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Research article

Effect of sub-inhibitory concentrations of antibiotics on biofilm formation and expression of virulence genes in *penicillin-resistant, ampicillin-susceptible Enterococcus faecalis*

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ABSTRACT

Biofilm formation is a key factor in the pathogenesis of enterococcal infections. Thus, the biofilm-forming ability and frequency of biofilm-related genes in *penicillin-resistant, ampicillin-susceptible Enterococcus faecalis* (PRASEF) compared to penicillin- and ampicillin-susceptible *E. faecalis* (PSASEF) were assessed in the present study. In addition, the effect of sub-inhibitory concentrations (sub-MICs) of antibiotics on biofilm formation and expression of virulence genes was evaluated. Twenty PRASEF and 21 PSASEF clinical isolates were used to determine the effect of sub-MICs of antibiotics (ampicillin, penicillin, and gentamicin) on biofilm formation, and ten selected isolates were subjected to RT-qPCR to detect the transcript levels of virulence genes (*efaA*, *asa1*, *esp*, and *ace*). Antibiotic susceptibility was evaluated by the microdilution broth method. Biofilm formation assay was performed using the microtiter plate method. All PSASEF and PRASEF isolates produced biofilms *in vitro*. Most isolates had three or four virulence genes. Sub-MICs of ampicillin significantly decreased biofilm production and expression of *ace* and *asa1* genes, although the transcript levels were significantly lower (−350% and −606.2%, respectively) among the PSASEF isolates only. Sub-MICs of gentamicin did not have any significant effect on biofilm formation, but slightly increased the transcript levels of *efaA*. In conclusion, this study showed that the biofilm-forming ability and frequency of the evaluated virulence genes were similar among the PRASEF and PSASEF isolates. Further, *in vitro* antibiotic sub-MICs were confirmed to interfere with the expression pattern of virulence genes and biofilm formation by *E. faecalis*. However, further studies are required to clarify the role of sublethal doses of antibiotics on enterococcal biofilms.

1. Introduction

Previously considered as commensal organisms with little clinical significance, in the last decades, enterococci have emerged as nosocomial pathogens and are becoming one of the main causes of healthcare-associated infections, such as urinary, wound, intra-abdominal and bloodstream infections, endocarditis, and more rarely, meningitis (Ramos et al., 2020). Metabolic versatility and resistance to hostile environments (including a wide pH range, ionizing radiation, osmotic and oxidative stresses, and high levels of heavy metals and antimicrobials) facilitate host colonization/infection by enterococcal strains (Ramsey et al., 2014).

Enterococci exhibit intrinsic resistance to some antibiotics, such as aminoglycosides and certain beta-lactams, and acquired resistance to other antibiotics, such as glycopeptides, penicillin, and high-level aminoglycosides (Hollenbeck and Rice, 2012). Notably, these microorganisms have been extraordinarily successful at acquiring resistance determinants to virtually any antimicrobial agent routinely used in therapy, either through chromosomal mutations or by acquiring mobile genetic elements (Hollenbeck and Rice, 2012). Enterococcal antibiotic resistance is a growing clinical challenge as it favors bacterial survival, particularly in the hospital environment, making it harder to treat and increasing the risk of disease spread and death (Hollenbeck and Rice, 2012; Kristich et al., 2014).

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Initially reported in Greece by Metzidie et al. (2006), the emergence of the phenotype of resistance to penicillin G and susceptibility to ampicillin in *Enterococcus faecalis* stresses the capacity of the evolution of enterococci, as acquired resistance to penicillin or ampicillin has rarely been observed in this enterococcal species. In Brazil, our research group first reported the phenotype of penicillin resistance among *E. faecalis* strains recovered from hospitalized patients (Conceição et al., 2011). Furthermore, the penicillin-resistant, ampicillin-susceptible *E. faecalis* (PRASEF) isolates were not found to produce β-lactamase (Metzidie et al., 2006; Conceição et al., 2012; Gawryszewska et al., 2021) and tended to show acquired resistance to other classes of antimicrobials, such as high-level gentamicin resistance (HLGR) (Metzidie et al., 2006; Costa et al., 2019). The emergence of this unusual resistance phenotype in the hospital environment may have been facilitated by the presence and accumulation of various enterococcal virulence factors.

Although enterococci do not possess a vast repertoire of extracellular toxins, several virulence factors that contribute to enterococcal survival in the hospital environment as well as in interindividual transmission and pathogenesis have been identified (Dunny et al., 2014; Goh et al., 2017). Among the main enterococcal virulence factors are the microbial surface components that recognize adhesive matrix molecules, such as adhesion of collagen from *E. faecalis* (encoded by *ace*), enterococcal surface protein (encoded by *esp*), aggregation substance (encoded by *agg* or *asa1*), and surface antigen known as *E. faecalis* antigen A (encoded by *efaA*) (Dunny et al., 2014; Garsin et al., 2014). These virulence determinants are associated with a greater ability of enterococci to adhere to host tissues, and have been implicated in biofilm formation, which is considered a key factor in the pathogenesis of enterococcal infections (Mohamed and Huang, 2007; Fisher and Phillips, 2009).

Biofilm is defined as a community of sessile microorganisms attached to a biotic or abiotic surface and surrounded by a matrix of extracellular polymeric substances, and can be formed in natural, industrial and medical environments (Srivastava and Bhargava, 2016). Clinically, enterococcal biofilms remain a challenge as they contribute to chronic infections and are highly resistant to host defenses and antibiotics, making their eradication extremely difficult (Mohamed and Huang, 2007; Di Domenico et al., 2019). Several studies have proposed that sub-inhibitory concentrations of antibiotics act as signaling molecules that mediate a variety of cellular processes, such as gene transcription and expression, quorum sensing, and biofilm formation in various bacterial species (Kaplan 2011; Laureti et al., 2013; Andersson and Hughes, 2014).

The effects of exposure to sub-inhibitory concentrations of antibiotics on biofilm formation or virulence gene expression in enterococci remain poorly explored. Moreover, to our knowledge, no information is currently available on the biofilm production ability or the presence of biofilm-related virulence genes among emerging PRASEF isolates. Thus, the present study aimed to evaluate (1) the biofilm production ability, (2) the frequency of biofilm-related genes, and (3) the effect of sub-inhibitory concentrations of antibiotics on biofilm formation and expression of biofilm-related genes in PRASEF compared to penicillin- and ampicillin-susceptible *E. faecalis* (PSASEF) isolates.

2. Material and methods

2.1. Bacterial isolates and species identification

Twenty PRASEF and 21 PSASEF clinical isolates were evaluated in this study. These isolates were recovered from hospitalized patients at a Brazilian tertiary hospital, between 2006 and 2016, in previous studies carried out by our research group (Conceição et al., 2012; Costa et al., 2019). The isolates were recovered from different clinical specimens, including secretions (abdominal, pleural, and ocular), urine, wounds, and blood. Species identification for each isolate was based on conventional biochemical tests (Teixeira et al., 2011) and was confirmed by PCR using species-specific primers (Dutka-Malen et al., 1995).

