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). *E. 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 1784 *E. faecalis* genomes from PATRIC database from different sources, time spans, clonal lineages and antibiotic resistance profiles. CHX minimum inhibitory concentrations (MIC_{CHX}) and minimum bactericidal concentrations (MBC_{CHX}) against *E. faecalis* presented normal distributions (0.5-64 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} (≤1mg/L). Distinct ST40-*E. faecalis* subpopulations carrying truncated and non-truncated EfrE were detected, 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}=8mg/L; MBC_{CHX}=64mg/L) remain lower than in-use concentrations of CHX (>500mg/L). However, CHX increasing use combined with concentration gradients occurring in diverse environments potentially selecting multidrug-resistant strains with different CHX

susceptibilities, alert to the importance of monitoring the trends of *E. faecalis* CHX tolerance within a One-Health approach.

INTRODUCTION

Chlorhexidine (CHX) is a 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, food industry, animal husbandry and pets (3). Currently, CHX is recommended in the prevention of skin or oral colonization and consequent health care-associated infections by multidrug-resistant (MDR) bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) (4, 5). 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, 6). 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, or changes in bacterial communities (e.g. in the oral microbiota towards a greater abundance of Firmicutes) (7-9).

Within Firmicutes, *Enterococcus* spp. is one of the most frequently found taxa in both humans and animals (10). They are members of the oral and gut microbiota of mammals, birds and reptiles, able to cause infections in animals, and one of the leading causes of human hospital-acquired infections globally (10). Their ability to tolerate different stresses facilitates their survival in the environment, being frequently recovered from plants and vegetables, water bodies and soil (10, 11). 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 (12).

Enterococcus faecium populations of clade A1, a cluster overrepresented by clinical isolates, have shown a trend towards CHX tolerance (13). 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 (13, 14). 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 (15-17). 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 (18). 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 (18, 19).

Our aim was to evaluate CHX susceptibility, the variability of the *chlR-efrEF* genes and to link 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 minimum inhibitory concentration(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) of isolates showed an MIC_{CHX} of 0.5-1 mg/L, corresponding in both cases to *E. faecalis* recovered from different sources and clonal lineages. MIC_{CHX} values

105 presented a normal distribution, with a selected $\log_2$ standard deviation (SD) of 0.52 and a
106 fitted curve overlapping the raw count distribution (Fig. 1A). The MIC $ECOFF_{CHX}$ proposed
107 for 99% of the population by the ECOFFinder tool was 8 mg/L. However, the MIC_{CHX}
108 distribution analysis using the NORM.DIST Excel function showed a 4% probability of a
109 wild-type isolate having an MIC_{CHX} of >8 and ≤16 mg/L and 0% >16 mg/L. Therefore, based
110 on the normal distribution data, a tentative MIC $ECOFF_{CHX}$ of ≤16 mg/L is suggested for *E.*
111 *faecalis*.
112 CHX digluconate minimum bactericidal concentration(s) (MBC_{CHX}) ranged from 4 to 64
113 mg/L, with an MBC_{50} of 16 mg/L and MBC_{90} of 32 mg/L. A normal MBC_{CHX} distribution
114 was also observed, being the selected $\log_2$ SD of 1.06 (Fig. 1B). The highest MBC_{CHX} of 32-
115 64 mg/L (30%; n=45/151) as well as the lowest MBC_{CHX} of 4-8 mg/L (38%; n=57/151)
116 comprised in both cases isolates from different sources and clonal lineages. The MBC
117 $ECOFF_{CHX}$ proposed for 99% of the population by the ECOFFinder tool was 64 mg/L, and
118 the NORM.DIST Excel function estimated an 12% probability of a wild-type isolate having
119 an MBC_{CHX} =64 mg/L and 0% >64 mg/L. Thus, both analysis point to a tentative MBC
120 $ECOFF_{CHX}$ of ≤64 mg/L for *E. faecalis*.
121 The analysis of CHX activity regarding isolates' antibiotic resistance profiles showed that
122 MDR *E. faecalis* had higher mean MIC_{CHX} but similar mean MBC_{CHX} comparing to non-
123 MDR ones [5.0 vs 4.2 (P≤0.05) and 16.1 vs 19.4 mg/L (P≥0.05), respectively]. The MIC_{CHX}
124 and MBC_{CHX} among VRE was variable and ranged, respectively, between 4-8mg/L and 4-32
125 mg/L (n=14; human infection, hospital sewage, human faecal samples at hospital admission
126 and dog faeces from 1996-2016). MIC_{CHX}/MBC_{CHX} of linezolid-resistant isolates varied
127 between 1-8 mg/L and 16-64 mg/L (n=6; raw frozen pet food in 2019-2020), respectively.
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131 **_E. faecalis_ isolates from the food chain and recent human samples express higher**
132 **tolerance to chlorhexidine**

