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Molecular Epidemiology and Virulence Gene Expression of *Enterococcus* spp. isolated from clinical samples in Southern Brazil

Moleculaire epidemiologie en virulentie genexpressie van Enterococcus spp. klinische isolaten uit Zuid-Brazilië

Dissertation submitted to obtain a PhD in Pharmaceutical Sciences at the University of Antwerp and in Health Sciences at the Federal University of Health Sciences of Porto Alegre defended by

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LIST OF ABBREVIATIONS

ºC - degrees Celsius
% - percentage
µg - microgram
µL – microliter
µM - micromolar
h – hour
nm - nanometer
pmol - picomol
s – second

A

Ace – Collagen Adhesin
AMP - ampicillin
AS – Aggregation Substance
ATCC – American Type Culture Collection

B

BHI – Brain Heart Infusion
BLAST – Basic Alignment Search Tool
BSI – Bloodstream Infection

C

CAPES - Coordination for the Improvement of Higher Level -or Education- Personnel
CAUTI – Catheter-associated urinary tract infection
CDC – Centers for Disease Control and Prevention
cDNA- Complementary DNA
CIP - ciprofloxacin
CLSI – Clinical and Laboratory Standards Institute
CNPq - National Council for Scientific and Technological Development
CT – clonal type

D

DNA – deoxyribonucleic acid

E

Esp – Enterococcal Protein Surface

F

FAPERGS - Foundation of Research Support of the State of Rio Grande do Sul

G

GI – Gastrointestinal tract

H

HAI – Healthcare associated infection
HA –Hospital A
HB –Hospital B
HC –Hospital C
HLG – high level of gentamicin
HS – human serum

I

IE – Infective Endocarditis
ICU – Intensive Care Unit

M

MIC – Minimum Inhibitory Concentration
MBC – Minimum Bactericidal Concentration
MSCRAMMS - Microbial Surface Components Recognizing Adhesive Matrix Molecules

P

PBP – Penicillin-Binding Protein
PCR – Polymerase Chain Reaction
PFGE – Pulsed Field Gel Electrophoresis

Q

qPCR - quantitative Polymerase Chain Reaction

R

RNA – ribonucleic acid

T

TBE - Tris/Borate/EDTA
TEI - teicoplanin
TSB – tryptic soy broth
TSA – tryptic soy agar
TSBg - tryptic soy broth plus glucose

V

VAN - vancomycin
VRE – Vancomycin resistant enterococci

U

UFCSPA - Federal University of Health Sciences of Porto Alegre
UTI - Urinary Tract Infection

SUMMARY

Enterococcus faecalis and *Enterococcus faecium* are opportunistic pathogens, able to cause different types of infections, from ordinary urinary tract infections to life-threatening healthcare-associated ones. This capacity is directly associated with their ability to form biofilms and the presence of many virulence factors and resistance genes, giving them selective advantages in adverse conditions. Vancomycin-Resistant Enterococci (VRE) have become increasingly common in some settings around the world and their rapid and accurate identification, as well as the understanding of their clonal spread, can improve treatment and infection control policies. In this study, we evaluated the performance of a selective chromogenic medium (chromID™ VRE agar) using 184 clinical isolates of *Enterococcus* spp. (susceptible and resistant to vancomycin) and reference strains. Secondly, we characterized the susceptibility profile and clonal relationships of 94 VRE, recovered from inpatients at three general Hospitals of Porto Alegre, Southern Brazil. Thirdly, we evaluated the biofilm formation of 123 *E. faecalis* clinical isolates in the presence and absence of 1% D-(+)-glucose and the expression of four biofilm-related genes (*ebpA, efaA, ace* and *gelE*) in eleven clinical isolates and *E. faecalis* ATCC 29212 by qPCR. Lastly, we evaluated the *E. faecalis* V583 biofilm formation capability in the absence and presence of human serum (1%, 5%, 25% and 50%) and the

expression of five virulence genes (*ebpA, efaA, ace, gelE* and *asa*) in planktonic and sessile cells by qPCR. Although ChromID™ VRE agar had a very good sensitivity (95.52%), it presented a very low specificity (30%). All VRE were identified as *E. faecium* and exhibited high-level resistance to vancomycin and resistance to teicoplanin, ampicillin, ciprofloxacin and 13.8% of them were resistant to high level of gentamicin. All VRE harbored *vanA* gene, 85.1% *esp* gene and 94.7% *acm* gene. PFGE profile analysis revealed a polyclonal distribution with 23 clonal types encompassing 79 isolates, while 15 isolates exhibited unique patterns. Considering biofilm formation, 1% glucose supplementation increased significantly the biofilm formation among *E. faecalis*. However, gene expression varied among them, showing that patterns of virulence gene expression may be dependent partially on the bacterial genetic background, rather than environmental conditions. On the other hand, an inhibition of the adhesion in the presence of 5% human serum was observed compared to the control group. Planktonic cells of *E. faecalis* V583 exhibited upregulation of *asa* gene in 5%, 25% and 50% of human serum, while in the sessile cells, *efaA*, *gelE, ace* and *asa* genes were significantly upregulated in 25% and 50% human serum. A better understanding of this process as well as the application of these findings *in vivo* may help in the search for strategies to control the biofilm formation by this microorganism.

SAMENVATTING

Enterococcus faecalis en *Enterococcus faecium* zijn opportunistische pathogenen, in staat om verschillende soorten infecties te veroorzaken, gaande van gewone urineweginfecties tot levensbedreigende ziekenhuisinfecties. Deze capaciteit is rechtstreeks verbonden met hun biofilmvermogen en de aanwezigheid van vele virulentiefactoren en resistentiegenen, waardoor ze selectieve voordelen bezitten in ongunstige omstandigheden. Vancomycine-Resistente Enterokokken (VRE) komen steeds vaker voor in sommige instellingen over de hele wereld. Een snelle en nauwkeurige identificatie, evenals een betere kennis van hun klonale verspreiding, kunnen een positief effect hebben op de behandeling en het infectie controlebeleid. In deze studie evalueerden we een selectief chromogeen medium (chromID™ VRE agar) met behulp van 184 klinische isolaten van *Enterococcus* spp. (gevoelig en resistent tegen vancomycine) en referentiestammen. Ten tweede, karakteriseerden we het gevoeligheidsprofiel en de klonale verwantschappen van 94 VRE, geïsoleerd uit patiënten van drie algemene ziekenhuizen van Porto Alegre, Brazilië. Ten derde, evalueerden we de biofilmvorming van 123 *E. faecalis* klinische isolaten in de aan- en afwezigheid van 1% D-(+)-glucose en de expressie van vier biofilm-gerelateerde genen (*ebpA*, *efaA*, *ace* en *gelE*) in elf klinische isolaten en *E. faecalis* ATCC 29212 door qPCR. Tenslotte evalueerden we de *E. faecalis* V583

biofilmvorming capaciteit in de aan- en afwezigheid van humaan serum (1%, 5%, 25% en 50%) en de expressie van vijf virulentiegenen (*ebpA, efaA, ace, gelE* en *asa*) in planktonische en sessiele cellen door qPCR. Hoewel ChromID™ VRE agar een zeer goede gevoeligheid (95.52%) heeft, is de specificiteit met 30% aan de lage kant. Alle VRE werden geïdentificeerd als *E. faecium* en vertoonden een hoge mate van resistentie tegen vancomycine en resistentie tegen teicoplanine, ampicilline, ciprofloxacine en 13,8% van hen waren resistent tegen hoge concentraties van gentamicine. Alle VRE bezaten het *vanA* gen, 85.1% het *esp* gen and 94.7% het *acm* gen. PFGE profiel analyse toonde een polyklonaal distributie aan met 23 klonale types die 79 isolaten bevatten, terwijl 15 isolaten unieke patronen bezaten. Supplementatie met 1% glucose verhoogde sterk de biofilmvorming bij *E. faecalis.* De genexpressie varieerde echter tussen hen, waaruit blijkt dat patronen van virulentie-afhankelijke genexpressie gedeeltelijk afhankelijk zijn van de bacteriële genetische achtergrond, eerder dan van milieu-omstandigheden. Anderzijds werd een remming van de adhesie waargenomen bij aanwezigheid van 5% humaan serum in vergelijking met de controlegroep. Planktonische cellen van *E. faecalis* V583 vertoonden een opregulatie van het asa gen in 5%, 25% en 50% humaan serum, terwijl in de sessiele cellen, *efaA, gelE, ace* en *asa* genen aanzienlijk werden opgereguleerd in 25% en 50% humaan serum. Een beter begrip van dit proces evenals de toepassing van deze bevindingen *in vivo* kunnen helpen bij het zoeken naar nieuwe strategieën om biofilmvorming van dit microorganisme tegen te gaan.

CHAPTER 1

INTRODUCTION

1.1 GENUS *Enterococcus*

The genus *Enterococcus* includes Gram-positive bacteria, non-spore forming, facultative anaerobes, arranged in pairs or short chains whose shape is spherical or ovoid. These bacteria can grow in high salt concentrations and in a range of temperatures (10-45ºC). The ability to hydrolyse esculin in the presence of 40% of bile salts and the absence of enzymes are also typical characteristics of this genus (LEBRETON et al., 2014).

Fifty-eight species of *Enterococcus* were recognized between 1992 and 2017 (EUZÉBY, 2018). Due to their ability to survive under adverse conditions, they can be found in a variety of environments such as soil, water, plants as wells as and colonizing the oral cavity, genitourinary and gastrointestinal (GI) tract of humans and animals (BYAPPANAHALLI et al., 2012; HIGUITA & HUYCKE, 2014).

Enterococcus faecalis and *Enterococcus faecium* are the main species associated whit human infections, being responsible for the majority of reported cases (ARIAS & MURRAY, 2012). Although in a small proportion, other enterococcal species including *E. gallinarum, E. casseliflavus, E.hirae, E.*

raffinosus, *E. avium*, *E. mundtii* and *E. durans* have also been reported in human infections. In some scenarios, however, these species may be underestimated due to the frequent misidentification (PRAKASH et al., 2005; ESCRIBANO et al., 2013; HIGUITA & HUYCKE, 2014; BOURAFA et al., 2015; SHARIFI-RAD et al., 2016).

1.1.1 E. faecalis and E. faecium

Infections caused by *E. faecalis* and *E. faecium* are considered a global health problem, since they are difficult to manage and treat (HIGUITA & HUYCKE, 2014; KRISTICH et al., 2014). It is known that *E. faecium* is intrinsically more resistant to antibiotics than *E. faecalis*. This species has emerged as one of the main multidrug-resistant pathogens in the hospital environment due to its intrinsic resistance characteristics plus its genomic plasticity, leading to the acquisition and dissemination of mobile genetic elements carrying resistance genes (GAO et al., 2018). In this context, *E. faecium* expressing resistance to vancomycin, ampicillin, and high-levels of aminoglycosides is the major phenotype among clinical isolates, impairing therapeutic options (PALMER et al., 2011; LEBRETON et al., 2013; HIGUITA & HUYCKE, 2014; RAZA et al., 2018).

These well-adapted *E. faecium* lineages designs a clonal dissemination pattern, where most strains of *E. faecium* causing nosocomial infections are part

of the same sub-population (CATTOIR & LECLERCQ, 2013; PRIETO et al., 2016).

Despite *E. faecium* importance (mainly because of their resistance patterns), *E. faecalis* is responsible for the largest number of cases of enterococcal infections, being 10 times more prevalent than other species of enterococci (ARIAS & MURRAY, 2012; SHOKOOHIZADEH et al., 2013). It is suggested that they are widely adapted to the host due to their commensal relationship. They also possess virulence characteristics, such as the ability to form biofilms, which facilitate the processes of colonization and opportunistic infections (SHEPARD & GILMORE, 2002; SURIYANARAYANAN et al., 2018).

1.2 ENTEROCOCCAL DISEASE

Usually, enterococci coexist with the host. However, different factors, such as broad-spectrum antimicrobials and host immunosuppression, may alter the role of this microorganism, from commensal to opportunistic pathogen, breaking the symbiotic relationship (SHEPARD & GILMORE, 2002; LITTMAN & PAMER, 2011).

Enterococci are able to cause different types of infections. The urinary tract is the most common site of enterococcal infection, i.e. cystitis, prostatitis and epididymitis in older man and uncomplicated cystitis in young women.

Intra-abdominal, pelvic, and soft tissue are also sites of enterococcal infections, but rarely presented as monomicrobial infection (ORSI & CIORBA, 2013; HIGUITA & HUYCKE, 2014).

Enterococci are the third cause of bloodstream infections (BSI) and infective endocarditis (IE), which are the most serious and often life-threatening infections caused by this microorganism (BEGANOVIC et al., 2018; PERICÁS et al., 2015). Rarely, they cause osteomyelitis, septic arthritis, and pneumonia (KOW & FERZANDI, 2013; HIGUITA & HUYCKE, 2014) as well as diseases involving central nervous system (WANG et al., 2014; PATEL et al., 2016).

Indeed, enterococci are among the main aetiological agents of healthcare-associated infections (HAI) (HIDRON et al., 2008; SIEVERT et al., 2013). In the 1970s and 1980s, the occurrence of antimicrobial resistance led to the emergence of this genus, being a subject of major concern among infection control staff (ARIAS & MURRAY, 2012). Between 2011 and 2014, 365,490 cases of HAIs in the United States were reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention (CDC). *E. faecalis, E. faecium* and other species of enterococci were responsible for 7.4% (30,034), 3.7% (14,942) and 3.6% (14,694) of the cases of nosocomial infections, respectively (WEINER et al., 2016).

Because of its opportunistic nature, the risk factors for enterococcal infections are mainly advanced age, being female, long-term hospitalization, organ transplantation, presence of medical devices, multiple antimicrobial

therapy and immunosuppression (CONWAY et al., 2016; MONTESERIN & LARSON, 2016; MEDINA-POLO et al., 2017; POULADFAR et al., 2017).

1.2.1 Treatment and antimicrobial infection

Treatment of enterococcal infections could be challenging and depends on several factors such as site and nature (monomicrobial or polymicrobial) of infection, susceptibility to β-lactams, aminoglycosides, and glycopeptides or to the combination of these antimicrobial classes (HIGUITA & HUYCKE, 2014).

For systemic infections, the combination of high doses of gentamicin with penicillin or ampicillin is recommended. For ampicillin-resistant isolates, glycopeptides (vancomycin or teicoplanin) in association with gentamicin are the alternative therapy, although eradication rates are lower. On the other hand, the treatment of vancomycin-resistant enterococci (VRE) will depend on the antibiogram and the *Enterococcus* species. Linezolid or daptomycin may be used to treat infections by *E. faecalis* and *E. faecium* resistant to vancomycin, however, the combination of quinupristin/dalfopristin may be administered only in case of *E. faecium* infections once *E. faecalis* is intrinsically resistant to this drug (SHEPARD & GILMORE, 2002; ARIAS & MURRAY, 2012).

As mentioned before, the main reason for the recognition of enterococci as an important nosocomial pathogen is their resistance to different antimicrobials, which give them selective advantages and contributes to their adaptation in adverse environments, colonization of the GI tract and spread

among patients. Such resistance may arise from mutations in the nucleic acid sequence or be acquired by horizontal gene transfer. In general, it involves transformation and acquisition of genetic determinants of resistance, in plasmids and transposons, from another microorganism carrying the phenotype (GILMORE et al., 2013; KRISTICH et al., 2014).

Enterococci are intrinsically resistant to cephalosporins, sulfonamides, clindamycin and low levels of β-lactam and aminoglycosides, due to chromosome-mediated genes. Acquired resistance may arise to virtually all commonly used antimicrobial agents classes: macrolides, chloramphenicol, tetracycline, fluoroquinolones, and glycopeptides, as well as high levels of resistance to aminoglycosides and β-lactams (Figure 1) (SHEPARD & GILMORE, 2002; ARIAS & MURRAY, 2012; KRISTICH et al., 2014).

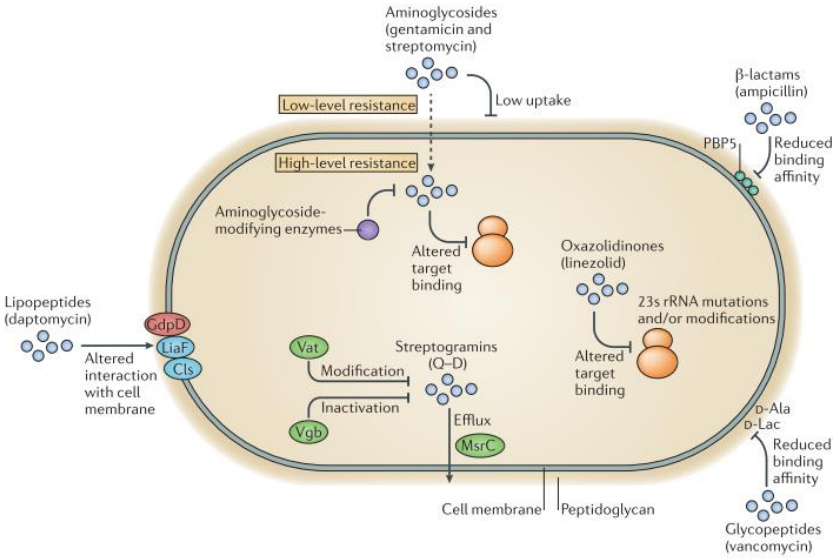

Fig 1. Mechanism of enterococcal antimicrobial resistance (Retrieved from ARIAS; MURRAY, 2012).

