INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

Mestrado em Tecnologias Clínico-Laboratoriais

2º Ano

Modulatory effect of epigallocatechin-3 gallate in Staphylococcus aureus toxin production genes transcription

Mestranda: Raquel Portanova de Almeida (2020209)

Orientadora: Professora Doutora Edna Soraia Gregório Ribeiro (ESTeSL, H&TRC)

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“Unless the Lord watches over the city, the guards stand watch in vain”

Ps127:1

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Abstract

Antimicrobial resistance of pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) is a major public health concern worldwide. Currently, several approaches are being developed to fight against these microorganisms, including the use of compounds with therapeutic potential such as green tea catechins.

Epigallocatechin-3 gallate (EGCG) is the most abundant catechin in tea and the most medically relevant, with anti-inflammatory, antioxidant, anti-carcinogenic properties, high antimicrobial potential and reported synergism with several antibiotics. It has been shown that EGCG exposure alters the transcription of numerous genes in *S. aureus*, including genes implicated in toxin, such as *hlgA, hlgB* which encode the gamma haemolysing subunits A and B, respectively and *hly,* that encodes for an alpha-haemolysin-precursor.

In this study, we aimed to assess the effects of EGCG exposure in the transcriptional patterns of *hlgA, hlgB* and *hly* in *S. aureus* strains with divergent resistance phenotypes.

Transcriptional levels of selected genes were determined, by bacterial RNA extraction, conversion into cDNA and quantification by qRT-PCR, followed by statistical treatment.

Overall, our results show that divergent resistance phenotypes are associated with differential transcriptional expressions of the studied genes: upregulation in nosocomial strains, when compared to commensal ones, and downregulation after exposure to EGCG, although the patterns were different in *hlgA* and *hlgB*/*hly.* Also, *hly* had higher transcriptional expression before exposure to EGCG than the other two analysed genes. Data suggests a correlation between epigenetic modulation and the expression of virulence factors namely haemolysins.

This study increases the scientific knowledge and has allowed to produce accumulating evidence to suggest that EGCG could be a novel therapeutic option in the fight against antibiotic resistance.

Keywords: EGCG, MRSA, Antibiotic resistance, transcription modulation, haemolysins.

Resumo

A resistência antimicrobiana de agentes patogénicos como o *Staphylococcus aureus* resistente à meticilina (MRSA) é uma enorme preocupação de saúde pública a nível mundial. Atualmente, várias abordagens de combate a estes microrganismos estão a ser desenvolvidas, incluindo a utilização de compostos com potencial terapêutico como as catequinas do chá verde.

A epigalocatequina-3-galato (EGCG) é a catequina do chá com maior relevância clínica, apresentando um forte poder anti-inflamatório, antioxidante, anticancerígeno, antimicrobiano e demonstrando sinergismo com diversos antibióticos. A exposição a EGCG afeta a transcrição de vários genes em *S. aureus*, incluindo genes relacionados com fatores de virulência, como *hlgA*, *hlgB* que codificam subunidades hemolisantes gama A e B, respetivamente e *hly*, que codifica um precursor de alfa-hemolisina.

O objetivo deste estudo foi avaliar os efeitos da exposição ao EGCG nos padrões de transcrição de *hlgA, hlgB* e *hly* em estirpes de *S. aureus* com fenótipos de resistência divergentes.

Foram utilizadas estirpes com diferentes origens, perfis de resistência, e sinergismo da EGCG com vários antibióticos. Os níveis transcricionais dos genes selecionados foram determinados por extração do RNA, conversão em cDNA e quantificação por qRT-PCR utilizando primers específicos, seguidas de tratamento estatístico.

A análise de resultados demonstra que fenótipos de resistência divergentes estão associados a expressões transcricionais diferentes: mais elevadas nas estirpes nosocomiais que comensais, e mais reduzidos após exposição. Também se observou que *hly* teve maior expressão transcricional pré-exposição. Os dados analisados sugerem uma correlação entre a modulação epigenética e a expressão de fatores de virulência.

Este estudo permite assim acumular evidências que sugerem que o EGCG pode ser uma nova opção terapêutica no combate à resistência a antibióticos.

Palavras-chave: EGCG, MRSA, Resistência a antibióticos, modulação da transcrição, hemolisinas.

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List of Abbreviations and Symbols

α- alfa

β- beta

γ- gamma

CA-MRSA – Community-acquired MRSA

Ct – cycle threshold

cDNA – complementary DNA

DNA –Deoxyribonucleic acid

EC – epicatechin

ECG - epicatechin-3-gallate

EGC - epigallocatechin

EGCG – Epigallocatechin-3-gallate

HA-MRSA – Healthcare-acquired MRSA

LA-MRSA – Land stock-acquired MRSA

MRSA – Methicillin-resistant Staphylococcus aureus

MSSA – methicillin-susceptible Staphylococcus aureus

PVL - Panton-Valentine leucocidin

qRT-PCR – quantitative Real-Time Polymerase Chain Reaction

RNA – ribonucleic acid

rRNA –ribosomal RNA

WHO – World Health Organization

1- Introduction

The World Health Organization describes antimicrobial resistance in human infections such as methicillin-resistant *Staphylococcus aureus* (MRSA) as a worldwide health issue. MRSA is known to cause numerous hospitalizations and deaths worldwide(1,2). The rise in antibiotic-resistant microorganisms has been, for decades, raising concerns about antibiotic prescribing and overuse and has prompted the need to develop new molecular targets with therapeutic potential(3, 4, 5, 6).

Antimicrobial resistance develops when bacteria, viruses, fungi, and parasites evolve over time and lose their ability to react to antibiotics, making infections more difficult to treat and raising the risk of disease transmission, severe illness, and death(1,7). The use of conventional antibiotics to treat these illnesses is usually fraught with complications, such as the emergence of multiple drug resistance and negative side effects. In some circumstances, the sole choice for treating multidrug-resistant bacteria is to use a synergistic antibiotic medication combination with bioactive substances(8).

S. aureus possesses a diverse set of virulence factors that enable it to thrive in harsh environments and allow for its great success as a pathogen(9,10,11). Among its many virulence factors, *S. aureus* secretes toxins into the extracellular matrix, during the post-exponential and early stationary phases. These toxins are proteins that cause haemolysis and typically participate in tissue penetration, allowing microorganisms to invade their host(11). That means overexpression of some virulence factors genes, like toxin production ones, can cause clinically relevant antibiotic resistance in *Staphylococcus aureus*(11,12). This multi-resistance developing in many microorganisms has been leading to a search for new effective natural antimicrobial substances(5,13–15).

Due to the presence of high content catechin, namely EGCG or epigallocatechin-gallate(17) a green tea extract from the leaves of *Camellia sinensis* (green tea) has been demonstrated to exhibit a wide range of antimicrobial activities(5, 16–28). Studies indicate the efficacy of EGCG in reversing the MRSA resistance phenotype in vitro and observed the antimicrobial potential and synergistic effect of EGCG against various antibiotics in strains isolated from hospital-acquired infections and nasopharyngeal colonization in vitro(11–23). EGCG is also an epigenetic modulator as it upregulates the transcription of several genes including *Agr*, *OrfX*, *SpdC* and *WalKR,* and signal transduction pathways including JAK/STAT, MAPK, PI3K/AKT, Wnt and Notch(11,12,17).

When compared to hospital-acquired MRSA strains (HA-MRSA), community-acquired MRSA strains (CA-MRSA) have significant differences in risk factors, antibiotic resistance, growth rate, toxins, and/or virulence characteristics(11). Since *Staphylococcus aureus* strains isolated from acute nosocomial infections show divergent resistance profiles to antibiotics commonly used as therapeutic options, epigenetic and drug resistance modulators, like EGCG are currently seen as potential targets for new therapeutic approaches for these pathogens(11).

The effectiveness of EGCG in reversing the phenotype of resistance in *S. aureus* both from nosocomial infections and commensal strains has been proved(30), as well as the fact that divergent resistance phenotypes are associated with divergent transcriptional expression of epigenetic modulator genes(31).

Moreover, the assessment of EGCG effects on virulence genes such as *hlgA, hlgB* and *hly*, associated with toxin production, which play a key role on *S. aureus*'s virulence, establishment, and health outcomes, are of foremost importance. Taking all this into account, it is essential to assess EGCG transcriptional effect in these genes and its correlation with divergent resistance phenotypes and strains.

2- Sate of the art

2.1 - Staphylococcus aureus

Staphylococcus aureus, a Gram-positive, cocci-shaped bacterium, was first described by the Scottish surgeon Alexander Ogaston in surgical abscess in 1881(31). In 1884 Anton J. Rosenbach, a German surgeon, isolated two strains of *staphylococci*, which he named for the pigmented appearance of their colonies*: Staphylococcus aureus*, from the Latin *aurum* for gold, and *Staphylococcus albus* (currently *epidermidis*), from the Latin *albus* for white(32).

S. aureus is one of the most frequent pathogenic microorganisms that cause hospital- and community-acquired infections, with substantial public-health effects. It is the second most common cause of bacteraemia in Europe and one of the leading causes of sepsis worldwide(33). In Portugal, it has been raising concerns about occupational health, and efforts are being made to standardize exposure assessment processes in workplaces (2).

S. aureus is a commensal microorganism that, meeting the ideal conditions, can cause serious infections such as endocarditis, toxic shock syndrome, among others, as well as cause food poisoning, causing vomiting, diarrhoea, and prostration(34). Toxin-mediated diseases such as food poisoning, scalded skin syndrome, toxic shock syndrome; skin and soft tissue infection (e.g., boils, cellulite, and impetigo); deep site infection (e.g., bone, joints, heart valve, spleen, and liver), and lung and urinary tract infections, are just a few of the clinical manifestations caused by *Staphylococcus aureus*(35).

2.2- Antibiotic resistance

Antibiotics can be bacteriostatic, that is, they inhibit growth by keeping it in a stationary phase, or bactericidal, killing microorganisms(36). Antibiotics are classified based on their mechanism of action, meaning the location where their antibacterial impact is exerted, changing cell structure and function, and ultimately leading to cell death (35,38,39).