133 The MIC_{CHX} and MBC_{CHX} distribution of the 151 *E. faecalis* isolates tested were analyzed
134 separately by source and time span (5-year intervals). The MIC_{CHX} distribution of the 151 *E.*
135 *faecalis* revealed that the mean MIC_{CHX} of isolates from humans (4.8 mg/L; 44 STs among
136 77 isolates) was higher than the associated with isolates from the food chain (4.1 mg/L; 47
137 STs among 59 isolates) ($P \leq 0.05$) but similar to those from the environment (4.8 mg/L; 11
138 STs among 12 isolates) ($P \geq 0.05$). Within the group of *E. faecalis* from humans, the mean
139 MIC_{CHX} was significantly higher among those associated with infection (5.4 mg/L; 27 STs
140 among 41 isolates) than colonization (4.2 mg/L; 29 STs among 36 isolates) ($P \leq 0.05$). In
141 contrast, mean MBC_{CHX} values were significantly higher among isolates from the food chain
142 (22.6 mg/L) than isolates from humans or the environment (15.3 mg/L and 13.0 mg/L,
143 respectively) ($P \leq 0.001$). MBC_{CHX} of *E. faecalis* from human infection or colonization
144 isolates were similar (17.1 mg/L vs 13.2 mg/L, respectively; $P \geq 0.05$).

145 Food chain *E. faecalis* from different time spans showed variable MIC_{CHX} and MBC_{CHX}, with
146 no apparent increasing trend over time (Fig. 2A). However, a significant increasing trend in
147 the mean MIC_{CHX} and MBC_{CHX} over the years was detected in isolates from human sources
148 (Fig. 2B) ($P \leq 0.05$). We also analyzed the MIC_{CHX}/MBC_{CHX} trends separately for strains
149 associated with human infection or colonization (including isolates mostly from faeces or the
150 urinary tract of healthy humans, but also faeces from long-term care facility patients and
151 individuals at hospital admission) (Table S1). The mean MIC_{CHX} and MBC_{CHX} of isolates
152 obtained from human colonization in 2001-2005 (3.8 and 10.8 mg/L, respectively; 15 STs
153 among 16 isolates) was statistically similar to that of more recent ones (2016-2020: 4.2 and
154 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 2001-2005 (10.5 mg/L; 12 STs among 13 isolates) and 2016-2020 (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 and 3, Table S2). In order to better recognize the impact of the incomplete ChlR, EfrE and EfrF proteins on the susceptibility to CHX, the MIC_{CHX} and MBC_{CHX} were also determined for all isolates with incomplete proteins which 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 (0.5-1 mg/L for 91% of the strains, n=30/33), the MBC_{CHX} values ranged from 1 mg/L to 64 mg/L, similarly to that observed for other isolates without frameshift, non-frameshift 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 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 5 ST268 *E. faecalis* human faecal isolates (GenBank acc. numbers:

NZ_CABGJG000000000; CABGJA000000000; BJTJ00000000; BJTS00000000; 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 (696-734 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. 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 392 amino acid position (Table S3).

Among the 632 isolates with a 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).

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205 **EfrE-truncated ST40 *E. faecalis* clustered separately from non-truncated ST40 ones in**
206 **the phylogenetic tree and were mostly recovered from humans**

207 To establish an association between clonal lineages and CHX phenotypes, we performed the
208 core-genome multi-locus sequence typing (cgMLST) based phylogeny of all sequenced *E.*
209 *faecalis* isolates with available phenotypic information (n=174). We identified 77 STs and
210 160 complex types (CTs) with variable MIC_{CHX} and MBC_{CHX} values for isolates of each ST
211 or CT (Fig. 3; Table S2). Nonetheless, it is of note that ST40 *E. faecalis* (18 CTs) and ST200
212 *E. faecalis* (5 CTs) isolates expressing lower MIC_{CHX} (0.5-1 mg/L) clustered separately,
213 while the few ST179, ST308 and ST319 with low MIC_{CHX} were dispersed throughout the
214 phylogenetic tree (Fig. 3).

215 To further analyze the ST40 *E. faecalis*, all 33 ST40 genomes from our collection and the 89
216 available at the PATRIC database (n=122) were separately analyzed in a new cgMLST-based
217 phylogenetic tree (Fig. 4). Isolates with operons encoding a truncated EfrE protein clustered
218 separately from those with operons encoding a complete EfrE protein. Cluster "A" grouped
219 20 of the 21 strains with a non-truncated EfrE (Fig. 4, Table S3), whereas ST40 *E. faecalis*
220 with a truncated EfrE grouped in clusters "B" (n=12 isolates), "C" (n= 39 isolates) or "D"
221 (n=50 isolates), the latter comprising also one isolate with non-truncated EfrE. The oldest *E.*
222 *faecalis* with a truncated EfrE was recovered from the food chain in 1900-1950. Overall,
223 ST40 *E. faecalis* with a truncated EfrE included in clusters C and D were isolated
224 predominantly from humans (81%; n=82/101; $P<0.0001$) of different geographical regions.
225 ST40 *E. faecalis* isolates from cluster A had an MIC_{CHX} of 8 mg/L while most ST40 isolates
226 of clusters B, C and D (n=24/26) had an MIC_{CHX} of 1 mg/L. The only ST40 *E. faecalis* with
227 non-truncated EfrE included in cluster D presented the same ChlR-EfrEF mutations as a
228 ST308 *E. faecalis* from a healthy human, which also had an MIC_{CHX} of 1 mg/L without