Ampicillin belongs to the β-lactams antibiotics, which inhibit the peptidoglycan synthesis by binding to the penicillin-binding proteins (PBPs). Resistance to β-lactams is associated with the production of a low-affinity PBP or, less frequently, the production of β-lactamases. While ampicillin resistance in *E. faecalis* is rare, it occurs in 90% of *E. faecium* and is associated with PBP5 production, a constitutive low-affinity enterococcal PBP (ARIAS & MURRAY, 2012; CATTOIR & GIARD, 2014).

Gentamicin is an aminoglycoside, which acts by binding the bacterial 30S small ribosomal subunit, inhibiting the protein synthesis. As enterococci are intrinsically resistant to low levels of aminoglycosides, the treatment includes a combination of an aminoglycoside (high-level) with β-lactams or glycopeptides, but the resistance to high levels of aminoglycosides impairs the synergism of treatment. This resistance occurs due to the production of aminoglycoside-modifying enzymes, which lead to loss of synergism and decrease the bactericidal effect (SHEPARD; GILMORE, 2002; ARIAS; MURRAY, 2012).

Ciprofloxacin is a fluoroquinolone that acts inhibiting cell division, commonly used to treat urinary tract infections. Resistance to this antimicrobial occurs by mutations in *gyrA* and *parC* genes that encode, respectively, DNA gyrase and Topoisomerase IV (LI et al., 2015).

Vancomycin and teicoplanin are glycopeptides, whose mechanism of action involves binding to the terminal D-alanine-D-alanine dipeptide of bacterial cell wall, thereby inhibiting the peptidoglycan synthesis. Resistance to

glycopeptides is due to the modification in the ending binding target D-alanine by D-lactate (high levels of resistance) or D-serine (low levels of resistance) (CATTOIR & LECLERCQ, 2013; FARON et al., 2016). So far, nine genes encoding glycopeptide resistance have been reported (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*) (Table 1). *vanA* and *vanB* are the most prevalent in HAI and highly transferable to other pathogens. VanA phenotype is characterized by high levels of vancomcyin and teicoplanin resistance, while VanB determines various levels of inducible resistance to vancomycin and susceptibility to teicoplanin. (DUTKA-MALEN et al., 1995; PERICHON et al., 1997; FINES et al., 1999; MCKESSAR et al., 2000; LEBRETON et al., 2011; BOYD et al., 2008; XU et al., 2010). Recently, it was first described in Europe an *E. faecium* isolated from a patient with bacteremia, harboring both *vanA* and *vanB* genes (PAPAGIANNITSIS et al., 2017).

Table 1. Summary of the Phenotypic and Genotypic Characteristics of the Alphabet VREs Operons (Retrieved from Ahmed & Baptiste, 2017)

Van-operon	*Common carrier spp.*	*Degree level of resistance vancomycin vs. teicoplanin*	*Phenotypic expressions*	*Location & mobility*
vanA	*E. faecium* *E. faecalis*	High for both	Inducible	Chromosome Transferable
vanB; vanB1, B2, B3	*E. faecalis* *E. faecium*	High-variable to vancomycin Susceptible to teicoplanin	Inducible	Chromosome Transferable
vanC; vanC1, C2, C3, C4	*E. gallinarum* *E. casseliflavus* *E. flavescens*	Low to vancomycin Susceptible to teicoplanin	Constitutive Inducible 	Chromosome —
vanD; vanD1, D2, D3, D4, D5	*E. faecium*	Low to high for both	Constitutive Inducible	Chromosome —
vanE	*E. faecalis*	Low-moderate to vancomycin Susceptible to teicoplanin	Inducible	Chromosome —
vanG; vanG1, G2	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome Transferable
vanL	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome —
vanM	*E. faecium*	High for both	Inducible	Unknown Transferable
vanN	*E. faecium*	Low to vancomycin Susceptible to teicoplanin	Constitutive	Plasmid Transferable

VRE, vancomycin resistance enterococci.

Linezolid is an oxazolidinone antibiotic that inhibits protein synthesis, commonly used to treat infections caused by Gram-positive bacteria, such as community-acquired and nosocomial pneumonia, skin and soft tissue infections and VRE infections resistant to penicillins. This antimicrobial has no activity against Gram-negative bacteria. Resistance to linezolid is associated with mutations in the *23S rRNA* gene, and it is considered an emerging problem (AGER & GOULD, 2012; KRISTICH et al., 2014; BI et al., 2018).

Quinupristin/dalfopristin is a combination of two streptogramins (A and B) that inhibit protein synthesis after their interaction with the 50S ribosomal subunit. This antimicrobial is active against *E. faecium*, while *E. faecalis* is intrinsically resistant to the drug. Acquired resistance among *E. faecium* is commonly associated with streptogramin A resistance genes and can occur by enzymatic modification of the antibiotic. It is commonly associated with the presence of *vatD* and *vatE*, which encode acetyltransferases that inactivate the antibiotic (ARIAS & MURRAY, 2012; KRISTICH et al., 2014).

Daptomycin is a lipopeptide antibiotic, derived from *Streptomyces roseosporus* fermentation, which is active only against Gram-positive bacteria. Its mechanism of action involves binding to bacterial membranes, in the presence of calcium ions, causing depolarization and inducing the rapid inhibition of protein, DNA, and RNA synthesis, resulting in bacterial cell death (STEENBERGEN et al., 2005). In enterococci, daptomycin resistance is associated with alterations in genes responsible for regulatory systems of cell-

envelope homeostasis and stress-response, as well as genes responsible for producing enzymes involved in the metabolism of phospholipids (TRAN et al., 2015).

1.2.2 Vancomycin Resistant Enterococci (VRE)

In most regions, the majority of enterococci causing HAI are resistant to vancomycin. Therefore, the treatment is a challenge once these bacteria are also resistant to the other antimicrobials (HIGUITA & HUYCKE, 2014). There is evidence that VRE may act as reservoir and source of other antimicrobial-resistant genes (AHMED & BAPTISTE, 2017).

VRE infection starts with the colonisation of the host GI tract, most commonly in critically ill patients. After the establishment as a member of the GI microbiota, they can take advantage of any disruption of the intestinal flora and quickly proliferate in the gut (MILLER et al., 2016). Previous treatment with vancomycin is a risk factor for gut colonization with VRE. Besides, the risk to develop VRE infection is 9 times higher in hospitalized patients colonized than in non-colonized, especially among children (FLOKAS et al., 2017).

Other risk factors for the development of VRE infection includes physical proximity with infected or colonized patients, long-term hospitalization, multiple antimicrobial therapy, patients undergoing transplantation, comorbidities such as diabetes and renal failure, and presence of medical devices (ARIAS & MURRAY, 2012; SHORMAN & AL-TAWFIQ, 2013; ADESIDA et al., 2017). The risk of death by VRE infection is higher

compared to VSE infection, once the therapeutic options are limited in the first case. For example, DA SILVA et al., (2014) reported a risk of death by VRE bacteremia 2.73-fold higher than by VSE bacteria.

It is known that enterococci can survive for up to 4 months on surfaces and persist for up to 60 min on the skin. In the hospital environment, hands of healthcare professionals, if improperly sanitized, may serve as a vector for the VRE spread between patients (FARON et al., 2016). Controlling the spread of these resistant bacteria are sorely needed. Hence, the implementation of a strong infection control program in the healthcare facilities, as well as, specific policies related to the bacterial prevalence and rates of resistance, may minimize the risk of transmission and outbreaks (HIGUITA & HUYCKE, 2014).

Clinical use of vancomycin started in the 1960s and the first report of VRE occurred in England in 1988 (UTTLEY et al., 1988). After that, VRE has been quickly spread among hospitals, people and animals, countries and continents (WILLEMS et al., 2005; ARIAS & MURRAY, 2012; CATTOIR & LECLERCQ, 2013).

In Brazil, the first case was described in 1996, in a hospital of Curitiba, Southern Brazil. Besides vancomycin resistance, the strain exhibited resistance to teicoplanin, ampicillin, and to high levels of gentamicin and streptomycin (DALLA COSTA et al., 1999). In 1997, a case of *E. faecium* carrying *vanA* was described in a patient with meningitis in São Paulo, Southeast Brazil (ZANELLA et al., 1999). In 2000, it was reported the first case of vancomycin

resistant *E. faecalis* (VRE_{fl}) isolated from an ICU patient, in Porto Alegre (D'AZEVEDO et al., 2000). However the epidemiology changed, and in 2014, in Porto Alegre, RESENDE et al., (2014) described a clonal outbreak of vancomycin resistant *E. faecium* (VRE_{fm}) carrying the *vanA* gene.

During 2015, the SENTRY Antimicrobial Surveillance Program evaluated 8,072 Gram-positive isolates collected from 69 medical centers in the U.S. Census Divisions and among the *Enterococcus* sp. isolates, 71.1% were VRE (PFALLER et al., 2017).

VEGA & DOWZICKY (2017) reinforce the importance of VRE in Brazil. They described the antimicrobial susceptibility profile of Gram-positive and Gram-negative bacteria collected between 2004 and 2015 in nine countries of Latin America, including Brazil. They observed 40.8% of VRE_{fm} among all countries, being Brazil the country with the highest rate of VRE (77.3%).

As a consequence of the increasing VRE isolation in many regions around the world, studies have been performed focusing on the epidemiological characterization of these isolates in order to understand the VRE spread. Different tools have been used to obtain the genetic relatedness of the strains, such as Pulsed Field Gel Electrophoresis (PGFE) and Multilocus Sequence Typing (MSLT), being the MLST the only methodology able to compare the data obtained from different laboratories (PRIETO et al., 2016).

The clonal complex 17 (CC17), identified by MLST, is a lineage of *E. faecium*, well-adapted to the hospital environment and responsible to the major

of VRE_{fm} infections worldwide (TOP et al., 2008b). This lineage has been reported in epidemiological studies performed in different countries that evaluated the genetic relatedness by MLST, such as Iran, China, USA and Brazil (PALAZZO et al., 2011; DA SILVA et al., 2012; CORREIA et al., 2014; YANG et al., 2015; ALVES et al., 2017; MICHAEL et al., 2017; SACRAMENTO et al., 2017). In contrast, *E. faecalis* are less associated with one dominant clone. Despite that, CC2, CC40, and CC87 are the most common lineages of *E. faecalis* present in hospital-associated infections (MIKALSEN et al., 2015; PRIETO et al., 2016).

In Brazil, most of the epidemiological studies were performed with VRE isolates from São Paulo. In 2008, D'AZEVEDO et al., (2008) evaluated the genetic relatedness by PFGE of 37 VREs (20 VRE_{fm} and 17 VRE_{fl}) isolated in a teaching hospital of São Paulo with the first VRE isolated in Brazil, and they observed seven distinct clonal types and all different from the first VRE. In 2011, PALAZZO et al., (2011) characterized by PFGE and MLST 22 VRE_{fm} isolated from different patients during an outbreak in a University Hospital in Southeast Brazil, and they identified new sequences types but all belonged to the CC17. The same was observed by DA SILVA et. al., (2012) that identified 53 VRE_{fm} isolated from two hospitals in Ribeirão Preto, São Paulo. All grouped in 9 STs, being 8 STs belonging to CC17 and one from a different clonal complex (ST658).

More recently, a study evaluated 2633 VRE from 26 hospitals, isolated during an 18-year period. PFGE was performed to 153 VRE_{fl} and 125 VRE_{fm} randomly selected and grouped both species in four clusters. The PFGE clusters were also evaluated by MLST, and VRE_{fl} isolates were identified as belonging to CC2, CC4 and CC9 and all VRE_{fm} belonging to CC17 (SACRAMENTO et al., 2017).

1.3 VIRULENCE FACTORS OF ENTEROCOCCI

Enterococci possess many virulence factors which give them pathogenic potential to break the stable relationship whit the host and become a pathogen in the human body, as well as, a selective advantage to survive under adverse conditions. Changes in the ecology and different virulence determinants have been associated with enterococcal infection. However, this virulence is complex and multifactorial without a major molecule or event (BALLERING et al., 2009; GILMORE et al., 2013).

As previously mentioned, *E. faecalis* is generally more virulent than *E. faecium*. However, with the advent of sequencing, new insights have reinforced the virulence potential of *E. faecium* (SOHEILI et al., 2014; AL-TALIB et al., 2015; GAO et al., 2018; JAHANSEPAS et al., 2018).

1.3.1 Virulence genes

Most virulence factors among enterococci are related to adhesion on biotic and abiotic surfaces. Enterococcal surface protein (Esp) is a cell wall-associated protein, coded by a gene located on a pathogenicity island, that contributes to the persistence of *Enterococcus* at the site of infection and can modulate inflammation. Firstly described in *E. faecalis*, *esp* gene has a homologue which is also highly disseminated among *E. facium* (esp_{fm}) (SHANKAR et al., 1999; EATON & GASSON, 2002; SAVA et al., 2010; ZOU & SHANKAR, 2016; GAO et al., 2018). Esp increases the *E. faecalis* and *E. faecium* ability to form biofilms and contributes to colonization of heart valves (TENDOLKAR et al., 2004; HEIKENS et al., 2007; HEIKENS et al., 2011).

Collagen Adhesin (Ace) is a protein that belongs to the MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules), which mediates adherence to collagen and laminin and contributes to the ability of *Enterococcus* sp. to colonize aortic valves and cause endocarditis. It is encoded by *ace* gene in *E. faecalis* and *acm* gene in *E. faecium* (NALLAPAREDDY & MURRAY, 2006; NALLAPAREDDY et al., 2008; SINGH et al., 2010).

Gelatinase (GelE) is a secreted factor, regulated by the Fsr quorum sensing in *E. faecalis*. This protease is able to hydrolyse gelatin, casein, collagen, hemoglobin and is the main mediator of pathogenesis in endocarditis. Its enzymatic activity is required in the first steps of the biofilm formation, as well

as its development (HANCOCK & PEREGO, 2004; THURLOW et al., 2010; GARSIN et al., 2014).

Enterococci change plasmids very easily mainly because of aggregation substance (AS) which is a group of surface proteins, encoded by pheromone-responsive gene, that mediates the transfer of plasmids favoring bacterial aggregation. Its adherence properties also enhance *E. faecalis* virulence by accelerating the biofilm formation (CHUANG-SMITH et al., 2010; GARSIN et al., 2014).

Endocarditis and biofilm-associated pili (*ebp* locus) is a pilin gene cluster of *E. faecalis* and *E. faecium* that comprises three structural subunits EbpA, EbpB and EbpC and a pilus-specific sortase. *ebp* pili encodes surface proteins, contributes to the colonization and infection of host tissues, and has a role in the initial adherence to platelets, fibrinogen and collagen and affects the biofilm biogenesis (NALLAPAREDDY et al., 2006; NIELSEN et al., 2013; SILLANPÄÄ et al., 2013).

Finally, *E. faecalis* antigen A (EfaA) is an adhesin, considered the major surface antigen of *E. faecalis.* It has been associated with infective endocarditis and also found in therapy-resistant endodontic infections (LOWE et al., 1995; PREETHEE; KANDASWAMY & HANNAH, 2012). It was recently reported an influence of the *efaA* in the biofilm formation induced by sub-MIC concentrations of gentamicin (KAFIL et al., 2016).

1.3.2 Biofilm

Enterococci are strongly associated with infections related to medical devices once they are capable to attach firmly to the surface of material and form biofilms, as a way to resist shear forces. Biofilms are populations of microbial cells attached to a biotic or abiotic surface, typically surrounded by a self-produced matrix of extracellular polymeric substances (EPS). Studies have demonstrated that bacteria use their ability to form biofilm in the colonization and infection processes, as a survival strategy in hostile environments. It is believed that most part of the microbial existence on Earth is within a biofilm community (MONDS & O'TOOLE, 2009; GARSIN & WILLEMS, 2010; GUPTA et al., 2015).

Biofilm formation is dependent on different factors, such as pH, temperature, CO_2, nutrients, surface type and cell density (PILLAI et al., 2004). Presence of supplements as carbohydrates and serum are examples of important factors that influence the enterococci adhesion at surfaces, as well as the metabolism of biofilm production of this bacteria (MOHAMED & HUANG, 2007).

The process of biofilm formation is complex and involves different stages: attachment and immobilization on a surface by physical forces, cell-to-cell interaction, accumulation, maturation, secretion of the EPS, dispersal and return to the planktonic form. Initially, the attachment step is reversible and starts with the adhesion of planktonic cells on a surface. After that, this adhesion

becomes irreversible, the microbial cells start to grow, and form a three-dimensional biofilm structure (Figure 2) (GUPTA et al., 2015; MONDS & O'TOOLE, 2009).

