

Research Article

Antibiotic Scuptibility, Biofim-Forming Capacity and Hemolysin and Gelatinase Production By of *Enterococcus Spp* Isolates from Vaginal Microbiota

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Abstract

Initially commensal bacteria of the gastrointestinal tract, *Enterococcus* species are opportunistic pathogens that can colonize the female vaginal tract, particularly in patients with aerobic vaginitis or after receiving antibiotic treatment. *Enterococcus* spp., are associated with a wide range of illnesses, particularly in patients with altered host microbiota or immunocompromised conditions. Here we assessed the antibiotic susceptibility, the biofilm formation ability and the production of hemolysin and gelatinase in enterococcal isolates from vaginal microbiota with phenotypic characterization methods. Our results revealed the prevalence of *E. faecalis* strains from vaginal samples. 12.9% of *E. faecalis* were resistant to glycopeptides while *E. faecium* isolates showed multi-resistant profile to β -lactamine antibiotics, aminoglycosides and macrolides. Biofilm was detected in thirty isolates, of which four were strongly biofilm-producing and thirteen moderately biofilm-producing. Moreover, hemolysin and gelatinase virulence factors were detected in 83.9% and 89.3% of *Enterococcus* spp strains respectively. In addition to biofilm formation, our results demonstrated that hemolysin and gelatinase production are significant virulence factors in *Enterococcus* spp isolated from vaginal microbiota.

INTRODUCTION

The gut, genitourinary tract and oral cavity are among the environments with low redox potential where Enterococci thrive [1]. The expression of different enterococcal characteristics eventually leads to virulence in unfavourable conditions [2]. Most of human enterococcal infections are caused by *E. faecalis*, which is followed by *E. faecium* among the *Enterococcus* species [3]. Because of their capacity to acquire and disseminate antibiotic resistance genes, enterococci are becoming significant nosocomial pathogens, with inherently resistant to antibiotics [4].

Increasing virulence may or may not be linked to increasing antibiotic resistance in bacterial cells, as cost-benefit studies have demonstrated that virulence and antibiotic resistance are two entirely distinct characteristics of bacterial cells [5]. More antibiotic resistance was shown by the *Enterococcus* strains that produced biofilms as opposed to those that did not [6,7].

For instance, a key component of the pathogenicity of *Enterococcus faecalis* isolated from chronic infections is the capability of biofilms production [8]. In addition to biofilm formation, it has been demonstrated that *E. faecalis* strains that produce hemolysin are virulent in both human and animal infections [9], and are linked to higher infection severity [10]. *E. faecalis* produces the protease gelatinase, which hydrolyzes a variety of peptides, including collagen, casein, hemoglobin, and gelatin [11]. In endocarditis, enterococcal gelatinase is the main pathogenic mediator [12].

Our finding aims to assess the antibiotic susceptibility, the biofilm formation ability and the presence of hemolysin and gelatinase in enterococcal isolates from vaginal samplings.

MATERIALS AND METHODS**Bacterial strains**

Our study was carried out on 31 *Enterococcus* spp

strains isolated from vaginal samplings undertaken in the microbiology laboratory of the Monastir Maternity and Neonatology Center in Tunisia during 2019.

Confirmation of bacterial identification

Bacteria were isolated on a chromogenic culture medium (CHROMagar™ StrepB, France). After incubation of each sample at 37°C for 24 h, the greenish-blue colonies corresponding to the *Enterococcus* genus (as opposed to the mauve colonies for *Streptococcus*) were plated on potassium tellurite agar medium (Bio-Rad, France). This medium distinguishes *E. faecalis* from other enterococci species. *E. faecalis* is the only species capable of reducing tellurite to tellurium, producing black colonies on this medium.

Confirmation of the Identification was based on morphological and biochemical characteristics. Selected colonies were identified using standard microbiological techniques, namely Gram staining and catalase enzyme testing, followed by biochemical confirmation using the VITEK® 2 compact automated system (BioMerieux®, France) based on advanced colometry technology.

