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Original article

Impact of sub-inhibitory antibiotic concentrations on biofilm formation among nosocomial isolates of *Enterococcus* species

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ABSTRACT

Background: *Enterococci*, particularly those that produce biofilm, can cause different infections ranging from simple infections to sepsis. This study aims to understand the effect of sub-inhibitory concentrations of antibiotics (ampicillin, vancomycin, ciprofloxacin and tetracycline) on biofilm formation of *Enterococcus* and its association with virulence genes (*asa1*, *esp* and *gel E*). **Methods:** This cross-sectional study included *Enterococci* strains isolated from different microbiological samples. Disk diffusion and microtiter plate techniques were used to examine the isolates antibiotic sensitivity and biofilm production respectively. Isolates were investigated for *asa1*, *esp* and *gel E* genes using multiplex PCR. Minimum inhibitory concentration (MIC) of ampicillin, vancomycin, ciprofloxacin, and tetracycline for strong biofilm forming isolates was determined by broth microdilution then the sub-MIC effect of these antibiotics on their biofilm formation was evaluated. **Results:** A total of 80 *Enterococcus* (65 *Enterococcus faecalis* and 15 *Enterococcus faecium***)** strains were identified. Compared to *Enterococcus faecalis*, *Enterococcus faecium* showed higher antibiotics resistance and lower biofilm formation. *asa 1*, *esp* and *gel E* genes were detected in 44.6%, 60.0% and 76.9% of *Enterococcus faecalis* and 40.0%, 33.3% and 26.7% of *Enterococcus faecium* respectively with higher frequency of these genes among biofilm forming isolates. Of the four selected antibiotics, sub-inhibitory concentrations of only ampicillin and vancomycin significantly enhanced biofilm density of *Enterococcus faecalis* while *Enterococcus faecium* was not significantly affected. **Conclusion:** Increased biofilm formation in some isolates at different sub-MIC ensures the significance of appropriate antibiotic use to reduce the danger of resistant bacteria selection.

Introduction

Enterococci, which are one of the normal flora of gastrointestinal system, can cause a variety of infections such as urinary tract infections, endocarditis, pelvic and wound infections [1]. In 2017, *Enterococci* were included in the World Health Organization "Global Priority list of antibiotic-resistant bacteria" [2].

E. faecalis and *E. faecium* are the primary cause of 90% of Enterococcal infections. The most crucial virulence factor of *Enterococci* is their ability to form biofilms. The bacterial biofilm

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development process begins with adhesion where the populations of cells permanently affixed to a variety of biotic and abiotic surfaces and coated in a hydrated matrix of proteins, polysaccharides, nucleic acids, and exopolymeric substance. These biofilms restrict phagocytosis and promote antibiotic resistance, making infection more difficult to eliminate [3].

Several virulence factors have been linked to the biofilm's production among *Enterococci* including *asa* 1 (aggregation substance), *Ebp* (endocarditis and biofilm-associated pili) and *Esp* (surface protein implicated in the colonization, persistence, and biofilms production). Some *Enterococci* can also produce GelEA (gelatinase), CylA (cytolysin) and Hyl (hyaluronidase) which are involved in their pathogenesis [4].

Antibiotic treatment is widely agreed to be the most effective approach for microbial infections management. However, antibiotic concentrations lower than the minimum inhibitory concentration (sub-MICs), have been associated with a greater capacity for biofilm formation leading to reduced susceptibility to antibiotics [5].

Sub-inhibitory concentrations of a number of antimicrobial classes with different targets and also disinfectants have been shown to stimulate biofilm formation in a dose-dependent way for a variety of Gram positive and Gram negative species; however, the underlying mechanisms are still unclear. The maximal stimulation of biofilm formation is typically observed at 1/2 and 1/4 MIC [6].

The most widely accepted theories regarding how sub-MICs of antibiotics could enhance biofilm include alterations in surface protein, up-regulation of adhesion- proteins, and induction of oxidative stress responses by release of extracellular DNA (eDNA) from susceptible bacteria leading to increase in biofilm formation by the remaining cells [6]. Also, antibiotics at sub-inhibitory doses may influence the expression of genes related to biofilms, resulting in emergence of resistant populations that are able to withstand stressful environments [7].

Therefore, the aim of this work is to identify the in vitro effect of sub-inhibitory concentrations of antibiotics (ampicillin, vancomycin, ciprofloxacin and tetracycline) on biofilm formation of *Enterococcus* and its association with virulence genes (*asa1*, *esp* and *gel E*).

Methods

Study design and setting

This cross-sectional study was conducted in Medical Microbiology and Immunology Department at the Faculty of Medicine, National Liver Institute, Menoufia University, Egypt from January 2022 to November 2023. The study protocol has received approval by the Ethical Committee, Faculty of Medicine, Menoufia University (IRB approval number 2023MICR1).