229 possessing an incomplete ChlR-EfrEF (Table S2; Fig. 3). Additionally, most of our isolates
230 of clusters A and B had an MBC_{CHX} ≥16 mg/L (92.3%, n=12/13; P<0.0001), while strains in
231 clusters C and D mostly had an MBC_{CHX} <16 mg/L (75%, n=15/20; P≤0.05).
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234 **DISCUSSION**
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236 The increasing challenge to control the growth and transmission of human and animal
237 pathogens in clinical settings, ambulatory care or in the food industry explains the rising use
238 of biocides in different sectors, namely of CHX. However, the scarcity of available data
239 concerning both wild-type bacterial phenotypes and subpopulations' adaptation to biocides
240 over the years limits the perception and the restraint of a potential biocide resistance threat.
241 In this study we showed that the MIC_{CHX} and MBC_{CHX} normal distributions for the *E.*
242 *faecalis* isolates analyzed were in accordance with the ranges previously reported for this
243 species (15, 20). However, the higher mean MBC_{CHX} values found in isolates from the food
244 chain as well as the increasing mean MIC_{CHX}/MBC_{CHX} values of recent isolates from human
245 infections suggest the adaptability of *E. faecalis* populations in settings where CHX is heavily
246 used. A tentative MIC $ECOFF_{CHX}$ and MBC $ECOFF_{CHX}$ of 16 mg/L and 64 mg/L,
247 respectively, proposed by the ECOFFinder tool and the NORM.DIST Excel function analysis
248 based on *E. faecalis* normal distribution, seems, therefore, limited because it comprises
249 isolates with heterogeneous phenotypes and genotypes. Although further molecular analysis
250 are needed to understand the significance of such diversity in bacterial populations classified
251 as "wild-type" for CHX, the MIC/MBC_{CHX} values found are considerably below the in-use
252 concentrations of CHX (500 to 40,000 mg/L) (2, 3). Nevertheless, they are within or higher
253 than the levels that have been detected in the skin of patients subjected to CHX bathing

(<4.69-600 mg/L), in cow milk (4-78 mg/L) or in sewage (28-1300 ng/L) (5, 21, 22). As CHX tends to persist in water, sediment and soils (23), diverse *E. faecalis* populations showing different CHX susceptibilities could hypothetically be selected and adapt within gradients of sub-inhibitory concentrations occurring not only in patients' skin but also in diverse environments (5, 21-25).

The detection of *E. faecalis* isolates falling into the upper borderline of the MBC_{CHX} distribution (32-64 mg/L), with many of them recently recovered from human infections or the food chain, and some showing resistance to vancomycin or linezolid, alerts for the possibility of MDR strains selection by CHX as well as an adaptation towards CHX tolerance in the following years. Such increase in CHX tolerance over time has been described for other relevant bacterial species, such as *Staphylococcus aureus, Klebsiella pneumoniae* or *Acinetobacter baumanni* (26-28), suggesting that the increasing use of CHX since the 2000s in community, veterinary and hospital contexts (22, 27) might have been contributing to this ecological adaptation. Moreover, other bacterial stresses, 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 (29, 30), 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 (18). We showed 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* affect *E. faecalis* growth 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 (31), but a divergent evolution among strains with truncated and non-

279 truncated EfrE was detected, being both selected across different time spans and geographical
280 regions. Most *E. faecalis* with truncated EfrE, presenting the same mutation, were of human
281 origin, being isolated from this source at least since the 1960's. However, whether this
282 truncated EfrE subpopulation reflects multiple evolved genomic regions of ST40 *E. faecalis*
283 with a better human host adaptation, namely to colonization, remains to clarify. More studies
284 are also needed to better understand the role of the EfrEF operon in the metabolism of *E.*
285 *faecalis* and specifically in the tolerance to CHX and other stresses, as this operon was
286 described to be involved in the transport of ethoxylated fatty amines, fluoroquinolones and
287 fluorescent dyes (18, 19, 32). Although changes in the *chlR-efrEF* genes seem to impact
288 strains' growth inhibition by CHX in most cases, a few isolates (ST40, ST59 and ST860)
289 with incomplete/deleted ChlR-EfrEF exhibited MIC_{CHX} levels >1 mg/L suggesting the
290 occurrence of other cellular mechanisms allowing bacteria growth under CHX exposure.
291 In conclusion, our study provides novel and comprehensive insights about CHX susceptibility
292 within the *E. faecalis* population structure context, revealing more CHX tolerant
293 subpopulations recovered from the food chain and recent human infections. Although a
294 functional EfrEF operon was previously described to be important to *E. faecalis* V583
295 response to CHX (18), we further show a detailed analysis of the genetic diversity of the
296 operon and the correlation with CHX phenotypes, namely the impact of incomplete ChlR-
297 EfrE proteins on isolates' growth (MIC_{CHX}). The recent strains with a higher tolerance to
298 CHX and the known multiple sources for CHX diffusion pollution (e.g., down-the-drain of
299 CHX containing products used in diverse society sectors) (23) alert for the potential
300 consequences of the growing CHX use and to the need of continuous monitoring *E. faecalis*
301 adaptation towards CHX tolerance within a One Health approach.
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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) (31, 33), 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, Brazil) and time spans (1996-2020) (33-35). 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 3 or more antibiotics from different families), 8% (n=14/181) as resistant to vancomycin and 3% (n=6/181) to linezolid, in previous studies (33-35). Of these, 151 *E. faecalis* 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 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/20h) (36). Using a 96-well microtiter plate containing serial two-fold dilutions of CHX (concentration range of 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 20h at 37ºC. 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 (36).