2.2. Antimicrobial susceptibility testing

The microdilution broth method was used in accordance with the CLSI guidelines (CLSI, 2015) to determine the minimal inhibitory concentration (MIC) of beta-lactams (ampicillin and penicillin) and aminoglycoside (gentamicin). Mueller-Hinton broth, brain heart infusion (BHI) broth (Difco, Becton, Dickinson and Company Sparks, France), and solutions of antibiotics prepared from powders of known potencies (Sigma–Aldrich Denmark A/S, Copenhagen, Denmark) were used for the antimicrobial susceptibility test. Overall, the antimicrobial concentrations ranged from 0.25-256 μg/mL; however, gentamicin concentration ranged from to 125–2,000 μg/mL for the HLGR strains.

The results were interpreted according to the CLSI guidelines (CLSI, 2017). MIC of $\geq$16 μg/mL indicated resistance for penicillin and ampicillin, while MIC of >500 μg/mL indicated resistance for gentamicin. Quality control testing was performed using *E. faecalis* ATCC®29212 and ATCC® 51299 as the reference strains.

2.3. Biofilm assay

The microtiter biofilm assay was performed as described by Kafil et al. (2016) and Stepanovic et al., 2007, with some modifications. Briefly, few colonies of each isolate were suspended in BHI broth to OD_{600} (optical density 600) of 0.2 and homogenized for 1 min. Each well of a sterile 96-well polystyrene plate was filled with 200 μL of the bacterial suspension. To evaluate the effect of antibiotics (ampicillin, penicillin, or gentamicin) on biofilm formation, sub-minimal inhibitory concentrations (sub-MICs) specific for each isolate were added to the wells. In general, one-half of the MIC was used; however, as some isolates did not grow at this penicillin or ampicillin concentration in the biofilm production assays, a lower sub-MIC was used (one-quarter or one-eighth of the MIC) (Supplementary Data Table). For gentamicin, 500 μg/mL was used as the sub-MIC only for HLGR strains. The plates were covered and incubated for 24 h at 37 °C under aerobic conditions, without shaking. Each isolate was evaluated in six wells.

After incubation, the supernatant was discarded and the wells were washed three times with 200 μL of sterile phosphate-buffered saline (PBS) to remove unattached cells. The plates were then left to dry for 1 h at 60 °C. After the formed biofilms were fixed with 150 μL methanol for 20 min, methanol was removed and the plate was air-dried overnight at room temperature in an inverted position. The biofilms were stained with 150 μL of crystal violet solution (2%) for 15 min at room temperature, and the wells were rinsed with PBS. Microtiter plates were inverted on a paper towel and air-dried. To detach the biofilms, 150 μL of acetic acid (33%) was added to each well, and the lidded plate was left at room temperature for 30 min without shaking.

The optical density of resolubilized crystal violet was measured at 570 nm using a microtiter plate reader (TP-Reader, ThermoPlate). BHI broth, which was dispensed into six wells per tray, was employed as the negative control. The final OD value for each isolate and the negative control was obtained as the average of six replicates. Duplicates were done for all plates. *E. faecalis* ATCC 29212 was used as the positive control.

Biofilm production was categorized as non-adherent, weakly, moderately, or strongly adherent, based on the mean of the OD values, as proposed by Stepanovic et al. (2000). Briefly, the cut-off OD (ODc) value was defined as three standard deviations above the mean OD of the negative control. Each microtiter plate had an ODc value. The final optical density value of each strain is expressed as the mean OD value obtained following subtraction of the ODc value. Negative results for this subtraction represented biofilm non-adhesion, whereas positive values indicated biofilm production.

2.4. Detection of virulence genes by conventional PCR

To verify the presence of the virulence genes *efaA*, *ace*, *esp*, and *asa1*, which are associated with the colonization of enterococci, DNA of the 41

E faecalis isolates was purified, and each specific gene was amplified by conventional PCR using specific primers, as previously described (Vankerckhoven et al., 2004; Martín-Platero et al., 2009; Moraes et al., 2012). Amplifications were conducted using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA). PCR was carried out with 50 ng/μL of bacterial DNA, 0.2 μM of each specific primer, 0.2 mM of mixed dNTPs, 1X reaction buffer, 2 mM $MgCl_2$, 0.625 U/μL Taq DNA polymerase, and MilliQ water to a final volume of 10 μL. The initial denaturation step (94 °C for 5 min) was followed by 30 cycles of denaturation (94 °C for 1 min), annealing at a specific temperature for each primer pair for 1 min, and extension (72 °C for 1 min), followed by a single final extension step (72 °C for 5 min). Each PCR set included a no-template control. The PCR products were separated by electrophoresis (50–90 min at 90 V) on a 1.5% agarose gel in 1X TAE buffer solution, stained with ethidium bromide, and visualized under UV light. A 100-bp DNA ladder was used as a molecular marker.

2.5. Quantitative RT-PCR assay (RT-qPCR)

The transcript levels of virulence genes (*ace*, *asa1*, *esp*, and *efaA*) in *E. faecalis* clinical isolates, exposed or not exposed (non-treated, NT) to the sub-MICs of ampicillin (a cell wall inhibitor) and gentamicin (a protein synthesis inhibitor), were detected by RT-qPCR. Briefly, the PRASEF and PSASEF isolates, which harbored the four genes simultaneously, were cultured overnight, diluted to 1% in fresh BHI medium (Liofilchem, Italy), and incubated at 37 °C until an OD590 of 0.4 (the exponential phase of growth) was achieved. The cultures were centrifuged (12,000 rpm, 10 min) and the pellet was suspended in 10 mL BHI-containing sub-MICs of antibiotics (ampicillin or gentamicin) or BHI broth only (control) for each isolate. After incubation with shaking for 1 h at 37 °C, the culture was centrifuged and then subjected to RNA extraction using TRIzol™ Reagent (Thermo Scientific, USA), according to the manufacturer's instructions.

The RNA concentration was measured using a NanoDrop™ Lite Spectrophotometer (Thermo Scientific™, USA). All RNA samples were subjected to DNase I treatment with RQ1 RNase-Free DNase (Promega, USA) according to the manufacturer's instructions, and then re-quantified. The RNA quality was measured using the OD260/280 ratio. Following extraction, reverse transcription was performed to obtain cDNA. The GoScript™ Reverse Transcription System kit (Promega) was used according to the manufacturer's instructions. The cDNA was stored at −20 °C.