Study population

Clinical isolates were obtained from patients admitted to Menoufia University Hospitals and exhibited clinical features of health-care associated infections (HAIs) which are infections that patients can get while receiving medical care in a healthcare facility [8]. For the majority of patients, HAIs appear 48 hours or more after admission. Before collecting samples, all patients gave written consent after being informed about the purpose and nature of our study. Patients who rejected to engage in our research, those who responded well to antibiotic therapy and those who were colonized without signs of infection were all excluded. Sample size was calculated according to Bernardi et al. [5] with power 80% and confidence level 95%.

Specimen collection and processing

Specimens including urine, blood, endotracheal aspirates and wound swabs were gathered following aseptic techniques then sent for processing and cultivation on various media according to sample type (Merck, Darmstadt, Germany). Plates were aerobically incubated at 37°C for twenty-four hours. BACT/ALERT 3D (Biomeriuex, France) was used to incubate blood culture bottles and those with positive growth were sub-cultured.

Identification of *Enterococcus*

Gram staining, colony morphology and biochemical testing were used to identify the obtained colonies [9]. An API 20 Strep biochemical test kit (BioMerieux, St. Louis, MO, USA) had been employed for validation of Enterococcal isolates biochemically. For confirmation and species identification, VITEK2 compact device system and Gram positive identification (GP ID) cards (Biomeriuex, France) were used.

Antibiotic susceptibility testing

Modified Kirby–Bauer disk diffusion method on Muller Hinton agar (MHA; Oxoid, UK) was used then confirmed through VITEK2 compact device system using Gram positive susceptibility card (AST-P580 and 586). The used antibiotic disks (Oxoid, UK) were penicillin (10µg), ampicillin (10µg), vancomycin (30µg), teicoplanin (30µg), erythromycin (15µg), tetracycline (30µg), ciprofloxacin (5µg), levofloxacin (5µg), nitrofurantoin (300µg), and linezolid (30µg). *E. faecalis* ATCC 29212 served as quality control strain. The CLSI (Clinical and Laboratory Standards Institute) criteria were followed for the interpretation of all antibiotic susceptibility data [10].

Detection of Enterococcal biofilm formation using microtiter plates (MTP)

The ability of *Enterococci* isolates to develop biofilms were assessed through MTP technique **(Figure 1)** as previously described by Christensen et al. [11], with slight modifications. In brief, overnight cultures of isolates were inoculated in trypticase-soy broth (TSB, Merck, Germany) that was newly-prepared and contained 1% glucose. Wells in a 96-well polystyrene microtiter plate with a flat bottom (Falcon® 3077, Becton Dickinson, New Jersy) were filled with 200 µl of diluted culture of isolates followed by incubation then contents of wells were removed through plate inversion, washed with 200 µL of phosphate buffered saline (PBS; pH 7.2) four times, fixed with 200 µL of methanol, stained with 100 µL of 1% crystal violet and then thorough cleanings with sterile distilled water. The plate was then allowed to dry in air and dye was re-solubilized by adding approximately 200 µl of 30% acetic acid. The negative control well was filled with sterile TSB containing 1% glucose while a previously identified strong biofilm producing *Enterococcus fecalis* was the positive control. ELISA reader (Thermo scientific Multiskan FC 357, Finland) was used to measure the optical density (OD) of at 570 nm. All tests were conducted in triplicate.

Isolates were grouped (non-producers, weak, moderate and strong) according to the OD readings of the bacterial biofilms, as reported by Stepanovic et al. [12].

Screening for genes involved in biofilm production in clinical isolates

Enterococci isolates were investigated for *asa1, esp* and *gel E* genes. Following the Manufacturer`s instructions, QIAamp DNA Micro Kit (50) tests (Qiagen, Germany, cat. no. 56304) was used for bacterial DNA extraction and purification. **Table 1** displayed the primer sequences that were employed. The following conditions were used for the PCR amplification using a pre-programmed thermal cycler (MultiGene Optimax, Labnet International Inc., USA): An initial activation step of DNA polymerase at 95°C for 15 minutes, then 30 cycles of denaturation for 1 minute at 94°C, annealing (56°C for 1 minute), and extension (72°C for 1 minute), followed by one cycle at 72°C for 10 minutes [13].

The amplification products were analyzed using gel electrophoresis (EC250-90 electrophoresis power supply, Thermo Electron Corporation, USA), over 1.5% agarose gel (Cleaver Scientific Ltd., Warwickshire, UK) then visualized by UV light trans-illuminator (IBI Kodak Ultralinker, France). A 100-bp DNA ladder (Fermentas; Thermo Fisher Scientific, Inc.) was utilized as a molecular marker **(Figure 2)**.