Their 5 mechanisms of action are: inhibition of cell wall synthesis; inhibition of protein synthesis; inhibition of nucleic acid synthesis; destruction of cell membrane function, and inhibition of folic acid synthesis (inhibition of metabolism)(40). Antibiotics with action at the level of bacterial wall synthesis belong to the β-lactams and are the most used antibiotics in clinical practice due to their therapeutic efficacy and low toxicity(41,42,43). They are bactericidal agents, since they damage the cell wall, compromising the bacteria's integrity(18). This class of antibiotics are penicillins, cephalosporins, carbapenems and monobatamics, and they have in common the β-lactam ring that confers the pharmacological activity(43).

Antibiotic resistance can develop in a variety of ways, but bacteria manage to avoid its effects due to 4 main mechanisms: a) modification or enzymatic destruction of the antibiotic (for example, destruction of β-lactams by β-lactamases which cause hydrolysis of the β-lactam ring prior to its binding to penicillin-binding proteins or PBPs); b) efflux pumps (promote the efflux of the antibiotic from the intracellular to the extracellular medium); c) change in antibiotic target molecules (due to total loss of affinity or simple reduction thereof); d) alteration of bacterial cell membrane permeability (modifying the antibiotic binding site)(39,40,43).

2.3- Antibiotic resistance in *Staphylococcus aureus*

In *Staphylococcus aureus*, antibiotic resistance has been developed by mutations in genes or by the acquisition of resistance genes from other bacteria of the same species. Studies describe that resistance to methicillin is determined by gene 13 - *mecA*, which codes for changes in the β-lactam receptor, stimulating the production of PBP with low affinity for the antibiotic, resulting in resistance, causing the bacteria to resist the treatment. This type of resistance is the basis of resistance for MRSA(44). Some *Staphylococcus aureus* strains are resistant to methicillin (MRSA), but also to a broad range of antibiotics, making it difficult to treat, and the source of dangerous infections both in hospitals and in the community, with a high morbidity and mortality rate(45–47). Even though methicillin is no longer used in clinical practice or even produced commercially, the label MRSA has persevered. Furthermore, except for the latest generation of cephalosporin-lactams, methicillin resistance emerges as resistance to practically all lactams. This makes infection treatment even more difficult(48). This resistance encompasses vancomycin(50,51), considered one of the last treatment

options for severe MRSA infections(52) and relatively new agents such as linezolid and daptomycin (50). These are sometimes also called VRSA (Vancomycin-resistant *Staphylococcus aureus*)(31). This resistance is also a huge challenge for medicine in terms of treatment and control of *Staphylococcus aureus* infections (28). The term *superbug* has also been used for years when referring to such strains of antibiotic resistant bacteria (not just MRSA)(53–58). The ability of *Staphylococcus aureus* to acquire 5 genes that confer antibiotic resistance combined with host gene mutation has facilitated the evolution of a multi-resistant pathogenic microorganism for which treatment options may be severely limited(59). Its antibiotic resistance has been developed by mutations in genes or by the acquisition of resistance genes from other bacteria of the same species.

2.4 - Methicillin-resistant Staphylococcus aureus strains

The scientific community defines MRSA strains into HA-MRSA, associated with healthcare and hospital infections; community-associated CA-MRSA and LA-MRSA associated with animal husbandry(2,62,63).

HA-MRSA affect more patients admitted to hospitals or nursing homes, the elderly, and newborns. Risk factors for the emergence of HA-MRSA are the prolonged use of antibiotics, long hospital stays, surgery, and medical devices (such as catheters, etc.). The transmission of this microorganism is essentially made through person-to-person contact (this includes health personnel, patients, visitors and handled hospital equipment)(2,59,63), and the most common mode of transmission being the lack of basic hand hygiene(63). Studies report that prolonged exposure to bio-aerosols, particularly in the workplace, can pose a health risk and potentially result in six infectious diseases both for workers and for the dissemination of these microorganisms in the community(2,35). In healthcare, bioaerosols and hand contact are the most common ways for MRSA to spread. The danger of colonization is substantial since clinical analysts and public health workers are in constant contact with patients colonized with these bacteria, particularly during the collection of biological samples(35).

CA-MRSA have been emerging as a pathogenic microorganism with clinical significance associated with infections particularly in young healthy, asymptomatic individuals. These MRSA strains combine methicillin resistance with increased virulence, causing highly invasive, progressive, and potentially fatal diseases and becoming a public health problem associated with the community(35). CA-MRSA strains mostly cause skin and soft tissue diseases, ranging from boils to necrotizing fasciitis. Sharing personal items, neglected skin lesions, and poor hygiene have been identified as risk factors for CA-MRSA infection(45,64,65). Despite these differences, CA-MRSA and HA-MRSA can coexist(66).

LA-MRSA, mostly associated with animal husbandry, will not be considered for this study.

Antibiotic resistance profile is an important feature: while HA-MRSA strains are characterized by broad resistance to various antibiotics, CA-MRSA strains demonstrate high sensitivity to various antibiotics such as gentamicin, clindamycin, sulfametaxazol / trimethoprim, ciprofloxacin, and vancomycin(66).

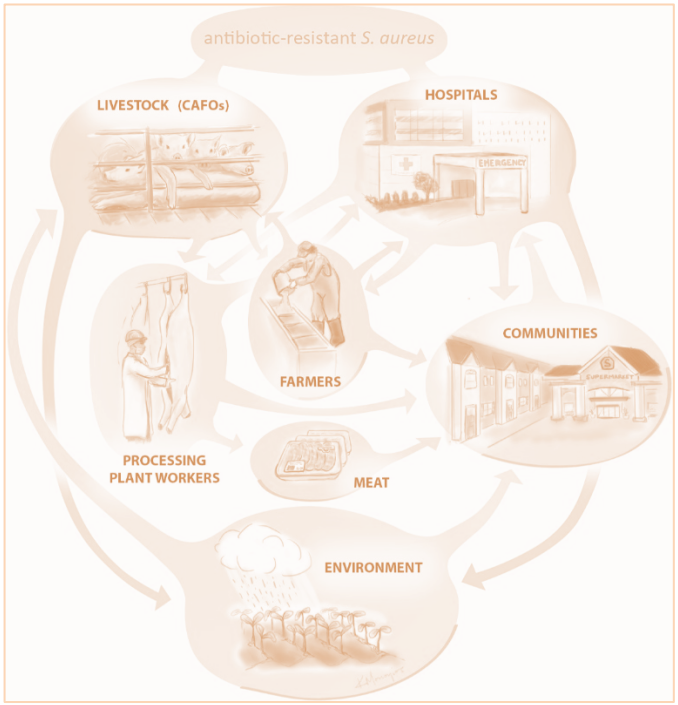

Figure 2 - The tangled web of MRSA: CA-MRSA, HA-MRSA, LA-MRSA. Adapted from (68)

2.5 - Virulence factors

S. aureus has been continuing to gain more antimicrobial resistance factors since the surfacing of MRSA in the 1970s. Virulence factors, as antimicrobial resistance factors, are thought to have evolved and spread across this species by gene transfer mediated by mobile genomic islands, bacteriophages, plasmids, transposons, and insertion sequences(69).

Agr (accessory gene regulator), *S. aureus* virulence regulator gene, is of immense importance in regulating such virulence, although not essential for its expression(67,68). *S. aureus* high success as a pathogen is largely determined by its capacity to adapt to varied environments by modulating the expression of a wide variety of virulence factors(72).

Virulence factors are:

- Adherence factors (surface proteins and antigens): Several adhesins help *S. aureus* adhere to the surface of the host cell. Proteins covalently linked to cell peptidoglycans are one of the most common types of *S. aureus* adhesins. The most prominent components of the extracellular matrix or blood plasma, such as fibrinogen, fibronectin, and collagens, are recognized by these molecules.

- Exoproteins (enzymes, toxins, and surface proteins): These are a group of exoproteins such as exotoxins and enzymes, including nucleases, proteases, lipases, hyaluronidase, and collagenase. These proteins convert local host tissue into nutrients required for bacterial growth. The proteins cause breakdown of the host cells and are thus said to be cytolytic: cytolytic toxins form pores of holes called β-barrel pores in the plasma membrane. This leads to leakage of the cell's content and lysis of the target cell.
- Other toxins include α-haemolysin, β-haemolysin, γ-haemolysin, leucocidin, and Panton-Valentine leucocidin (PVL). *S. aureus* produces an additional group of toxins called the toxic shock syndrome toxin. It also secretes staphylococcal enterotoxins and the exfoliative toxins A and B. These cause toxic shock syndrome and food poisoning and are also involved in staphylococcal scalded skin syndrome(73)(31,68–76). Toxins such as α-haemolysin are encoded in the genome, but others, such as PVL, are encoded on mobile genetic elements like prophage(82).

2.6- New approaches for antimicrobial compounds: Epigallocatechin-3-gallate

Over the last few years, with the appearance of multi-resistant microorganisms, the WHO has encouraged the search for new natural antimicrobial compounds that can be used against these microorganisms(5). Several studies have tried to find new approaches focused on the use of natural products or compounds with therapeutic properties(15,25,83). One such approach was the use of green tea, and the study of its beneficial properties(16,17,19,21,24). Green tea is mainly produced in Asian countries from the leaves of the *Camellia sinensis* plant. Its chemical composition includes, among other components, polyphenols (flavonoids and catechins), responsible for its beneficial properties. The four main catechins in this tea are: epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate or EGCG(22). EGCG is the most abundant and most relevant catechin from a medical point of view, presenting a strong anti-inflammatory, antioxidant, anti-carcinogenic power, high antimicrobial power (fighting and preventing infections), and demonstrating synergism with various antibiotics(16,18,20,21,25,27,81,87,88).

-2.6.1 EGCG and antibiotic synergism

Previous studies have shown that there is synergism between EGCG and different antibiotics(21,26,28,78,79). Many of these studies investigated the possible synergism between green tea catechins with β-lactams and focused their target on *S. aureus*, in particular MRSA. It has been shown that EGCG causes damage to the bacterial cell wall, compromising its integrity(83).

