Antibiotherapy in association with Epigallocatechin-3 gallate (EGCG) is an effective alternative for infections caused by methicillin-resistant *Staphylococcus aureus*?

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ABSTRACT: Antimicrobial resistance of human pathogens such as Methicillin-Resistant *Staphylococcus aureus* (MRSA), is globally defined as a major public health concern. Currently, several new therapeutic approaches are being developed with the aim find an alternative to treat these infections, including the use of natural compounds with epigenetic modulation potential such as green tea catechins. In green tea, Epigallocatechin-3 gallate (EGCG) is the most abundant and medically relevant catechin, with anti-inflammatory, antioxidant, anti-carcinogenic, and antimicrobial properties as well as synergistic effects reported for several antibiotics. The search for new therapeutic alternatives has led to the development of studies regarding the EGCG effect in *S. aureus* virulence factors and transcriptional modulation. Several studies, including from our research group, have demonstrated that EGCG exposure is able to affect the bacteria transcriptional pattern in numerous genes. Transcriptional effects were reported in genes implicated in toxin production, such as *hly,* which encodes for an alpha-haemolysin-precursor and *hlgA, hlgB,* the gamma haemolysin subunits A and B, respectively, in the epigenetic modulator *orfx* (a *staphylococci methyltransferase*) and in genes involved in resistance responses (*spdC* and *WalKR).* Moreover, increasing evidence has demonstrated potential correlations between epigenetic modulation and the expression of virulence factors including haemolysins. It is clear that EGCG should be considered as a new compound for antimicrobial treatment and/or therapeutic adjuvant against antibiotic-resistant microorganisms even in divergent phenotypic resistance strains.

Keywords: EGCG; MRSA; Antibiotic resistance; Transcription modulation; Haemolysins.

Antibioterapia em associação com Epigalocatequina-3 galato (EGCG) é uma alternativa eficaz para infeções causadas por *Staphylococcus aureus* resistente à meticilina?

RESUMO: A resistência antimicrobiana de patógenos humanos, como *Staphylococcus aureus* resistente à meticilina (MRSA), é globalmente definida como uma grande preocupação de saúde pública. Atualmente, várias novas abordagens terapêuticas estão a ser desenvolvidas com o objetivo de encontrar uma alternativa para tratar essas infeções, incluindo o uso de compostos naturais com potencial de modulação epigenética, como as catequinas do chá verde. No chá verde, a Epigalocatequina-3 galato (EGCG) é a catequina mais abundante e clinicamente relevante, com propriedades anti-inflamatórias, antioxidantes, anticancerígenas e antimicrobianas, bem como efeitos sinérgicos relatados para vários antibióticos. A busca por novas alternativas terapêuticas tem levado ao desenvolvimento de estudos sobre o efeito do EGCG em fatores de virulência e modulação transcricional de *S. aureus*. Vários estudos, inclusive do nosso grupo de investigação, demonstraram que a exposição ao EGCG é capaz de afetar o padrão transcricional da bactéria em vários genes. Efeitos de transcrição foram relatados em genes implicados na produção de toxinas,

como *hly*, que codifica para um precursor da alfa-hemolisina e *hlgA*, *hlgB*, as subunidades A e B da gama-hemolisina, respetivamente, no modulador epigenético *orfx* (um estafilococo metiltransferase) e em genes envolvidos em respostas de resistência (*spdC* e *WalKR*). Além disso, evidências crescentes demonstraram correlações potenciais entre a modulação epigenética e a expressão de fatores de virulência, incluindo hemolisinas. Assim, o EGCG deve ser considerado como um novo composto para tratamento antimicrobiano e/ou adjuvante terapêutico contra microrganismos resistentes a antibióticos, mesmo em estirpes com fenótipos de resistência divergentes.

Palavras-chave: EGCG; MRSA; Resistência a antibióticos; Modulação da transcrição; Hemolisinas.

Introduction

The World Health Organization currently describes antimicrobial resistance of human pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), as a global health hazard. MRSA is associated with high rates of hospitalizations and deaths worldwide[1-2]. The emergence of antibiotic-resistant microorganisms has raised numerous concerns regarding the current indiscriminate use and overuse of prescribed antibiotics and sustains the urge to develop new molecular targets with therapeutic potential as well as new therapeutic approaches[1,3-4].

The development of antimicrobial resistance is associated with the capacity of bacteria not to be affected by antibiotics mechanisms of action which leads to the incapacity to treat infections and the increased risk of disease transmission, severe illness, and death[1,5]. Currently, conventional antibiotics including penicillin, gentamicin, and erythromycin, among others, are associated with the emergence of multiple drug-resistant microorganisms and hazardous side effects. Multidrug-resistant bacteria are been treated through a therapy model of synergistic antibiotic medication combination with bioactive substances[6].