Antibiotics and MIC values determination

Ampicillin, vancomycin, ciprofloxacin and tetracycline were selected for evaluating their sub-MIC effect on *Enterococcus* capacity to produce biofilm. They were selected according to the following: Antibiotics which are routinely tested and reported with *Enterococcus* infection, effectiveness on strong biofilm producing isolates and different mechanism of action (cell wall, DNA and ribosomes). Selected isolates for the test were the strong biofilm-forming as detected by MTP assay. In compliance with CLSI standards [10, 14], broth microdilution was performed to assess the MICs of the selected antibiotics within 96-well plates with Mueller Hinton broth (Oxoid, UK). The reference strains for quality control tests were *E. faecalis* ATCC 29212.

Impact of sub-MIC on biofilm formation

Bacterial suspensions from strong biofilm-producing isolates were made as mentioned under the heading: Detection of Enterococcal biofilm formation using microtiter plates. About 100 µl of bacterial suspensions and 100 µl of the corresponding antibiotic sub-MIC concentrations (specific to each isolate) were added to the test

wells. For control wells (antibiotic-free controls), 200 µl of TSB without antibiotic was the negative control and 200 µl of the diluted culture without antibiotic was the positive control. Plates were incubated for 24 hours at 37 °C. All experiments were conducted in triplicate and repeated at least three times. At 570 nm, the plate reader was used to determine isolates O.D [15].

Statistical analysis

The SPSS version 20 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Mean and SD were used to express continuous variables. Frequency and percentage were used to express categorical variables. ANOVA, repeated measures ANOVA, and chi-square testing were applied. For every test, a significance level of $P<0.05$ was applied.

Results

Identification and antibiogram of *Enterococcus*

Our study enrolled 80 isolates of *Enterococcus*, of which 65 were *E. faecalis* and 15 were *E. faecium*. While *E. faecalis* was primarily recovered from urine samples (69.2%), *E. faecium* was primarily recovered from blood (53.3%) (P value <0.001). *E. faecium* showed much greater resistance to penicillin, ampicillin, vancomycin and teicoplanin compared to *E. faecalis* (P value <0.05) **(Table 2)**.

Enterococcal biofilm formation and biofilm related genes

Biofilm forming ability was significantly (P value 0.003) higher among *E. faecalis* (78.5%) compared to *E. faecium* (40.0%). Regarding investigated genes, *asa 1, esp* and *gel E* genes were detected in 44.6%, 60.0% and 76.9% respectively among *E. faecalis* isolates compared to 40.0%, 33.3% and 26.7% respectively among *E. faecium* isolates with statistically significant difference only for *gel E* gene **(Table 2)**.

Among *E. faecalis* and *E. faecium* isolates, *asa 1, esp* and *gel E* were more prevalent in biofilm forming isolates with statistically significant difference only in *E. faecalis* isolates, however, the number of *E. faecium* isolates was too low to have a reliable conclusion. There was significant positive correlation between number of these genes and biofilm forming capacity among *Enterococcus* isolates. Urine included the majority of the isolates that produced biofilms: 74.5% of *E. faecalis* and 50.0% of *E. faecium* **(Table 3)**.

Antibiotics and determination MIC values

Among the strong biofilm forming isolates, ampicillin MIC ranged from 8 to 64 µg/ml while that of vancomycin ranged from 1 to 4 µg/ml. Regarding ciprofloxacin and tetracycline, their MIC ranged from 2 to 16 µg/ml and 4 to 64 µg/ml respectively.

Assessment of the impact of antibiotics sub-MIC on biofilm development

Mean OD increased at different sub-inhibitory concentrations of ampicillin and vancomycin especially at concentrations of 1/2 and 1/4 MIC with statistically significant difference only among strong biofilm forming *E. faecalis* whereas in ciprofloxacin and tetracycline there was slight increase of mean OD of strong biofilm forming *Enterococcus* isolates at concentrations of 1/2 MIC with statistically insignificant difference **(Table 4 , Figure 3)**.

At sub-inhibitory concentrations of ampicillin and vancomycin, there was a significant positive correlation between mean OD of strong biofilm forming *Enterococcus* isolates and number of detected biofilm related genes. All isolates positive for the three genes under investigation showed an increase in biofilm production at the corresponding 1/2, 1/4, and 1/8 MIC of vancomycin. In contrast, the two isolates with a single gene showed a decrease, increase and no effect at the corresponding 1/2, 1/4, and 1/8 MIC of vancomycin. For ampicillin, all four isolates with all 3 genes showed an increase in biofilm mass at concentrations of 1/2 and 1/4 MIC of ampicillin while one of the two isolates that tested positive for only one gene showed decreased biofilm development at 1/2 MIC of ampicillin and both isolates increased biofilm formation at 1/4 MIC of ampicillin **(Table 5)**.