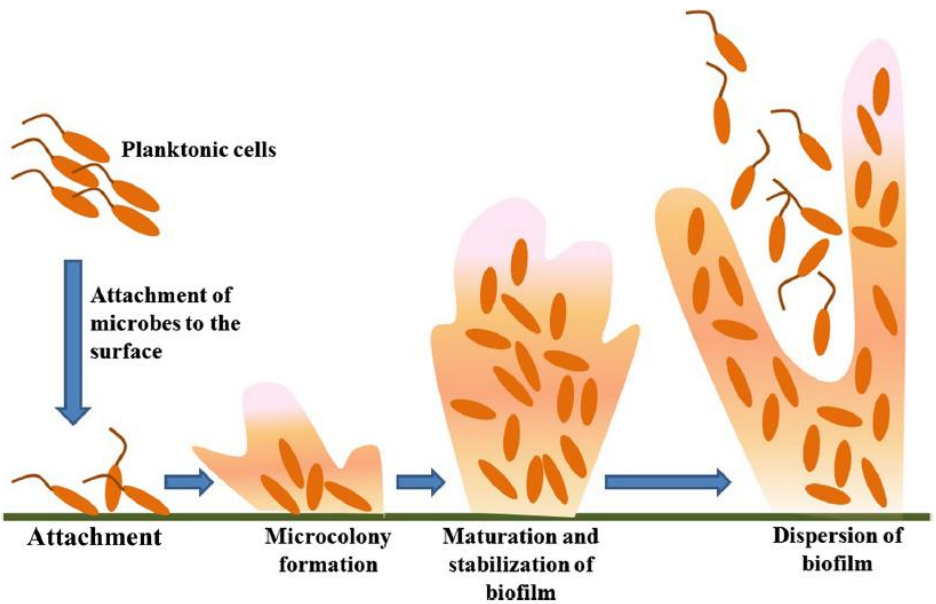

Fig.2 Stages of biofilm formation (Retrieved from GUPTA et al., 2015)

Several genetic determinants have been reported in the literature as contributing to biofilm formation, predominantly in *E. faecalis,* and the number of genetic factors required for biofilm formation has increased, reinforcing the complexity of these communities. Among these biofilm-related genes, there are surface proteins located in the genome or plasmids, enzymes as sortase, autolysin and proteases, regulatory genes and genes of the enterococci metabolism (Figure 3) (MOHAMED & HUANG, 2007; PAGANELLI et al., 2012).

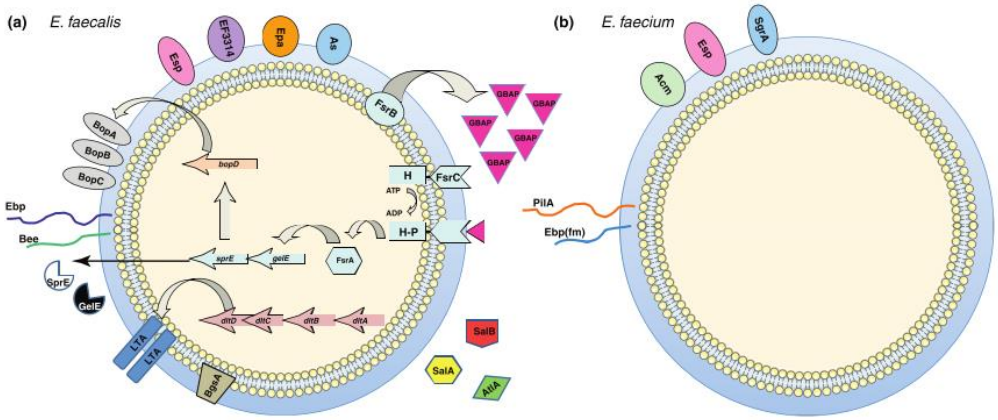

Fig. 3 Schematic representation of biofilm-associated proteins and structures of (a) *E. faecalis* and (b) *E. faecium*. (Retrieved from Paganelli, Willems, & Leavis, 2012)

Several types of chronic infections are strongly associated with biofilm formation, making these infections difficult to treat and eradicate (Figure 4). It is a mode of bacteria to survive in adverse environments, protection against stress conditions, phagocytosis, dehydration and antimicrobial therapy and also can increase the bacterial pathogenicity (MOHAMED & HUANG, 2007; GUPTA et al., 2015).

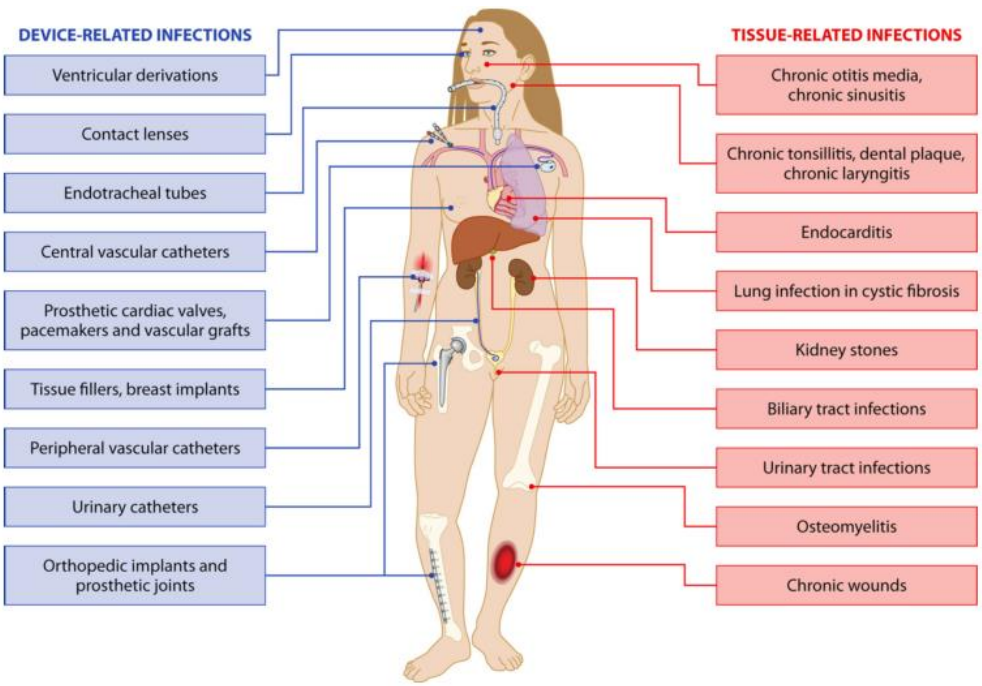

Fig. 4 Biofilm-related infections (Retrieved from LEBEAUX; GHIGO; BELOIN, 2014).

LEBEAUX et al., (2014), described some strategies that can avoid the biofilm formation: reduction in the number of implanted devices, early removal of unnecessary devices, systemic antibiotic prophylaxis during device implantation, antibiotic coating of implanted devices and antibiotic lock therapy. Indeed, different lock therapy regimens have been tested against *E. faecalis* biofilm, however, in some cases, the biofilm was not completely eradicated by the tested concentrations (LUTHER et al., 2016, 2017; ALONSO et al., 2018).

When a biofilm is established, the treatment of the infection is a challenge and involves, in most cases, the removal of the indwelling devices, or chronic biofilm suppressive treatment with antibiotic combination therapy (WU

et al., 2015). In biofilms, the Minimum Bactericidal Concentration (MBC) required for sessile cells can be approximately 10-1000 times higher than the MBC for planktonic cells, impacting the treatment when an effective antimicrobial therapy is impossible due to toxicity (MOHAMED & HUANG, 2007; WU et al., 2015).

Bacteria can naturally exhibit resistance or tolerance in the presence of the antimicrobial. Resistance is often due to genetic alterations and occurs by numerous mechanisms, such as efflux pumps, reduced permeability to antibiotics, enzymatic activity and modification of the antibiotic target. On the other hand, the antimicrobial tolerance can be genetic, when there are genes associated with the reduced affinity by the antibiotic, or phenotypic when the environment affects the antibiotic action. Biofilm is a mixture of resistance and tolerance, resulting in decreased antibiotic penetration, slow growth rate, altered metabolism and presence of persister cells (Figure 5) (ABDALLAH et al., 2014; KESTER; FORTUNE, 2014; LEBEAUX et al., 2014; OLSEN, 2015).

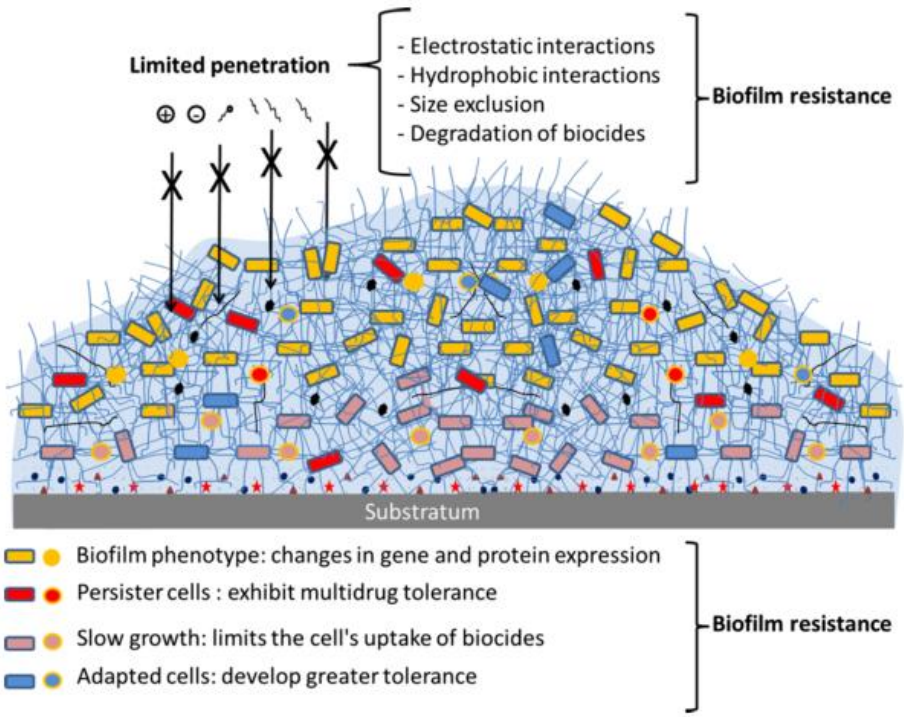

Fig. 5 Mechanisms of biofilm resistance to antimicrobial agents (Retrieved from ABDALLAH et al., 2014).

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CHAPTER 2

AIMS OF THE STUDY

2.1 GENERAL AIM

The general aim of this research was to characterize epidemiologically and genotypically isolates of *Enterococcus* spp. from the Gram-Positive Laboratory Microorganism Bank (Federal University of Health Sciences of Porto Alegre, Brazil). The isolates were recovered from clinical samples of patients attended in hospitals of Porto Alegre, Brazil.

2.2 SPECIFIC AIMS

- To evaluate the performance of a commercial agar to detect VRE.
- To evaluate the susceptibility profile of VRE isolates.
- To detect the presence of vancomycin resistant genes in Vancomycin Resistant Enterococci by conventional PCR.
- To determine clonal relationship among VRE isolates by PFGE.
- To detect the presence of virulence factors in *E. faecalis* and *E. faecium* by conventional PCR.

- To analyse the influence of environmental factors, such as presence and absence of 1% glucose and presence of different concentrations of human serum (1%, 5%, 25% and 50%) on biofilm formation of *E. faecalis.*

- To quantify the expression of virulence genes associated with biofilms in different environmental conditions.

CHAPTER 3

EVALUATION OF A SELECTIVE CHROMOGENIC MEDIUM FOR DETECTING VANCOMYCIN-RESISTANT ENTEROCOCCI

Soares RO, Rossato AM, Sambrano GE, Tolfo NCC, Caierão J, Paim TGDS, d'Azevedo PA. Evaluation of a selective chromogenic medium for detecting vancomycin-resistant enterococci. Braz J Microbiol. 2017 Oct - Dec; 48(4):782-784. doi: 10.1016/j.bjm.2017.03.005.

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Evaluation of a selective chromogenic medium for detecting vancomycin-resistant enterococci

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ABSTRACT

Rapid identification of vancomycin-resistant enterococci (VRE) can assist in choosing the appropriate treatment and preventing VRE spread. The performance of chromID™ VRE agar was evaluated using 184 clinical isolates of *Enterococcus* spp. and reference strains. The test had a sensitivity of 95.52% but a low specificity of 30%.

Vancomycin-resistant enterococci (VRE) are among the major agents of healthcare-associated infections and are considered a public health problem. Rapid VRE identification can assist in choosing the appropriate treatment and preventing VRE spread.[1,2] The aim of this study was to evaluate the performance of a selective chromogenic medium for the detection and differentiation of vancomycin-susceptible and -resistant *Enterococcus faecium* and *Enterococcus faecalis*.

Vancomycin-susceptible enterococci (VSE) isolated from cases of infection ($n = 50$) and VRE clinical isolates ($n = 134$), including those collected from surveillance rectal swab cultures ($n = 62$) and from cases of infection ($n = 72$), were evaluated (Table 1). The following reference strains were also included: VRE strain (*E. faecium*, $n = 1$) and VSE strains (*E. hirae*, $n = 1$; *E. gallinarum*, $n = 2$; *E. faecium*, $n = 1$; and *E. faecalis*, $n = 4$).

All isolates were previously identified by phenotypic methods (hydrolysis of esculin in the presence of bile, production of pyrrolidonyl arylamidase, growth in broth containing 6.5% NaCl, and negative catalase test evidentiated by the absence of effervescence).[3] Polymerase chain reaction was also used to confirm the presence of the genus *Enterococcus* and distinguish the species according to methods previously described by Ke et al.[5] and Karyama et al.[4] All isolates were obtained from the culture collection of the Gram-positive Cocci Laboratory – UFCSPA and stored in skim milk (Difco™) at −20 °C. Vancomycin minimum inhibitory concentration was determined by broth microdilution according to CLSI guidelines (2015)[6] and by Etest® according to the manufacturer's instructions. The chromID™ VRE (bioMérieux, Brazil S/A) assays were performed in two steps. First, the isolates that were previously stored in skim milk were grown in bile-esculin agar to confirm the presence of enterococci and check culture purity. Second, pure samples were grown in trypticase soy agar for 24 h, followed by single-colony growth in chromID™ VRE agar at 37 °C. After 24 h, plates with any growth were considered VRE positive. A negative result was defined as a 48-h incubation period

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Table 1 – Clinical isolates used to evaluated the performance of the chromID™ VRE.

Species	Susceptibility to vancomycin[a]		Origin	
	S	R	Infection	Surveillance culture
E. faecalis	43	47	47	43
E. faecium	7	87	75	19
Total	50	134	122	62

[a] S, susceptible; R, resistant.

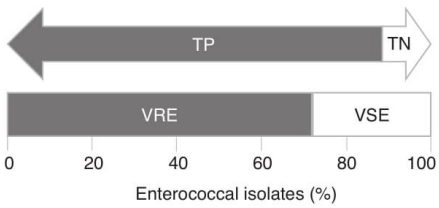

Fig. 1 – Schematic representation of the results obtained using chromID™ VRE.

without any bacterial growth. According to the manufacturer's instructions, chromID™ VRE agar allows the identification of species based on the detection of enzyme activity. Therefore, *E. faecium* was stained purple and *E. faecalis* was stained blue-green.

Of the *E. faecalis* VRE tested ($n = 47$), 41 were stained blue-green, 5 were stained gray, and 1 isolate did not grow. All *E. faecium* VRE tested ($n = 87$) were stained purple. Among VSE isolates ($n = 50$), 15 did not grow (10 *E. faecalis* and 5 *E. faecium*) and 35 (33 *E. faecalis* and 2 *E. faecium*) showed some growth at the edges of the plates in the corresponding color of each species, which may suggest false-positive results. All VSE-reference strains tested ($n = 8$) did not show any visible growth in the chromogenic medium. Fig. 1 shows a schematic representation of the results obtained with chromID™ VRE agar.

The chromID™ VRE agar had a sensitivity of 87.23% and 100% for detecting *E. faecalis* VRE and *E. faecium* VRE, respectively, and a combined sensitivity and specificity of 95.52% and 30.00%, respectively, for detecting VRE. No difference was observed in the specificity and sensitivity at 24 and 48 h. The positive predictive value (corresponding to the percentage of VRE that tested positive in chromID™ VRE) was 78.53% (95% confidence intervals; C.I. = 72.22–84.83%) and the negative predictive value was 71.43% (95% C.I. = 52.10–90.75%) (Table 2). Regarding sensitivity, similar results have been obtained in previous studies evaluating the performance of chromID™ VRE.[1,7–14] In relation to the specificity, previous studies have obtained values higher than 95%,[1,7,8,11] in contrast to the low specificity observed in this study.

Colonies with non-discriminatory staining (gray, dark, or colorless) have been reported in some studies.[10,15] We also observed VRE colonies with a grayish shade, which may lead to false-negative results.

Among all VRE isolates, 100% of the *E. faecium* grew within 24 h. The same was observed in previous studies.[11,14] According to Grabsch et al.,[8] 24-h identification of VRE allows earlier confirmation of colonization by these strains, facilitates infection control, and helps to avoid the spread of microorganisms. However, one *E. faecalis* VRE isolate did not grow even after 48 h, which may suggest that some strains can exhibit a different behavior and/or require more time to grow in the medium.

Delmas et al.[1] compared growth before and after an enrichment step in bile-esculin agar supplemented with vancomycin in order to select only VRE strains. The enrichment step improved the performance of chromID™ VRE at 24 h of incubation. Other studies have shown that strains incubated overnight in an enrichment broth containing vancomycin as a first step followed by the use of chromID™ VRE resulted in improved specificity or sensitivity.[1,10,11,14,16] However, this method is only useful for fecal specimens due to the large number of different microorganisms that can be present in these samples.

Most studies evaluating chromID™ VRE performance have used only fecal specimens (stool samples and rectal swabs) or only resistant strains. In our study, we evaluated well-characterized vancomycin-resistant and -susceptible isolates in order to observe the possible occurrence of false-positive results, because incorrectly prescribed antibiotics have a negative clinical impact. Despite the data presented here, a possible limitation of this study is that fecal samples were not included,

Table 2 – Evaluation of chromID™ VRE in detecting true positive vancomycin-resistant enterococci.