After identification, *Enterococcus* spp strains were stored at -20°C in eppendorfs tubes containing Brain Heart Broth (BHI, Biorad) supplemented with 30% of glycerol.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out using a Vitek® 2 Compact automated system (BioMerieux®, France) according to the recommendation of EUCAST [13]. The principle of this method is based on the determination of the minimum inhibitory concentration (MIC) comparing with standard MIC values listed in the database of the apparatus. Thus, from an overnight bacterial culture of each tested strain, a suspension was prepared in NaCl solution (0.45%) and optic density of the bacterial inoculum was adjusted to 0.5 McFarland using a DensiCHEK densitometer. The antibiogram card (AST-P592) was then inoculated with the prepared bacterial suspension and incubated in the automated system at 35-37°C for 24 hours. The MIC result for each antibiotic was finally obtained with an Advanced Expert System (AESTM) software. Results from this test are interpreted using clinical categorization (S: sensitive, I: intermediate and R: resistant). The antibiotics tested are Ampicillin (AMP), Imipenem (PM), Teicoplanin (TEC), Vancomycin (VAN), Gentamicin (GEN), Streptomycin (STR), Erythromycin (ERY), Tigecycline (TGC), Ciprofloxacin (CIP), Linezolid (LZD), Trimethoprim/Sulfamethoxazole (T/S)

Biofilm formation

Enterococcus spp strains were incubated in Brain Heart Broth (BHI, Biorad) at 37°C for 24h. A 1:100 dilution in BHI broth supplemented with 2% glucose (m/v) was then performed for each strain tested. A total volume of 200µl of each dilution was transferred to 96-well U-shaped polystyrene plates (Nunc; Nunc, Denmark). Each strain was tested three times. Wells containing only BHI broth with 2% sterile glucose were considered as negative controls. The plate was then incubated aerobically for 24 hours at 37°C. The broth from each well was then aspirated and the plate rinsed three times with phosphate-buffered saline (PBS) to remove non-adhered bacteria. The plate was then dried at room temperature. After the drying step, the biofilm formed by the bacteria was stained with 100µl of 1% crystal violet solution (Merck, France), for 20 min. Excess dye was removed by three successive washes, with 300µl of sterile distilled water. The optical density of each well was measured at 570nm using a microplate reader (Automated Multi skanreader, Italy). The ability of strains to form biofilm was classified into three categories [14]:

- "Strongly biofilm-forming": (OD570 ≥ DOc)
- "Moderately biofilm-forming": (2DOc ≤ DO570 < 4DOc)
- "Weakly biofilm-forming" (DOc ≤ DO570 < 2DOc)
- "Non-biofilm-forming": (OD570 < DOc)

ODc: Optical density control [14].

Gelatinase production

The gelatinase production capacity of isolated bacteria was determined after inoculation of strains onto TSA media (Trypticase Soy Agar; Biorad) supplemented with 0.8% (m/v) gelatin. The presence of a clear halo around the colonies indicates the presence of the desired enzyme. After detecting the presence of this enzyme, we quantified the gelatinase activity. To do this, we sterilely dug 5mm diameter wells in the gelatin agar (0.8%) prepared beforehand. Then, using the 24h cultures of the enterococci strains to be tested, on TSB liquid medium (Trypticase Soy Broth; Biorad), a volume of 50µl was transferred to the wells already prepared on the agar. After incubation at 37°C for 24 hours, we measured the diameter of the halo around each well [15].

Hemolytic potency

Hemolytic activity was tested on blood agar. Each strain was inoculated by circular streaking using a metal loop. After incubation for 24 hours at 37°C, 3 types of hemolysis can be observed:

- Hemolysis α : greenish in colour, resulting from partial lysis of red blood cells.

- β hemolysis: light yellowish colour, resulting from complete red cell lysis.

- Hemolysis γ : no hemolysis and blood agar retain its red color [16].

After detecting the type of hemolysis, we quantitatively estimated this hemolytic activity. Sterile 5mm diameter wells were dug in the blood agar. Then, using the 24h cultures of enterococci on TSB liquid medium, a volume of 50 μ l was transferred to the wells already prepared on blood agar. After incubation at 37°C for 24h, we measured the diameter of the hemolysis halo around each well.

RESULTS

Confirmation of bacterial species

100% of the isolated strains were gram-positive with cocci form after Gram staining. All of these strains were catalase producer. Confirmation of enterococcal species identification was carried out biochemically using the Vitek® 2 Compact automated system. 28 strains were identified as *E. faecalis* (90%) and 3 strains of *E. faecium* (10%) as presented in Table 1.