To determine the MBC_{CHX}, 10µl of each well without visible growth from the 96-well MIC_{CHX} plate were incubated onto brain heart infusion (BHI) agar plates at 37ºC for 24h, as defined by the CLSI (37). 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 (37). Each experiment was repeated 3-6 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 (38). 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 v.16.44 function was used to calculate the probability of occurrence of isolates at higher concentrations and, consequently, evaluate the potential presence of an

353 acquired tolerance mechanism if such probability was too low, using the mean, the standard
354 deviation and with the cumulative normal distribution function option set to TRUE (38).
355 Finally, the statistical significance of the differences between MIC_{CHX} and MBC_{CHX} of
356 isolates from the diverse sources, time spans and with disparate antibiotic resistance profiles
357 was assessed using the two-tailed unpaired Student's *t*-test (Excel v.16.44), and the
358 differences associated with the source and MBC_{CHX} distribution among *E. faecalis* ST40
359 populations were analyzed by the Fisher exact test using GraphPad Prism software, version
360 9.0., with *P* values ≤0.05 considered significant.
361
362 **Whole-genome sequence analysis**
363 The genomic search of *chlR*, *efrE* and *efrF* genes (reference strain: *E. faecalis* V583;
364 GenBank accession no. AE016830.1; locus-tag EF_2225 to EF_2227) was performed in the
365 666 *E. faecalis* sequenced genomes by using the MyDBfinder tool available at the Center for
366 Genomic Epidemiology (www.genomicepidemiology.org). The *chlR-efrEF* genes identified
367 in each genome were translated into the corresponding amino acid sequences by the DNA
368 translate tool of ExPASy SIB Bioinformatics Resource Portal
369 (https://web.expasy.org/translate/) and the occurrence of incomplete ChlR-EfrEF proteins
370 was evaluated.
371 For the sequenced *E. faecalis* included in the phenotypic assays, a comparison of the amino
372 acid sequences with the reference strain *E. faecalis* V583 was performed using the Clustal
373 Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/) to identify specific mutations.
374 Their clonal relationship was also established by MLST and cgMLST ((39);
375 http://pubmlst.org; (40); Ridom SeqSphere+, version 7.2). A phylogenetic tree based on their
376 cgMLST allelic profiles was constructed using Ridom SeqSphere+ software and isolates'
377 information was added to the tree using the 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 1784 *E. faecalis* genomes of the PATRIC database, representing a timespan between 1900-2020 (last update on the 18th of December 2020). In addition, to assess if *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 the 18th of December 2020), using the Ridom SeqSphere+ software. Isolates' information was added to the tree using the iTol software (https://itol.embl.de).

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406 Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Patrícia
407 Antunes: Formal analysis, Investigation, Writing – review & editing, Funding acquisition.
408 Rob Willems: Formal analysis, Writing – review & editing; Jukka Corander: Formal analysis,
409 Writing – review & editing; Teresa M. Coque: Formal analysis, Writing – review & editing.
410 Luísa Peixe: Supervision, Funding acquisition, Formal analysis, Writing – review & editing.
411 Ana R. Freitas: Conceptualization, Methodology, Formal analysis, Supervision, Writing –
412 review & editing, Funding acquisition. Carla Novais: Conceptualization, Methodology,
413 Software, Formal analysis, Investigation, Supervision, Writing – original draft, Writing –
414 review & editing, Funding acquisition, Project administration.