ChromID™ VRE agar	Gold standard (VRE)[a]	Gold standard (VSE)[a]	Total
Test positive	128	35	163
Test negative	6	15	21
Total	134	50	184
Sensitivity		95.52% (95% C.I. = 92.02–99.02%)	
Specificity		30.00% (95% C.I. = 17.29–42.70%)	
Positive predictive value		78.53% (95% C.I. = 72.22–84.83%)	
Negative predictive value		71.43% (95% C.I. = 52.10–90.75%)	
Accuracy		77.72% (95% C.I. = 71.70–83.73%)	

[a] Vancomycin resistance was determined using both Etest® and CLSI guidelines; C.I., confidence intervals.

because the density of microorganisms in a clinical specimen may affect the correct diagnosis.

In conclusion, the chromID™ VRE agar is a rapid and useful tool for the screening and identification of VRE, with a good sensitivity of about 96.00%. However, because specificity (30.00%) was limited by false positive VRE mainly, we recommend further VRE identification by conventional tests to avoid misinterpretation.

Conflicts of interest

The authors declare no conflicts of interest.

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CHAPTER 4

HIGH DIVERSITY OF VANCOMYCIN-RESISTANT *Enterococcus faecium* ISOLATED IN SOUTHERN BRAZIL

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Manuscript in Preparation

Abstract

Vancomycin-resistant enterococci (VRE) are common in some hospital settings and their clonal spread has been described in different regions of the world. We determined the antimicrobial susceptibility profile and the clonal relationship of VRE isolates recovered from inpatients at three general hospitals of Porto Alegre, Brazil. Ninety-four VRE were characterized as *Enterococcus faecium* and exhibited resistance to teicoplanin, ampicillin, ciprofloxacin, and susceptibility to linezolid, quinupristin-dalfopristin and daptomycin. High level resistance to gentamicin was detected in 13.8% of them. All VRE_{fm} harbored *vanA* gene, while 85.1% and 94.7% harbored respectively *esp* and *acm* virulence genes. PFGE profile analysis revealed 23 clonal types including 79 isolates,

while 15 isolates exhibited unique pattern type, showing a polyclonal distribution of VRE_{fm} in Southern Brazil. These findings contribute to the local understanding regarding the characteristics of the circulating VREs in the region.

Key-words: VRE, *acm* gene, *esp* gene, PFGE, clonal types

Introduction

The ability of *Enterococcus faecium* to rapidly acquire mobile genetic elements associated to antimicrobial resistance is well-established (Gilmore et al., 2013; Cattoir and Giard, 2014; García-Solache et al., 2016). Vancomycin-resistant *Enterococcus faecium* (VRE_{fm}) has become increasingly common in some hospital settings and their clonal spread has been described worldwide (Freitas et al., 2016; Mahony et al., 2018), including Brazil (Alves et al., 2017; Resende et al., 2014; Sacramento et al., 2017). Most VRE_{fm} isolated from Brazilian hospitals belong to clonal complex 17 (CC-17), i.e. a well-adapted lineage to the hospital environment and responsible for the majority of VRE_{fm} infections worldwide (Top et al., 2008; Palazzo et al., 2011; Alves et al., 2017; Sacramento et al., 2017). VanA-related VRE_{fm} is the most prevalent phenotype around the world and frequently presents virulence factors which facilitates the infection process and multiresistance features that considerably reduce the therapeutic options (Ahmed and Baptiste, 2017).

The aim of this work was to determine genetic relatedness of VRE_{fm}, focusing on virulence and resistance characteristics.

Materials and Methods

Bacterial Strains

Ninety-four vancomycin resistant enterococci from the Gram-Positive Laboratory Microorganism Bank (Federal University of Health Sciences of Porto Alegre, Brazil) were evaluated. The isolates were recovered from clinical samples of patients attended in hospitals of Porto Alegre, Brazil, from September 2012 to April 2017, as part of an epidemiological surveillance study. Only one isolate per patient was considered. The project was approved by the Ethics Committee of Human Research of Federal University of Health Sciences of Porto Alegre, under the number 1.283.544.

Identification of *Enterococcus* species, vancomycin resistance and virulence genes

Primary genus identification was performed through the observation of specific phenotypic characteristics by the respective hospital's microbiology laboratory. The genus confirmation, species identification, detection of the vancomycin resistant determinants *vanA* and *vanB* genes and virulence genes *acm* (adhesin of collagen) and *esp* (enterococcal protein surface) were determined by PCR as previously described (Kariyama et al., 2000; Rathnayake

et al., 2012; Kafil and Mobarez, 2015;). Primers used in this study are described in Supplementary Table 1.

Antimicrobial Susceptibility testing

The antimicrobial susceptibility profile was done using disk diffusion for ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (120 µg), linezolid (30 µg), quinupristin-dalfopristin (15 µg) and teicoplanin (30 µg) and interpreted according to CLSI 2017 guidelines (Clinical and Laboratory Standards Institute, 2017). Minimum Inhibitory Concentrations (MIC) were determined by Etest® strips (bioMeriéux) for daptomycin. Multidrug resistance (MDR) strains were defined as those presenting resistance to three or more different antimicrobial classes (Magiorakos et al., 2012). *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were used as quality control.

Chromosomal Analysis of Genomic DNA by PFGE

Pulsed-field gel electrophoresis (PFGE) was performed as previously described (Saeedi et al., 2002), with the following modifications: agarose plugs were prepared and treated with 1 mg/mL of lysozyme (Sigma Co., 48000U/mg), 5U/mL of mutanolysin (Sigma Co., 3000U/mL). Digestion of chromosomal DNA was achieved with 20 U of Anza™ 22 *SmaI* (Thermo Fisher Scientific®) and restriction fragments were separated using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA).

Results were analyzed with Bionumerics software version 7.1 (Applied Maths) using the unweighted-pair group method with arithmetic mean (UPGMA). Dendrogram was constructed using dice coefficients with optimization and tolerance set to 0.5% and 1%, respectively. Clustering above 80% similarity were considered as a clone type (CT) (Alves et al., 2017).

Results

All 94 VRE were identified as *Enterococcus faecium.* Enteroccci were recovered from urine 42.6% (n=40), blood 29.8% (n=28), rectal swab 14.9% (n=14), body fluids 11.7% (n=11) and catheters 1.1% (n=1).

All VRE_{fm} exhibited vancomycin MICs higher than 256 μg/mL and resistance to teicoplanin (all carrying *vanA* gene). They were resistant to ampicillin, ciprofloxacin, and susceptible to linezolid, daptomycin (MIC $\leq$ 4 μg/mL) and quinupristin-dalfopristin. High-level resistance to Gentamicin was detected in 13 (13.8%) isolates. Considering virulence genes, 80 (85.1%) and 89 (94.7%) isolates harbored *esp* and *acm* genes, respectively. Seventy-six isolates carried both genes and one isolate did not possess any of the those.

PFGE defined 23 clone types (CTs) which included 79 of the 94 isolates, and 15 were singletons (Figure 1, Table 1). There was one dominant cluster, CT8, including 17,7% of VRE_{fm}, recovered either from infection (blood, urine) and surveillance cultures.

Discussion

VRE_{fm} has become one of the leading causes of nosocomial infections, especially among severely ill patients (Howden et al., 2013). We described the clonal relationship of 94 VRE_{fm} recovered from inpatients in Porto Alegre, Southern Brazil. Besides vancomycin, all *E. faecium* exhibited resistance to ampicillin and ciprofloxacin, and 13.8% high level resistance to gentamicin.

Around the world, studies have reported the spread of CC-17 (Alves et al., 2017; Brilliantova et al., 2010; López et al., 2012; Palazzo et al., 2011), a lineage that exhibits resistance to most antibiotics clinically used for the treatment of enterococcal infections. It is well adapted to the hospital environment and has been associated with most of the reported hospital outbreaks worldwide (Panesso et al., 2010; Willems et al., 2005). Our isolates showed phenotypic characteristics similar to the CC-17 lineage, such as ampicillin and ciprofloxacin resistance and presence of *esp* gene (Gao et al., 2018). Indeed, most VRE_{fm} harboured *esp* and *acm* genes, both related with biofilm formation and adherence to extracellular matrix, giving *E. faecium* selective advantages in the hospital environment (Hendrickx et al., 2007).

Similar to our findings, Akpaka et al., (2017) performed a study between 2009 to 2014 with twelve hospitals from eight Caribbean countries and they found 31.4% of VRE strains. Among these, 70 were *E. faecium*, harboring *vanA* and *esp* genes, with 100% of resistance to ciprofloxacin, 92.8% resistance to

ampicillin and 100% of susceptibility to daptomycin, linezolid and quinupristin/dalfopristin.

In a study performed in 2011 evaluating antimicrobial susceptibility patterns of isolates from 11 countries in Latin America, Brazil presented the highest rate of VRE (27%) (Jones et al., 2013). In 2016, a SENTRY study reported a rate of 71.7% of VRE_{fm} in Brazil (Sader et al., 2016).

Although *E. faecalis* is more prevalent in enterococcal infections, VRE_{fm} has been increasing in Brazilian hospitals. Conceição et al. (2011) observed an increase of 13% in VRE rate in a hospital in Southeastern Brazil between 2006-2009, being 89.5% *vanA-E. faecium*. Another study conducted with 29 isolates from a hospital in Southern Brazil observed that all isolates were VRE_{fm} carrying *vanA* gene and were part of a main clone (Resende et al., 2014).

In our study, VRE_{fm} were classified into 38 types (23 clonal types and 15 singletons), demonstrating a high genetic heterogeneity. A similar polyclonal distribution of VRE_{fm} has also been observed in other studies (Landerslev et al., 2016; Pourshafie et al., 2008; Somily et al., 2016; J. Top et al., 2008).

Finally, our study contributes to the local understanding about the characteristics of the circulating VREs in the region, since there are few publications on this topic in the last 5 years in Brazil.

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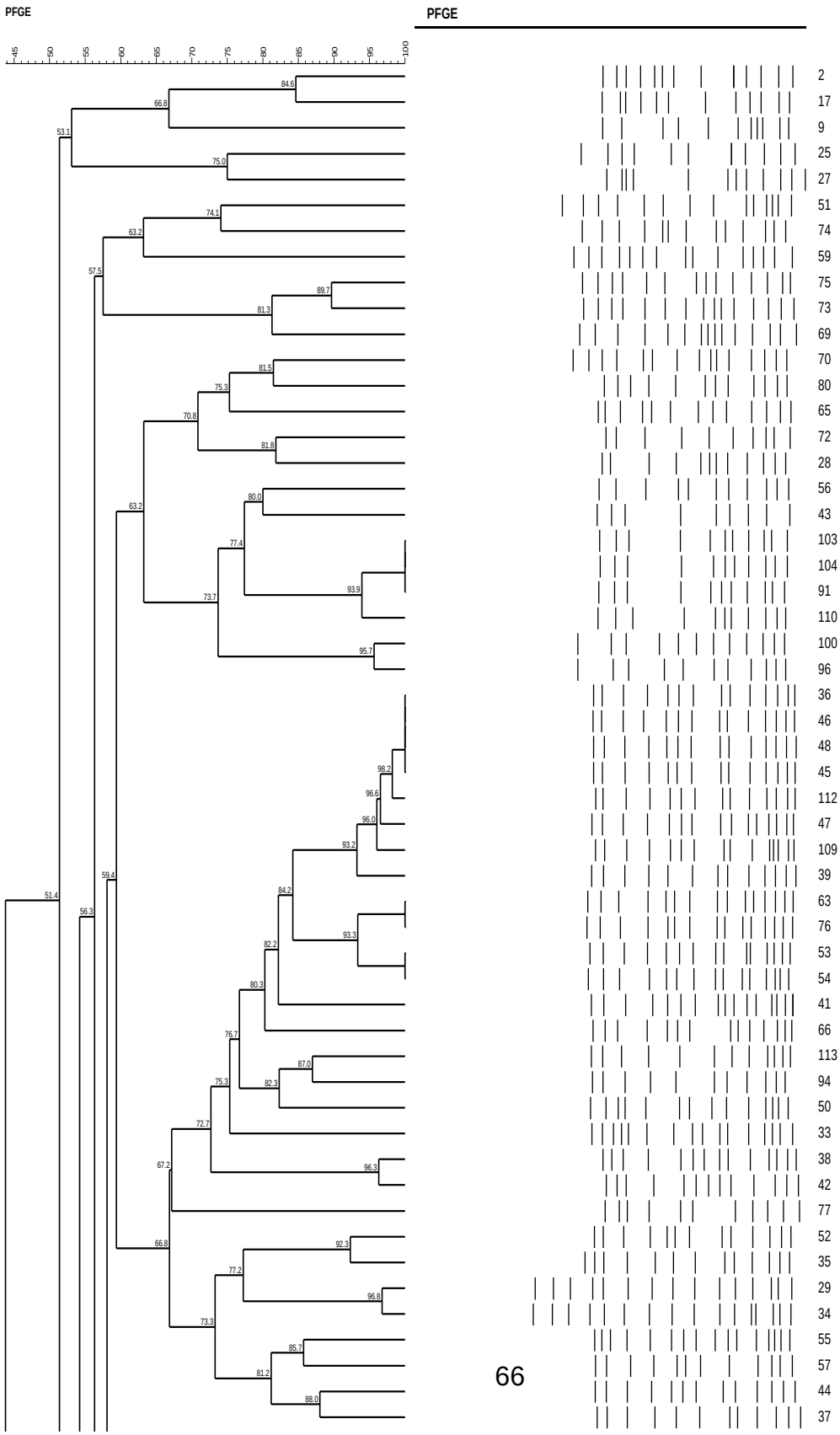
Supplementary Table 1. Description of primers used in PCR for the detection of species and vancomycin-resistance genes and virulence factors of *Enterococcus faecium*:

Target gene	Sequence of primer	Amplicon Size (pb)	Reference
Enterococcus faecium	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	658	[18]
Enterococcus faecalis	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941	[18]
vanA	5'-CATGAATAGAATAAAAGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	1030	[18]
vanB	5'-GTGACAAACCGGAGGCGAGGA-3' 5'-CCGCCATCCTCCTGCAAAAAA-3'	433	[18]
esp	5'-GGAACGCCTTGGTATGCTAAC-3' 5'-GCCACTTTATCAGCCTGAACC -3'	95	[17]
acm	5'-GGCCAGAAACGTAACCGATA-3' 5'-AACCAGAAGCTGGCTTTGTC-3'	135	[26]


PFGE
PFGE
ID
45
50
55
60
65
70
75
80
85
90
95
100
84.6
66.8
53.1
75.0
74.1
63.2
57.5
89.7
81.3
81.5
75.3
70.8
81.8
63.2
80.0
77.4
73.7
93.9
95.7
98.2
96.6
96.0
93.2
59.4
51.4
56.3
84.2
93.3
82.2
80.3
76.7
87.0
75.3
82.3
72.7
67.2
96.3
66.8
92.3
77.2
96.8
73.3
85.7
81.2
88.0
2
17
9
25
27
51
74
59
75
73
69
70
80
65
72
28
56
43
103
104
91
110
100
96
36
46
48
45
112
47
109
39
63
76
53
54
41
66
113
94
50
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38
42
77
52
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29
34
55
57
44
37

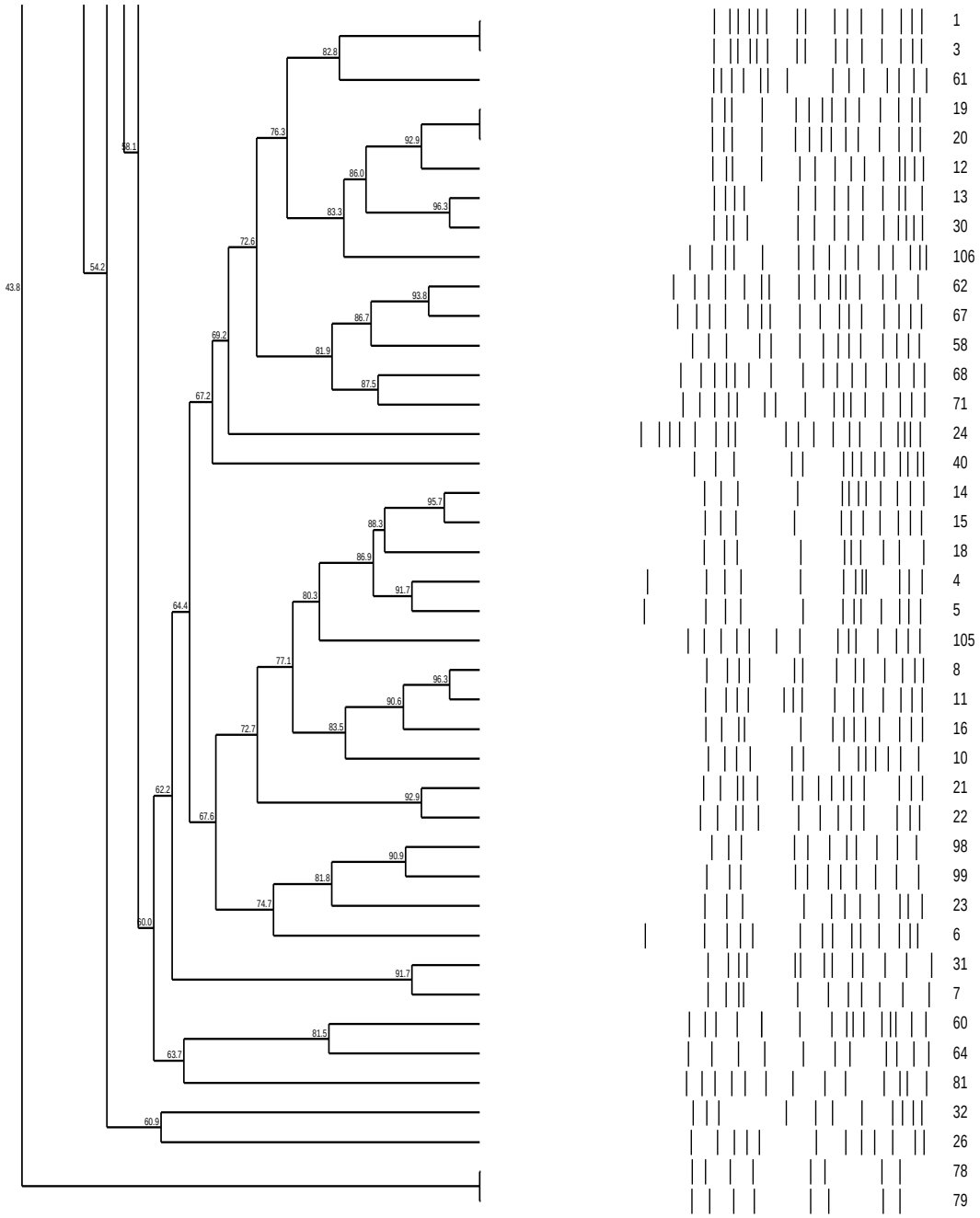

Fig.1 PFGE dendrogram and PFGE profile images of 94 *vanA E. faecium* from Porto Alegre, Brazil.