Table 1. Primers used for detection of biofilm related genes

Target gene	Primer sequence (5'-3')	Size (bp)	Reference
asa1	Forward GCACGCTATTACGAACTATATGA	375	**Vankerckhoven et al.** [13]
	Reverse TAAGAAAGAACATCACCACGA		
esp	Forward GGAACGCCTTGGTATGCTAAC	510	
	Reverse GCCACTTTATCAGCCTGAACC		
gelE	Forward TATGACAATGCTTTTTGGGAT	213	
	Reverse AGATGCACCCGAAATAATATA		

Table 2. Clinical characteristics and virulence determinants of isolated *Enterococcus*

Clinical characteristics	Total (N=80)	Studied groups				χ2	P value
		Enterococcus faecalis (N=65)		*Enterococcus faecium* (N=15)			
		N	%	N	%		
Specimen type:							
Urine	48 (60.0)	45	69.2	3	20.0	23.83	<0.001
Blood	13 (16.3)	5	7.7	8	53.3		HS
Endotracheal aspirates	12 (15.0)	8	12.3	4	26.7		
Wound swab	7 (8.8)	7	10.8	0	0.0		
Antibiotic resistance rate:							
Penicillin (10µg)	21 (26.3)	8	12.3	13	86.7	34.80	<0.001 HS
Ampicillin (10µg)	17 (21.3)	6	9.2	10	66.7	25.12	<0.001 HS
Vancomycin (30µg)	3 (3.8)	1	1.5	2	13.3	4.69	0.03 S
Teicoplanin (30µg)	3 (3.8)	1	1.5	2	13.3	4.69	0.03 S
Erythromycin (15µg)	65 (81.3)	52	80	13	86.7	0.35	0.55 NS
Tetracycline (30µg)	60 (75.0)	49	75.4	11	73.3	0.02	0.86 NS
Ciprofloxacin (5µg)	61 (76.3)	48	73.8	13	86.7	1.10	0.29 NS
Levofloxacin (5µg)	57 (71.3)	46	70.8	11	73.3	0.03	0.84 NS
Nitrofurantoin[a] (300µg)	14/48	12	26.7	2	66.7	0.22	0.63 NS
Linezolid (30µg)	(29.2)	1	1.5	2	13.3	4.69	0.03 NS
	3 (3.8)						
Biofilm formation:							
Yes	57 (71.2)	51	78.5	6	40.0	8.80	0.003
No	23 (28.8)	14	21.5	9	60.0		S
Biofilm strength:							
Strong	11 (13.8)	10	15.4	3	20.0	10.57	0.01
Moderate	18 (22.5)	17	26.2	1	6.7		S
Weak	26 (32.5)	24	36.9	2	13.3		
None	25 (31.2)	14	21.5	9	60.0		
Virulence genes:							
asa 1	35 (43.7)	29	44.6	6	40.0	0.10	0.74 NS
esp	44 (55.0)	39	60.0	5	33.3	3.50	0.06 NS
gel E	54 (67.5)	50	76.9	4	26.7	14.03	<0.001 HS

χ2: Chi square test, [a]: For urinary isolates only, HS: Highly significant (P value < 0.001) S: Significant (P value < 0.05), NS: Not significant (P value > 0.05), N: Number

Table 3. Association between biofilm formation and clinical characteristics of isolates

Clinical characteristics	Biofilm formation		$\chi 2$ (P value)	Strength of biofilm formation		
	Biofilm forming	None biofilm forming		Strong	Moderate	Weak
	N (%)	N (%)		N (%)	N (%)	N (%)
Enterococcus faecalis						
Virulence genes:						
asa 1	26 (50.9)	3 (21.4)	3.88 (0.04[a]) S	7 (70.0)	11 (64.7)	8 (33.3)
esp	34 (66.7)	5 (35.7)	4.38 (0.03[b]) S	7 (70.0)	13 (76.5)	19 (79.2)
gel E	43 (84.3)	7 (50.0)	7.28 (0.006[c]) S	9 (90.0)	16 (94.1)	18 (75.0)
Number of genes						
One	11 (21.6)	9 (64.3)		1 (10.0)	4 (23.5)	6 (25.0)
Two	23 (45.1)	3 (21.4)	20.0 (<0.001) HS	5 (50.0)	3 (17.7)	15 (62.5)
Three	17 (33.3)	0 (0.0)		4 (40.0)	10 (58.8)	3 (12.5)
None	0 (0.0)	2 (14.3)		0 (0.0)	0 (0.0)	0 (0.0)
Specimen type:						
Urine	38 (74.5)	7 (50.0)	11.08 (0.01) S	8 (80.0)	13 (76.5)	17 (70.9)
Blood	2 (3.9)	3 (21.4)		0 (0.0)	0 (0.0)	2 (8.3)
Endotracheal aspirates	4 (7.8)	4 (28.6)		0 (0.0)	1 (5.8)	3 (12.5)
Wound swab	7 (13.7)	0 (0.0)		2 (20.0)	3 (17.7)	2 (8.3)
Enterococcus faecium						
Virulence genes:						
asa 1	4 (66.7)	2 (22.2)	2.96 (0.08[a]) NS	2 (66.7)	1 (100.0)	1 (50.0)
esp	3 (50.0)	2 (22.2)	1.25 (0.26[b]) NS	1 (33.3)	1 (100.0)	1 (50.0)
gel E	3 (50.0)	1 (11.1)	2.78 (0.09[c]) NS	2 (66.7)	0 (0.0)	1 (50.0)
Number of genes						
One	2 (33.3)	5 (55.6)		1 (33.3)	0 (0.0)	1 (50.0)
Two	4 (66.7)	0 (0.0)	9.04 (0.01) S	2 (66.7)	1 (100.0)	1 (50.0)
Three	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)
None	0 (0.0)	4 (44.4)		0 (0.0)	0 (0.0)	0 (0.0)
Specimen type:						
Urine	3 (50.0)	0 (0.0)		2 (66.7)	0 (0.0)	1 (50.0)
Blood	1 (16.7)	7 (77.8)	7.18 (0.02) S	0 (0.0)	0 (0.0)	1 (50.0)
Endotracheal aspirates	2 (33.3)	2 (22.2)		1 (33.3)	1 (100.0)	0 (0.0)
Wound swab	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)