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416

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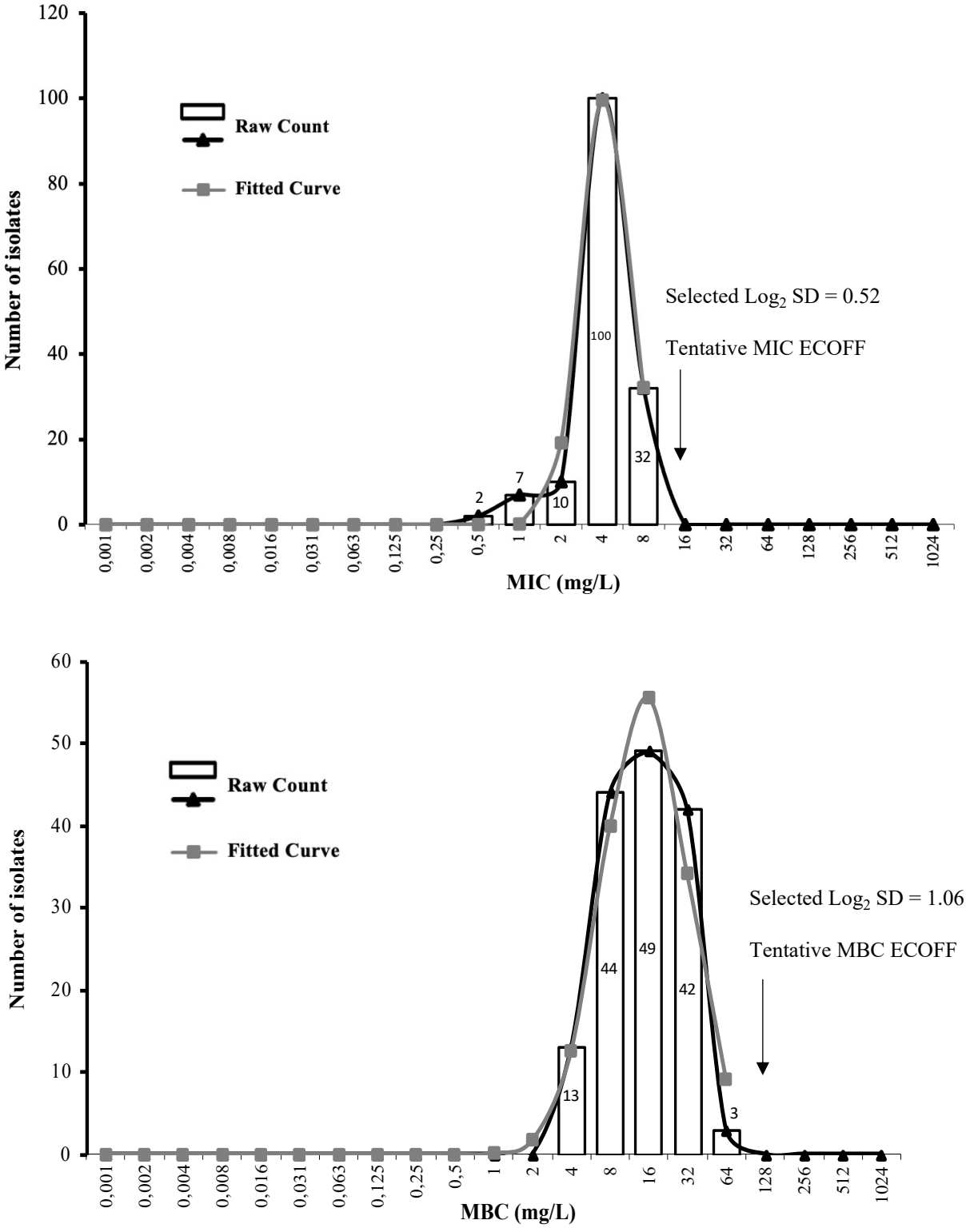
Figure legends

Fig 1. Distribution of the *Enterococcus faecalis* studied by different chlorhexidine minimum inhibitory concentrations-MIC (A) and minimum bactericidal concentrations-MBC (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 ≤16mg/L is 4% and 0% >16mg/L and with an MBC≤64mg/L is 100% and 0% >64mg/L. The tentative ECOFFs for MIC and MBC suggested are, therefore, 16 and 64 mg/L, respectively.

Fig 2. Chlorhexidine mean minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) distribution over the years (five-year intervals, from 2001 to 2020) of *E. faecalis* from independently analysed 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 faecal samples at hospital admission) between 2001-2005 and 2016-2020 (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 2006-2015 (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-squared (R^2) value were added to each distribution using Excel 16.44.

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 accordingly to the *E. faecalis* cgMLST scheme (40), using Ridom SeqSphere+ software version 7.2. *E. faecalis* isolates' features, marked with different colours and shapes using the 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 minimum inhibitory concentrations (MIC), chlorhexidine minimum bactericidal concentrations (MBC), the source and date of isolation. For more isolates' 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 the 18th of December 2020) (n=122). The clonal relationship of the strains was established from the sequence analysis of 1,972 gene targets accordingly to the *E. faecalis* cgMLST scheme (40), 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 minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), the source and date of isolation as well as the CT (Complex Type) of each isolate were marked with different colours, shapes or by text, using the iTol software (https://itol.embl.de). For more isolates' details see Table S3.