Other studies showed that catechins interact synergistically with tetracycline against *S. aureus*(17,80,81). Several other studies have also shown a synergistic effect with penicillin, oxacillin, ampicillin / sulbactam and imipenem on MRSA(24–26,80,81).

-2.6.2 EGCG, antibacterial activity, epigenetic potential, and changes in gene regulation

EGCG displays the strongest antibacterial activity of all tea catechins(16,21,24,27,89), disrupts the bacterial membrane(63)(89), is also an epigenetic and drug resistance modulator and can be a potential target for new therapeutic approaches(11,82)(91). Data from ongoing studies(12) suggest the efficacy of EGCG in reversing the MRSA resistance phenotype *in vitro* and observed the antimicrobial potential and synergistic effect of EGCG against various antibiotics in strains isolated from hospital-acquired infections and nasopharyngeal colonization in vitro.

EGCG has been found to upregulate transcription of several genes(11,12,83,85). In(86) it was demonstrated that in *S. aureus* treated with EGCG there is upregulation of transcription genes involved in membrane transport (to recover membrane function); and downregulation of genes involved in toxin production and stress response. EGCG has also been shown to cause significant harm to *S. aureus.* at a concentration of 500 mg/L, which is about the same as the EGCG content of green tea (a cup of regular tea has about 800 mg/l of EGCG)(87).

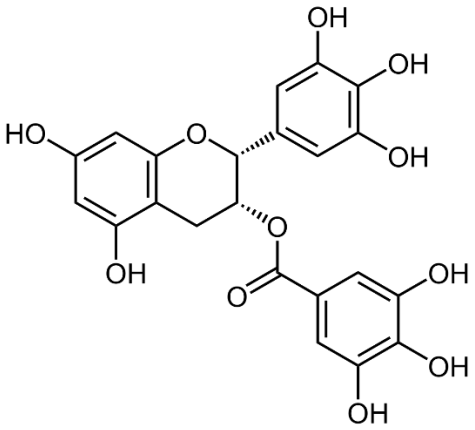

Figure 3 – EGCG molecule. Adapted from (93)

-2.6.3 Transcriptional effects of EGCG in toxin associated genes

In(86) authors demonstrated that EGCG exposure altered the transcription of several genes, in *S. aureus*. Genes implicated in toxin generation and stress response had lower transcription levels, including the tree genes chosen for this study: *hlgA*, *hlgB* and *hly*. Genes involved in membrane transport had higher transcription levels (to recover membrane function), and that as a result, EGCG can potentially operate as a natural antibacterial agent, inhibiting *S. aureus* growth and toxin

generation – which will result in adherence to the advice of the WHO(3, 4, 5, 6), as previously mentioned.

The various haemolysins known as α, β, ∆, and gamma (PVL) haemolysins primarily mediate *S. aureus* lysis of red blood cells(93). Also, *S. aureus* produces two types of two-component pore-forming toxin, g-hemolysin (Hlg) and Leukocidin, which consist of Hlg2 and LukF, and LukS and LukF, respectively(73). The genes of the Hlg/Luk components, *hlg2*, *lukS* and *lukF*, form a gene cluster in this order and transcribe into hlg2 and lukS-F mRNAs(84). "The *lukF*/*S-hlg*, *hlgA*, and *hla* genes encode for hemolysins and leucocidin components (…) *HlgA* is a valid virulence factor and plays a role for the non-canonical pairing of leukotoxins in the pathogenesis of *S. aureus* strains"(96).

Alpha-toxin, or alpha-hemolysin (Hla): is the principal cytotoxic agent produced by *S. aureus* and the first member of the pore-forming beta-barrel toxin family to be discovered. Alpha-toxin binds to the membrane of eukaryotic cells resulting in the release of low-molecular weight molecules and leading to an eventual osmotic lysis(37,85).

Gamma-hemolysin is a toxin that also seems to act by forming pores in the membrane of the cell(86)(99) (or beta-barrel pore-forming toxins that are secreted from the bacteria as monomers(94). It has a hemolytic and a leucotoxic activity(86)(99)

HlgA – gamma haemolysing subunit A

HlgB - gamma haemolysing subunit B

The *hly* gene on the *S. aureus* chromosome is an alpha-haemolysin-precursor(99) and encodes the 293-residue protein monomer, which forms heptameric units on the cellular membrane to form a complete beta-barrel pore. This structure allows the toxin to perform its major function, development of pores in the cellular membrane, eventually causing cell death(84).

β-toxin, or sphingomyelinase, is coded by the *hlb* gene and delta-haemolysin is a 26 amino acid peptide encoded by the *hld* gene(97).

Studies refer that alpha-toxin is a major virulence factor in some infections(100), causing lethality and tissue necrosis(101), and that *hly*+ mutant strains are more infectious than *hly*- strains(102), making the study of these genes fundamental to the understanding of several infectious processes.

3- Objectives

Main Objective:

In this study, we aimed to assess EGCG transcriptional effects in genes associated with virulence factors, namely toxin production genes *hlgA*, *hlgB* and *hly,* in MRSA strains with divergent resistance phenotypes.

3.1- Specific research objectives

- Analysis of *hlgA*, *hlgB* and *hly* gene expression by measuring transcriptional levels, using quantitative Real-Time Polymerase Chain Reaction or qRT-PCR, in commensal MRSA strains from healthy volunteers, with no exposure to EGCG and after 24-hour exposure to a concentration of 100µg/ml EGCG.
- Analysis of *hlgA*, *hlgB* and *hly* gene expression by measuring transcriptional levels, using quantitative Real-Time Polymerase Chain Reaction or qRT-PCR, in MRSA strains from hospital infection (bronchoalveolar lavage, blood culture and pressure ulcer biopsy), with no exposure to EGCG and after 24-hour exposure to a concentration of 100µg/ml EGCG.
- Observation of haemolysis phenotype of MRSA strains before and after EGCG exposure.

4- Materials and Methods

This study aimed to assess the differences in gene transcription that EGCG induces in genes associated with virulence factors, namely toxin production genes *hlgA*, *hlgB* and *hly*, in divergent MRSA strains. Thus, we analysed MRSA bacterial strains coming from commensal nasopharynx flora of healthy volunteers and hospital infections, before and after 24-hour exposure to 100µg/ml of EGCG, therefore Real-Time Polymerase Chain Reaction or qRT-PCR was used to find the transcriptional levels of the epigenetic modulators. RNA was extracted from the samples, quantified, and converted to cDNA for the qRT-PCR; the transcriptional levels obtained were evaluated, compared, and correlated with data from(12,31,84). The most resistant strains for commensal and nosocomial infections samples were chosen according to(31), since the utilised samples were the same. Blood agar cultures were performed to allow haemolysis “visualization”.

4.1- Study type

This study is:

- Observational, since there was no manipulation, only observation and analysis.
- Descriptive correlational, since the primary interest was describing relationships among variables, namely, to describe the changes in gene transcription and correlate them with the different resistance profiles of the samples, their origin and synergism previously seen with EGCG and various antibiotics.
- Transversal, since only the transcriptional levels of the selected genes were measured

The variables are:

- Origin of strains, classifying as nominal and bivariate, obtaining as values Commensal Flora and Nosocomial Infections.
- Synergism of EGCG and antibiotics, classified as nominal and bivariate, with values like observed and not observed.
- Transcriptional levels of the toxin production genes, *hlgA*, *hlgB* and *hly*, classifying them as quantitative variables, on the ratio scale, with values obtained after the statistical treatment of the data obtained from the qRT-PCR.

4.2- Characterization of samples of MRSA strains

The sampling method is non-probabilistic, of rational selection as the strains were selected based on the results obtained by Zeferino (2020)(30) that had characteristics considered relevant to the study.

The samples consist of 14 isolates: 7 of them with no exposure to EGCG; and the other 7 the same strains, after a 24hour exposure to a concentration of 100µl/ml ECGC.
These 7 strains are described on the table below:

Table 1 - sample strains, their source, what type of biological sample they are, and their phenotype as synergism with EGCG. Adapted from* (30)

source	strain	Biological sample	Synergism with EGCG
MRSA commensal	MB2	nasopharyngeal exudate	IMIP S; TETRA S; GENTA S; AMOX R full reversal
	MB12		IMIP R full reversal; TETRA S; GENTA S; AMOX R no reversal
	VFXB14		**IMIP S; TETRA S; GENTA S; AMOX R no reversal**
	VFXB16		IMIP S; TETRA S; GENTA S; AMOX R reversal with EGCG ≥100µg/mL
MRSA nosocomial	18	Bronchoalveolar lavage	IMIPS; TETRA S; GENTA R full reversal; AMOX R no reversal
	22	blood culture	**IMIP R no reversal; TETRA S; GENTA S; AMOX R no reversal**
	31	blood culture	IMIP S; TETRA no reversal; GENTA R reversal with EGCG ≥100µg/mL; AMOX no reversal

Phenotypes refer to Zeferino *et al.* (2020)**(63) **IMIP – imipenem; TETRA -Tetracycline; GENTA – gentamicin; AMOX – Amoxicillin; S – sensitive to; R – resistant to. Highlighted in purple are the most resistant strains in each MRSA type - *VFXB14: sensitive to Imipenem, tetracycline, and gentamycin, resistant with no reversal after EGCG to amoxicillin. 22: sensitive to tetracycline and gentamicin, resistant with no reversal after EGCG to imipenem and amoxicillin.

4.3- Laboratory procedures for defrost

Samples had been frozen at -80 ºC. So, they were first slowly defrosted, on dry ice, as to cause the least possible damage.

-4.3.1 Haemolysis phenotype analysis

Samples were cultivated in blood agar, with 24h of incubation, at 37ºC. Each Petri dish was divided in half, with the pre-EGCG treatment on one side and the after-EGCG treatment on the other.