Table 1. Description of 94 VRE_{fm} clinical isolates from Porto Alegre, Brazil, recovered from Sept-2012 to Apr-2017.

Strain ID	Source	Date	PFGE	Resistance Profile	Virulence Profile
2	Urine	Oct-12	CT1	AMP, CIP, TEI, VAN	*esp+*, *acm+*
17	Urine	May-13	CT1	AMP, CIP, TEI, VAN	*esp+*, *acm+*
69	Blood	Dec-16	CT2	AMP, CIP, TEI, VAN	*esp+*, *acm+*
73	Urine	Jan-17	CT2	AMP, CIP, TEI, VAN	*esp+*, *acm+*
75	Urine	Jan-17	CT2	AMP, CIP, TEI, VAN	*esp+*, *acm+*
70	Body Fluids	Dec-16	CT3	AMP, CIP, TEI, VAN	*esp+*
80	Urine	Apr-17	CT3	AMP, CIP, TEI, VAN	*acm+*
28	Urine	May-15	CT4	AMP, CIP, TEI, VAN	*esp+*, *acm+*
72	Urine	Jan-17	CT4	AMP, CIP, TEI, VAN	*esp+*, *acm+*
43	Body Fluids	Sep-15	CT5	AMP, CIP, TEI, VAN	*esp+*, *acm+*
56	Body Fluids	Mar-16	CT5	AMP, CIP, TEI, VAN	*esp+*, *acm+*
91	Rectal Swab	Sep-14	CT6	AMP, CIP, TEI, VAN	*esp+*, *acm+*
103	Rectal Swab	Dec-14	CT6	AMP, CIP, TEI, VAN	*esp+*, *acm+*
104	Rectal Swab	Jan-15	CT6	AMP, CIP, TEI, VAN	*esp+*, *acm+*
110	Rectal Swab	Jun-15	CT6	AMP, CIP, TEI, VAN	*esp+*, *acm+*
96	Urine	Nov-14	CT7	AMP, CIP, TEI, VAN	*esp+*, *acm+*
100	Rectal Swab	Dec-14	CT7	AMP, CIP, TEI, VAN	*esp+*, *acm+*
36	Blood	Aug-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
39	Blood	Aug-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
41	Blood	Sep-15	CT8	AMP, CIP, TEI, VAN	*esp+*
45	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
46	Urine	Nov-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
47	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
48	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
53	Urine	Jan-16	CT8	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*
54	Blood	Jan-16	CT8	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*
63	Blood	Oct-16	CT8	AMP, CIP, TEI, VAN	-

66	Blood	Nov-16	CT8	AMP, CIP, TEI, VAN	*esp+*
76	Urine	Jan-17	CT8	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*
109	Rectal Swab	May-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
112	Rectal Swab	Jul-15	CT8	AMP, CIP, TEI, VAN	*esp+*, *acm+*
50	Body Fluids	Dec-15	CT9	AMP, CIP, TEI, VAN	*acm+*
94	Urine	Nov-14	CT9	AMP, CIP, TEI, VAN	*esp+*, *acm+*
113	Rectal Swab	Aug-15	CT9	AMP, CIP, TEI, VAN	*esp+*, *acm+*
38	Urine	Aug-15	CT10	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*
42	Urine	Sep-15	CT10	AMP, CIP, TEI, VAN	*esp+*, *acm+*
35	Urine	Jul-15	CT11	AMP, CIP, TEI, VAN	*esp+*, *acm+*
52	Urine	Dec-15	CT11	AMP, CIP, TEI, VAN	*esp+*, *acm+*
29	Urine	May-15	CT12	AMP, CIP, TEI, VAN	*esp+*, *acm+*
34	Urine	Jul-15	CT12	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*
37	Urine	Aug-15	CT13	AMP, CIP, TEI, VAN	*esp+*, *acm+*
44	Urine	Sep-15	CT13	AMP, CIP, TEI, VAN	*esp+*, *acm+*
55	Urine	Jan-16	CT13	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*
57	Urine	Apr-16	CT13	AMP, CIP, TEI, VAN	*esp+*, *acm+*
1	Blood	Sep-12	CT14	AMP, CIP, TEI, VAN	*acm+*
3	Blood	Oct-12	CT14	AMP, CIP, TEI, VAN	*esp+*, *acm+*
61	Body Fluids	Nov-16	CT14	AMP, CIP, TEI, VAN	*esp+*, *acm+*
12	Catheter	Apr-13	CT15	AMP, CIP, TEI, VAN	*esp+*, *acm+*
13	Blood	May-13	CT15	AMP, CIP, TEI, VAN	*acm+*
19	Urine	Jun-13	CT15	AMP, CIP, TEI, VAN	*esp+*, *acm+*
20	Blood	Jul-13	CT15	AMP, CIP, TEI, VAN	*esp+*, *acm+*
30	Blood	May-15	CT15	AMP, CIP, TEI, VAN	*esp+*, *acm+*
106	Rectal Swab	Mar-15	CT15	AMP, CIP, TEI, VAN	*esp+*, *acm+*
58	Urine	Mar-16	CT16	AMP, CIP, TEI, VAN	*esp+*, *acm+*
62	Urine	Oct-16	CT16	AMP, CIP, TEI, VAN	*acm+*
67	Urine	Nov-16	CT16	AMP, CIP, TEI, VAN	*esp+*, *acm+*

68	Urine	Dec-16	CT16	AMP, CIP, TEI, VAN	*acm*+
71	Urine	Dec-16	CT16	AMP, CIP, TEI, VAN	*acm*+
4	Urine	Oct-12	CT17	AMP, CIP, TEI, VAN	*esp*+, *acm*+
5	Blood	Oct-12	CT17	AMP, CIP, TEI, VAN	*esp*+, *acm*+
14	Blood	May-13	CT17	AMP, CIP, TEI, VAN	*esp*+, *acm*+
15	Body Fluids	May-13	CT17	AMP, CIP, TEI, VAN	*esp*+, *acm*+
18	Blood	May-13	CT17	AMP, CIP, TEI, VAN	*esp*+, *acm*+
105	Rectal Swab	Jan-15	CT17	AMP, CIP, TEI, VAN	*esp*+, *acm*+
8	Urine	Feb-13	CT18	HLG, AMP, CIP, TEI, VAN	*esp*+, *acm*+
10	Urine	Mar-13	CT18	AMP, CIP, TEI, VAN	*esp*+, *acm*+
11	Urine	Apr-13	CT18	HLG, AMP, CIP, TEI, VAN	*esp*+, *acm*+
16	Blood	May-13	CT18	AMP, CIP, TEI, VAN	*acm*+
21	Blood	Jul-13	CT19	HLG, AMP, CIP, TEI, VAN	*esp*+, *acm*+
22	Urine	Jul-13	CT19	AMP, CIP, TEI, VAN	*esp*+, *acm*+
23	Rectal Swab	Jul-13	CT20	AMP, CIP, TEI, VAN	*esp*+, *acm*+
98	Rectal Swab	Nov-14	CT20	AMP, CIP, TEI, VAN	*esp*+, *acm*+
99	Rectal Swab	Dec-14	CT20	AMP, CIP, TEI, VAN	*esp*+, *acm*+
7	Blood	Dec-12	CT21	AMP, CIP, TEI, VAN	*esp*+, *acm*+
31	Blood	May-15	CT21	AMP, CIP, TEI, VAN	*esp*+, *acm*+
60	Body Fluids	May-16	CT22	AMP, CIP, TEI, VAN	*acm*+
64	Urine	Oct-16	CT22	AMP, CIP, TEI, VAN	*esp*+, *acm*+
78	Blood	Feb-17	CT23	AMP, CIP, TEI, VAN	*esp*+, *acm*+
79	Blood	Apr-17	CT23	AMP, CIP, TEI, VAN	*esp*+, *acm*+
6	Blood	Dec-12	*Singleton*	AMP, CIP, TEI, VAN	*esp*+, *acm*+
9	Body Fluids	Feb-13	*Singleton*	AMP, CIP, TEI, VAN	*esp*+, *acm*+
24	Body Fluids	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*esp*+, *acm*+
25	Blood	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*esp*+, *acm*+
26	Urine	Aug-13	*Singleton*	HLG, AMP, CIP, TEI, VAN	*acm*+
27	Rectal Swab	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*acm*+

32	Urine	May-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+*, *acm+*
33	Urine	May-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+*, *acm+*
40	Blood	Sep-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+*, *acm+*
51	Blood	Dec-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+*, *acm+*
59	Urine	Apr-16	*Singleton*	AMP, CIP, TEI, VAN	*acm+*
65	Urine	Oct-16	*Singleton*	AMP, CIP, TEI, VAN	*acm+*
74	Body Fluids	Jan-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+*
77	Urine	Feb-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*
81	Body Fluids	Apr-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+*, *acm+*

PFGE, pulsed-field gel electrophoresis; CT, clonal type; HLG, high level of gentamicin; AMP, ampicillin; CIP, ciprofloxacin; TEI, teicoplanin; VAN, vancomycin; *esp*, enterococcal protein surface gene; *acm*, collagen adhesin gene.

CHAPTER 5

INFLUENCE OF GLUCOSE SUPPLEMENTATION ON BIOFILM FORMATION AND EXPRESSION OF ASSOCIATED-BIOFILM GENES IN *Enterococcus faecalis* CLINICAL ISOLATES

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Manuscript in Preparation

Abstract

Enterococcus faecalis is an important cause of medical device-associated infections. Hence, it is crucial to understand the mechanisms of enterococcal biofilm formation. It is known that glucose and expression of different virulence genes could affect the enterococci biofilm formation. We evaluated the ability to form biofilms for 123 *E. faecalis* clinical strains in the presence and absence of 1% D-(+)-glucose. Real-time quantitative PCR was performed to evaluate the relative expression of biofilm-related genes (*ebpA, efaA, ace* and *gelE*) in eleven

clinical isolates and *E. faecalis* 29212 at the same conditions. It was observed that 1% glucose supplementation increased significantly the biofilm capability, however, the gene expression varied among clinical isolates, showing that patterns of virulence gene expression are dependent on the bacterial isolate.

Keywords: *Enterococcus faecalis*, biofilm, virulence genes, glucose

Abbreviations: TSB, tryptic soy broth; DPBS, Dulbecco's Phosphate-Buffered Saline; PCR, Polymerase Chain Reaction; CV, Crystal Violet; NC, Negative Control; SD, Standard Deviation; ODc, Optical density cut-off

1. Introduction

Enterococci are natural inhabitants of the human gastrointestinal tract. However, they are also able to cause a variety of infections, like urinary tract infections, bacteraemia, endocarditis and medical device-associated infections. Enterococci can form biofilms allowing them to survive under difficult conditions and to tolerate more easily antibacterial treatments [1,2]. Furthermore, *Enterococcus faecalis* can harbour different virulence genes that may play a crucial role in the pathogenesis and biofilm formation. The regulation of these genes during biofilm formation is not totally understood [3]. Endocarditis and biofilm-associated pilus (*ebpA*), *E. faecalis* endocarditis antigen (*efaA*), collagen-binding adhesin (*ace*) and

gelatinase (*gelE*) are some of the enterococcal virulence genes that have an important role in the biofilm development [4–7].

Nutritional conditions can also influence the *in vitro* biofilm formation. Glucose is a supplement that has been evaluated for biofilm formation by different microorganisms because it can increase the pathogenicity of different microbes [8–11]. There are also several studies associating the presence of virulence genes with the ability to form biofilms in *E. faecalis,* as well as different essential conditions for biofilm formation [12–14]. However, to our knowledge, this is the first study to report the influence of 1% glucose on the expression of *ebpA, efaA, gelE* and *ace* genes in *E. faecalis* biofilms.

Thus, the aim of this study was to quantify *E. faecalis* biofilm formation in the presence and absence of 1% D-(+)-glucose and to evaluate the influence of 1% glucose in biofilm-related genes expression.

2. Materials and methods

2.1 Bacterial Strains

One hundred and twenty-three clinical isolates of *Enterococus faecalis* obtained from the collection of the Laboratory of Gram-positive cocci were selected for this study. The origins of the isolates were as follows: 95 (77.24%) from urine, 19 (15.45%) from blood and 9 (7.32%) from body fluids. All isolates were previously classified as biofilm formers by Soares et al. [15]. *E. faecalis*

ATCC 29212, purchased from the American Type Culture Collection (ATCC®, USA), was used in all assays as reference strain.

2.2 Virulence genes

The presence of four virulence genes, *ebpA* (5′-AAAAATGATTCGGCTCCAGAA-3′ and 5′-TGCCAGATTCGCTCTCAAAG -3′), *efaA* (5′-TGGGACAGACCCTCACGAATA -3′ and 5′-CGCCTGTTTCTAAGTTCAAGCC -3′), *ace* (5′-GGAGAGTCAAATCAAGTACGTTGGTT -3′ and 5′-TGTTGACCACTTCCTTGTCGAT -3′) and *gelE* (5′-TATGACAATGCTTTTTGGGAT -3′ and 5′-AGATGCACCCGAAATAATATA -3′) were investigated by PCR as previously described [4–6,16].

2.3 Biofilm formation assays

The biofilm assays were performed in 96-well cell culture microtiter plates (KASVI®). Briefly, bacterial suspensions in tryptic soy broth (TSB, KASVI®) supplemented with and without 1% D-(+)-glucose (MERCK®) were added to each well (8 wells per isolate). Plates were covered and incubated for 24h at 35°C under static conditions. Biofilm quantification was performed according to an adapted protocol from O'Toole [17] and Stepanovic et al. [18]

through the quantification of total biomass with 0.01% crystal violet (CV) staining (Merck®). The absorbance was measured in octuplicate, in a microtiter plate reader (KASUAKI) at 492 nm. *E. faecalis* ATCC 29212 was used as positive control and non-bacterial-inoculated wells were used as negative control. For each strain and negative control (NC), average ($\overline{X}$) and standard deviation (SD) values were calculated from the OD492 values obtained in the absence and presence of glucose 1%. Optical density cut-off (ODc) was calculated by the formula:

$$\overline{X}_{NC} + 3\,(SD_{NC})$$

2.4 Biofilm induction for gene expression assays

Eleven isolates of *E. faecalis* and one control (*E. faecalis* ATCC 29212), harbouring all the four virulence genes, were randomly selected for gene expression assays. Biofilm induction was performed in 6-well microplates (Greiner CELLSTAR®, flat-bottomed sterile cell culture), in a final volume of 3 mL per well, by adding 300 µl of inoculum (1.5×10^8 CFU/mL). One treatment was tested per isolate: The control contains only TSB medium and the treatment contains TSB medium plus 1% D-(+)-glucose. All biofilm assays were performed in triplicate with two biological replicates (at different days). The microplates were incubated at 35°C for 8 hours, achieving the mid-log phase of

growth. After that, the TSB medium was removed and the wells were washed twice with Dulbecco's phosphate-buffered saline (DPBS 1X, Gibco®) for removal of the non-attached cells. One milliliter of deionized water (Sigma-Aldrich®) was added to the well, and the attached cells were harvested with a cell scraper (240 mm, TPP®). Then, 500 µl of the suspension was transferred into a sterile microtube of 2mL, followed by vigorous vortexing and sonication to break up cell aggregation. One milliliter of RNAprotect Bacteria Reagent (Qiagen®) was added into a microtube for stabilization the RNA prior to RNA isolation procedures. After RNA stabilization, bacterial cells were pelleted by centrifugation (10 min at 5000g). The supernatant was decanted and the pellet was frozen at -20°C until the RNA extraction.

2.5 RNA extraction and cDNA synthesis

RNA was extracted by enzymatic lysis using lysozyme (Sigma-Aldrich®) and proteinase K (Sigma-Aldrich®) digestion followed by purification using the RNeasy Mini Kit (Qiagen®), according to manufacturer procedures. After the extraction, the amount of the total RNA extracted was determined using the Thermo Scientific NanoDrop™ 2000 spectrophotometer, as the 260nm/280nm ratio with expected values between 1.8 and 2.