[a]**:** Association between biofilm formation and presence of *asa 1* gene among isolate
[b]**:** Association between biofilm formation and presence of *esp* gene among isolates
[c]**:** Association between biofilm formation and presence of *gel E* gene among isolates

Table 4. Impact of sub-inhibitory antibiotic concentrations on biofilm formation among strong biofilm producing isolates of *Enterococcus*

	Mean ± SD optical density of strong biofilm producing Enterococcal isolates			**Repeated measures ANOVA**	**P value**
	Total (N=13)	***Enterococcus faecalis* (N=10)**	***Enterococcus faecium* (N=3)**		
	Mean ± SD	**Mean ± SD**	**Mean ± SD**		
Ampicillin:					
Control	0.575±0.11	0.567±0.13	0.605±0.06	19.33[a]	<0.001[a] HS
1/2 MIC	0.603±0.14	0.594±0.15	0.631±0.09	12.37[b]	0.002[b] S
1/4 MIC	0.612±0.12	0.603±0.13	0.640±0.07	7.45[c]	0.10[c] NS
1/8 MIC	0.578±0.12	0.570±0.13	0.605±0.06		
Vancomycin:					
Control	0.575±0.11	0.567±0.13	0.605±0.06	16.09[a]	<0.001[a] HS
1/2 MIC	0.628±0.17	0.616±0.19	0.668±0.12	9.02[b]	0.01[b] S
1/4 MIC	0.658±0.12	0.642±0.13	0.714±0.11	9.39[c]	0.08[c] NS
1/8 MIC	0.585±0.12	0.577±0.14	0.609±0.07		
Ciprofloxacin:					
Control	0.575±0.11	0.567±0.13	0.605±0.06	2.21[a]	0.16[a] NS
1/2 MIC	0.582±0.12	0.571±0.13	0.619±0.08	1.03[b]	0.33[b] NS
1/4 MIC	0.575±0.11	0.567±0.13	0.605±0.06	1.0[c]	0.42[c] NS
1/8 MIC	0.575±0.11	0.567±0.13	0.605±0.06		
Tetracycline:					
Control	0.575±0.11	0.567±0.13	0.605±0.06	2.25[a]	0.15[a] NS
1/2 MIC	0.583±0.12	0.572±0.13	0.618±0.08	1.09[b]	0.32[b] NS
1/4 MIC	0.575±0.11	0.567±0.13	0.605±0.06	1.0[c]	0.42[c] NS
1/8 MIC	0.575±0.11	0.567±0.13	0.605±0.06		

[a]: For comparison of mean OD of total strong biofilm forming *Enterococcus* isolates at different antibiotic concentrations
[b]: For comparison of mean OD of strong biofilm forming *Enterococcus faecalis* isolates at different antibiotic concentrations
[c]: For comparison of mean OD of strong biofilm forming *Enterococcus faecium* isolates at different antibiotic concentrations

Table 5. Association between effect of sub-inhibitory antibiotic concentrations on biofilm formation among total strong biofilm producing isolates of *Enterococcus* and biofilm related genes.