Gene expression analysis was performed using specific primers mentioned elsewhere (Kafil et al., 2016), on 96-well real-time PCR plates (Applied Biosystems™) sealed with an optical adhesive (Applied Biosystems™). All experiments were performed in triplicate. The final volume of each reaction was 20 μL; each reaction contained 10 μL of QuantiNova SYBR Green Master Mix (2X) (Qiagen, Germany), 2 μL of QN ROX Reference Dye, 0.2 μL of yellow buffer, 2.8 μL of nuclease-free water, 2 μL of the forward primer (0.7 μM), 2 μL of the reverse primer (0.7 μM), and 1 μL of the cDNA sample (100 ng). The amplification reaction and data acquisition in real-time were performed using the StepOne™ System equipment (Applied Biosystems™) under the following conditions: PCR initial heat activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, and combined annealing/extension at 60 °C for 10 s.

The cycle threshold (Ct) value of each transcript was normalized to that of the 23S rRNA gene. The melting curve was analyzed to verify the specificity and identity of the amplified products. The differences between the means of the normalized Cts of the *ace*, *esp*, *asa1*, and *efaA* transcripts of the isolates exposed to gentamicin or ampicillin and the controls (NT) were analyzed in pairs (NT: gentamicin and NT: ampicillin).

2.6. Statistical analysis

Data were tabulated and analyzed using IBM SPSS Statistics 21 and GraphPad Prism 7.0. The data were analyzed for distribution, and the variances were compared (D'Agostino & Pearson normality test and Bartlett's test to compare variances). Mann-Whitney, Wilcoxon matched-pairs signed rank, or Kruskal-Wallis test with Dunn's multiple comparisons was used to assess the biofilm continuous variables independently, and the data are expressed as medians with 95% confidence intervals or maximum and minimum values. The paired t-test was used to assess the difference between the means of the normalized Cts of the isolate transcripts, and the data are expressed as mean and standard deviation. Categorical variables associated with biofilm synthesis were assessed using the Chi-square, Fisher's exact, or Chi-square with Yates' correction tests. The differences were considered significant when was <0.05.

3. Results

The 41 clinical *E. faecalis* isolates included in this study were recovered from various clinical samples, primarily wounds (29.3%), urine (24.4%), blood (17.1%), and others (29.2%) (Supplementary Data Table). All 41 *E faecalis* isolates were susceptible to ampicillin, whereas 85% and 47.6% of the PRASEF and PSASEF isolates, respectively, showed HLGR. The MICs of gentamicin ranged from 8 to >2,000 μg/mL. Ampicillin and penicillin MICs ranged from 0.25–8 μg/mL and 1–32 μg/mL, respectively. The antibiotic MIC values obtained for each isolate and the respective sub-MICs tested are shown in the Supplementary Data Table.

All *E. faecalis* isolates produced biofilms, and most PSASEF and PRASEF isolates (100% and 95%, respectively) were moderate biofilm producers in the absence of antibiotics (Table 1). Figure 1 shows the OD values for each PSASEF (Figure 1A) or PRASEF (Figure 1B) isolate in the absence and presence of sub-MICs of antibiotics. Based on the results, gentamicin did not have any significant effect on biofilm formation in the PRASEF or PSASEF isolates compared with the control (non-treated). Conversely, in the presence of ampicillin or penicillin, biofilm formation was significantly lower in the PRASEF and PSASEF isolates.

All 41 clinical *E. faecalis* isolates had the *efaA* gene. In addition, 37 (90.2%) isolates had the *asa1* gene, 33 (80.5%) had the *esp* gene, and 32 (78.0%) had the *ace* gene. Taken together, our results indicate that each isolate possessed at least two virulence genes associated with biofilm production. In brief, 7.3% (n = 3) of the *E. faecalis* isolates had two genes, 36.6% (n = 15) had three genes, and 56.1% (n = 23) had the four virulence genes. No significant differences were observed when the isolates were stratified according to penicillin susceptibility profile, despite higher rates of *esp* (90.0%) and *asa1* (100.0%) genes in the PRASEF isolates than the PSASEF isolates (71.4% and 81.0%, respectively) (Table 2). Figure 2 illustrates the biofilm formation of the PSASEF (Figure 2A) and PRASEF (Figure 2B) isolates according to the virulence gene pattern and the absence or presence of sub-MIC of antibiotics.

RT-qPCR was used to determine the effects of sub-MICs (gentamicin and ampicillin) on the mRNA levels of virulence genes. Ten PRASEF and PSASEF isolates that were moderate biofilm producers, HLGR, and harbored four virulence genes were selected for this assay. The normalized Ct means of the transcripts of each virulence gene of the PRASEF and PSASEF isolates were analyzed in pairs when the isolates were exposed to sub-MICs of gentamicin and ampicillin, or when they were not treated with antibiotics. A decrease in the levels of *ace* and *asa1* transcripts was observed in isolates exposed to the sub-MICs of ampicillin; however, this decrease was only significant ($p = 0.023$ and $p = 0.008$, respectively) in the PSASEF group (Table 3). Relative quantification revealed that the levels of the *ace* and *asa1* transcripts were approximately 4.4-fold (2^2.17; −350%) and 7.1-fold (2^2.82; −606.2%) lower in the PSASEF isolates than in the non-treated isolates.

4. Discussion

Enterococci have an extraordinary ability to form biofilms, which is a remarkable pathogenesis strategy that allows their survival in adverse conditions and persistence at the site of infection (Mohamed and Huang, 2007). Biofilms have been considered essential in the pathogenesis of enterococcal infections, mainly urinary tract infections, endocarditis, and

Table 1. Classification of the *penicillin-resistant, ampicillin-susceptible Enterococcus faecalis* (PRASEF), and *penicillin- and ampicillin-susceptible E. faecalis* (PSASEF) isolates according to their ability to produce biofilm in absence (non-treated) or exposed to sub-minimal inhibitory concentrations of antibiotics.

Groups of isolates		Number (%) of isolates				*P*-value*
		Non-producer	Weak	Moderate	Strong	
PRASEF (n = 20)	Non-treated	0	1 (5.0)	19 (95.0)	0	<0.001
	Ampicillin	0	11 (55.0)	9 (45.0)	0	
	Penicillin	0	20 (100.0)	0	0	
	Gentamicin	0	3 (15.0)	17 (85.0)	0	
PSASEF (n = 21)	Non-treated	0	0	21 (100.0)	0	<0.001
	Ampicillin	0	11 (52.4)	10 (47.6)	0	
	Penicillin	2 (9.5)	14 (66.7)	3 (14.3)	2 (9.5)	
	Gentamicin	0	1 (4.8)	20 (95.2)	0	

* $p < 0.001$, chi-square test. The dependency of the distribution of the number of isolates was assessed by the type of adherence and exposure to antibiotics for the two groups of isolates, PRASEF and PSASEF. However, when the frequencies of the biofilm production categories were compared between the PRASEF and PSASEF isolates in a paired manner, no statistically significant differences were found ($p = 0.44$, Wilcoxon matched-pairs signed rank test).