The amount of 40 ng of high-quality total RNA was reverse-transcribed to complementary DNA (cDNA) in 10 µl volume using the AccuScript High Fidelity RT-PCR System (Agilent Technologies) according the manufacturer's

instructions. After that, cDNAs were diluted (1:20) and frozen at -20ºC until qPCR reaction.

2.6 Reference genes and target genes

Two reference and four virulence genes were chosen for the expression assays. The elongation factor Tu, a GTP binding protein involved in peptide chain formation, encoded by *tuf* gene [19] and the 23S Ribossomal RNA, part of the large 50S subunit of the ribosome in prokaryotes, encoded by *23SrRNA* gene [20] were selected as reference genes. Primers for reference genes *tuf* (5′-ATTAATGGCTGCAGTTGACG -3′ and 5′- AGCAACAGTACCACGTCCAG -3′) and *23SrRNA* (5′- CAGTGTCAGATGGGCAGTTT -3′ and 5′-GCTCCCTTCTGCCTTTACAC -3′) were obtained using the Primer3Plus software version: 2.4.0 [21]. Primers specificity was evaluated *in silico* with genome of *Enterococcus faecalis* V583 (NC_004668.1) using the BLAST® software (National Centre for Biotechnology Information).

The target genes used in this assay were the same evaluated in the conventional PCR: *ebpA*, *efaA*, *ace* and *gelE*. PCR efficiency was evaluated for each primer and the amplification efficiency (E) was calculated from the slope of the standard curve using the formula $E = 10^{-1/slope}$ (ideal value between 1.9 and 2.1).

2.7 Quantitative real-time PCR

Quantitative real-time PCR was performed in the StepOnePlus™ Real-Time PCR System (Applied Biosystems™). Master-mix for each PCR run was prepared in 20 µl volume as follows: 10 µl SYBR® Green PCR Master Mix (Applied Biosystems™), 5.6 µl water (Sigma-Aldrich®), 0.2 µl of each primer (0,1 µM) and 4 µl of cDNA. The following amplification program was used: 10 min of denaturation at 95°C, 40 cycles of real-time PCR consisting of 15 s at 95°C for denaturation, 15 s at (56°C to *tuf*, *efaA*, *ebp*, *gelE* and *ace* genes and 60°C for *23SrRNA* gene), 45 s at 60°C for annealing, and a final melting curve. All reactions were performed in triplicate with two biological replicates.

2.8 Data analysis

Data were described as mean ± SD. Differences in the biofilm formation comparing medium with and without glucose was calculated by percentage of increase and decrease of the optical density. Statistical analyses were performed by GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). All tests were performed with a confidence level of 95%. Relative expression analysis of target and reference genes in the treated-well was performed comparing to the non-treated sample by the REST 2009 software (PFAFFL, 2001 and Qiagen) using the crossing points (CP) of each gene studied. Results of expression greater than 2-fold were considered significant.

3. Results

3.1 Effects of glucose supplementation on biofilm formation

The biofilm-forming capacity of *E. faecalis* in the presence and absence of 1% glucose after 24 hours of incubation was evaluated by measuring the total biomass using CV staining and then classified according to Stepanovic criteria [18]. In the absence of glucose, 10 (8.1%) of the isolates were classified as non-producers, 60 (48.8%) were weak producers, 45 (36.6%) were moderate producers and 8 (6.5%) were strong producers. On the other hand, in the presence of 1% glucose, all *E. faecalis* tested were biofilm producers, being 18 (14.6%) classified as weak producers, 44 (35.8%) were moderate producers and 61 (49.6%) were strong producers (Figure 1A).

Among the 10 non-biofilm forming isolates in the absence of glucose, all these isolates were able to form biofilm in the presence of 1% glucose. Considering the 18 isolates previously classified as weak biofilm formers in the presence of glucose, 5 isolates were non-biofilm producers and 13 were weak biofilm producers in the absence of this supplement. On the other hand, among the 44 isolates classified as moderate biofilm formers in the presence of glucose, 4 were non-biofilm producers and 30 were weak biofilm producers in the absence of the supplement, showing a clear increase in the capability to form biofilms when glucose was supplemented. Finally, among the 61 isolates classified as strong biofilm formers in the presence of glucose, only 6 also

exhibited strong capacity to form biofilms in the absence of the supplement. This confirms that glucose is important for the expression or increase of the biofilm-forming capacity.

Figure 1B shows the OD 492 obtained in the absence and presence of 1% glucose. In the presence of 1% glucose, the basal OD 492 values were significantly higher than without glucose ($p<0.02$) for strong biofilm producers, representing an increase of 41%. However, no significant differences were observed in the basal OD 492 values for the weak and moderate biofilm producers in both conditions.

3.2 Presence of the virulence genes

Overall, 118 (95.9%) isolates possessed at least two of the four virulence genes evaluated, while 42 (34.1%) isolates possessed all the four genes. Eighty-five (69.1%) *E. faecalis* contained *ebpA*, 118 (95.9%) *efaA,* 79 (64.2%) *ace* and 87 (70.7%) *gelE*. Among the isolates from urine all genes had the highest frequency of occurrence (73.7% *ebpA;* 96.8% *efaA;* 72.6% *ace* and 70.5% *gelE*), followed by blood (52.6% *ebpA;* 100% *efaA;* 42.1% *ace* and 73.7% *gelE*) and body fluids (55.6% *ebpA;* 77.8% *efaA;* 33.3% *ace* and 66.7% *gelE*).

Among the 42 isolates containing all the four genes (*ebpA, efaA, gelE and ace*) and the 46 isolates that have at least 3 of the genes, respectively 95.2% and 87% of the isolates exhibited moderate or strong ability to form biofilms in the presence of glucose.

3.3 Effects of glucose on *ebpA, efaA, ace* and *gelE* genes expression

The relative expression of virulence genes was studied using qPCR. Expression of each gene was evaluated for eleven *E. faecalis* clinical isolates and the reference strain *E. faecalis* ATCC 29212 after biofilm induction with 1% glucose. Overall, the levels of transcription of all evaluated genes increased when 1% glucose was added to the medium. However, considering the average of gene expression for all twelve isolates (clinical strains and control), the effects were not statistically significant (Figure 2). Moreover, the clinical source like urine, blood or other body fluid did not influence the expression of the virulence genes.

For all clinical isolates, gene expression was affected by the presence of D-(+)-glucose. Only three clinical strains exhibited high levels of *epbA* transcription (> 2.6-fold upregulation EFL5, EFL8 and EFL17) and *gelE* transcription (> 2.9-fold upregulation – EFL8, EFL15 and EFL17) in 1% glucose. Transcriptions of *efaA* and *ace* were upregulated in five (> 2.2-fold upregulation – EFL5, EFL8, EFL12, EFL15 and EFL17) and six (> 2-fold upregulation – EFL3, EFL5, EFL8, EFL12, EFL15 and EFL17) clinical strains, respectively (Table 1). For all other strains, no significant differences were observed in the transcription levels between the treatment and the control conditions.

Comparing the biofilm formation capacity in the absence or presence of 1% glucose with the biofilm-related gene expression, it was observed that 6

isolates exhibited at least one gene positively regulated by the presence of glucose and they also increased the biofilm production in the presence of the supplement. In contrast, 4 isolates (EFL 6, EFL 7, EFL 13 and EFL 14) increased their capacity to form biofilms, but their biofilm-related genes expression unchanged in the presence of glucose. Finally, one isolate (EFL 4) had not alterations in the gene expression and it remained a moderate biofilm former in the presence and absence of glucose.

4. Discussion

This study evaluated the *in vitro* effects of glucose on biofilm formation. The process of biofilm formation involves different steps: attachment, accumulation, maturation and dispersal. Initially, the attachment step is reversible and starts with the adhesion of planktonic cells to a surface. After that, this adhesion becomes irreversible, the microbial cells start to growth and form aggregates. The maturation step involves the biofilm wrapped by extracellular polymeric substance [23,24].

The presence of glucose seems to be an essential factor for biofilm formation by different bacteria, and it also contributes to bacterial growth, reproduction and metabolic activities. However, the optimal concentration varies among different publications [10,25,26]. In *E. faecalis*, biofilm formation decreases when exposed to low glucose concentrations (0.05 and 0.15%) [10]. Therefore, high or low glucose concentrations can influence positively or

negatively the biofilm formation and thus we tested the optimal concentration. In previous experiments, we observed that 1% glucose gave the highest biofilm formation in *E. faecalis* (data not published).

Pillai et al. [27] also showed that 1% glucose supplementation increased the optical density and can regulate, directly or indirectly, the transcription of some glucose-dependent genes associated with biofilm formation in *E. faecalis*. All isolates in this study were classified before as biofilm formers in the presence of glucose [data not shown]. Glucose supplementation significantly increased biofilm formation already at 8 hours. A similar result was observed by Seneviratne et al. [25] who evaluated the biofilm formation of *E. faecalis* in 2% glucose and with different culture media compositions.

In general, studies have shown a correlation between the presence of some virulence genes with the ability of *Enterococcus* to form biofilms [12,13,15,28]. In our study, the *efaA* gene was the most prevalent, present in 95.9% of the *E. faecalis* isolates, followed by *gelE* (70.7%), *ebpA* (69.1%) and *ace* (64.2%). Our results provide the idea that some genes may not be mandatory for biofilm formation in enterococci, since they are not present in all strains. In a study performed with 196 isolates of enterococci from urine, it was demonstrated that the presence of *efaA* increases the biofilm formation while *ace* and *gelE* genes had not effect on biofilm production in this specie [29].

Our study also investigated the direct effect of glucose on the biofilm-related genes expression. Isolates evaluated in the expression assays possessed

all the four genes (*ebpA, efaA, ace* and *gelE*). Interestingly, our findings demonstrated that the expression of some genes did not alter in the presence of glucose, showing that patterns of virulence gene expression are dependent on the bacterial isolate.

The biofilm formation in *E. faecalis* involves transcriptional regulatory systems or cell-surface components [30]. Many studies prove that *epbA, efaA, ace,* and *gelE* genes are strongly associated with pathogenesis of *Enterococcus* spp., even as they play an individual role in the biofilm biogenesis [31]. Nevertheless, it was not possible to obtain a correlation between the effect of glucose supplementation on biofilm formation and the expression of the genes evaluated.

In this study, all four genes were upregulated in 1% glucose in a subgroup of isolates and no significant differences were observed for the others. Upregulation of *ace* gene transcription in the presence of 1% glucose was observed in 6 (50%) of the 12 isolates of *E. faecalis* evaluated, followed by the upregulation of *efaA* gene in 5 (41.7%), *ebpA* gene in 3 (25%) and *gelE* gene in 3 isolates (25%). Ran et al. [10] also evaluated the transcription levels of *ace* and *gelE* genes of *E. faecalis* ATCC 33186, in the presence and absence of glucose, and they observed upregulation of the *ace* gene in medium without glucose and upregulation of the *gelE* gene in 0.15% glucose. We observed that the levels of gene expression are clinical strain specific. According to Nallaparedy and

Murray [6], expression of *ace* may be regulated in a variety of environmental conditions by different mechanisms.

In conclusion, this study demonstrated a positive effect of 1% glucose on *E. faecalis* biofilm formation, however significant differences in biofilm-related gene expression were observed only for some strains. Biofilm formation is a multifactorial process influenced by different factors such as gene expression and conditions of induction. A better understanding of this process may help in the search for possible targets to prevent biofilm formation by this microorganism.

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Disclosure

There are no conflicts of interest to declare.

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Fig. 1. (A) and (B) Optical density values of CV solutions (OD492), obtained from 24 h biofilms of *E. faecalis* strains formed in the presence (TSBG) and absence of 1% glucose (TSB). Error bars represent the standard error. Statically differences obtained when compared with strong biofilm formed without glucose using one-way ANOVA ($^{*}p < 0.05$).

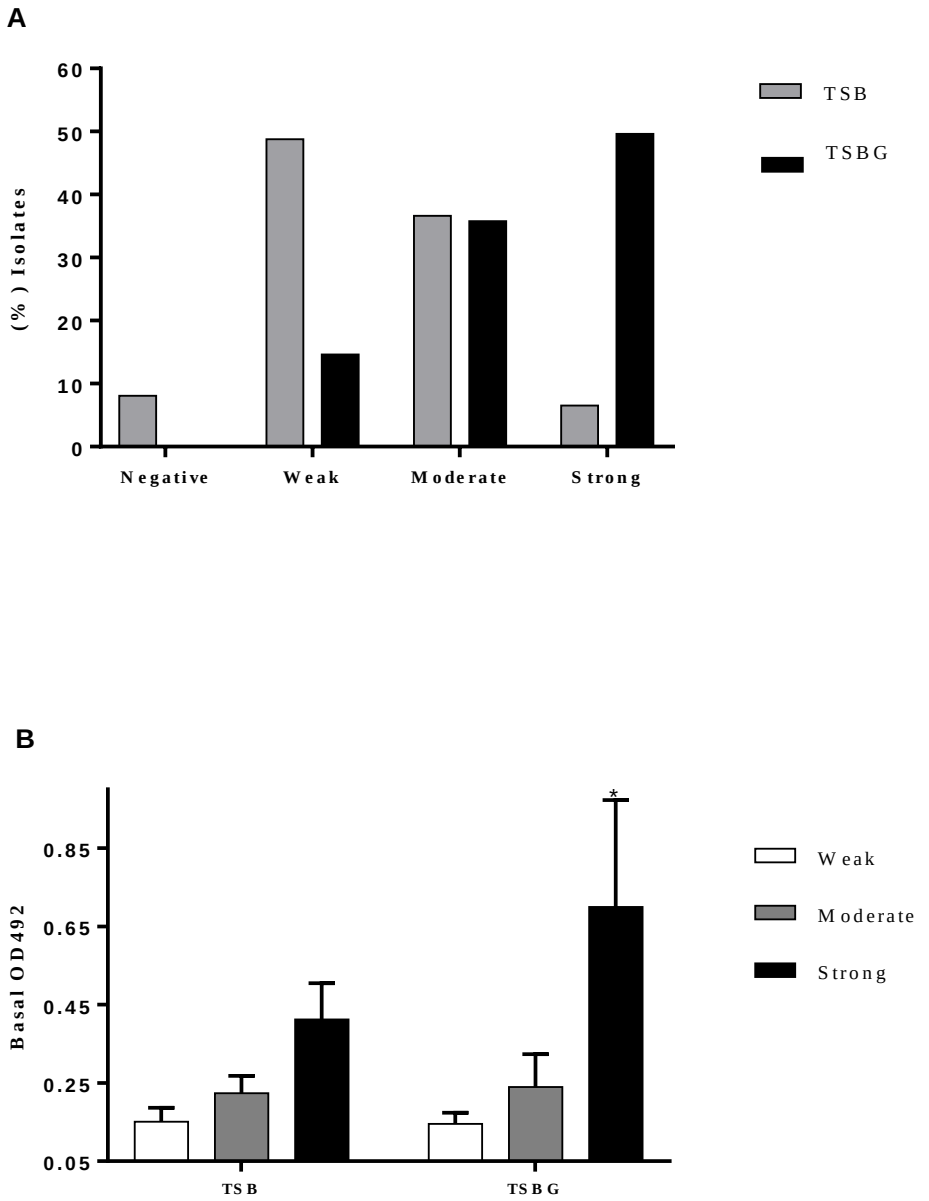

Fig. 2. Level of gene expression of *ebpA*, *efaA*, *ace* and *gelE* in 8 h biofilms of *E. faecalis* with 1% glucose. The values were calculated using Pfaffl method, normalized by the expression of the housekeeping gene and compared with the gene expression without glucose. Error bars represent standard deviation.