-4.3.2 RNA extraction

Total RNA was extracted using the *nzytech™ NZY Total RNA Isolation kit* (Annex I and Annex II). This kit is intended to be used with bacterial strains.

To ensure that undamaged RNA molecules are retrieved, a denaturing lysis buffer containing guanidine thiocyanate, which inactivates RNases cells, is used. The use of ethanol enables for the selective binding of whole RNA to the silica membrane while contaminants are eliminated. The DNase solution is applied to the silica membrane to prevent DNA contamination. Finally, a pure RNA eluate in an RNase-free aqueous solution is achieved.

-4.3.3 RNA quantification

Using *NanoDrop One*© from *ThermoFisher*™, against water, microvolume spectrophotometry was employed to quantify the quantity of RNA in all isolates. This equipment can compute the absorbance ratios A260/280, which control the quality of RNA extraction, in addition to determining the RNA concentration (ng/L) - Close optimal values of 2.0 for “pure” RNA, and A260/230 which may indicate contamination with various compounds (phenols, guanidine, magnetic particles, carbohydrates and proteins).

-4.3.4 Conversion to cDNA

Firstly, the appropriate RNA concentrations for each strain were obtained, and then the volume necessary for the normalization of the RNA content used in 100ng, diluted in RNase-free water, was calculated. 100ng of total RNA from each strain were reverse transcribed into cDNA using *Applied Biosystems™ TaqMan™ Reverse Transcription* (*Invitrogen™, Thermo Fisher Scientific Inc.*) reagents, in a reduced Reverse-Transcriptase reaction, with random hexamers as primers, and according to manufacturer’s instructions (Annexe I) (Annex II). Reverse-Transcriptase reactions with the following conditions: 25°C for 10 min, 37°C for 30 min, 95°C for 5 min, maintained at 4ºC, in the thermal cycler.

-4.3.5 qRT-PCR

Done in *Connect™ Real-Time PCR Detection System* (*Bio-rad*), to quantify gene expression. Each reaction was performed 3 times, using *SYBR Green Mastermix* (Bio-rad), Forward Primer, Reverse Primer and free-RNAse water, in a final volume of 20µl. For each triplicate, 3.25µL of cDNA were used, and 0.3µL for the 16sRNA. Control PCRs were performed for all primer combinations without template (figure 1). Cycle conditions were as follows: 95˚C for 3 minutes; 40 cycles of 95˚C for 10 seconds, 60˚C for 30 seconds, with fluorescence reading.

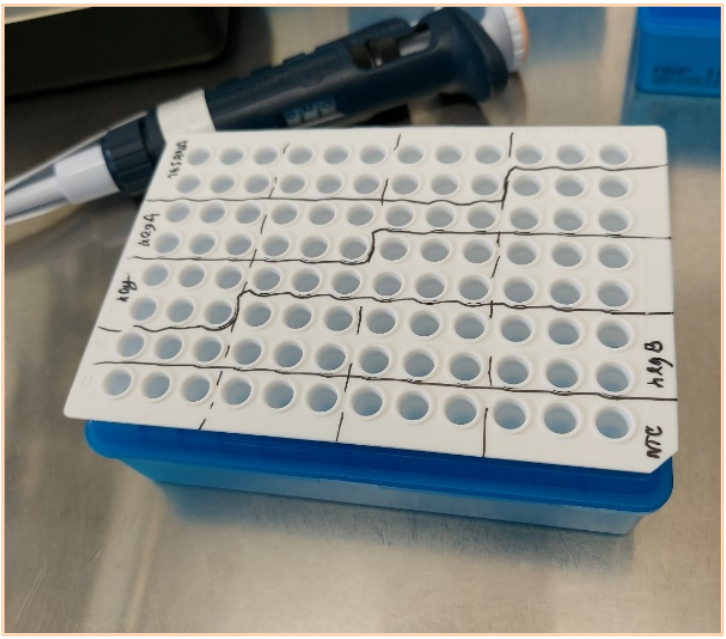

Figure 4 - PCR microplate (NTC – No-template control, 16sRNA housekeeping gene). The same design was used for both pre- and post-treatment with EGCG samples.

Relative quantification was performed by normalizing the threshold cycles (Ct) of the target genes with the average Ct of 16S rRNA, by calculating ΔCt, for each of the measurements. 16S rRNA was used as control since it is a housekeeping gene, so the transcription of the target genes was compared with this one.

Transcriptional levels were analyzed in Microsoft Excel software by calculating **ΔΔCt** as: **ΔCt of treated genes − average of ΔCt of the control gene**. The groups of genes considered for this analysis were: commensal MRSA pre-treatment, commensal MRSA post-treatment, nosocomial MRSA pre-treatment and nosocomial MRSA post-treatment. According to (31), we chose the most resistant strain for each group and compared the transcriptional levels against it. The control is in the aforementioned mathematical expression (average ΔCt control). The averages of Cts obtained for each gene and respective strain contributed to the calculation of ΔCt with treatment. Lastly, the significant differences were obtained by performing a t-test.

Table 2 – primers used in qRT-PCR

Genes	Forward primer (5'→ 3')	Reverse Primer (3'→ 5')	References
16S rRNA	5′-GATCAGCATGCTACGGTGAA-3′	5′-TCGACGGCTAGCTCCTAAAA-3′	(22)
hlgA	5′-AGGCAGTGGCTCATTCAACT-3′	5′-CTTGACCATTCGGTGTAACG-3	(86)
hlgB	5′-GGCAGACAAAGCAGTGCATA-3′	5′-TTAGCGCCATCTTGTCTGTG-3′	(86)
hly	5′-TCTTGGAACCCGGTATATGG-3′	5′-AGCGAAGTCTGGTGAAAACC-3′	(86)

Transcript levels were expressed as a relative percentage and the calculation of the relative difference between gene expressions was performed using the following method:

ΔΔCq(sample) = Cq(gene of interest) – Cq(GAPDH) ΔCq(reference) = Cq(calibrator)-Cq (GAPDH)* ΔΔCq = ΔCq(sample) - ΔCq(reference) Relative quantification = 2-ΔΔCq.

As previously stated, each reaction was performed in triplicate in order to increase the statistical power of the Cq value obtained for each sample.

4.4- Ethics

This work stems from Zeferino, (2020)(30), approved by the Ethics Committee of the ESTeSL, IPL, (ref: CE-ESTeSL-Nº.18-2019.). This study is based on the set of samples given by the aforementioned work.

4.5- Financing

This work was supported by Instituto Politécnico de Lisboa, Lisbon, Portugal for funding the Project "Resistance modulation and epigenetic divergence in resistant phenotypic profiles of Staphylococcus Aureus" (IPL/2020/ EpiResistanceSA _ESTeSL). The study was performed at the H&TRC Health and Technology Research Centre. Authors and H&TRC are grateful for the support of FCT/MCTES through UIDB/05608/2020 and UIDP/05608/2020.

5. Results

5.1– Transcription

For transcriptional analysis, qRT-PCR results were paired together as commensal or nosocomial, pre and post 24-hour exposure to 100 µg/ml EGCG, making four groups, and their transcription compared with data from the most resistant strains, VFXB14 (commensal) and 22 (nosocomial), chosen accordingly to the antibiogram and synergism with EGCG (30).

First, transcriptional levels for the 3 genes were calculated through **ΔCt** (for every gene and sample), normalised with 16sRNA. Then, **ΔΔCt as: ΔΔCt = ΔCt with treatment - ΔCt of control** (most resistant strains, (30)). Finally, a **t-student test** was performed to compare the averages of the samples and ascertain if there were significant differences. Annex IV.

Data is represented by the average of ΔΔCt and the respective standard deviation for each strain, allowing assessment of the distribution of data within the same group for the most resistant strain.

-5.1.1 Transcriptional analysis of MRSA strains with no EGCG exposure

The most resistant commensal strain is VFXB14, and nosocomial is strain 22. Strain MB2 with “sensitive to Imipenem, Tetracycline and gentamicin, and total reversion to amoxicillin” phenotype and strain MB12 with “sensitive to tetracycline and gentamicin, resistant to amoxicillin, and total reversion to imipenem” phenotype. Strain 18 phenotype is “sensitive to imipenem and tetracycline, full reversion to gentamicin and no reversion to amoxicillin” and strain 31 is “sensitive to imipenem, reversion to gentamicin at com EGCG ≥100µg/mL, and no reversion to amoxicillin”.

Table 3 - Average ΔΔCt and its standard deviation for commensal flora and nosocomial infection samples, without treatment, genes hlgA, hlgB and hly.

strain	Commensal (VFXB14)			Nosocomial (22)	
	VFXB16	**MB2**	**MB12**	**18**	**31**
hlgA					
ΔΔCt avg	0.031	2.108	0.005	8.428	12.173
ΔΔCt std	0.004	0.397	0.001	1.884	1.958
hlgB					
ΔΔCt avg	0.033	1.575	0.037	30.403	9.035
ΔΔCt std	0.003	0.186	0.006	9.241	1.826
hly					
ΔΔCt avg	0.015	0.000	0.153	50.342	5.892
ΔΔCt std	0.013	0.000	0.016	17.637	0.655

-*hlgA* gene, commensal flora

It can be observed strain MB2 (which phenotype is “total reversion to amoxicillin after exposure to EGCG”) has transcription levels about two times higher than the most resistant strain, whereas the other strains have slightly lower levels, similar to each other: hlgA (Log2FC (MB2) =-2.108 ± 0,397). Both strains VFXB16 (hlgA (Log2FC (VFXB16) =-0.031 ± 0.004)) and MB12 (hlgA (Log2FC (MB12) =-0.005 ± 0.001)) have quite similar transcriptional levels to the most resistant strain.

-*hlgB* gene, commensal flora

It is observable strain MB2 has transcriptional levels about 1 time higher than the most resistant strain (hlgB (Log2FC (MB2) =-1.575 ± 0.186)), whereas the other strains have slightly lower levels that are comparable to one another (hlgB (Log2FC (VFXB16) =-0.033 ± 0.003); (hlgB (Log2FC (MB12) =-0.037 ± 0.006)).