Antibiotic resistance profile

The results of the resistance profile of enterococcal isolates showed that 4 strains (12.9%) of *E. faecalis* were resistant to vancomycin and teicoplanin (Glycopeptide family), which are considered a treatment of last resort against these species. In addition, 3 strains of *E. faecium* (9.67%) are multi-resistant to β -lactam antibiotics, aminoglycosides and macrolides (Table 2).

Biofilm formation

The results of biofilm formation abilities were presented in Figure 1. The *Enterococcus* spp strains exhibited different level of biofilm formation capability. We have found that 4 out of 28 of *E. faecalis* strains were highly biofilm-forming ($DO_{570} \geq 0.48$), 12/28 strains were moderately biofilm-forming ($0.24 \leq DO_{570} < 0.48$), 11/28 strains were weakly biofilm-forming ($0.12 \leq DO_{570} < 0.24$), and only one strain that was unable to form a biofilm, representing 14.3%, 42.9%, 39.3%, and 3.5% of the total tested strains respectively. Regarding *E. faecium* strains, only one strain tested was moderately biofilm-forming (33.3%). However, the two other strains were found to be weakly biofilm-forming.

Gelatinase production

Gelatin hydrolysis was detected by the appearance of a clear halo around the wells on gelatin agar (Figure 2). Our results showed that 100% of *E. faecium* strains were able to produce gelatinase, compared with *E. faecalis* (89.3%). Moreover, it was also found that 20 isolated *Enterococcus* strains presented diameters exceeding 12mm. For instance, the Strain 58620 showed a hemolysis diameter of 14.5mm (Table 3).

Hemolysin production

Firstly, the Hemolysin production was revealed qualitatively by the presence of a clear halo around colonies seeded on blood agar (Figure 2). We noted that 26 strains of enterococci exhibited β -type hemolysis (83.9%) versus 5 γ -type strains (16.1%). Then, the results of the quantitative test showed that 11 strains of *E. faecalis* had hemolysis diameters exceeding 12mm, and up to 17mm (Strain 56234). In contrast, the 3 *E. faecium* strains had smaller diameters, ranging from 8 to 11.5 mm (Table 3).

DISCUSSION

Enterococci, typically found in intestinal and skin microbiota, are microorganisms with a remarkable

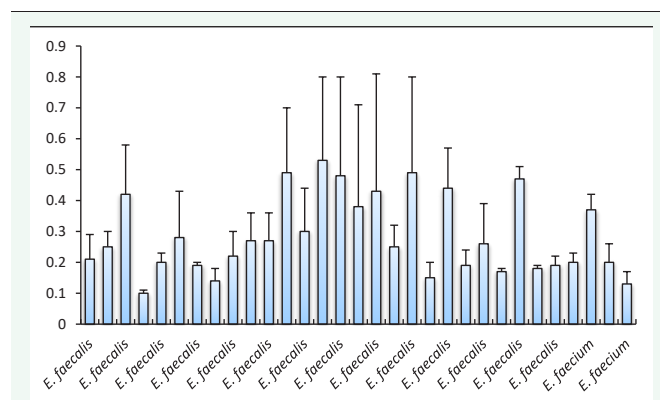


Figure 1 Biofilm formation abilities of *Enterococcus* spp isolates from vaginal microbiota, assed by the crystal violet staining methods. Values are the average of at least three independent determinations.

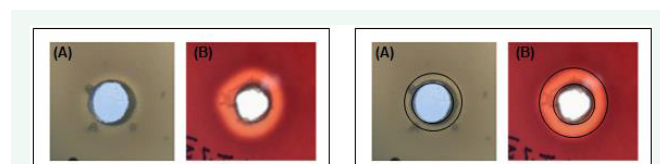


Figure 2 Gelatinase (A) and Hemolysin production by tested *Enterococcus* spp strain. Graph in the right showed the diameter of the well (initially containing the bacterial suspension) and the zone of inhibition (degradation of the substrate) revealing the secretion of the enzyme or toxin by the tested strain.