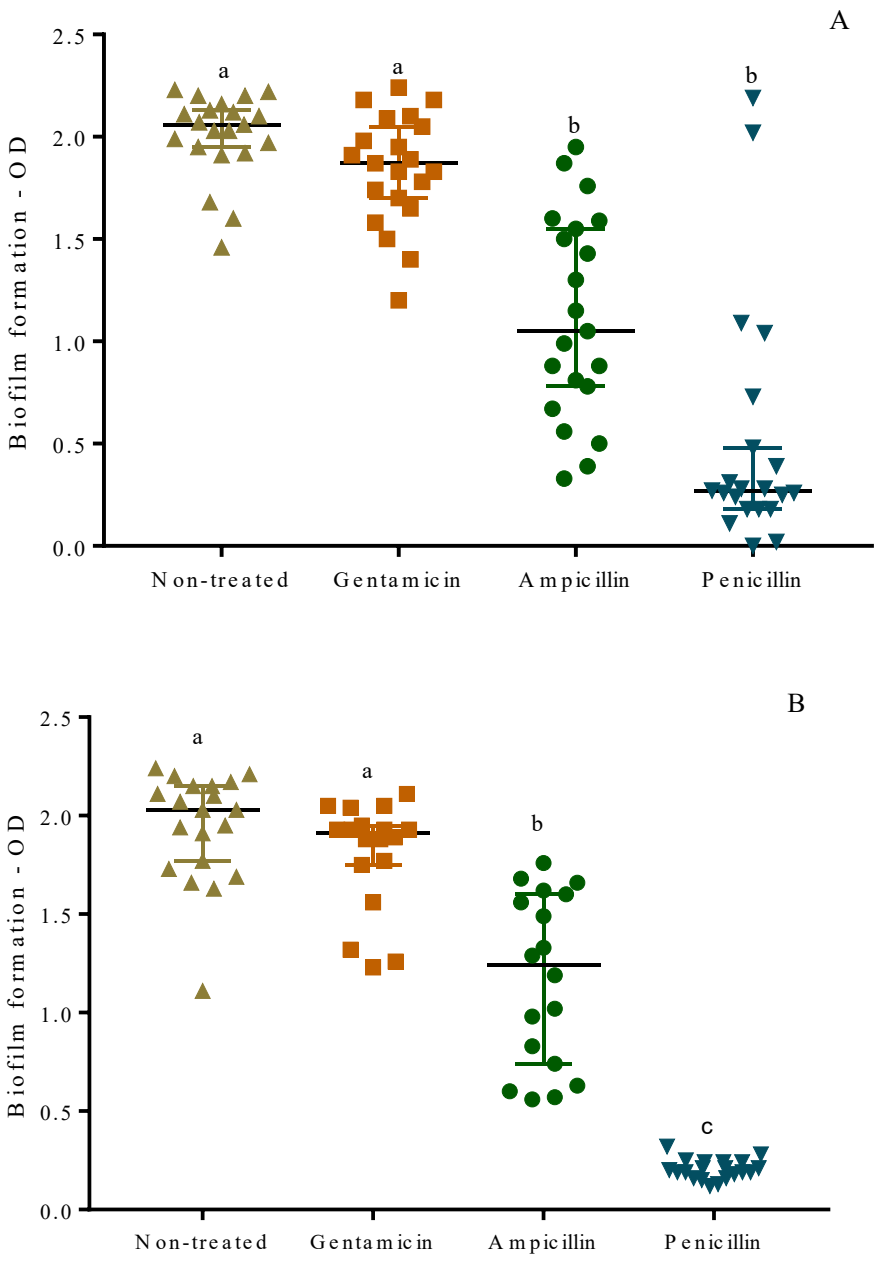

Figure 1. Biofilm formation by (A) *penicillin- and ampicillin-susceptible Enterococcus faecalis* (PSASEF) and (B) *penicillin-resistant, ampicillin-susceptible E. faecalis* (PRASEF) isolates in absence (non-treated) or exposed to sub-minimal inhibitory concentrations of antibiotics. The letters a, b, and c represent statistically significant differences ($p < 0.05$) of the optical density (OD) between groups based on the Kruskal-Wallis with Dunn's multiple comparisons test. Data are expressed as medians and 95% confidence intervals.

Table 2. Presence of the virulence genes (*ace, asa1, esp*, and/or *efaA*) among the *penicillin-resistant, ampicillin-susceptible Enterococcus faecalis* (PRASEF) and *penicillin- and ampicillin-susceptible E. faecalis* (PSASEF) isolates.

Genes	Number (%) of isolates			*P*-value*
	PSASEF (n = 21)	PRASEF (n = 20)	Total (n = 41)	
ace	18 (85.71)	14 (70.00)	32 (78.05)	0.28
*asa*1	17 (80.95)	20 (100.00)	37 (90.24)	0.11
esp	15 (71.43)	18 (90.00)	33 (80.49)	0.24
*efa*A	21 (100.00)	20 (100.00)	41 (100.00)	-
ace + *asa1*	15 (71.43)	14 (70.00)	29 (70.73)	-
ace + *esp*	12 (57.14)	12 (60.00)	24 (58.54)	-
asa1 + *esp*	13 (61.90)	18 (90.00)	31 (75.61)	0.20
ace + *asa1* + *esp*	11 (52.38)	12 (60.00)	23 (56.10)	-
ace + *asa1* + *esp* + *efaA*	11 (52.38)	12 (60.00)	23 (56.10)	-

* *P-values* were determined using Chi-square, Fisher's exact, or Chi-square with Yates' correction tests (significance level was 5%).

wound infections, in addition to ensuring their successful establishment in hospital settings (Srivastava and Bhargava, 2016; Vestby et al., 2020). Biofilm formation is a complex and multifactorial event, but may be attributable in part to specific virulence factors, such as those associated with enterococci colonization/adhesion of the host (Fisher and Phillips, 2009).

Notably, all PRASEF and PSASEF isolates tested in the present study produced biofilm, with all being moderate biofilm producers, except one isolate. In contrast, Weng et al. (2019) found that only 49.0% of the clinical *E. faecalis* isolates recovered from a tertiary hospital in Malaysia were producers of biofilms. Further, among those producers, 28% were identified as strong biofilm producers. However, higher rates of biofilm formation, similar to those found in our study, have been reported in clinical *E. faecalis* isolates recovered in Germany (73.3%), Italy (80%), Spain (98%), and the United Kingdom (100%) (Baldassarri et al., 2001; Sandoe et al., 2003; Maestre et al., 2012; Anderson et al., 2016). A previous Brazilian study reported a high rate (87.3%) of biofilm-producing *E. faecalis* isolates, similar to the present study (Soares et al., 2014). Biofilm formation among enterococci has been proposed to vary according to the nature of specimens, type of strains, and geographical location (Mohamed et al., 2004; Maestre et al., 2012; Tsikrikonis et al., 2012; Weng et al., 2019). The high prevalence of biofilm-producing isolates in the present study might be due to the fact that all isolates are of clinical origin and many cause invasive infection. In addition, these isolates were recovered from a hospital where *E. faecalis* lineages enriched for some virulence-associated and antimicrobial resistance genes are common (Raven et al., 2016; Pöntinen et al., 2021). Of note, although the *E. faecalis* isolates studied originated from a single hospital, they were recovered from different clinical specimens over a long period (2006–2014).