-*hly* gene, commensal flora

We can see that MB2 had no difference in its transcriptional levels (hly (Log2FC (MB2) =0.000 ± 0.000)), whereas the other two strains had lower transcriptional levels, when compared to the most resistant strain, in particular VFXB16 (which phenotype is “no reversion with exposure to EGCG≥100µg/ml”): hly (Log2FC (VFXB16) =-0.015 ± 0.013). (hly (Log2FC (MB12) =-0.153 ± 0.016)).

-*hlgA* gene, nosocomial infections

It can be observed a generalized increase in transcriptional levels, especially strain 31 (“sensitive to imipenem, reversion to gentamicin at com EGCG ≥100µg/mL, and no reversion to amoxicillin”) with hlgA (Log2FC (31) =-12.173 ± 1.958). (hlgA (Log2FC (18) =-8.428 ± 1.884)).

-*hlgB* gene, nosocomial infections

Both strains have higher transcriptional levels than the most resistant strain, especially strain 18 which is 3 times higher (“no reversion to amoxicillin”), as can be seen: (hlgB (Log2FC (18) =-30.403 ± 9.241)). (hlgB (Log2FC (31) =-9.035 ± 1.826)).

-*hly* gene, nosocomial infections

Again, transcriptional levels of strain 18 are much higher than the ones of the most resistant strain: (hly (Log2FC (18) =-50.342 ± 17.637), and also about 10 times higher than strain 31: (hly (Log2FC (31) =-5.892 ± 0.655)).

-5.2.1 Statistical analysis of transcriptional-MRSA strains with no EGCG exposure

A Student's t test was used to check for statistical differences between genetic expression of samples and the most resistant strain. Student's t test was used to compare means, considering $p<0.001$ significantly different.

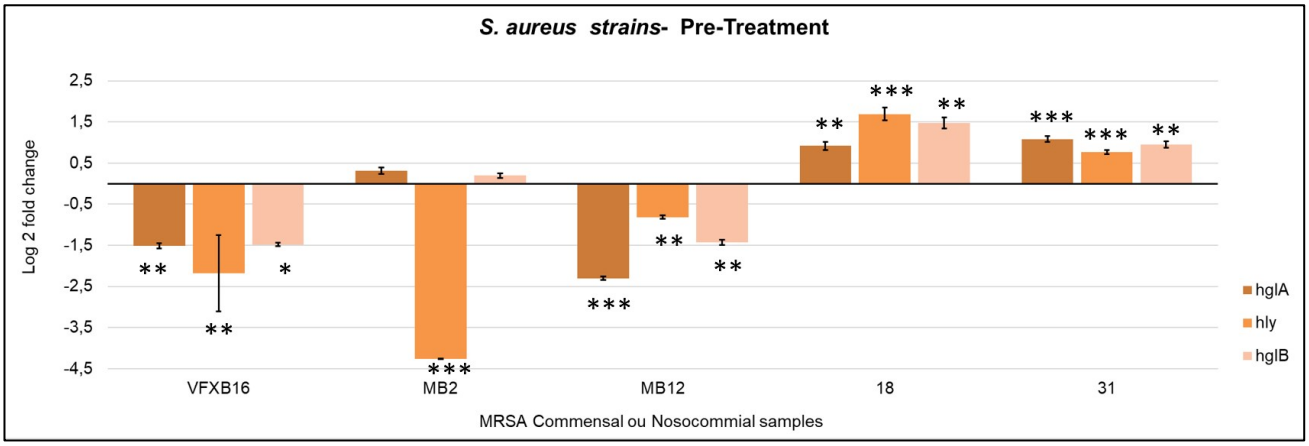

*Figure 5 - Statistical analysis of transcriptional-MRSA strains with no EGCG exposure. 16S rRNA was used as a cell maintenance gene and the relative quantification was performed for normalization of Cts. The error bars represent the standard deviation between independent treatments and triplicates of qRT-PCR. The values significant statistics, which were compared with the most resistant strains and calculated with Student's t test, they are illustrated as: ***p <0.01; ** p <0.001; *p<0.05.).*

In commensal strains, the most sensitive phenotype shows different patterns of gene expression when compared to the more resistant strains.

Strain MB2 which is the most sensitive of commensal strains (phenotype “sensitive to Imipenem, tetracycline, and gentamicin; and full reversal to amoxicillin at 25µg”) had the highest variation in transcriptional levels among genes, with *hly* having the highest and statistically significant increase in transcriptional levels ($p<0,001$) in comparation to the other genes.
In nosocomial strains this did not happen. Both strains 18 (“full reversal to gentamicin and no reversal to amoxicillin”) and 31 (“reversion to gentamicin, no reversion to amoxicillin”) had similar increased levels of transcription in all genes, and always statistically significant ($p<0,001$). Still, *hly* was again the gene with the highest increase in transcriptional levels, though only slightly.
Strain MB12 (“sensitive to tetracycline and gentamicin, full reversion to imipenem, and no reversal to amoxicillin”) had increased transcriptional levels in all genes, and statistically significant ($p<0,001$). *HlgA*, not *hly,* was the gene with the highest increase in transcriptional levels for this strain.
Strain VFXB16 (“reversal to amoxicillin with EGCG ≥100µg/mL, sensitive to others”) had also increased transcriptional levels, similar for all genes.

-5.2.2 Transcriptional analysis of MRSA strains, with 24 hour, 100µg/ml EGCG treatment.
The most resistant commensal strain is VFXB14, and nosocomial is strain 22. Strain MB2 with “sensitive to imipenem, tetracycline and gentamicin, and total reversion to amoxicillin” phenotype and strain MB12 with “sensitive to tetracycline and gentamicin, resistant to amoxicillin, and total reversion to imipenem” phenotype. Strain 18 phenotype is “sensitive to imipenem and tetracycline, full reversion to gentamicin and no reversion to amoxicillin” and strain 31 is “sensitive to imipenem, reversion to gentamicin at com EGCG ≥100µg/mL, and no reversion to amoxicillin”.

Table 4 - Transcriptional analysis of MRSA strains, with 24 hour, 100µg/ml EGCG treatment

strain	**Commensal** (VFXB14)			**Nosocomial** (22)	
	VfXB16	**MB2**	**MB12**	**18**	**31**
hlgA					
ΔΔCt avg	1.210	0.705	0.529	0.155	0.060
ΔΔCt std	0.030	0.083	0.084	0.035	0.006
hlgB					
ΔΔCt avg	0.943	0.201	0.578	161.871	145.450
ΔΔCt std	0.042	0.018	0.129	7.138	14.647
hly					
ΔΔCt avg	0.488	0.001	0.731	334.115	334.340
ΔΔCt std	0.012	0.000	0.111	58.649	22.717

-*hlgA* gene commensal flora strains

The transcriptional levels of strain VFXB16 are about 1 time higher than the most resistant strain: hlgA (Log2FC (VFXB16) =-1.210 ± 0.030), and the others are only slightly higher: hlgA (Log2FC (MB2) =-0.705 ± 0.083), hlgA (Log2FC (MB12) =-0.529 ± 0.084).

-*hlgB* gene commensal flora strains

In general, transcriptional levels slightly higher than those of the most resistant strain, are observable: hlgB (Log2FC (MB2) =-0.201 ± 0.018), hlgB (Log2FC (MB12) =-0.578 ± 0.129), hlgB (Log2FC (VFXB16) =-0.943 ± 0.042).

-*hly* gene commensal flora strains

In this case, strain MB2 has almost similar transcriptional levels, then the ones of the most resistant strain hly (Log2FC (MB2) =-0.001 ± 0.000), while the others have slightly higher ones: hly (Log2FC (MB12) =-0.731 ± 0.111) and hly (Log2FC (VFXB16) =-0.488 ± 0.012).

-*hlgA* gene nosocomial infection samples

Transcriptional levels of sample 18 (which phenotype is “no reversion to amoxicillin”) are slightly higher than the ones from the most resistant strain: hlgA (Log2FC (18) =-0.155 ± 0.035), and in the case of sample 31 (“sensitive to imipenem, reversion to gentamicin at com EGCG ≥100µg/mL, and no reversion to amoxicillin”), are mostly similar: hlgA (Log2FC (31) =-0.060 ± 0.006).

-*hlgB* gene nosocomial infection samples

In this case there was a vast increase in transcription, for both strains, in comparison to the most resistant strain, but higher for sample 18 (“no reversion to amoxicillin” phenotype): hlgB (Log2FC (18) =-161.871 ± 7.138); hlgB (Log2FC (31) =-145.450 ± 14.647).

-*hly* gene nosocomial infection samples

This last group had the highest increase in transcriptional levels in comparison to the most resistant strain’s ones (334 times higher, and similar for both strains): hly (Log2FC (18) =-334.115 ± 58.649), hly (Log2FC (31) =-334.340 ± 22.717).

-5.2.2.1 Statistical analysis of transcriptional-MRSA strains with 24 hour, 100µg/ml EGCG exposure

A Student's t test was used to check for statistical differences between genetic expression of samples and the most resistant strain. Student's t test was used to compare means, considering p<0.001 significantly different. Tables with values can be found on Annex IV.