Table 1: Confirmation of the bacterial species identification

Number	Code	CHROMagar™ StrepB	Potassium Tellurite	Gram staining	Catalase test	Vitek (strain)
1	16325	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
2	16382	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
3	14635	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
4	15971	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
5	53945	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
6	54011	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
7	54113	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
8	54758	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
9	53977	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
10	54091	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
11	54085	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
12	55630	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
13	56234	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
14	55929	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
15	56170	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
16	55386	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
17	55507	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
18	55680	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
19	57060	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
20	56727	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
21	57327	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
22	58620	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
23	59137	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
24	59987	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
25	17133	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
26	53964	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
27	58560	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
28	59197	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecalis</i>
29	14502	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecium</i>
30	54709	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecium</i>
31	54520	Blue-green	Black colonies	Gram (+) cocci	+	<i>E. faecium</i>

capacity to develop resistance to antibiotics [17]. The prevalence of vancomycin resistance in these organisms is rising in hospitals, which limits the available treatments [18]. Despite their relatively modest virulence, they can seriously infect susceptible patients, particularly with impaired immune systems and/or those in need of complex long-term care. *Enterococcus faecalis* and *Enterococcus faecium* are the two primary species. In the present study 90.3 % of the isolates were identified as *E. faecalis* while 9.7% belongs to *E. faecium* species. This is in agreement with previous reports showing that the prevalence of *E. faecalis* strains from vaginal origin compared to *E. faecium* [7,19].

The antibiotic susceptibility test from our study revealed that 4 strains of *E. faecalis* were resistant to vancomycin and teicoplanin (12.9%) which belong to Glycopeptide family, considered as a treatment of last resort against this species. Although the epidermis, oropharynx and gastrointestinal tract are the most common sites of colonization by vancomycin-resistant enterococci [20], our results underlined that the vagina is another possible

niche for the colonization with this bacterial phenotype. More generally, the clinical effectiveness of vancomycin is at risk due to the emergence and widespread dissemination of two types of complicated resistance mechanisms, each consisting of a multi-enzyme route, in pathogenic species [21]. Additionally, the tested strains of *E. faecium* showed multi-resistant to β -lactam aminoglycosides and macrolides antibiotics. It was reported that unlike *E. faecalis*, *E. faecium* is more frequently drug-resistant [22]. In line with our research, several studies on *E. faecium* strains show that these strains are very sensitive to tigecycline, highlighting their potential as suitable therapeutic options for treating resistant infections caused by this bacterium [23, 24].

Because of its propensity to build biofilm highly than *E. faecium*, the treating of *E. faecalis* infections is particularly difficult and may be a factor in the stagnant mortality rates [25,26]. This is an agreement with our finding since we have found that the majority of *E. faecalis* isolates were found to be biofilm forming strain. A recent report showed that 68.27% of *E. faecalis* strains, collected from different

Table 2: Antibiotic resistance profiles of *E. faecalis* and *E. faecium* strains

Strain	AMP	PM	TEC	VAN	GEN	STR	ERY	TGC	CIP	LZD	T/S
<i>E. faecalis</i>	S	S	S	S	S	S	I	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	R	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	S	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	S	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	I	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	s	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	R	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	S	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	I	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	I	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	I	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	R	S	S	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	I	S	*	S	I
<i>E. faecalis</i>	S	S	S	S	S	S	I	S	*	S	I
<i>E. faecalis</i>	I	S	R	R	S	S	R	S	*	S	I
<i>E. faecalis</i>	I	S	R	R	S	S	R	S	*	S	I
<i>E. faecalis</i>	I	S	R	R	S	S	R	S	*	S	I
<i>E. faecalis</i>	I	S	R	R	S	S	R	S	*	S	I
<i>E. faecium</i>	R	R	S	S	R	R	R	S	R	S	I
<i>E. faecium</i>	R	R	S	S	R	R	R	S	R	S	I
<i>E. faecium</i>	R	R	S	S	R	R	R	S	R	S	I

S: sensitive, I: intermediate; R: resistant; *: untested

Table 3: Biofilm formation, gelatinase and hemolysin production by vaginal *Enterococcus* spp strains