The high biofilm formation ability demonstrated in this study may be due to the simultaneous presence of three or four of the investigated virulence genes (*ace*, *asa1*, *efaA*, and *esp*), which have been linked to biofilm formation by enterococci in many studies, among the isolates. Toledo-Arana et al. (2001) and Soares et al. (2014) found significant associations between *esp* and the ability of *E. faecalis* isolates of clinical origin to form biofilms. Chuang-Smith et al. (2010), Soares et al. (2014), and Anderson et al. (2016) observed an association between the presence of *asa1* and biofilm formation, whereas Seno et al. (2005) reported that *E. faecalis* isolates from urinary tract infections harboring both *asa1* and *esp* genes formed biofilms at significantly higher rates than those with neither gene. In the present study, as virtually all isolates were moderate biofilm producers and harbored the four investigated virulence genes, the association between biofilm-forming ability and biofilm-related virulence genes could not be verified. However, conflicting results have been reported regarding the role of these virulence genes in biofilm formation by enterococci, highlighting the need for further studies on this subject, as virulence factors can act as targets for new drugs to prevent bacterial biofilms (Mohamed et al., 2004; Kristich et al., 2004; Johnson et al., 2004; Paganelli et al., 2012). In addition, other virulence determinants not investigated in this study, such as *gelE* (gelatinase) and *ebp* (endocarditis and biofilm-associated pilus), may also contribute to the high biofilm-forming ability of the PRASEF and PSASEF isolates.

The effect of sub-MICs of antibiotics remains controversial, as some studies have shown that they can induce or decrease biofilm formation and can downregulate or upregulate gene expression *in vitro* in various gram-positive and gram-negative bacterial species (Kaplan 2011; Laureti et al., 2013; Andersson and Hughes, 2014). In this study, the sub-MICs of ampicillin significantly decreased biofilm production and downregulated the expression of *ace* and *asa1*, mainly in the PSASEF isolates, whereas the sub-MICs of gentamicin did not have any significant effect on biofilm formation or expression of the investigated virulence genes. Interestingly, similar to the sub-MICs of ampicillin, penicillin sub-MICs significantly decreased biofilm formation even among the PRASEF isolates whose planktonic cells were resistant to this antibiotic. Conversely, Kafil et al. (2016) reported that the exposure of clinical *E. faecalis* isolates to ampicillin sub-MICs did not interfere with biofilm formation, but strongly increased the expression of *ace* and *esp* genes, whereas exposure of the isolates to gentamicin sub-MICs increased the biofilm formation and the expression of *efaA* and *esp* genes, especially at 8 and 16 μg/mL. To our knowledge, no other published study evaluated the effects of the sub-MIC of these clinically important antibiotics on *E. faecalis*. Of note, ampicillin, which acts on the bacterial wall, is widely used to treat enterococcal infections, alone or in combination with aminoglycosides, such as gentamicin, to obtain a bactericidal effect that is essential for the treatment of more severe infections (Hollenbeck and Rice, 2012).

The effects of other anti-enterococcal drugs at sub-MIC levels were also evaluated. Moura et al. (2015) showed that the sub-MICs of vancomycin, despite having no effect on biofilm formation, increased the expression of several VRE genes, such as *ace* and *vanA* in a selected strain of *E. faecalis*. Maestre et al. (2012) reported that the sub-MICs of tigecycline (0.25 × MIC and 0.5 × MIC) decreased biofilm formation by 85% and 60%, respectively, in the *E. faecalis* strains evaluated (n = 20). In a study that evaluated two biofilm-producing *E. faecium* strains (84 and 95), specific sub-MIC values of erythromycin, streptomycin, and vancomycin induced maximum biofilm production and enhanced *esp* gene expression, while sub-MICs of ampicillin, chloramphenicol, and gentamicin did not induce biofilm formation in the 95 strain (Yuksel et al., 2018). In contrast, only erythromycin (MIC/4) increased biofilm formation in *E. faecium* 84. Likewise, other studies with diverse bacterial species suggested that the inhibition or induction of biofilm formation is antibiotic dose-dependent (Hoffman et al., 2005; Davies et al., 2006; Haddadin et al., 2010).

The effect of multiple sub-MICs of each antibiotic on biofilm formation was not evaluated in the present study, thereby serving as a limitation. Therefore, differences related to the sub-MICs of ampicillin and gentamicin, in addition to the type of *E. faecalis* strains and the assay conditions, such as the culture medium used for biofilm formation and the expression of virulence genes, may explain the conflicting findings of this study and those of Kafil et al. (2016). Indeed, different environmental factors can affect the expression of virulence genes in enterococci. Hew et al. (2007) found that *efaA* is significantly upregulated in *E. faecalis* upon exposure to BHI medium. Moreover, *E. faecalis* can upregulate virulence factors and stress response genes, which are often associated with virulence, in response to various types of food- and host-associated stress (Lenz et al., 2010).

In conclusion, this study revealed that both the PRASEF and PSASEF isolates of clinical origin have remarkable ability to form biofilms and a high frequency of virulence genes (*ace*, *asa1*, *esp*, and *efaA*). Moreover, the sub-MICs of antibiotics was confirmed to interfere with the expression patterns of virulence genes and biofilm formation by *E. faecalis*.