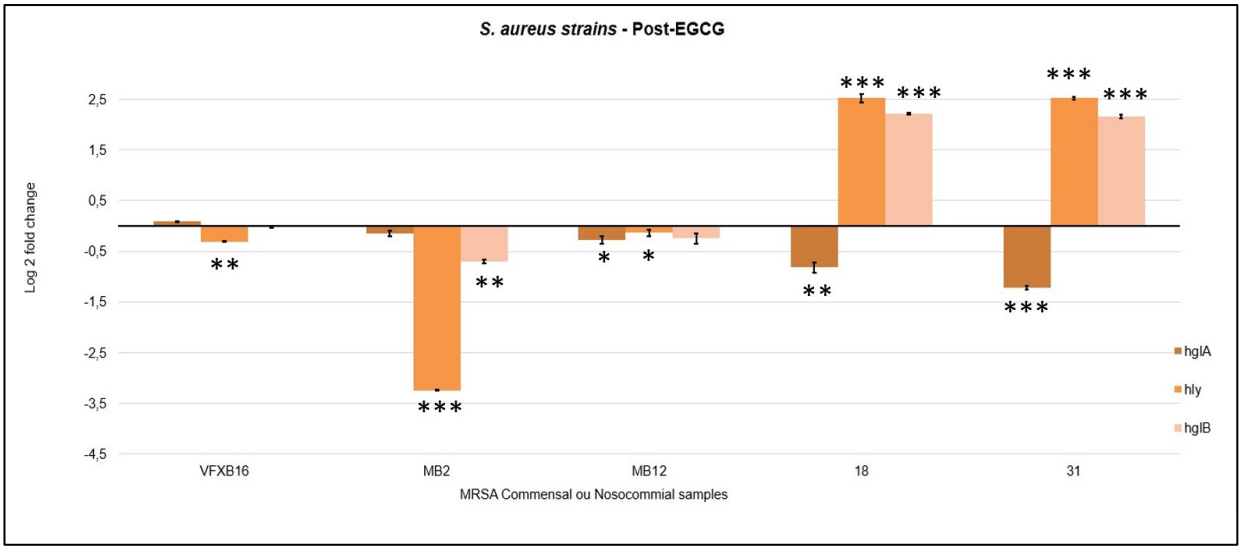

*Figure 6 - Statistical analysis of transcriptional-MRSA strains with 24 hour, 100µg/ml EGCG exposure. 16S rRNA was used as a cell maintenance gene and the Relative quantification was performed for normalization of Cts. The error bars represent the standard deviation between independent treatments and triplicates of qRT-PCR. The values significant statistics, which were compared with the most resistant strains and calculated with Student's t test, they are illustrated as: ***p <0.01; ** p <0.001; *p<0.05.).*

After exposure to 100µg EGCG, the pattern was similar: Again, strain MB2 which is the most sensitive of commensal strains (phenotype “sensitive to imipenem, tetracycline, and gentamicin; and full reversal to amoxicillin at 25µg”) had the highest variation in transcriptional levels among genes with *hly* having the highest and statistically significant increase in transcriptional levels (p<0,001). Again,

both strains 18 (“sensitive to imipenem and tetracycline, full reversal after EGCG of resistant phenotype for gentamicin and no reversal after EGCG to resistant to amoxicillin”) and 31 (“sensitive to imipenem, reversion to gentamicin at com EGCG ≥100µg/mL, and no reversion to amoxicillin”) had similar increased levels of transcription in all genes, and always statistically significant ($p<0,001$) (except for *hlgA* in strain 18).

Strain MB12 (“full reversal to amoxicillin, sensitive to others”) had increased transcriptional levels on all genes, higher and statistically significant for *hly* ($p<0,001$).

Strain VFXB16 (“reversal to amoxicillin with EGCG ≥100µg/mL, sensitive to others”) also had increased transcriptional levels on all genes, higher in *hly*.

When comparing before and after EGCG treatment in commensal strains, there was a decrease in transcriptional levels, with the exception of strain MB2, for genes *hlgA* and *hlgB*.

In nosocomial strains the performance was similar. In strain 18 there was a general decrease in transcription levels, statistically significant.

Strain 31 behaved similarly, having a decrease in transcription levels, statistically significant, apart from gene *hlgA* which had an increase in transcription levels from pre- to post-treatment with EGCG.

The highest decrease in transcriptional levels occurred in gene *hlgA* in strain MB12.

5.3 – Haemolysis phenotype analysis

In this study, we also performed a blood agar culture of the studied MRSA strains, with and without EGCG exposure. As previously mentioned, in *Staphylococcus aureus* haemolysin is a key virulence factor, causing for instance, the typical β-haemolytic phenotype, also known as the complete haemolytic phenotype(82,94).

After 24 hours, it is possible to observe a tendency of reduction of the phenotype, which may demonstrate EGCG is reducing the expression of haemolysis inducing genes. Below (Figure 7) is an example picture of the blood agar cultures:

Figure 7 -Front and back images from bacteria blood agar culture in petri dishes: “T0” stands for pre-treatment and “EGCG” stands for after treatment.

6- Discussion

WHO describes resistance to antibiotics as a persistent global challenge, and has been urging for the development of new, naturally based substances able to efficiently fight infection(1,7,38,103–106). World epidemiological surveillance data shows that MRSA are a word wide problem, with high associated mortality and/or health rates(1,104) and a major health concern in our country(2,36,107)
The assessment of EGCG transcriptional effects in these genes associated with divergent resistance phenotypes is of foremost importance, since virulence genes such as *hlgA, hlgB* and *hly*, are highly relevant, are toxin production genes, and have haemolytic and leucotoxic activity(37)(84)(86)(99).
Authors of(86) used *S. aureus* NCTC 8325 strains attained from the National Collection of Type Cultures of Public Health England, cultured in Tryptic Soy Broth. These were treated with EGCG at 500mg/L, for 1 and 4 hours. Their results showed after 1 hour of EGCG administration, the transcription levels of 75 genes increased while those of 72 genes decreased. The transcription levels of 109 genes increased after a 4-hour treatment with EGCG, while the levels of 154 genes decreased. The genes that exhibited reduced transcriptional levels were genes involved in toxin production and stress response, including the tree genes chosen for this study: *hlgA, hly*, and *hlgB;* while the genes that displayed increased transcriptional levels were associated with membrane transport. Thus, it is possible to hypostasise that EGCG has the potential of being a natural antibacterial agent, inhibiting *S. aureus* growth and toxin generation.
In this context, we aimed to assess the effects of EGCG exposure in the transcriptional patterns of *hlgA, hlgB* and *hly* in *S. aureus* strains with divergent resistance phenotypes.
Our results show that divergent resistance phenotypes are linked to differential transcriptional expressions of the studied genes. In pre-treatment commensal strains, the most sensitive strain had a higher variation on transcriptional levels, with *hly* being the gene with the highest and statistically significant variation. This did not happen in nosocomial strains which is probably because these commensal strains have less multi resistance and, in general, lesser pathogenicity since they are the source of colonisations rather than infections(108,109). Antibiotic resistance in nosocomial stains poses a problem to current medicine as it results in "*higher medical costs, longer hospital stays, and an increase in mortality*"(1). It poses a threat to our ability to treat prevalent infectious diseases, mostly because without efficient antibiotics for infection prevention and treatment, organ transplants, chemotherapy, and procedures such as caesarean sections become significantly riskier(1,38,103,104)
Also, it is known that as *S. aureus* progresses from asymptomatic colonisation to an invasive pathogen responsible for nosocomial infections, the bacteria accumulate virulence factors such as genes for adhesin synthesis, immune evasion genes, and toxins(110).

Regarding *hlgA* gene transcription data, we observed higher transcription levels on nosocomial strains, than commensal ones, which is to be expected since genes of virulence factors are probably more expressed on more pathogenic strains(108,109). When comparing the transcriptional levels of *hlgA* before and after treatment with EGCG, on commensal strains there was, on average, a statistically significant decrease, but a (slight) increase on nosocomial strains.

Furthermore, *hlgB* transcriptional levels were also upregulated in commensal strains than nosocomial ones. When comparing the transcriptional levels of *hlgB* before and after treatment with EGCG, there was, on average, a decrease on transcriptional levels after EGCG exposure, also statistically significant. This decrease was not the highest on the most sensitive strains, though. This may mean that EGCG effect is not as robust on nosocomial strains as it is on commensal ones, once again because commensal strains are the source of colonisations rather of infections, they have less multi resistance and, in general, lower pathogenicity(108,109).

Before EGCG exposure, *hly* gene transcription had higher levels than the other two analysed genes. Again, this gene's transcriptional levels were also higher for commensal strains than nosocomial ones. Also, when comparing the transcriptional levels of *hly* before and after treatment with EGCG, there was, on average, a statistically significant decrease on transcriptional levels after EGCG exposure ($p \leq 0.001$). As in *hlgB*, this decrease was also not the highest on the most sensitive strains. The higher effect of exposure to EGCG is shown in the transcriptional expression of *hlgA* in strain MB12 (with the decrease in transcriptional levels being the most substantial).

In general, *hly* had higher transcriptional levels than the other two genes, and *hlgA* had higher transcriptional levels than *hlgB*. These results are like (Kitichalermkiat *et al*)(86), were *hlgA* also had higher transcriptional values than *hlgB* (about double). In a study about the repressive effect of haemin on the transcription of haemolysins(111), it was also found that most haemolysin-associated genes varied around 120-fold, while *hlgA* transcription varied only by a factor of 3. Also, in(112), a study of the genomic analysis of MRSA exposed to manuka honey, it was found that the most notable changes in gene expression (provided by the microarray data) indicated that manuka honey had a marked effect on the expression of important MRSA virulence determinants, such as haemolysins. *HlgA* was among the most downregulated genes (with fold change −54), (although *hlgB* and *hly* were not studied). So, it seems possible *hlgA* has more changes in transcription than *hlgB*, while *hly* seems to be more expressed than the other two.

