. Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline, CLSI document M26-A. Clinical and Laboratory Standards Institute, Wayne, PA.
45. Turnidge J, Kahlmeter G, Kronvall G. 2006. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clin Microbiol Infect 12:418–425. https://doi.org/10.1111/j.1469-0691.2006.01377.x.
46. Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C, Coque TM, Canton R, Baquero F, Murray BE, del Campo R, Willems RJ. 2006. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol 44:2220–2228. https://doi.org/10.1128/JCM.02596-05.
47. Neumann B, Prior K, Bender JK, Harmsen D, Klare I, Fuchs S, Bethe A, Zuhlke D, Gohler A, Schwarz S, Schaffer K, Riedel K, Wieler LH, Werner G. 2019. A core genome multilocus sequence typing scheme for *Enterococcus faecalis*. J Clin Microbiol 57:e01686-18. https://doi.org/10.1128/JCM.01686-18.