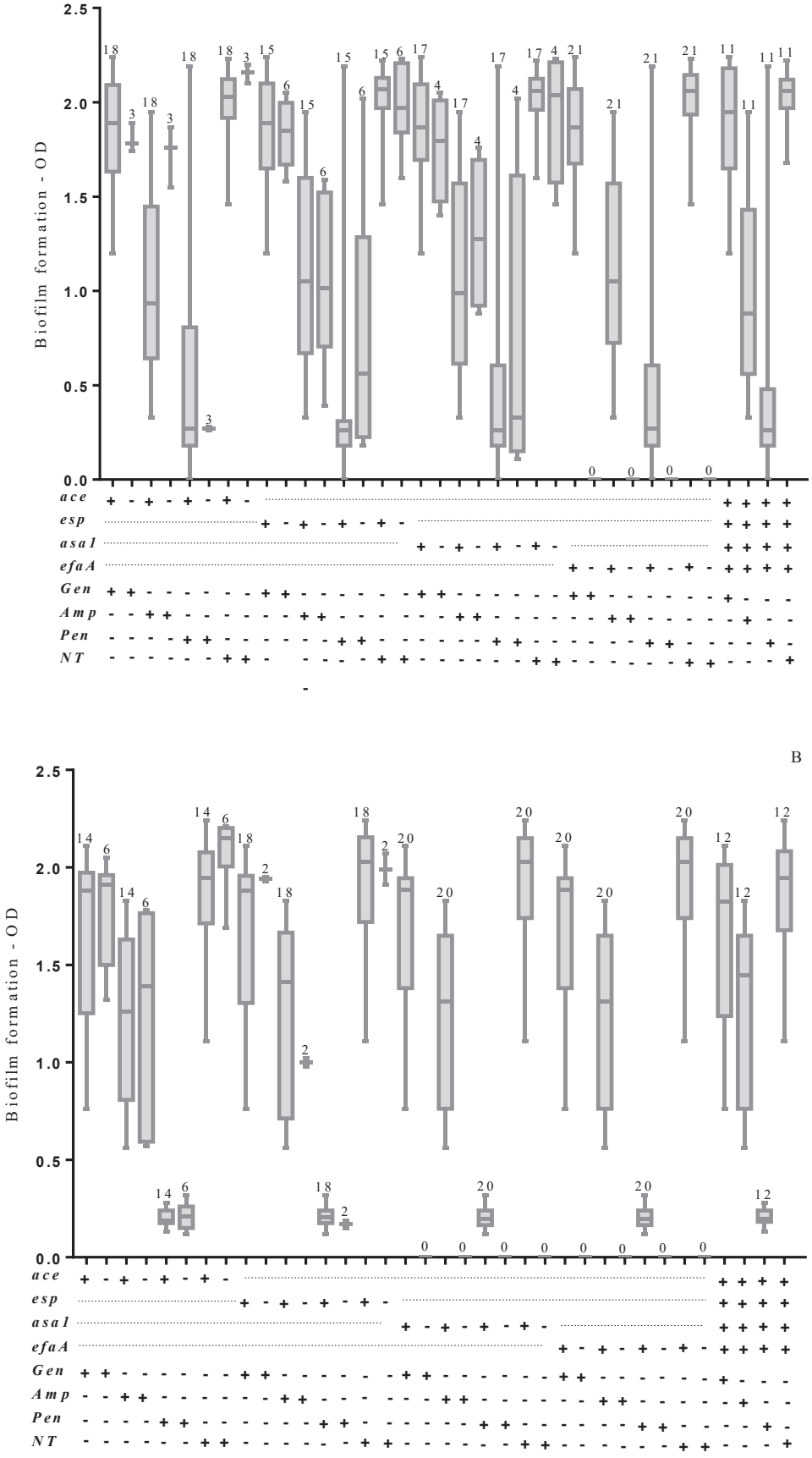

Figure 2. Biofilm formation by (A) *penicillin- and ampicillin-susceptible Enterococcus faecalis* (PSASEF) and (B) *penicillin-resistant, ampicillin-susceptible E. faecalis* (PRASEF) according to the presence of virulence genes (*ace*, *esp*, *asa1*, and/or *efaA*) and the absence (non-treated, NT) or presence of sub-minimal inhibitory concentrations of gentamicin (Gen), ampicillin (Amp), and penicillin (Pen). Biofilm production was evaluated based on the optical density (OD). The data are expressed as median, minimum, and maximum values. The numbers above each box-plot represent the absolute values for each bacterial profile.

Table 3. ddCts and percentage of the *ace*, *esp*, *asa1*, and *efaA* transcripts levels in *penicillin-resistant, ampicillin-susceptible Enterococcus faecalis* (PRASEF), and *penicillin- and ampicillin-susceptible E. faecalis* (PSASEF) exposed to sub-minimal inhibitory concentrations of gentamicin or ampicillin.

Transcript	Antibiotics	ddCT PRASEF (Treated–Non-treated)		ddCT PSASEF (Treated–Non-treated)	
		Mean ± SD (%)	*P*-value	Mean ± SD (%)	*P*-value
ace	Gentamicin	+0.16 ± 1.86 (−11.73%)	0.874	−0.12 ± 1.13 (+7.98%)	0.842
	Ampicillin	+1.06 ± 1.33 (−108.49%)	0.210	+2.17 ± 1.01 (−350.02%)	0.023
esp	Gentamicin	+0.05 ± 1.72 (−3.81%)	0.948	+0.43 ± 0.83 (−34.72%)	0.376
	Ampicillin	−0.51 ± 1.37 (−42.41%)	0.448	−0.12 ± 2.48 (+7.98%)	0.929
asa1	Gentamicin	+0.37 ± 1.40 (−29.24%)	0.636	+1.60 ± 1.53 (−203.14%)	0.128
	Ampicillin	+1.16 ± 1.40 (−123.46%)	0.198	+2.82 ± 0.90 (−606.16%)	0.008
efaA	Gentamicin	−1.37 ± 1.84 (+61.31%)	0.170	−0.90 ± 0.75 (+46.41%)	0.098
	Ampicillin	−0.37 ± 1.96 (+22.62%)	0.695	−0.96 ± 1.10 (+48.59%)	0.177

ddCt was determined considering the means of standardized Cts for PRASEF and PSASEF according to the absence or presence of antibiotics.
%, percentage of increase (+) or decrease (−) in the transcript level calculated from the ddCt.
SD, Standard Deviation.

Nonetheless, further studies are required to clarify the role of sub-lethal doses of antibiotics on biofilms, which is a hallmark of enterococcal infections. A better understanding of this phenomenon may have important clinical implications in the treatment of biofilm-related infections caused by this multidrug-resistant pathogen.

Declarations

Author contribution statement

Aline Andréia Caixeta Magalhães Tibúrcio: Performed the experiments; Analyzed and interpreted the data.

Aline Dias Paiva: Contributed reagents, materials, analysis tools or data; Wrote the paper.

André Luiz Pedrosa: Analyzed and interpreted the data; Wrote the paper.

Wellington Francisco Rodrigues: Analyzed and interpreted the data.

Raíssa Bernardes de Silva: Performed the experiments.

Adriana Gonçalves Oliveira: Conceived and designed the experiments; Contributed reagents; materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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References

Anderson, A.C., Jonas, D., Huber, I., Karygianni, L., Wölber, J., Hellwig, E., Arweiler, N., Vach, K., Wittmer, A., Al-Ahmad, A., 2016. *Enterococcus faecalis* from food, clinical specimens, and oral sites: prevalence of virulence factors in association with biofilm formation. Front. Microbiol. 6, 1534. PMID: 26793174; PMCID: PMC4707231.

Andersson, D.I., Hughes, D., 2014 Jul. Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol. 12 (7), 465–478. PMID: 24861036.

Baldassarri, L., Cecchini, R., Bertuccini, L., Ammendolia, M.G., Iosi, F., Arciola, C.R., Montanaro, L., Di Rosa, R., Gherardi, G., Dicuonzo, G., Orefici, G., Creti, R., 2001. *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. Med. Microbiol. Immunol. 190 (3), 113–120. PMID: 11827199.

Chuang-Smith, O.N., Wells, C.L., Henry-Stanley, M.J., Dunny, G.M., 2010 Dec 30. Acceleration of *Enterococcus faecalis* biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. PLoS One 5 (12), e15798. PMID: 21209892; PMCID: PMC3012704.

CLSI, 2015. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard – Tenth Edition. CLSI Document M07-A10. Clinical and Laboratory Standards Institute, Wayne, PA, p. 110.