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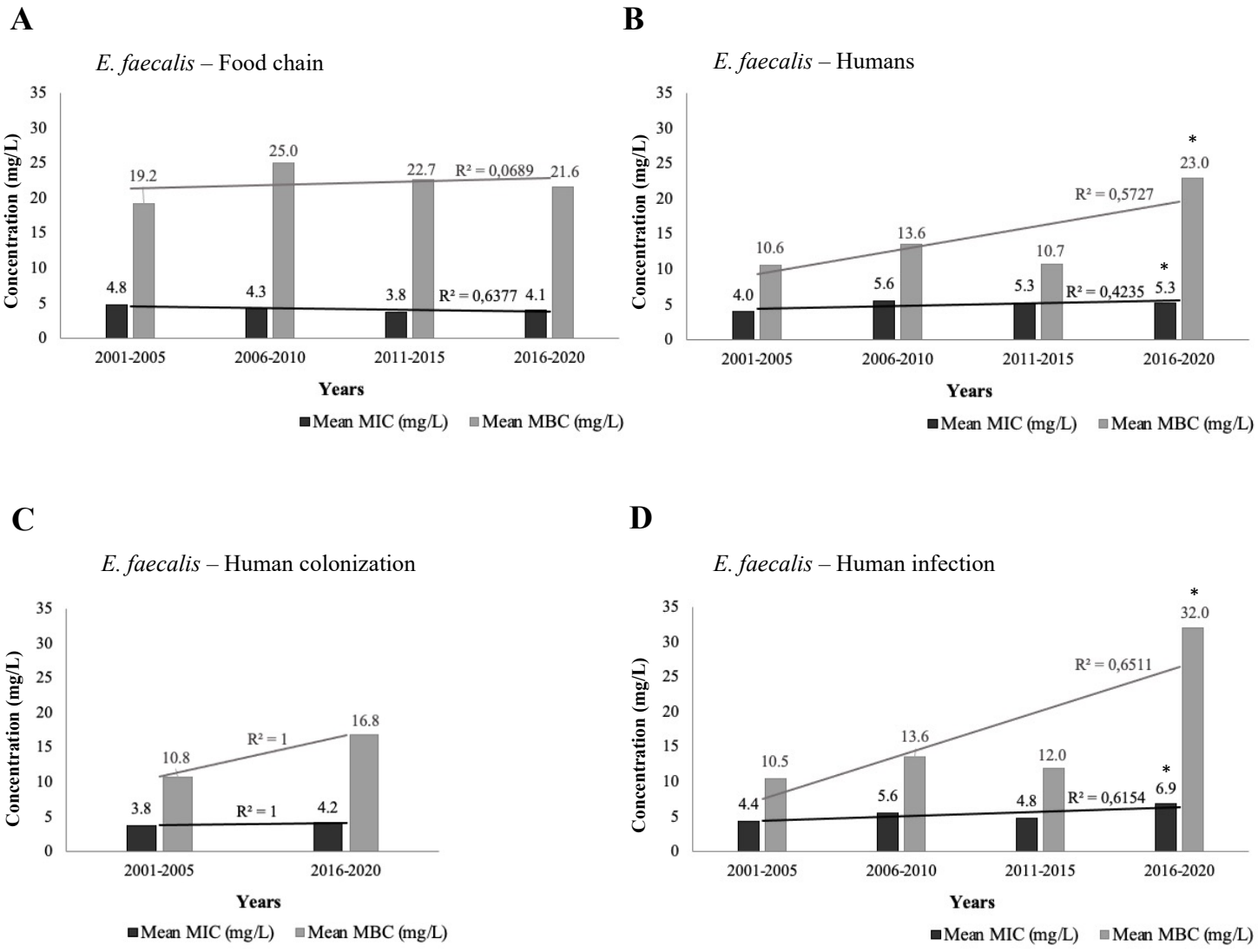

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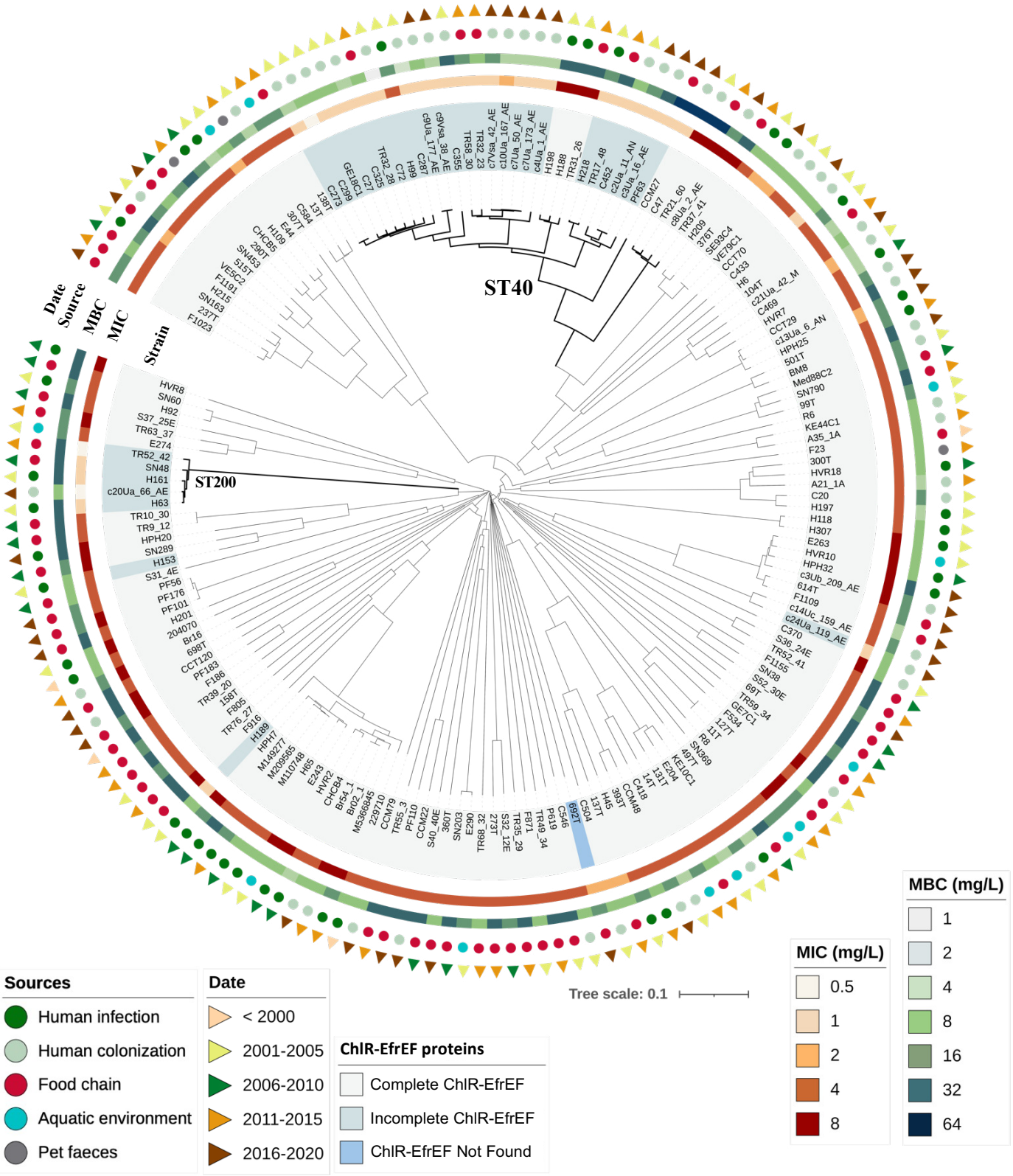