Sub-inhibitory antibiotic concentrations	**Effect on biofilm formation and biofilm related genes**								
	***asa 1* (N=9)**			***esp* (N=8)**			***gel E* (N=11)**		
	Increased	**No effect**	**Decreased**	**Increased**	**No effect**	**Decreased**	**Increased**	**No effect**	**Decreased**
	N(%)	**N(%)**	**N(%)**	**N(%)**	**N(%)**	**N(%)**	**N(%)**	**N(%)**	**N(%)**
Ampicillin:									
1/2 MIC	7 (77.8)	1 (11.1)	1 (11.1)	6 (75.0)	1 (12.5)	1 (12.5)	7 (63.6)	3 (27.3)	1 (9.1)
1/4 MIC	9 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	11 (100.0)	0 (0.0)	0 (0.0)
1/8 MIC	3 (33.3)	6 (66.7)	0 (0.0)	3 (37.5)	5 (62.5)	0 (0.0)	3 (27.3)	8 (72.7)	0 (0.0)
Vancomycin:									
1/2 MIC	8 (88.9)	0 (0.0)	1 (11.1)	6 (75.0)	0 (0.0)	2 (25.0)	8 (72.7)	0 (0.0)	3 (27.3)
1/4 MIC	9 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	11 (100.0)	0 (0.0)	0 (0.0)
1/8 MIC	5 (55.6)	4 (44.4)	0 (0.0)	4 (50.0)	4 (50.0)	0 (0.0)	5 (45.5)	6 (54.5)	0 (0.0)
Ciprofloxacin:									
1/2 MIC	4 (44.4)	4 (44.4)	1 (11.1)	1 (12.56)	6 (75.0)	1 (12.5)	4 (36.4)	6 (54.5)	1 (9.1)
1/4 MIC	0 (0.0)	9 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	11 (100.0)	0 (0.0)
1/8 MIC	0 (0.0)	9 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	11 (100.0)	0 (0.0)
Tetracycline:									
1/2 MIC	4 (44.4)	4 (44.4)	1 (11.1)	1 (12.56)	6 (75.0)	1 (12.5)	4 (36.4)	6 (54.5)	1 (9.1)
1/4 MIC	1 (11.1)	8 (88.9)	0 (0.0)	1(12.5)	7 (87.5)	0 (0.0)	1 (9.1)	10 (90.9)	0 (0.0)
1/8 MIC	0 (0.0)	9 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	11 (100.0)	0 (0.0)

Effect on biofilm formation and number of biofilm related genes										
	Ampicillin Mean ±SD (OD)	Vancomycin Mean ±SD (OD)	ANOVA	P value	Ampicillin*			Vancomycin *		
					Incr. N (%)	No eff. N (%)	Decr. N (%)	Incr. N (%)	No eff. N (%)	Decr. N (%)
At 1/2 MIC										
One gene	0.454±0.10	0.409±0.16	6.54[a]	0.01[a]	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	2
Two genes	0.563±0.12	0.594±0.14	6.77[b]	0.01[b]	4 (57.1)	2 (28.6)	1 (14.3)	5 (71.4)	0 (0.0)	(100.0)
Three genes	0.747±0.04	0.796±0.04			4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	2 (28.6)
										0 (0.0)
At 1/4 MIC										
One gene	0.494±0.08	0.536±0.07	6.46[a]	0.01[a]	2 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)
Two genes	0.575±0.10	0.627±0.12	4.30[b]	0.04[b]	7 (100.0)	0 (0.0)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)
Three genes	0.735±0.03	0.776±0.03			4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
At 1/8 MIC										
One gene	0.464±0.08	0.464±0.08	7.20[a]	0.01[a]	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)
Two genes	0.538±0.09	0.540±0.09	8.11[b]	0.008[b]	0 (0.0)	7 (100.0)	0 (0.0)	1 (14.3)	6 (85.7)	0 (0.0)
Three genes	0.706±0.04	0.724±0.04			3 (75.0)	1 (25.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)

[a]: For comparison of ampicillin mean OD and number of biofilm related genes among of total strong biofilm forming *Enterococcus* isolates
[b]: For comparison of vancomycin mean OD and number of biofilm related genes among of total strong biofilm forming *Enterococcus* isolates
*: Percent in row

Figure 1. Detection of Enterococcal biofilm formation using microtiter plates. 1: Positive control; 2: Negative control; 3: Strong biofilm producing isolate; 4: Moderate biofilm producing isolates; 5:Weak biofilm producing isolate.

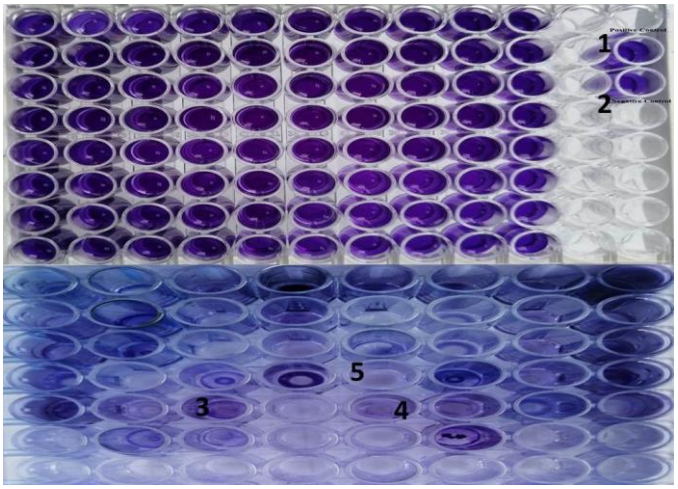


Figure 2. Gel electrophoresis showing the amplified product of the *gel E* gene (213 bp), *asa 1* (375 bp) and *esp* gene (510 bp). Lane 1: 100 bp DNA ladder. Lane 2 and 7: *gel E* and *asa 1* positive isolates; Lanes 3 and 5: *gel E* and *esp* positive isolates; Lane 4 and 6: *gel E* positive isolates; Lane 8: *esp* positive isolate.