CLSI, 2017. Performance Standards for Antimicrobial Susceptibility Testing. Twenty-seventh Edition. Informational Supplement. CLSI document M100-S27. Clinical and Laboratory Standards Institute, Wayne: PA, p. 282p.

Conceição, N., Oliveira, C.C., Silva, P.R., Avila, B.G., Oliveira, A.G., 2011. Trends in antimicrobial resistance among clinical isolates of enterococci in a Brazilian tertiary hospital: a 4-year study. Rev. Soc. Bras. Med. Trop. 44 (2), 177–181. Epub 2011 Apr 1. PMID: 21468473.

Conceição, N., Oliveira, C.C., Silva, L.E., Souza, L.R., Oliveira, A.G., 2012. Ampicillin susceptibility can predict in vitro susceptibility of penicillin-resistant, ampicillin-susceptible *Enterococcus faecalis* Isolates to amoxicillin but not to imipenem and piperacillin. J. Clin. Microbiol. 50 (11), 3729–3731. Epub 2012 Aug 15. PMID: 22895041; PMCID: PMC3486203.

Costa, L.B., Corá, L.F., Correa, F.E.L., Gabrielli, L.C., Oliveira, M.R., Conceição, N., Oliveira, A.G., 2019. High prevalence of the *aac(6')-Ie-aph(2")-Ia* gene in hospital isolates of *Enterococcus faecalis* Co-resistant to gentamicin and penicillin. Microb. Drug Resist. 25 (9), 1275–1281. Epub 2019 Jul 30. PMID: 31361553.

Davies, J., Spiegelman, G.B., Yim, G., 2006. The world of subinhibitory antibiotic concentrations. Curr. Opin. Microbiol. 9 (5), 445–453. Epub 2006 Aug 30. PMID: 16942902.

Di Domenico, E.G., Rimoldi, S.G., Cavallo, I., D'Agosto, G., Trento, E., Cagnoni, G., Palazzin, A., Pagani, C., Romeri, F., De Vecchi, E., Schiavini, M., Secchi, D., Antona, C., Rizzardini, G., Dichirico, R.B., Toma, L., Kovacs, D., Cardinali, G., Gallo, M.T., Gismondo, M.R., Ensoli, F., 2019 Oct 21. Microbial biofilm correlates with an increased antibiotic tolerance and poor therapeutic outcome in infective endocarditis. BMC Microbiol 19 (1), 228. PMID: 31638894; PMCID: PMC6802308.

Dunny, G.M., Hancock, L.E., Shankar, N., 2014. Enterococcal biofilm structure and role in colonization and disease. In: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N. (Eds.), Enterococci: from Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston: Massachusetts Eye and Ear Infirmary. PMID: 24649508.

Dutka-Malen, S., Evers, S., Courvalin, P., 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33 (1), 24–27. PMID: 7699051; PMCID: PMC227872.

Fisher, K., Phillips, C., 2009. The ecology, epidemiology and virulence of *Enterococcus*. Microbiology (Read.) 155 (Pt 6), 1749–1757. Epub 2009 Apr 21. PMID: 19383684.

Garsin, D.A., Frank, K.L., Silanpää, J., Ausubel, F.M., Hartke, A., Shankar, N., Murray, B.E., 2014 Feb 7. Pathogenesis and models of enterococcal infection. In: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N. (Eds.), Enterococci: from Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston: Massachusetts Eye and Ear Infirmary. PMID: 24649512.

Gawryszewska, I., Żabicka, D., Hryniewicz, W., Sadowy, E., 2021. Penicillin-resistant, ampicillin-susceptible *Enterococcus faecalis* in polish hospitals. Microb. Drug Resist. 27 (3), 291–300. Epub 2020 Jul 8. PMID: 32640911.

Goh, H.M.S., Yong, M.H.A., Chong, K.K.L., Kline, K.A., 2017. Model systems for the study of Enterococcal colonization and infection. Virulence 8 (8), 1525–1562. PMID: 28102784; PMCID: PMC5810481.

Haddadin, R.N., Saleh, S., Al-Adham, I.S., Buultjens, T.E., Collier, P.J., 2010. The effect of subminimal inhibitory concentrations of antibiotics on virulence factors expressed by

Staphylococcus aureus biofilms. J. Appl. Microbiol. 108 (4), 1281–1291. Epub 2009 Aug 19. PMID: 19778348.

Hew, C.M., Korakli, M., Vogel, R.F., 2007. Expression of virulence-related genes by *Enterococcus faecalis* in response to different environments. Syst. Appl. Microbiol. 30 (4), 257–267. Epub 2006 Sep 28. PMID: 17010551.

Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., Miller, S.I., 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436 (7054), 1171–1175. PMID: 16121184.

Hollenbeck, B.L., Rice, L.B., 2012. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence 3 (5), 421–433. Epub 2012 Aug 15. PMID: 23076243; PMCID: PMC3485979.

Johnson, J.R., Clabots, C., Hirt, H., Waters, C., Dunny, G., 2004. Enterococcal aggregation substance and binding substance are not major contributors to urinary tract colonization by *Enterococcus faecalis* in a mouse model of ascending unobstructed urinary tract infection. Infect. Immun. 72 (4), 2445–2448. PMID: 15039379; PMCID: PMC375174.

Kafil, H.S., Mobarez, A.M., Moghadam, M.F., Hashemi, Z.S., Yousefi, M., 2016. Gentamicin induces *efaA* expression and biofilm formation in *Enterococcus faecalis*. Microb. Pathog. 92, 30–35. Epub 2015 Dec 25. PMID: 26724739.

Kaplan, J.B., 2011. Antibiotic-induced biofilm formation. Int. J. Artif. Organs 34 (9), 737–751. PMID: 22094552.

Kristich, C.J., Li, Y.H., Cvitkovitch, D.G., Dunny, G.M., 2004. *Esp*-independent biofilm formation by *Enterococcus faecalis*. J. Bacteriol. 186 (1), 154–163. PMID: 14679235; PMCID: PMC365672.

Kristich, C.J., Rice, L.B., Arias, C.A., 2014 Feb 6. Enterococcal infection—treatment and antibiotic resistance. In: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N. (Eds.), Enterococci: from Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston: Massachusetts Eye and Ear Infirmary. PMID: 24649502.

Laureti, L., Matic, I., Gutierrez, A., 2013. Bacterial responses and genome instability induced by subinhibitory concentrations of antibiotics. Antibiotics (Basel) 2 (1), 100–114. PMCID: PMC4790301.

Lenz, C.A., Hew Ferstl, C.M., Vogel, R.F., 2010. Sub-lethal stress effects on virulence gene expression in *Enterococcus faecalis*. Food Microbiol 27 (3), 317–326. Epub 2009 Nov 10. PMID: 20227595.