Strains	Biofilm formation		Gelatinase production		Hemolysin production	
	OD (570)	Ability	Qualitative	Quantitative	Qualitative	Quantitative
<i>E. faecalis</i>	0.21±0.08	F	+	14±1.4	Hemolysisß	6.5±0.40
<i>E. faecalis</i>	0.25±0.05	M	+	12±0	Hemolysisß	8.33±0.47
<i>E. faecalis</i>	0.42±0.16	M	+	13±1.4	Hemolysisß	15±0
<i>E. faecalis</i>	0.10±0.01	NF	+	12±0	Hemolysisß	15±0
<i>E. faecalis</i>	0.20±0.03	F	-	5±0	Hemolysisß	13.66±0.94
<i>E. faecalis</i>	0.28±0.15	M	+	12.5±0.7	Hemolysisß	14.33±0.47
<i>E. faecalis</i>	0.19±0.01	F	+	12±1.4	Hemolysisß	13.5±0.5
<i>E. faecalis</i>	0.14±0.04	F	+	13±1.4	Hemolysisß	15±0
<i>E. faecalis</i>	0.22±0.08	F	+	12±0	Hemolysisß	17±2
<i>E. faecalis</i>	0.27±0.09	M	+	12±1.4	Hemolysisß	15±1
<i>E. faecalis</i>	0.27±0.09	M	+	14±	Hemolysisß	15.5±0.5
<i>E. faecalis</i>	0.49±0.21	H	+	11.5±0.7	Hemolysisß	15±1
<i>E. faecalis</i>	0.30±0.14	M	+	12±1.4	Hemolysisß	14±0
<i>E. faecalis</i>	0.53±0.27	H	+	11±0	Hemolysisß	15±1
<i>E. faecalis</i>	0.48±0.32	H	-	5±0	Hemolysisß	8±0
<i>E. faecalis</i>	0.38±0.33	M	+	12.5±0.7	Hemolysisß	7±0
<i>E. faecalis</i>	0.43±0.38	M	+	10.5±0.7	Hemolysisß	7±0
<i>E. faecalis</i>	0.25±0.07	M	+	10.5±0.7	Hemolysisß	7±0
<i>E. faecalis</i>	0.49±0.31	H	+	11.5±0.7	Hemolysisß	14.66±0.47
<i>E. faecalis</i>	0.15±0.05	F	+	13±0	Hemolysisß	15.66±0.47
<i>E. faecalis</i>	0.44±0.13	M	+	12.5±0.7	Hemolysisß	13±0
<i>E. faecalis</i>	0.19±0.05	F	+	14.5±0.7	Hemolysisß	13±0±0.47
<i>E. faecalis</i>	0.26±0.13	M	+	13.5±0.7	Hemolysisß	8.66±0.47
<i>E. faecalis</i>	0.17±0.01	F	+	13±0	Hemolysisß	13.33±0.47
<i>E. faecalis</i>	0.47±0.04	M	+	11±0	Hemolysisß	13.66±0.47
<i>E. faecalis</i>	0.18±0.01	F	-	5±0	Hemolysisß	12.33±0.47
<i>E. faecalis</i>	0.19±0.03	F	+	12.5±0.7	Hemolysisß	11.33±0.47
<i>E. faecalis</i>	0.20±0.03	F	+	12.5±0.7	Hemolysisß	11.33±0.47
<i>E. faecium</i>	0.37±0.05	M	+	10.5±0.7	Hemolysisß	12.66±0.47
<i>E. faecium</i>	0.20±0.06	F	+	13.5±0.7	Hemolysisß	15.33±0.94
<i>E. faecium</i>	0.13±0.04	F	+	11.5±0.7	Hemolysisß	15.66±0.47

H: Highly biofilm-forming. M: Moderately biofilm-forming. F: Low biofilm-forming ; N-F/ Non-forming

sources environmental and clinical samples, were biofilm producers [27]. More generally, biofilm-associated illnesses are challenging to cure, because inside biofilms bacteria are resistant to phagocytosis, antibiotics and environmental stress [28,29].

Hemolysin, aggregation substance, and gelatinase have all been identified as enterococci's virulence factors [15]. Our finding revealed that more than 89.3% of clinical strains of *Enterococcus* spp isolated from vagina were gelatinase producer. We also noted that 83.9% of the tested strains exhibited β -hemolysis. It was previously showed that 64% blood isolates of *E. faecalis* from patients with bacteremia were gelatinase producer [9]. Additionally, increased infection severity has been linked to hemolysin producing strains. This cytolytic protein has the ability to lyse human erythrocytes causing cell damages [30].

CONCLUSION

The identification of virulence factors linked to enterococcal infections in women will be a crucial focus of future research due to the growing significance of *Enterococcus* species as nosocomial pathogens and the rising incidence of glycopeptide resistance among enterococci. In the context of antibiotic resistance, the search for new molecules with preventing or suppressing properties against enterococcal virulence factors could offer therapeutic alternatives for combating these pathogenic bacteria.

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