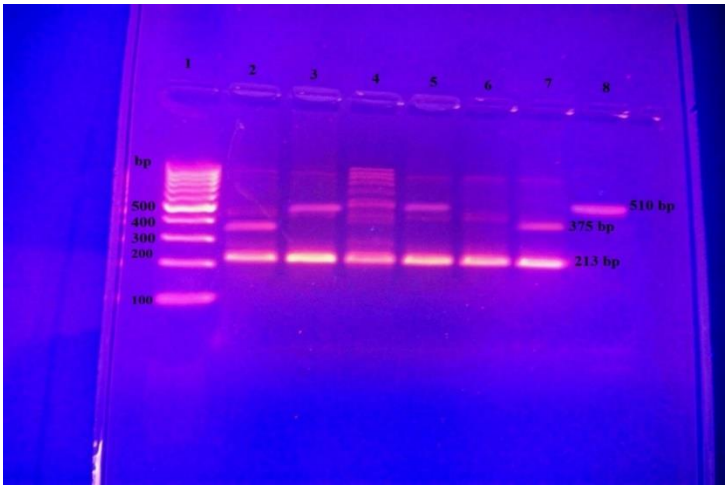
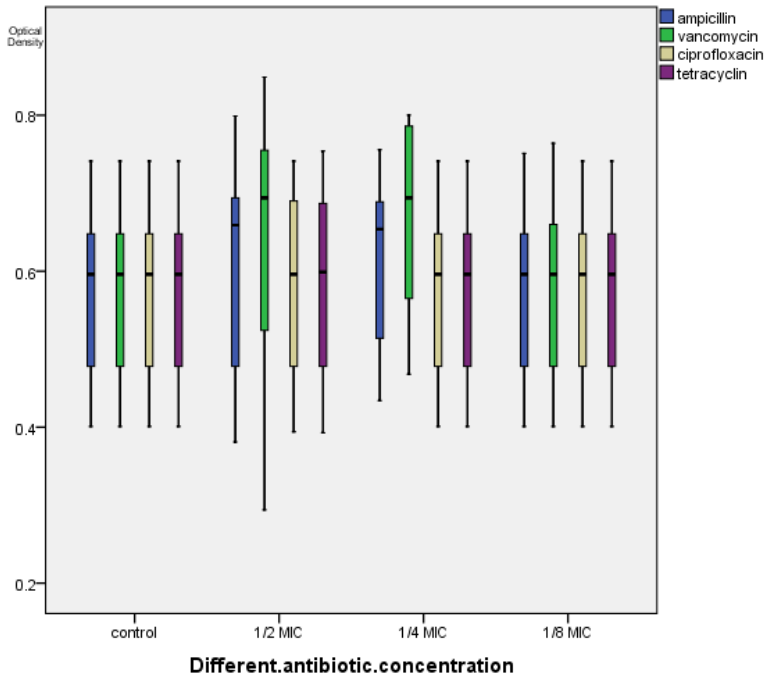


Figure 3. Impact of sub-inhibitory antibiotic concentrations on biofilm formation among strong biofilm producing isolates of *Enterococcus*



Discussion

Enterococci, especially *E. faecalis*, have become one of the common nosocomial pathogens in the last decades with increased production of biofilm that creates a favorable environment for microbial survival within the host [16].

In our study, *E. faecalis* represented 81.2% of enterococcal infections which is consistent with Maestre et al., [17] and Walaa et al., [18].

In this study, frequency of biofilm production was significantly higher among *E. faecalis* isolates and most biofilm forming isolates were from urine which is comparable to results of previous researches [3, 19, 20]. In contrast, Weng et al., [21] noted that *E. faecium* developed greater biofilms compared to *E. faecalis* (59.3% vs. 49.0%). *Enterococci* capacity to form biofilm varies, worldwide. About 90%, 80%, 59% and 57% of *E. faecalis* isolates could form biofilm in Japan, Italy, Poland, and Spain respectively [22].

In the present study, distribution of *asa 1*, *esp* and *gel E* genes were encountered more frequently in *E. faecalis* than in *E. faecium* isolates. This result was confirmed previously by Strateva et al., [23]. In contrary, Weng et al., [21] reported that *E. faecium* acquired the *esp* gene more frequently than *E. faecalis* (78.6% versus 46.2%).