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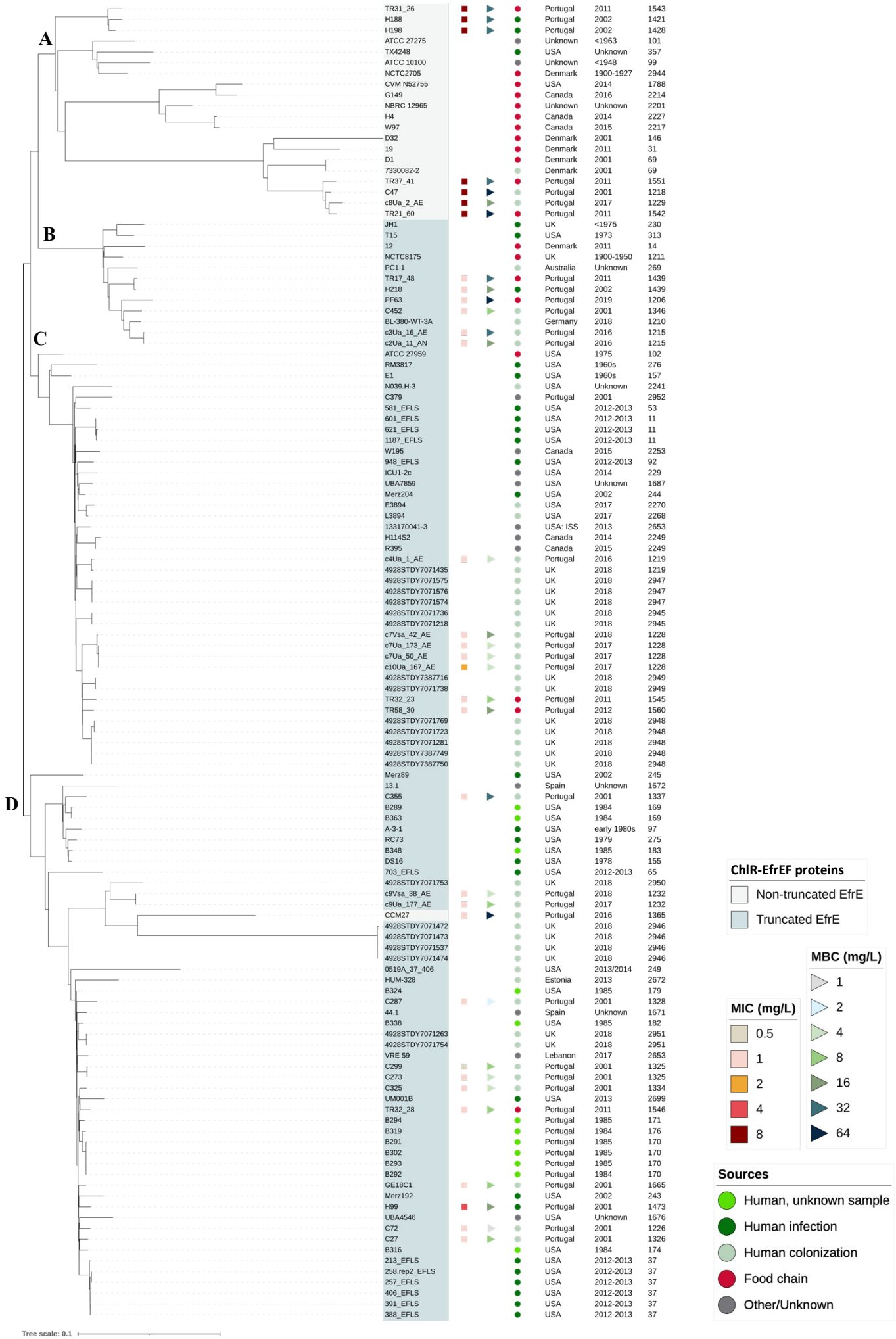
Strain MIC MBC Source Country Date CT
A
B
C
D
TR31_26 Portugal 2011 1543
H188 Portugal 2002 1421
H198 Portugal 2002 1428
ATCC 27275 Unknown <1963 101
TX4248 USA Unknown 357
ATCC 10100 Unknown <1948 99
NCTC2705 Denmark 1900-1927 2944
CVM N52755 USA 2014 1788
G149 Canada 2016 2214
NBRC 12965 Unknown Unknown 2201
H4 Canada 2014 2227
W97 Canada 2015 2217
D32 Denmark 2001 146
19 Denmark 2011 31
D1 Denmark 2001 69
7330082-2 Denmark 2001 69
TR37_41 Portugal 2011 1551
C47 Portugal 2001 1218
c8Ua_2_AE Portugal 2017 1229
TR21_60 Portugal 2011 1542
JH1 UK <1975 230
T15 USA 1973 313
12 Denmark 2011 14
NCTC8175 UK 1900-1950 1211
PC1.1 Australia Unknown 269
TR17_48 Portugal 2011 1439
H218 Portugal 2002 1439
PF63 Portugal 2019 1206
C452 Portugal 2001 1346
BL-380-WT-3A Germany 2018 1210
c3Ua_16_AE Portugal 2016 1215
c2Ua_11_AN Portugal 2016 1215
ATCC 27959 USA 1975 102
RM3817 USA 1960s 276
E1 USA 1960s 157
N039.H-3 USA Unknown 2241
C379 Portugal 2001 2952
581_EFLS USA 2012-2013 53
601_EFLS USA 2012-2013 11
621_EFLS USA 2012-2013 11
1187_EFLS USA 2012-2013 11
W195 Canada 2015 2253
948_EFLS USA 2012-2013 92
ICU1-2c USA 2014 229
UBA7859 USA Unknown 1687
Merz204 USA 2002 244
E3894 USA 2017 2270
L3894 USA 2017 2268
133170041-3 USA: ISS 2013 2653
H114S2 Canada 2014 2249
R395 Canada 2015 2249
c4Ua_1_AE Portugal 2016 1219
4928STDY7071435 UK 2018 1219
4928STDY7071575 UK 2018 2947
4928STDY7071576 UK 2018 2947
4928STDY7071574 UK 2018 2947
4928STDY7071736 UK 2018 2945
4928STDY7071218 UK 2018 2945
c7Vsa_42_AE Portugal 2018 1228
c7Ua_173_AE Portugal 2017 1228
c7Ua_50_AE Portugal 2017 1228
c10Ua_167_AE Portugal 2017 1228
4928STDY7387716 UK 2018 2949
4928STDY7071738 UK 2018 2949
TR32_23 Portugal 2011 1545
TR58_30 Portugal 2012 1560
4928STDY7071769 UK 2018 2948
4928STDY7071723 UK 2018 2948
4928STDY7071281 UK 2018 2948
4928STDY7387749 UK 2018 2948
4928STDY7387750 UK 2018 2948
Merz89 USA 2002 245
13.1 Spain Unknown 1672