Overall, our data results demonstrated that divergent expression patterns of *hlgA*, *hlgB* and *hly* genes associated with divergent resistance phenotypes of *S. aureus* previously identified, where observed. There were also differences in transcriptional expression associated with the bacteria's origin (commensal vs acute infection)(31), with pre-treatment commensal strains having higher transcriptional levels than nosocomial ones. Overall, we observed a decrease in transcription levels

after EGCG exposure, and the effect of EGCG was stronger on nosocomial strains than commensal ones, although it was not more observable in more sensitive strains than the most resistant ones.
This tendency has also been reposted in several published studies demonstrating the clear molecular divergence in these strains which may explain the obtained results(109,110,112–115). Furthermore, bacteria virulence factor genes are likely to be more expressed on more pathogenic strains(109,110,112–115), because of the bacteria's ability to adapt to harsher conditions. Also, the higher variation in transcriptional levels happened in the most sensitive pre-treatment commensal strain (with *hly* being the gene with the highest and statistically significant variation).
Additionally, when comparing our results with (Mira, 2020)(31) it is possible to see similar patterns associated with epigenetic modulators. Mira, 2020 findings showed that divergent resistance phenotypes are associated with different transcriptional expressions of the epigenetic modulators tested, more apparent in the most sensitive strains. When compared to nosocomial strains, commensal strains are more sensitive to EGCG exposure, with the latter showing transcription levels with more divergent values significantly elevated or decreased. Also, in some cases, exposure to EGCG had no effect on transcriptional patterns in nosocomial strains, implying that they are highly virulent. Studies have found that in bacteria like *S. aureus*, *S. epidermidis, E. coli*, and other pathogenic ones, nosocomial strains are much more virulent than commensal ones(109,110,112–115). When comparing our results with(31) the similarities, such as the stronger influence of EGCG in changing the expression of both epigenetic modulators and virulence factor genes in nosocomial strains when compared to commensal strains may indicate a correlation between epigenetic modulation and the expression of virulence factors. These results agree with published studies that associate epigenetic modulators to virulence factors(116)(31).
Finally, regarding the strains in which EGCG exposure resulted in a reversion of the previous resistance phenotype, for gene *hlgA*, the transcription levels, in the strain with more reversion of previous phenotype after EGCG exposure, decreased ((hlgA (Log2FC (VFXB16) =-0.031 ± 0.004)) to (hlgA (Log2FC (VFXB16) =-1.210 ± 0.030)); but increased in the nosocomial one (strain 18 - full phenotype reversal to gentamicin), (hlgA (Log2FC (18) =-8.428 ± 1.884) to hlgA (Log2FC (18) =-0.155 ± 0.035). These results emphasize the genomic divergences between commensal and nosocomial strains(109,110,112–115). On the other hand, data analysis of genes *hlgB* and *hly* demonstrated an overall similar behaviour: transcriptional levels (strains with higher resistance phenotype reversion after EGCG exposure) decreased, after EGCG exposure in comparation with pre-treatment data, in both commensal and nosocomial strains (except for gene *hlgB* in strain MB2). These results are in agreement with previous published data in which *hlgB* and *hly* genes also showed similar pattern (downregulation, after EGCG exposure, in similar patterns, with *hlgB* (-2.09), and *hly* (-2.51)), while *hlgA* was also downregulated but by (-4.23) ((1-log2-fold))(86).

7 – Conclusions

In this study, the analysed data demonstrated a clear effect on S. *aureus hlgA, hlgB* and *hly* transcriptional expression associated with EGCG exposure, particularly a decrease in transcriptional levels although with divergent patterns in *hlgA* and *hlgB*/*hly,* which suggests a lower susceptibility of *hlgA* 's expression to EGCG than *hlgB*/*hly.*

Overall, our results show that divergent resistance phenotypes are associated with differential transcriptional expressions of the studied genes: upregulation in nosocomial strains, when compared to commensal ones, and downregulation after exposure to EGCG. Data suggests a correlation between epigenetic modulation and the expression of virulence factors namely haemolysins.

This study increases the scientific knowledge and has allowed to produce accumulating evidence to suggest that EGCG could be a novel therapeutic option in the fight against antibiotic resistance.

8 - Study limitations and future perspectives

- As one of the study's drawbacks, we note the reduced number of used strains. Ideally, we would raise this number in future studies, allowing for a more comprehensive statistical analysis. We would also like to include more EGCG concentrations and time of exposure (more than 24hours), as, for example, in(13,65).
- As an innovation, we refer this study's in vitro observation of differences in transcription in commensal and nosocomial strains of *S. aureus* resistant phenotypic profiles, with a focus on genes associated with toxin production and haemolysis and connected to EGCG exposure.
- Since studies refer EGCG exposure damages the cell wall(63)(89) we suggest broadening the spectrum of used antibiotics, especially the ones more clinically relevant and which harm the cell wall and so increase the success in reversing the resistance phenotype in synergism with the EGCG.
- Taking our results into consideration, we recommend the study of more genes, and their interaction with EGCG, especially all mentioned in(89) but not limited to them, to better understand the mechanisms of EGCG influence in *S. aureus*.
- Since this study suggests a correlation between epigenetic modulation and the expression of virulence factors, we endorse these two "characteristics" together in future research.
- Because there is still a lot to learn about the relationship between *S. aureus* antibiotic resistance and virulence genes and human diseases, and there is the necessity to determine which of the bacteria's virulence factors are expressed in the context of infection to better understand pathogenesis(72), we also recommend the study of the other many genes involved in virulence and their interactions. Especially since during the infection process genes associated with virulence factors are usually upregulated.
- (117) cited in(87), suggests, in a study with *E. coli*, "EGCG affects the multidrug efflux pump system, and that this system is important for recovering from injury caused by EGCG", so studying how EGCG affects the efflux pump system in S. aureus would also be recommended.
- The extreme need to identify new solutions to fight bacterial infection, namely *S. aureus* infections, justifies the study of new and emerging compounds, with few or no side-effects, that can have an impact on infections in the hospital setting, such as a decrease in the occurrence of illnesses in the community. Incidentally, the major future prospect is related to the identification of EGCG as an antibacterial chemical and/or coadjuvant therapeutic agent by medicine regulatory agencies on a national and international scale.
- With this in mind, the research of pathogen genomics and the diagnosis and detection of virulence factors is also fundamental, leading to novel treatments for infectious diseases.

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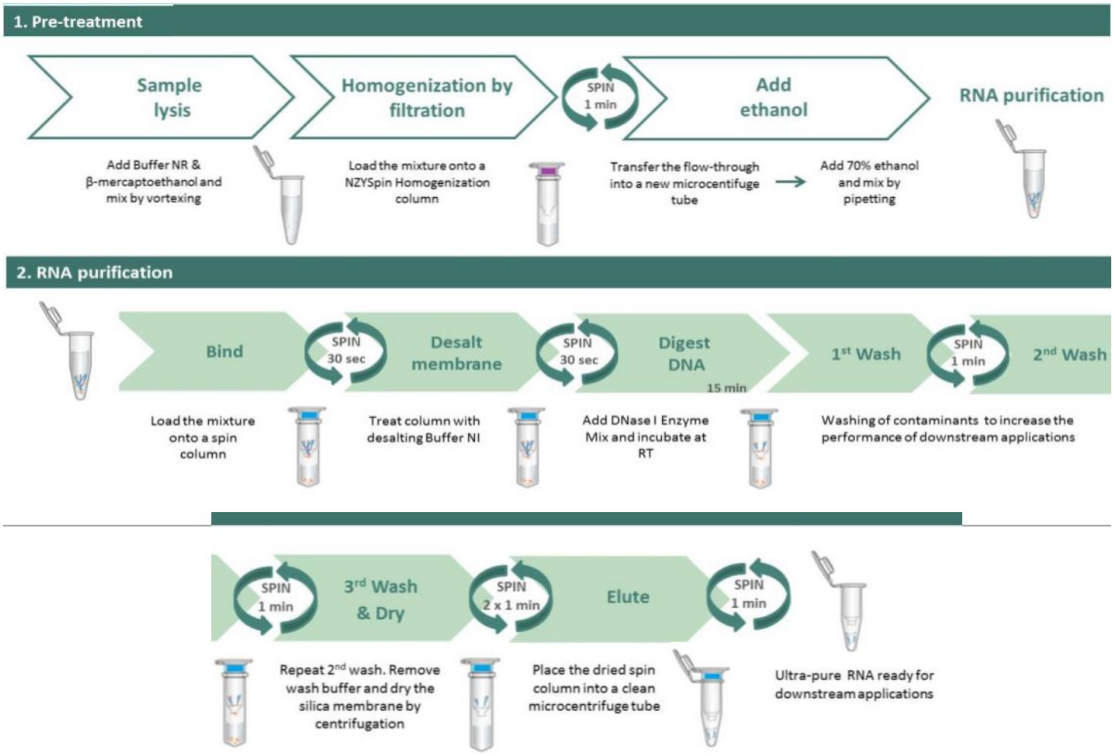
10- Annexes

Annex I - RNA Extraction Protocol, NZY Total RNA Isolation kit, nzytech™



Annex II – NZY TOTAL RNA ISOLATION KIT

Description

NZY Total RNA Isolation kit is designed for the easy purification of total RNA of highest integrity (longer than 200 bases) from a variety of sources such as animal tissues, bacteria cells and cell cultures. This method uses a denaturing lysis buffer containing guanidine thiocyanate, which inactivates cellular RNases, to ensure the recovery of intact RNA molecules. Ethanol is added to provide selective binding of total RNA into the silica membrane column and impurities are efficiently washed away. To prevent the contamination with DNA, a DNase I solution is directly added onto the silica membrane of the binding column. High-quality RNA is then eluted in RNase-free water. RNA is ready to use for applications like Reverse Transcriptase (RT) PCR, qPCR, in vitro translation or cDNA synthesis.

The NZY Total RNA Isolation kit is optimized to isolate up to 70 µg of RNA/column with an $A_{260/280}$ ratio between 1.9 and 2.1 from up to 30 mg of animal tissue, 1×10^9 bacteria cells or 5×10^6 of cultured cells. We suggest not exceeding the maximum recommended starting material to prevent a reduction in yield and purity.

Storage conditions and reagents preparation

All kit components can be stored at room temperature (20-25 °C), and are stable till the expiry date.

Before use, add 0.550 mL of RNase-free water to the DNase I vial. DNase I is sensitive to physical denaturation so do not vortex but instead mix gently by inverting the tube. The reconstituted DNase I should be stored at -20 °C and is stable for 6 months. Add 50 mL of 100% molecular biology grade ethanol to the NWR2 bottle. Buffers NR and NWR1 contain guanidine salts. Wear gloves and goggles when using this kit.