In the current study, frequency of *asa 1*, *esp*, *gel E* genes were higher among biofilm forming isolates. In agreement, Hashem et al., [3, 15], Kafil and Mobarez [24], Soares et al., [20] linked *esp*, *gel E* and *asa 1* genes to biofilm formation. In addition, Tibúrcio et al., [25] demonstrated higher biofilm production with simultaneous presence of three or four of the investigated virulence genes (*ace*, *asa1*, *efa A*, and *esp*) in isolates which matched our results.

Studies proposed that antibiotic sub-MICs are signaling compounds that regulate a range of cellular activities including expression of genes, quorum sensing, biofilm development and horizontal transfer of antimicrobial resistance genes among bacterial populations [26].

In agreement with our results, Ranieri et al., [6] reported that beta lactams can stimulate biofilm production via cell lysis induction, while antibiotics that affect DNA replication or ribosomes are less likely to perform this. Similarly, Yu et al., [27] reported increase in biofilm density among *E. faecalis* isolates with sub-MIC of antibiotics that inhibit cell wall synthesis (ampicillin, ceftriaxone, fosfomycin and oxacillin) but not with drugs targeting synthesis of protein, DNA, RNA or folic acid. They explained this enhancement effect by cell lysis and increased eDNA and eRNA which might modulate expression of biofilm-associated genes.

They also observed biofilm enhancement among isolates when grew with a non-antibiotic surfactant that is known cell lysis inducer. Additionally, previous studies reported biofilm promotion in *Staphylococcus aureus*, *Streptococcus mutans* and *Pseudomonas aeruginosa* following challenge with sub-MIC of antibiotics inhibiting cell wall synthesis [7, 28, 29, 30]. Additionally, Hagras et al., [7] study showed that there was time, strain as well as concentration-dependent changes in biofilm formation which was stronger on exposure to 1/2 and 1/4 MIC levels of cefepime after 48 hours than that at 24 h in strains with weak and non-biofilm producers. Bernardi et al., [5] discovered a significant enhancement in biofilm formation of *E. faecalis* with sub-inhibitory doses of both antibiotics inhibiting cell wall and protein synthesis particularly at their 1/2 MIC. In Yuksel et al., [26] study, vancomycin, streptomycin and erythromycin sub-MIC enhanced biofilm formation and *esp* gene expression in *E. faecium*, while biofilm development was unaffected by the sub-MICs of gentamycin, ampicillin, and chloramphenicol. In contrast, Hashem et al., [15] observed different results in which biofilm production and gelatinase activity were inhibited among *Enterococcus* when exposed to vancomycin and tigecycline at sub-MICs. Maestre et al., [17] reported the same effect of tigecycline. In the same line, Tibúrcio et al., [25] observed that biofilm production among *E. faecalis* isolates significantly decreased when subjected to sub-MICs of penicillin and ampicillin and the expression of *asa 1* and *ace* was down-regulated. The sub-MICs of gentamicin had no significant effect on biofilm or virulence genes expression. Kafil et al., [31] and Moura et al., [32] reported that ampicillin and vancomycin sub-MICs did not affect biofilm density among *E. faecalis* isolates however they could enhance expression of some genes involved in biofilm and antibiotic resistance. Kafil et al., [31] also found that gentamycin could enhance biofilm and its related genes.

Improper antibiotics prescriptions to the patients and antibiotic misuse play crucial role in this issue .Different strains even of the same species but isolated from various geographical locations may exhibit varying virulence factors and different genotypic and phenotypic response to sub-inhibitory concentrations of antimicrobial substance [33].

The impact of numerous sub-MICs of antibiotics on biofilm development and gene expression were not assessed in our study, thereby acting as a limitation of this research.

Conclusion

The results of our investigation highlight the significance of using antibiotics sensibly and appropriately so the microbial biofilm would not be exposed to sub-MIC, which would prevent additional or enhanced biofilm development. In order to combat biofilms and get a greater knowledge of biofilm development, it is important to investigate the expression of biofilm-related genes in response to sub-MICs of antibiotics.

Abbreviations

Minimum inhibitory concentration (MIC)

Extracellular DNA (eDNA)

Health-care associated infections (HAIs)

Cystine–Lactose–Electrolyte Deficient (CLED)

Microtiter Plates (MTP)

CLSI (Clinical and Laboratory Standards Institute)

Optical density (OD)

Trypticase-soy broth (TSB)

GelEA (gelatinase)

CylA (cytolysin)

Hyl (hyaluronidase)

asa 1 (aggregation substance)

Ebp (endocarditis and biofilm-associated pili)

Esp (surface protein implicated in the colonization, persistence, and biofilms production)

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Conflicts of interest

The authors declare that they do not have any conflict of interest.

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