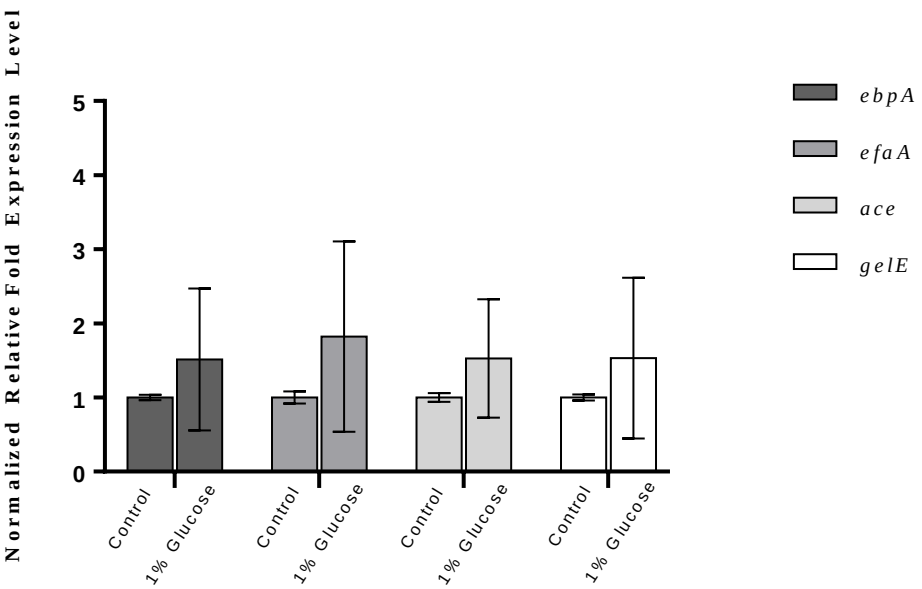

Table 1. Gene expression rate and percentage of biofilm expression change rate after glucose supplementation (Gene expression assay and biofilm induction performed at 8 h with 1% glucose)

Isolate	**Source**	***ebpA***	***efaA***	***ace***	***gelE***	**Biofilm****
EFL3	Body fluids	1.02 ± 0.26	1.39 ± 0.24	2.42 ± 0.57 *	1.52 ± 0.35	462%
EFL4	Body fluids	0.4 ± 0.39	0.48 ± 0.01	0.39 ± 0.02	0.42 ± 0.06	-8%
EFL5	Urine	2.96 ± 0.08 *	4.07 ± 0.18 *	2.22 ± 0.12 *	1.1 ± 0.09	413%
EFL6	Blood	1.16 ± 0.05	1.37 ±0.12	1.26 ± 0.05	1.18 ± 0.12	167%
EFL7	Blood	0.33 ± 0.02	0.51 ± 0.02	0.45 ± 0.05	1.09 ± 0.15	503%
EFL8	Urine	2.69 ± 0.09 *	2.82 ± 0.35 *	2.33 ± 0.19 *	2.93 ± 0.22 *	197%
EFL12	Blood	1.79 ± 0.24	2.27 ± 0.12 *	2.02 ± 0.12 *	1.06 ± 0.13	158%
EFL13	Urine	0.65 ± 0.03	0.44 ± 0.09	0.69 ± 0.03	0.82 ± 0.08	372%
EFL14	Blood	1.69 ± 0.08	1.67 ± 0.06	1.84 ± 0.06	1.77 ± 0.21	351%
EFL15	Body fluids	0.9 ± 0.08	2.82 ± 0.3 *	2.4 ± 0.25 *	3.07 ± 0.19 *	379%
EFL17	Blood	3.08 ± 0.06 *	3.59 ± 0.10 *	3.21 ± 0.30 *	3.52 ± 0.06 *	291%
ATCC29212	Urine***	1.39 ± 0.2	0.42 ± 0.01	1.19 ± 0.27	0.14 ± 0.11	238%

CHAPTER 6

HUMAN SERUM ENHANCES VIRULENCE FACTORS EXPRESSION AND INHIBITS ADHESION OF *Enterococcus faecalis* V583

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Manuscript in Preparation

Abstract

The *in vitro* effects of different concentrations (1%, 5%, 25% and 50%) of human serum (HS) on biofilm formation and virulence gene expression (*ebpA, efaA, ace, gelE* and *asa*) of *E. faecalis* V583 were evaluated. No differences were observed in the planktonic growth. However, an inhibition of the adhesion step of biofilm formation was observed in 5% HS. Planktonic cells of *E. faecalis* V583 exhibited upregulation of the asa gene in 5%, 25% and 50% HS, while in the sessile cells, *efaA, gelE, ace* and *asa* genes were significantly upregulated in high concentrations of HS (25% and 50%).

Keywords: *Enterococcus faecalis*, biofilm, virulence genes, human serum

1. Introduction

Enterococci are part of the normal microbiota of humans and animals. They are also recognized as an important opportunistic agent of urinary tract infections, endocarditis, bacteraemia and medical-device related infections [1,2]. Currently, 57 species of enterococci are described, being *Enterococcus faecalis* and *Enterococcus faecium* the most common species in health-care associated infections. Both are capable of forming biofilms and contain different virulence factors [3]: endocarditis and biofilm-associated pilus (EbpA), *E. faecalis* endocarditis antigen (EfaA), collagen-binding adhesion (Ace), gelatinase (GelE) and aggregation substance (Asa) are some of the enterococci virulence genes that have an important role in the complex process of biofilm development [4–7].

Serum from humans and animals is a supplement that has been evaluated for biofilm formation by different microorganisms [8–11]. Therefore, the aim of this study was to evaluate the *in vitro* effects of human serum on *E. faecalis* V583 biofilm formation. The expression of five virulence genes was analysed for both planktonic and sessile cells of *E. faecalis* V583. To our knowledge, this is the first study to report the influence of different concentrations of human

serum on the expression of *ebpA*, *efaA*, *gelE*, *ace* and *asa* genes in *E. faecalis* V583 biofilms.

2. Materials and Methods

2.1 Bacterial strain and growth conditions

Enterococcus faecalis V583 (ATCC 700802) from human blood was purchased from the American Type Culture Collection (ATCC®, USA) and stored at -20°C in Mueller-Hinton broth (MHB, KASVI) containing 10% (v/v) glycerol. Prior to each experiment, the strain was grown aerobically in tryptic soy agar (TSA, KASVI) at 35º C for 24 h.

2.2 Biofilm formation of E. faecalis V583

The biofilm-forming ability of *E. faecalis* V583 in different conditions (absence and presence of 1%, 5%, 25% and 50% HS) was tested in 96-well cell culture microtiter plates (Greiner CELLSTAR®). In each experiment, the 96-well microplates were filled with tryptic soy broth (TSB, Lab™, Neogen®), 1% D-(+)-glucose (Merck®) and different concentrations of previously heat-inactivated human serum (HS). Human serum (from male AB plasma, sterile-filtered, H4522) was purchased from Sigma Aldrich®.

The plates were incubated for 8 and 24 h at 37°C under static conditions. Biofilm quantification was performed according to the protocol from Extremina

et al. [12] with 0.2% crystal violet staining (Merck®). The absorbance was measured at 560 nm using GloMax® Discover System (Promega®). Two independent experiments were performed in triplicate.

2.3 Effect of human serum on planktonic growth of E. faecalis V583

A cell suspension of 10^5 cells/ml was prepared in Erlenmeyer flasks with TSBg (TSB plus 1% glucose) and different concentrations of HS (1%, 5%, 25% and 50%). The suspensions were incubated at 35°C under static conditions. Growth was determined spectrophotometrically by measuring the OD 600nm at different times (0, 2, 4, 6, 12 and 24 h) in order to determine the time-growth curve.

2.4 Expression of virulence genes

In the expression assays, the biofilm induction was performed in six-well microplates (Greiner CELLSTAR®), in a final volume of 3 mL per well, including 300 µl inoculum (1.5 X 10^8 CFU/mL). A control group and 4 different treatments were tested as follows: Control: only TSBg; Treatment 1: TSBg plus 1% HS; Treatment 2: TSBg plus 5% HS; Treatment 3: TSBg plus 25% HS and Treatment 4: TSBg plus 50% HS. All the treatments were performed in triplicate.

The microplates were incubated at 37°C for 8 hours, in the mid-log phase. Then, the medium was removed for RNA extraction of the planktonic

(non-attached) cells and the wells were washed twice with DPBS 1X (Gibco®). One-thousand microliters of deionized water (Sigma Aldrich®) was added in the well, and the sessile (attached) cells were harvested with a cell scraper (240 mm, TPP®). Furthermore, 500 µl volume of each suspension (planktonic and sessile) was transferred into a sterile microtube of 2mL, followed by vigorous vortexing and sonication to break up cell aggregation. One mL of RNAprotect Bacteria Reagent (Qiagen®) was added into a microtube for stabilization of RNA prior to RNA isolation procedures. After the RNA stabilization, bacterial cells were pelleted by centrifugation (10 min at 5000 g). The supernatant was decanted and the pellet was frozen at -20ºC until the RNA extraction.

RNA was extracted by enzymatic lysis using lysozyme and proteinase K digestion followed by purification using the RNeasy Mini Kit (Qiagen®), according to manufacturer's instructions. After extraction, the amount of the total RNA extracted was determined using the Thermo Scientific NanoDrop™ 2000 Spectrophotometer, with the 260nm/280nm ratio expected values between 1.8 and 2. Forty ng of high-quality total RNA was reverse-transcribed to complementary DNA (cDNA) in 10 µl volume using the AccuScript High Fidelity RT-PCR System (Agilent Technologies) according the manufacturer's instructions. After that, cDNAs were diluted in the proportion 1:20 and frozen at -20ºC until the performance of the qPCR.

2.5 Reference genes and target genes

Two reference and five virulence genes were chosen for the expression assays. The elongation factor Tu, a GTP binding protein involved in peptide chain formation, encoded by *tuf* gene [13] and the 23S Ribossomal RNA, part of the large 50S subunit of the ribosome in prokaryotes, encoded by *23SrRNA* gene [14] were selected as reference genes. Primers for reference genes were obtained using the Primer3Plus software version 2.4.0 [15]. Primers specificity was evaluated *in silico* with genome of *E. faecalis* V583 (NC_004668.1) using the BLAST® software (National Center for Biotechnology Information). The target genes used in this study were *E. faecalis* endocarditis antigen encoded by *efaA* gene [4], endocarditis and biofilm-associated pilus encoded by *epbA* gene [5], adhesion of collagen encoded by *ace* gene [6], gelatinase encoded by *gelE* gene and aggregation substance encoded by *asa* gene [7].

2.6 Quantitative real-time PCR

Quantitative real-time PCR in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) was performed. Master-mix for each PCR run was prepared in 20 µl volume as follows: 10 µl SYBR® Green PCR Master Mix (Applied Biosystems), 5.6 µl water (Sigma-Aldrich®), 4 µl of cDNA and 0.2 µl (0,1 µM) of each primer (sequences in Supplementary table 1). The following amplification program was used: 10 min of denaturation at 95°C, 40 cycles consisting of 15 s at 95°C for denaturation, 15 s at (56°C to *tuf, efaA, ebp, gelE*

and *ace* genes; 58°C to *asa* gene and 60°C for *23SrRNA* gene) and 45 s at 60°C for annealing. All reactions were performed in triplicate with three biological replicates.

2.7 Data analysis

Relative expression analysis of each target gene and reference gene in the different treatments was performed in relation to the non-treated samples, through of the Rest 2009 software (M. Pfall and Qiagen) [16] using the crossing points (CP) of each gene studied. Statistical analyses were performed by GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. Results

3.1 Effect of human serum supplementation on biofilm formation and planktonic growth

The biofilm-forming ability of *Enterococcus* was evaluated in the absence and presence of 1%, 5%, 25% and 50% of HS after 8 and 24 hours of incubation. At 8 hours, the group supplemented with 5% HS exhibited a decrease of 67% in the biofilm density, compared to the control group. However, no significant differences were observed at 24 hours, when a similar biofilm density was achieved in all conditions (Table 1). The effect of HS on the planktonic

growth was evaluated by time growth curve. Compared to the control group (without HS), the presence of different concentrations of HS (1%, 5%, 25% and 50%) did not affect the growth of *E. faecalis* V583 (Fig. 1).

3.3 Effects of human serum supplementation on the expression of the virulence genes ebpA, efaA, ace, gelE and asa

The relative expression of virulence genes was determined using qPCR. Expression of each gene was evaluated for *E. faecalis* V583 after biofilm induction with 1%, 5%, 25% and 50% of HS. Both sessile and planktonic cells were evaluated in each condition (Fig. 2; Table 1). Among planktonic cells a significant upregulation of the *asa* gene was observed in the presence of 5%, 25% and 50% of HS. For other genes, however, no significant differences were observed compared to the control group. In contrast, among sessile cells a significant upregulation of all genes was found for the high concentrations of HS (25 and 50%). Comparing the gene expression of planktonic cells and sessile cells, *ebpA, efaA, ace* and *gelE* genes were upregulated only in biofilms, while *asa* gene showed upregulation in both planktonic and sessile cells.

4. Discussion

This study evaluated the *in vitro* effects of HS on biofilm formation for the reference strain *E. faecalis* V583, which is commonly used in different

studies of virulence and resistance of enterococci. The process of biofilm formation is complex and involves different steps, being the attachment step critical and dependent on different factors [17,18].

In this study, bacteria supplemented with 5% HS, after 8 hours of incubation, produced 67% less biofilm than non-supplemented strains, demonstrating a certain inhibition from HS during the attachment step. Similar results were observed in experiments with *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* [19–21]. In contrast, Thompson et al. [21] reported an increased biofilm formation capacity in the presence of 5% HS supplementation in *S. aureus*. Recently, it was reported that HS in association with N-acetylcysteine also increases biofilm formation in *E. faecalis* and other bacteria [22].

In our experiments, the effect of HS on biofilm formation was fully reversed after 24 hours of incubation. On the other hand, the planktonic growth had no influence of the supplement.

HS possesses a complex composition rich in proteins and other nutrients [10], being commonly used as growth factor. Therefore, some components in HS can promote or inhibit biofilm formation. Apo-transferrin, for example, was reported as a HS component that could reduce the biofilm formation without affecting the planktonic cell growth of *S. epidermidis* [19,23]. Another study also performed the characterization of the inhibitory component of the HS, and they proposed that a non-protein component may inhibit the adhesion and

biofilm formation of fungi and bacteria [10]. Abraham and Jefferson [8] characterized the inhibitory components of HS in *S. aureus* biofilm formation and they found a low density non-proteinaceous substance that influences the transcription of biofilm-related genes.

Besides the environmental conditions, the type of surface may also affect the biofilm formation. A study evaluated the effect of the culture medium supplemented with 10% HS on the adhesion of *E. faecalis* ATCC 29212 to glass and silicone rubber, and the addition of serum did not affect the interaction between bacteria and the two surfaces [24].

This study also investigated the direct influence of HS on the biofilm-related genes expression. Planktonic cells are free-floating in the medium, while sessile cells are part of the biofilm structure [25]. It is known that there is a physiological difference between these two cell states and that biofilm production is regulated by quorum sensing systems [3,26]. When we compared the transcript levels of each gene among treatments and between cell states, there were differences compared to the control group. Sessile cells had more genes upregulated than planktonic cells. To understand the role of serum on *E. faecalis* biofilm formation is important, since they can form biofilms on medical devices and therefore come into contact with blood.

The biofilm formation of *E. faecalis* involves transcriptional regulatory systems or cell-surface components [27]. Many studies prove that *epbA*, *efaA*, *ace*, *gelE* and *asa* genes are strongly associated with pathogenesis of

Enterococcus and also play an individual role in the biofilm biogenesis [28]. In our study, with the exception of the *asa* gene, *epbA, efaA, ace* and *gelE* were upregulated only in sessile cells and with high concentrations of HS. It is known that these genes are influenced by the presence of serum [4,29–31]. However, our study showed a higher increase of the expression in the sessile cells than planktonic cells, which may be directly associated with the biofilm formation process.

Although HS inhibited *E. faecalis* biofilm formation at the concentration of 5%, our hypothesis is that this HS concentration was not enough to affect the gene expression. In the literature, there is no consensus about the optimal concentrations of supplements that promote or inhibit the biofilm formation in different species. For instance, several studies observed effects in gene expression and biofilm formation by HS varying between 3% and 50% [8,10,21].

In conclusion, this study demonstrated an *in vitro* inhibitory effect on biofilm formation among *E. faecalis* in low concentration of HS and a significant increase of the biofilm-related gene expression under high concentration of HS. A better understanding of this process as well as the application of these findings *in vivo,* may help in the search for strategies to prevent biofilm formation.

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Disclosure

There are no conflicts of interest to declare.

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Supplementary Table 1. Primers used in this study:

Gene	Sequence	Strand	Reference
tuf [a]	ATTAATGGCTGCAGTTGACG	forward	This study
	AGCAACAGTACCACGTCCAG	reverse	
23SrRNA [a]	CAGTGTCAGATGGGCAGTTT	forward	This study
	GCTCCCTTCTGCCTTTACAC	Reverse	
efaA [b]	TGGGACAGACCCTCACGAATA	forward	Lowe et al. (1995)
	CGCCTGTTTCTAAGTTCAAGCC	reverse	
epbA [b]	AAAAATGATTCGGCTCCAGAA	forward	Bourgogne et al. (2007)
	TGCCAGATTCGCTCTCAAAG	reverse	
ace [b]	GGAGAGTCAAATCAAGTACGTTGGTT	forward	Nallaparedy and Murray. (2006)
	TGTTGACCACTTCCTTGTCGAT	reverse	
	GCCACTTTATCAGCCTGAACC	reverse	
gelE [b]	TATGACAATGCTTTTTGGGAT	forward	Vankerckhoven et al. (2004)
	AGATGCACCCGAAATAATATA	reverse	
asa [b]	GCACGCTATTACGAACTATATGA	forward	Vankerckhoven et al. (2004)
	TAAGAAAGAACATCACCACGA	Reverse	

tuf, Translation, Elongation Factor Tu; *23SrRNA*, 23S Ribossomal RNA; *efaA*, *Enteroccoccus faecalis* endocarditis antigen; *ebpA*, Endocarditits and biofilm-associated pilus; *ace*, Adhesin of collagen; *gelE*, Gelatinase; *asa*, Aggregation substance

[a] Reference gene; [b] Target gene

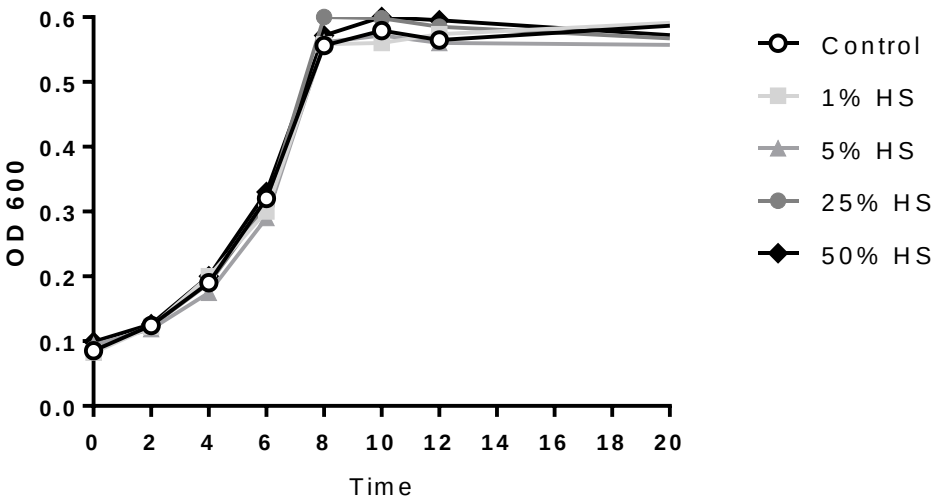

Fig. 1. Effect of human serum on planktonic growth of *Enterococcus faecalis* V583. Control (tryptic soy broth Plus 1% glucose - TSBg), 1% HS (TSBg plus 1% Human Serum), 5% HS (TSBg plus 5% Human Serum), 25% HS (TSBg plus 25% Human Serum) and 50% HS (TSBg plus 50% Human Serum).