System Components

Component	Volume (50 preps)
Buffer NR	25 mL
Buffer NI	25 mL
Buffer NWR1	15 mL
Buffer NWR2 (concentrate)	12.5 mL
RNase-free Water	15 mL
Digestion buffer	5 mL
DNase I (lyophilized)	1 vial
NZYSpin Homogenization Columns (purple rings)	50
NZYSpin Binding Columns (blue rings)	50
Collection tubes (2 mL)	150
Collection tubes (1.5 mL)	50

Guidelines for using NZY Total RNA Isolation kit

- RNA preparation using NZY Total RNA isolation kit can be performed at room temperature. However, isolated RNA should be treated with care because RNA is very sensitive to trace contaminations of RNases. Be certain not to introduce any RNases during the whole purification process. **Wear gloves at all times during RNA preparation and change gloves frequently**. To ensure RNA stability store pure RNA at -20 °C for short-term or at -70 °C for long-term.

- We suggest preparing the Digestion Mix before starting the RNA isolation protocol. For each isolation prepare the exact amount of Digestion mixture required as follows:

DNase I (reconstituted)	10 µL
Digestion buffer	90 µL

Mix by gentle pipetting. Store this mixture on ice.

- Optimal amounts of sample material to use in the preparation of RNA using the NZY Total RNA isolation kit are presented in Table 1.

Table 1. Amount of samples material.

Sample	Amount
Tissue	Up to 30 mg
Cultured cells	Up to 5×10^6 cells
Bacterial cells	Up to 10^9 cells

Protocol for purification of total RNA from animal tissues or cells

I. Sample preparation

Tissue samples: Cut up to 30 mg tissue sample (see table 1) into small pieces, and place it in a RNase-free microcentrifuge tube. Proceed with step II.

Cultured cells: Pellet up to 5×10^6 cultured cells (see table 1) at 6,000 x*g* for 5 min at 4 °C. Discard supernatant and add buffer NR directly to cell pellet. Proceed with step II.

Bacterial cells: Pellet up to 10^9 bacteria cells (see table 1) at 6,000 x*g* for 5 min at 4 °C. Discard the supernatant completely and add buffer NR directly to cell pellet. Proceed with step II.

II. Preparation of total RNA

1. Add 350 µL of buffer NR and 3.5 µL β-mercaptoethanol to the cell pellet or to the disrupted tissue. Vortex vigorously.

Note: *The lysate may be passed through a needle fitted to a syringe to reduce the viscosity.*

2. Apply the lysate into an NZYSpin Homogenization column (purple ring) placed in a 2 mL collection tube. Centrifuge for 1 min at 11,000 x*g*. **Save the flow-through**.
3. Transfer the flow-through into a new 1.5 mL microcentifuge tube. Add 350 µL of 70% ethanol and mix immediately by pipetting up and down. **Do not centrifuge**.
4. Pipette the lysate and load in an NZYSpin Binding column (blue ring). Centrifuge at 11,000 x*g* for 30 s. Discard the flow-through and place the column into a new collection tube.
5. Add 350 µL of Buffer NI and centrifuge at 11,000 x*g* for 30 s. Discard the flow-through and place the column back into the collection tube.
6. For each isolation, prepare the Digestion Mix in a sterile 1.5 mL microcentrifuge tube (as previously explained). Apply 95 µL of the Digestion Mix directly into the centre of the silica membrane of NZYSpin Binding column (blue ring) and incubate at room temperature for 15 min.
7. Add 200 µL of Buffer NWR1 and centrifuge for 1 min at 11,000 x*g*. Discard the flow-through and place the column in a new collection tube.
8. Add 600 µL of Buffer NWR2 and centrifuge at 11,000 x*g* for 1 min. Discard the flow-through and place the column back in the collection tube.

Note: *Ensure that absolute ethanol was added to Buffer NWR2 before use.*

9. Repeat wash with 250 µL of Buffer NWR2 and centrifuge at 11,000 x*g* for 2 × 1 min to dry the column membrane. Discard the flow-through.

10. Place the NZYSpin Binding Column in a clean 1.5 mL RNase-free microcentifuge tube. Add 40-60 µL RNase-free water directly to the column membrane. Centrifuge at 11,000 x*g* for 1 min to elute the RNA. Store the RNA at -20 °C for short-term or at -70 °C for long-term.

Quality control assay

Functional assay

All components of NZY Total RNA isolation kit are tested following the isolation protocol described above. The purification system must isolate 50-70 µg of total RNA/column.

V1901

Certificate of Analysis

Test	Result
Functional assay	Pass

Approved by:

Patrícia Ponte
Senior Manager, Quality Systems

For research use only

nzytech
genes & enzymes

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Annex III – APPROVAL OF THE HIGHER SCHOOL'S ETHICS BOARD OF ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA, ESTESL

CE-ESTeSL-Nº. 81-2021 – Raquel Portanova de Almeida

From Comissão Ética on 2021-11-22 10:32

Details Plain text

REFERÊNCIA INTERNA DO PROJETO: CE-ESTeSL-Nº. 81-2021 – Raquel Portanova de Almeida

TÍTULO DO DE PROJETO: "Efeito modelador da epigalatocatequina-3-galato na transcrição de genes de bombas de efluxo de S. aureus"

TIPO DE PROJETO/ESTUDO: Dissertação Mestrado Tecnologias Clinico-Laboratoriais

REQUERENTE: Raquel Portanova de Almeida

EQUIPA: Prof.ª Edna Ribeiro (ESTeSL-IPL)

INSTITUIÇÃO PROMOTORA: Escola Superior de Tecnologia da Saúde de Lisboa; IPL

Exma. Senhora Professora Doutora Edna Ribeiro

Exma. Senhora Dra. Raquel Portanova de Almeida, Estudante de Mestrado

A Comissão de Ética da ESTeSL (CE-ESTeSL) considera que tendo referido tratar-se de um estudo que prossegue do projeto CE-ESTeSL Nº.18-2019, (com parecer favorável da CE-ESTeSL), na medida em que utiliza as amostras deste. Dado que no corrente projeto são analisadas unicamente as estirpes bacterianas presentes nessas amostras, e não o material biológico propriamente dito dos doentes, não se levantam questões éticas, pelo que a CE-ESTeSL aprovou por unanimidade a emissão de parecer favorável.

O presente parecer tem em consideração a versão submetida da documentação enviada. Eventuais alterações nestes documentos determinam a necessidade de revisão do presente parecer.

Lembramos que todos os estudos que envolvem a autorização dos participantes e a recolha de amostras e dados anonimizados e/ou codificados têm de cumprir com o estabelecido no Regulamento Geral sobre a Proteção de Dados de 27 de abril de 2016.

Por último, solicita-se que, ao abrigo do artº 19 da Lei 21/2014 de 16 de abril e do disposto no nº 23 da atual versão da Declaração de Helsínquia, seja dado conhecimento à CE-ESTeSL do relatório final, com as conclusões do estudo, bem como de eventuais alterações ao protocolo de investigação e demais informações tidas por relevantes.

Aproveitamos ainda para desejar o maior sucesso no desenvolvimento deste trabalho.

A CE-ESTeSL encontra-se inteiramente disponível.

Com os melhores cumprimentos

Rute Borrego

Rute Borrego | Professora Adjunta
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Annex IV – T-STUDENT'S STATISTICS RESULTS TABLES

Table 1: Pre-treatment, commensal control = ΔCt VFXB14

strain	ΔCt VFXB16		
Gene	T test between control and treatment	avg	std
hlgA	0,006	19,00	0,15
hlgB	0,010	18,74	0,10
hly	0,003	19,94	0,03

Table 2: Pre-treatment, commensal control = ΔCt VFXB14

strain	ΔCt MB2		
Gene	T test between control and treatment	avg	std
hlgA	0,051	12,83	0,22
hlgB	0,197	13,18	0,14
hly	0,000	28,62	0,02

Table 3: Pre-treatment, commensal control = ΔCt VFXB14

strain	ΔCt MB12		
Gene	T test between control and treatment	avg	std
hlgA	0,000	21,55	0,08
hlgB	0,001	18,58	0,18
hly	0,001	17,18	0,12

Table 4: Pre-treatment, nosocomial control = ΔCt 22

strain	ΔCt 18		
Gene	T test between control and treatment	avg	std
hlgA	0,001	12,05	0,23
hlgB	0,001	14,34	0,32
hly	0,000	14,67	0,37

Table 5: Pre-treatment, nosocomial control = ΔCt 22

strain	ΔCt 31		
Gene	T test between control and treatment	avg	std
hlgA	0,001	11,64	0,05
hlgB	0,001	16,23	0,03
hly	0,000	17,72	0,13

Table 6: Pre-treatment, nosocomial control = ΔCt 22

strain	ΔCt 31		
Gene	T test between control and treatment	avg	std
hlgA	0,001	11,64	0,05
hlgB	0,001	16,23	0,03
hly	0,000	17,72	0,13

Table 7: Post-treatment, commensal control = ΔCt VFXB14

strain	ΔCt VFXB16		
Gene	T test between control and treatment	avg	std
hlgA	0,066	14,69	0,02
hlgB	0,452	14,19	0,04
hly	0,004	15,72	0,03

Table 8: Post-treatment, commensal control = ΔCt VFXB14

strain	ΔCt MB2		
Gene	T test between control and treatment	avg	std
hlgA	0,067	15,47	0,12
hlgB	0,003	16,42	0,09
hly	0,000	25,46	0,03

Table 9: Post-treatment, commensal control = ΔCt VFXB14

strain	ΔCt MB12		
Gene	T test between control and treatment	avg	std
hlgA	0,013	15,89	0,18
hlgB	0,017	14,72	0,02
hly	0,072	15,14	0,18

Table 10: Post-treatment, nosocomial control = ΔCt 22

strain	ΔCt 18		
Gene	T test between control and treatment	avg	std
hlgA	0,013	15,89	0,18
hlgB	0,017	14,72	0,02
hly	0,072	15,14	0,18

Table 11: Post-treatment, nosocomial control = ΔCt 22

strain	ΔCt 31		
Gene	T test between control and treatment	avg	std
hlgA	0,000	15,66	0,11
hlgB	0,000	11,67	0,12
hly	0,000	11,68	0,08