Maestre, J.R., Aguilar, L., Mateo, M., Giménez, M.J., Méndez, M.L., Alou, L., Granizo, J.J., Prieto, J., 2012. In vitro interference of tigecycline at subinhibitory concentrations on biofilm development by *Enterococcus faecalis*. J. Antimicrob. Chemother. 67 (5), 1155–1158. Epub 2012 Feb 9. PMID: 22323499.

Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M., 2009. Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. Int. J. Food Microbiol. 132 (1), 24–32. Epub 2009 Mar 27. PMID: 19375810.

Metzidie, E., Manolis, E.N., Pournaras, S., Sofianou, D., Tsakris, A., 2006. Spread of an unusual penicillin- and imipenem-resistant but ampicillin-susceptible phenotype among *Enterococcus faecalis* clinical isolates. J. Antimicrob. Chemother. 57 (1), 158–160. PMID: 16308417.

Mohamed, J.A., Huang, D.B., 2007. Biofilm formation by enterococci. J. Med. Microbiol. 56 (Pt 12), 1581–1588. PMID: 18033823.

Mohamed, J.A., Huang, W., Nallapareddy, S.R., Teng, F., Murray, B.E., 2004 Jun. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. Infect. Immun. 72 (6), 3658–3663. PMID: 15155680; PMCID: PMC415661.

Moraes, P.M., Perin, L.M., Todorov, S.D., Silva Jr., A., Franco, B.D., Nero, L.A., 2012. Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese. J. Appl. Microbiol. 113 (2), 318–328. PMID: 22587647.

Moura, T.M., Campos, F.S., Caierão, J., Franco, A.C., Roehe, P.M., d'Azevedo, P.A., Frazzon, J., Frazzon, A.P., 2015. Influence of a subinhibitory concentration of vancomycin on the in vitro expression of virulence-related genes in the vancomycin-resistant *Enterococcus faecalis*. Rev. Soc. Bras. Med. Trop. 48 (5), 617–621. PMID: 26516976.

Paganelli, F.L., Willems, R.J., Leavis, H.L., 2012. Optimizing future treatment of enterococcal infections: attacking the biofilm? Trends Microbiol 20 (1), 40–49. Epub 2011 Dec 12. PMID: 22169461.

Pöntinen, A.K., Top, J., Arredondo-Alonso, S., Tonkin-Hill, G., Freitas, A.R., Novais, C., Gladstone, R.A., Pesonen, M., Meneses, R., Pesonen, H., Lees, J.A., Jamrozy, D., Bentley, S.D., Lanza, V.F., Torres, C., Peixe, L., Coque, T.M., Parkhill, J., Schürch, A.C., Willems, R.J.L., Corander, J., 2021. Apparent nosocomial adaptation of *Enterococcus faecalis* predates the modern hospital era. Nat. Commun. 12 (1), 1523. PMCID: PMC7943827.

Ramos, S., Silva, V., Dapkevicius, M.L.E., Igrejas, G., Poeta, P., 2020. Enterococci, from harmless bacteria to a pathogen. Microorganisms 8 (8), 1118.

Ramsey, M., Hartke, A., Huycke, M., 2014 Feb 15. The physiology and metabolism of enterococci. In: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N. (Eds.), Enterococci: from Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston: Massachusetts Eye and Ear Infirmary. PMID: 24649507.

Raven, K.E., Reuter, S., Gouliouris, T., Reynolds, R., Russell, J.E., Brown, N.M., Török, M.E., Parkhill, J., Peacock, S.J., 2016. Genome-based characterization of hospital-adapted *Enterococcus faecalis* lineages. Nat Microbiol 1 (3), 15033. Epub 2016 Feb 8. PMID: 27213049; PMCID: PMC4872833.

Sandoe, J.A.T., Witherden, I.R., Cove, J.H., Heritage, J., Wilcox, M.H., 2003. Correlation between enterococcal biofilm formation in vitro and medical-device-related infection potential in vivo. J. Med. Microbiol. 52 (Pt 7), 547–550. PMID: 12808074.

Seno, Y., Kariyama, R., Mitsuhata, R., Monden, K., Kumon, H., 2005. Clinical implications of biofilm formation by *Enterococcus faecalis* in the urinary tract. Acta Med. Okayama 59 (3), 79–87. PMID: 16049560.

Soares, R.O., Fedi, A.C., Reiter, K.C., Caierão, J., d'Azevedo, P.A., 2014. Correlation between biofilm formation and *gelE, esp*, and *agg* genes in *Enterococcus* spp. clinical isolates. Virulence 5 (5), 634–637. Epub 2014 Apr 29. PMID: 24782231; PMCID: PMC4105314.

Srivastava, S., Bhargava, A., 2016. Biofilms and human health. Biotechnol. Lett. 38 (1), 1–22. Epub 2015 Sep 19. PMID: 26386834.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., Svabic-Vlahovic, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods 40 (2), 175–179. PMID: 10699673.

Stepanovic, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115 (8), 891–899. PMID: 1769694.

Teixeira, L.M., Carvalho, M.G., Shewmaker, P.L., Facklam, R.R., 2011. Enterococcus. In: Versalovic, J., Carroll, K.C., Funke, G., Jorgensen, J.H., Landry, M.L., Warnock, D.W. (Eds.), Manual of Clinical Microbiology, tenth ed. ASM Press, Washington, DC, pp. 350–364.

Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M., Amorena, B., Leiva, J., Penadés, J.R., Lasa, I., 2001. The enterococcal surface protein, *Esp*, is involved in *Enterococcus faecalis* biofilm formation. Appl. Environ. Microbiol. 67 (10), 4538–4545. PMID: 11571153; PMCID: PMC93200.

Tsikrikonis, G., Maniatis, A.N., Labrou, M., Ntokou, E., Michail, G., Daponte, A., Stathopoulos, C., Tsakris, A., Pournaras, S., 2012. Differences in biofilm formation and virulence factors between clinical and fecal enterococcal isolates of human and animal origin. Microb. Pathog. 52 (6), 336–343. Epub 2012 Mar 15. PMID: 22445820.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H., 2004. Development of a multiplex PCR for the detection of *asa1, gelE, cylA, esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. J. Clin. Microbiol. 42 (10), 4473–4479. PMID: 15472296; PMCID: PMC522368.

Vestby, L.K., Grønseth, T., Simm, R., Nesse, L.L., 2020. Bacterial biofilm and its role in the pathogenesis of disease. Antibiotics (Basel) 9 (2), 59. PMCID: PMC7167820.

Weng, P.L., Ramli, R., Hamat, R.A., 2019. Antibiotic susceptibility patterns, biofilm formation and *esp* gene among clinical enterococci: is there any association? Int. J. Environ. Res. Publ. Health 16 (18), 3439. PMCID: PMC6765802.

Yuksel, F.N., Karatug, N.T., Akcelik, M., 2018. Does subinhibitory concentrations of clinically important antibiotic induce biofilm production of *Enterococcus faecium* strains? Acta Microbiol. Immunol. Hung. 65 (1), 27–38. Epub 2017 Dec 15. PMID: 29243494.