C355 Portugal 2001 1337
B289 USA 1984 169
B363 USA 1984 169
A-3-1 USA early 1980s 97
RC73 USA 1979 275
B348 USA 1985 183
DS16 USA 1978 155
703_EFLS USA 2012-2013 65
4928STDY7071753 UK 2018 2950
c9Vsa_38_AE Portugal 2018 1232
c9Ua_177_AE Portugal 2017 1232
CCM27 Portugal 2016 1365
4928STDY7071472 UK 2018 2946
4928STDY7071473 UK 2018 2946
4928STDY7071537 UK 2018 2946
4928STDY7071474 UK 2018 2946
0519A_37_406 USA 2013/2014 249
HUM-328 Estonia 2013 2672
B324 USA 1985 179
C287 Portugal 2001 1328
44.1 Spain Unknown 1671
B338 USA 1985 182
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4928STDY7071754 UK 2018 2951
VRE 59 Lebanon 2017 2653
C299 Portugal 2001 1325
C273 Portugal 2001 1325
C325 Portugal 2001 1334
UM001B USA 2013 2699
TR32_28 Portugal 2011 1546
B294 Portugal 1985 171
B319 Portugal 1984 176
B291 Portugal 1985 170
B302 Portugal 1985 170
B293 Portugal 1985 170
B292 Portugal 1984 170
GE18C1 Portugal 2001 1665
Merz192 USA 2002 243
H99 Portugal 2001 1473
UBA4546 USA Unknown 1676
C72 Portugal 2001 1226
C27 Portugal 2001 1326
B316 USA 1984 174
213_EFLS USA 2012-2013 37
258.rep2_EFLS USA 2012-2013 37
257_EFLS USA 2012-2013 37
406_EFLS USA 2012-2013 37
391_EFLS USA 2012-2013 37
388_EFLS USA 2012-2013 37
Tree scale: 0.1
ChlR-EfrEF proteins
Non-truncated EfrE
Truncated EfrE
MIC (mg/L)
0.5
1
2
4
8
MBC (mg/L)
1
2
4
8
16
32
64
Sources
Human, unknown sample
Human infection
Human colonization
Food chain
Other/Unknown

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 the 18th of December 2020) (n=122). The clonal relationship of the strains was established from the sequence analysis of 1,972 gene targets accordingly to the *E. faecalis* cgMLST scheme (40), 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 minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), the source and date of isolation as well as the CT (Complex Type) of each isolate were marked with different colours, shapes or by text, using the iTol software (https://itol.embl.de). For more isolates' details see Table S3.