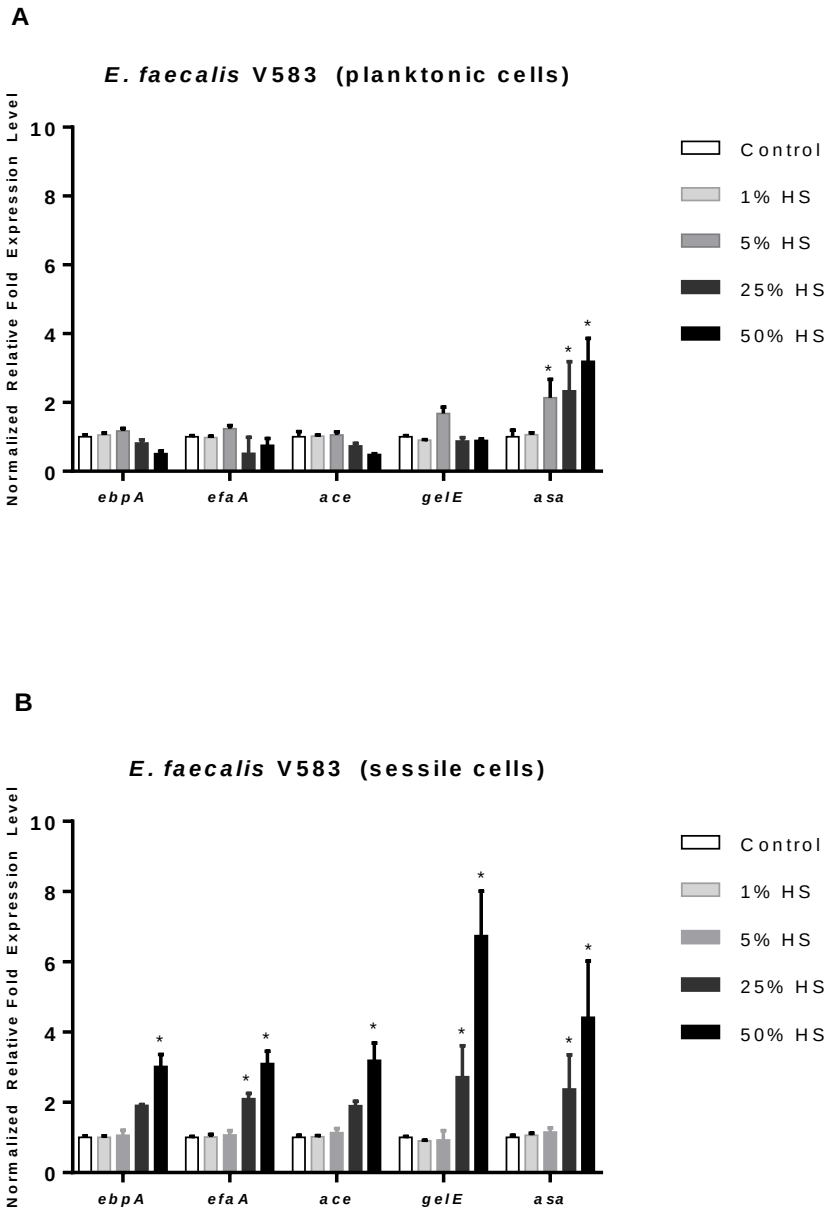

Fig. 2. Expression of *E. faecalis* V583 biofilm-related genes by qPCR. Control (tryptic soy broth plus 1% glucose - TSBg), 1% HS (TSBg plus 1% Human Serum), 5% HS (TSBg plus 5% Human Serum), 25% HS (TSBg plus 25% Human Serum) and 50% HS (TSBg plus 50% Human Serum). Housekeeping genes *tuf* and *23SrRNA* were used as control. A. Expression of planktonic cells of *E. faecalis* V583. B. Expression of sessile cells of *E. faecalis* V583. Error bars represent standard deviation. Statically differences obtained when compared with control ($* P < 0.05$).

Table 1. Gene expression rate and optical density after human serum supplementation (Gene expression assay and biofilm induction performed at 8 h).
The values are means ± standard deviations.

		ebpA	***efaA***	***ace***	***gelE***	***asa***	**OD** 8 hours**	**OD ** 24 hours**
Planktonic	**Contol**	1.0 ± 0.06	1.0 ± 0.04	1.0 ± 0.15	1.0 ± 0.04	1.0 ± 0.19	0.556 ± 0.08	0.598 ± 0.06
	1%HS	1.05 ± 0.06	0.98 ± 0.04	1.02 ± 0.03	0.9 ± 0.02	1.06 ± 0.06	0.548 ± 0.04	0.600 ± 0.08
	5%Hs	1.17 ± 0.08	1.23 ± 0.09	1.05 ± 0.1	1.68 ± 0.18	2.13 ± 0.54 *	0.561 ± 0.04	0.556 ± 0.06
	25%HS	0.82 ± 0.09	0.52 ± 0.05	0.74 ± 0.08	0.88 ± 0.1	2.34 ± 0.85 *	0.630 ± 0.07	0.585 ± 0.07
	50%HS	0.51 ± 0.08	0.75 ± 0.21	0.49 ± 0.02	0.9 ± 0.05	3.2 ± 0.67 *	0.572 ± 0.03	0.561 ± 0.07
Sessile	**Control**	1.0 ± 0.05	1.0 ± 0.03	1.0 ± 0.06	1.0 ± 0.3	1.0 ± 0.06	0.811 ± 0.05	1.144 ± 0.09
	1%HS	0.98 ± 0.03	1.01 ± 0.08	1.05 ± 0.03	0.87 ± 0.08	1.12 ± 0.04	0.761 ± 0.08	1.296 ± 0.12
	5%Hs	1.07 ± 0.14	1.07 ± 0.12	1.14 ± 0.12	0.93 ± 0.26	1.16 ± 0.11	0.260 ± 0.09	1.137 ± 0.10
	25%HS	1.92 ± 0.02	2.1 ± 0.15 *	1.91 ± 0.12	2.73 ± 0.89 *	2.34 ± 0.48 *	0.808 ± 0.04	1.105 ± 0.15
	50%HS	3.02 ± 0.34 *	3.11 ± 0.35 *	3.19 ± 0.5 *	6.74 ± 1.2 *	4.42 ± 1.6 *	0.874 ± 0.13	1.127 ± 0.12

* Significant; **Optical density, OD600 for planktonic growth and OD560 for biofilm growth.

CHAPTER 7.

GENERAL DISCUSSION

With regard to the results already published in the article "**Evaluation of a selective chromogenic medium for detecting vancomycin-resistant enterococci",** the correct identification of Vancomycin Resistant Enterococci (VRE) in surveillance culture is important. Colonization and infection with VRE is an increasing problem worldwide and is associated with the risk of developing Healthcare Associated Infections (HAI) (FLOKAS et al., 2017; ÖZSOY & İLKI, 2017). Screening for VRE is a routine procedure, and the use of good tools can improve the diagnosis, thus avoiding the spread of this pathogen and the incorrect prescription of antibiotics. In our study, we evaluated the performance of a selective chromogenic medium for the detection and differentiation of vancomycin-susceptible and vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. The chromID™ VRE agar had a high sensitivity, similar to previous studies evaluating the performance of the same medium (DELMAS et al., 2007; CUZON et al., 2008; GRABSCH et al., 2008; ASIR et al., 2009). In contrast, the specificity of the method was significantly lower compared to the same studies. We also observed colonies with non-

discriminatory staining that may lead to false-negative results. Therefore, we recommended the chromID™ VRE, since it is a rapid and useful tool for the screening and identification of VRE, as long as it is combined with conventional tests.

With regard to the manuscript "**High diversity of Vancomycin-resistant *Enterococcus faecium* isolated in Southern Brazil**", VRE_{fm} has become one of the leading causes of HAI, such as bacteremia and infective endocarditis (O'DRISCOLL & CRANK, 2015; TEDIM et al., 2017) and is responsible for hospital outbreaks worldwide (WILLEMS et al., 2005). In this context, we described the susceptibility profile and the clonal relationship of 94 VRE_{fm} consecutively recovered from inpatients of three general hospitals in Southern Brazil, from September 2012 till April 2017. All *E. faecium* isolates exhibited resistance to vancomycin (harboring *vanA* gene), teicoplanin, ampicillin and ciprofloxacin, and 13.8% high level resistance to gentamicin, showing a multidrug-resistant profile. Most of the VRE_{fm} isolates possessed *esp* and *acm* genes, both related with biofilm formation and adherence to extracellular matrix (HENDRICKX et al., 2007). Some of these characteristics found in our isolates are similar to the CC-17 lineage, i.e. a well-adapted lineage to the hospital environment and associated with most of the reported hospital outbreaks worldwide (WILLEMS et al., 2005; PANESSO et al., 2010). The VRE_{fm} isolates were separated by PFGE into 38 types (23 clonal types and 15 *singletons*),

showing a polyclonal distribution of VRE_{fm} in Southern Brazil, also observed in other studies (POURSHAFIE et al., 2008; TOP et al., 2008a; LANDERSLEV et al., 2016; SOMILY et al., 2016).

In the manuscript **"Influence of glucose supplementation on biofilm formation and expression of associated-biofilm genes in *Enterococcus faecalis* clinical isolates**", the *in vitro* effects of glucose on biofilm formation and gene expression of *Enterococcus faecalis* clinical isolates were assessed. The presence of glucose seems to be an essential factor for biofilm formation by different bacteria and contributes to bacterial growth, reproduction and metabolic activities (PILLAI et al., 2004; MARINHO et al., 2013; SENEVIRATNE et al., 2013; RAN et al., 2015). Biofilm formation was evaluated in the presence and absence of glucose, and the 1% glucose supplementation significantly increased biofilm formation in all isolates after 8 hours of induction. The direct influence of glucose on the biofilm-related genes expression was also investigated for eleven *E. faecalis* clinical isolates and one reference strain (*E. faecalis* ATCC 29212), possessing all the four genes (*ebpA, efaA, ace* and *gelE*). PILLAI et al. (2004) showed that 1% glucose supplementation increased the optical density and can regulate, directly or indirectly, the transcription of some glucose-dependent genes associated with biofilm formation in *E. faecalis*. In our study, all four biofilm-associated genes were upregulated in 1% glucose in a subgroup of isolates and no significant differences were observed for the others.

Interestingly, our findings demonstrated that the expression of some genes did not alter in the presence of glucose during the biofilm formation. This shows that other biofilm-related genes must be affecting the biofilm formation in these isolates, and that the patterns of virulence gene expression are dependent on individual characteristics of the strain.

Finally, in the manuscript **"Human serum enhances virulence factors expression and inhibits adhesion of *E. faecalis* V583"**, we evaluated the *in vitro* effects of human serum on biofilm formation of a reference strain (*E. faecalis* V583). Human serum presents a complex composition rich in proteins and other nutrients (DING et al., 2014), being commonly used as growth factor. Some of these components (protein or non-protein) may promote or inhibit biofilm formation (ARDEHALI et al., 2003; ABRAHAM & JEFFERSON, 2010; DING et al., 2014; SHE et al., 2016). In our study, the group supplemented with 5% human serum, at 8 hours incubation, produced 67% less biofilm than without supplementation. This demonstrates a certain inhibition from human serum during the attachment step, which was also observed in experiments performed with *S. aureus* and *P. aeruginosa* (ABRAHAM; JEFFERSON, 2010; HAMMOND et al., 2010; SHE et al., 2016). No influence of human serum was observed in the planktonic growth of *E. faecalis* V583. Our study also investigated the direct effect of human serum on the biofilm-related genes expression in planktonic and sessile cells. It is well-known that there is a

physiological difference between these two cells states (MOHAMED; HUANG, 2007; STEWART & FRANKLIN, 2008). The transcript levels of each gene among treatments and between cell states differed in comparison to the control group. Sessile cells had more genes upregulated than planktonic cells, which may be directly associated with the biofilm formation process. A better understanding of the interaction of *E. faecalis* with serum as a triggering factor of biofilm formation is important, since they can form biofilms on medical devices and therefore come into contact with blood.

CHAPTER 8

CONCLUSION

Responding to the specific aims of this thesis, we evaluated the performance of ChromID VRE® in the identification of 184 well characterized *Enterococcus* spp. and reference strains. ChromID VRE® is a commercial agar to detect VRE, commonly used in routine laboratories, and in our experiments had a good sensitivity of 95.52% but a low specificity of 30% due to false positive results. We recommend further VRE identification to avoid misinterpretation and unnecessary antimicrobial therapy.

We verified the multidrug resistance profile of all 94 VRE clinical isolates, similar to the VRE profile spread around the world. Besides vancomycin, all the VRE strains were resistant to ampicillin, ciprofloxacin and teicoplanin. Some of these were also resistant to high levels of gentamicin. However, all the VRE strains were susceptible to linezolid, daptomycin and quinupristin-dalfoprisitin, i.e. the therapeutic options to treat multidrug-resistant enterococcal infections, showing that so far, the resistance to these antimicrobials did not spread yet in Southern Brazil.

We verified that all VRE isolates possessed *vanA* gene, that encodes resistance to high levels of vancomycin and resistance to teicoplanin. The *vanB* gene was not present in none of the VRE strains. A high prevalence of the

virulence genes *esp* and *acm* was observed among them, being present in 85.1% and 94.7%, respectively. Resistance to vancomycin, ampicillin and ciprofloxacin and presence of *vanA* and *esp* genes are features of a genetic lineage of *E. faecium* designated as clonal complex-17, associated with most of the reported hospital outbreaks worldwide.

We performed the molecular characterization of 94 VRE isolates by PFGE and we verified a high clonal diversity among them. Seventy-nine isolates were grouped in 23 clonal types and 15 isolates exhibited unique pattern type.

We verified the presence of the biofilm-associated genes *ebpA*, *efaA*, *ace* and *gelE* in 123 *E. faecalis* previously characterized as biofilm formers. Forty-two (34.1%) isolates possessed the four genes. The *efaA* was the most prevalent gene, present in 95.9% of the *E. faecalis* isolates, followed by, *gelE* gene (70.7%), *ebpA* gene (69.1%) and *ace* gene (64.2%).

We evaluated the *in vitro* biofilm formation in the absence and presence of glucose supplementation for 123 *E. faecalis* strains previously characterized as biofilm formers, and we verified that 1% glucose increases significantly the biofilm capability. In view of this, we also evaluated the effect of 1% glucose in the expression of biofilm-associated genes in 11 isolates and one control, whose possessed all the four genes and were affected by the presence of glucose. The gene expression varied among the isolates, showing that patterns of virulence gene expression are not the same for all isolates from the same species.

Finally, we evaluated the *in vitro* influence of different concentrations of human serum (1%, 5%, 25% and 50%) on the biofilm formation and the virulence gene expression (*ebpA, efaA, ace, gelE* and *asa*) of *E. faecalis* V583 (ATCC 700802). We found an inhibition for 5% human serum in the initial step of biofilm biogenesis, however this concentration did not affect the gene expression. On the other hand, high concentrations of human serum (25% and 50%) increased the expression of *ebpA, efaA, ace* and *gelE* genes in sessile cells and the expression of *asa* gene in both planktonic and sessile cells, showing a directly effect of the human serum on the expression of the virulence genes during biofilm formation.

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Research - Publications

1. GRASSOTTI, TIELA TRAPP; ZVOBODA, DEJOARA DE ANGELIS; COSTA, LETÍCIA DA FONTOURA XAVIER; ARAÚJO, ALBERTO JORGE GOMES; PEREIRA, REBECA INHOQUE; **SOARES, RENATA OLIVEIRA;** WAGNER, PAULO GUILHERME CARNIEL; FRAZZON, JEVERSON; FRAZZON, ANA PAULA GUEDES. Antimicrobial Resistance Profiles in *Enterococcus* spp. Isolates from Fecal Samples of Wild and Captive Black Capuchin Monkeys (*Sapajus nigritus*) in South Brazil. Front. Microbiol. 2018; Doi: <https://doi.org/10.3389/fmicb.2018.02366>

2. **SOARES, RENATA OLIVEIRA;** ROSSATO, ADRIANA MEDIANEIRA; SAMBRANO, GUSTAVO ENCK; TOLFO, NEIDIMAR CEZAR CORRÊA; CAIERÃO, JULIANA; PAIM, THIAGO GALVÃO DA SILVA; D'AZEVEDO, PEDRO ALVES . Evaluation of a selective chromogenic medium for detecting vancomycin-resistant enterococci. Braz J Microbiol. 2017; 48(4): 782–784. Doi: <10.1016/j.bjm.2017.03.005>

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