Relevantly, the expression of virulence factors is crucial for microorganisms' resistance mechanisms. For *S. aureus*, virulence factors enable the development and establishment of these microorganisms in harsh environments and are linked to its success as a human pathogen[7-9]. Among its numerous virulence factors, the secretion of toxins into the cell-extracellular matrix, during the post-exponential and early stationary phases is one of the most relevant. These toxins are proteins with the ability to cause haemolysis and tissue penetration, which allows the microorganisms to invade their host[9]. Thus, overexpression of virulence factors genes, such as toxin production ones, can be associated with clinically relevant antibiotic resistance in *S. aureus*[9-10].

The increase of multi-resistance strains development has sustained the search for new therapeutic alternatives and effective natural antimicrobial compounds[3,10-12]. Among the newly studied compounds, Epigallocatechin-gallate (EGCG), green tea catechin from *Camellia sinensis* (green tea) has been demonstrated to exhibit a wide range of antimicrobial potential[3,13-24] as several studies have reported the ability of EGCG in reversing the MRSA resistance phenotype *in vitro* and reported an antimicrobial potential and synergistic effect with several antibiotics, including gentamicin, imipenem, tetracycline and amoxicillin in strains isolated from hospital-acquired infections and nasopharyngeal colonization[9-20]. Relevantly, it is acknowledged that EGCG is also an effective epigenetic modulator as it affects the transcription pattern of several genes including *Agr*, *OrfX*, *SpdC*, and *WalKR,* and signal transduction pathways including JAK/STAT, MAPK, PI3K/AKT, Wnt and Notch[9-10,14].

In our research group, EGCG effectiveness in the reversion of resistance phenotype in *S. aureus* both from nosocomial infections and commensal strains has been proved[25], as well as the fact that divergent resistance phenotypes are associated with divergent transcriptional expression of epigenetic modulator genes[26]. Considering the fact that hospital-acquired MRSA strains (HA-MRSA), and community-acquired MRSA strains (CA-MRSA) enclose divergences in risk factors, antibiotic resistance, growth rate, toxins, and/or virulence characteristics[23], epigenetic and drug resistance modulators such as EGCG can be seen as a valuable potential target for new therapeutic approaches for these pathogens[9].

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a Gram-positive, cocci-shaped bacterium, described in 1881 by the Scottish surgeon Alexander Ogaston in a surgical abscess[26]. In 1884 Anton J. Rosenbach, a German surgeon, also isolated two strains of *staphylococci*, named after the pigment observed in their colonies*: Staphylococcus aureus*, from the Latin *aurum* for gold, and *Staphylococcus albus* (currently *epidermidis*), from the Latin *albus* for white[27].

Currently, *S. aureus* is one of the most relevant human pathogens associated with high rates of hospital- and community-acquired infections, with significant public-health effects. It is described as the second most common cause of bacteraemia in Europe and one of the leading causes of sepsis worldwide[28]. In Portugal, several concerns have been raised for the past years, regarding *S. aureus* infections and colonization risks associated with occupational health, regarding health professionals and others such as livestock staff, and several efforts are being made in order to standardise human exposure assessment processes in workplaces[2].

These microorganisms can be transitory commensal bacteria that, in ideal growing conditions, may cause severe infections including endocarditis, and toxic shock syndrome, among others, and are also associated with food poisoning, causing vomiting, diarrhoea, and prostration[29]. Relevantly, toxin-mediated diseases such as food poisoning, scalded skin syndrome, toxic shock syndrome; skin and soft tissue infec-

tion (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 some of the clinical manifestations related to *S. aureus*[30].

Antibiotherapy resistance mechanisms

Since its discovery and development, antibiotherapy has saved millions of lives worldwide. Antibiotics can act through inhibition of bacterial growth (bacteriostatic) or by endorsing bacteria death (bactericidal)[31]. Antibiotics classification is based on their mechanism of action[30,32-33]. Currently, antibiotics are classified accordingly to five mechanisms of action: inhibition of cell wall synthesis; inhibition of nucleic acid synthesis; inhibition of protein synthesis destruction of cell membrane function, and inhibition of folic acid synthesis (inhibition of metabolism)[34].

β-lactams antibiotics associated with bacterial wall synthesis inhibition are the most utilized antibiotics in clinical practice due to their therapeutic efficacy and low toxicity[35]. β-lactams antibiotics which include carbapenems, penicillin, cephalosporins, monobactams, and carbapenems, have in common a β-lactam ring that confers the therapeutic activity, by damaging the cell wall which compromises the bacteria's integrity[15,35]. Antibiotic resistance to these compounds can emerge in different forms, however, the bacteria have managed to overcome its effects due to four 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 (enhance antibiotic efflux from the intracellular to the extracellular medium); c) alteration of the antibiotic target molecules (due to total loss of affinity by the expression of new PBP); d) changes in bacterial cell membrane permeability (modifying the antibiotic binding site)[33-35].

Antibiotic resistance in *Staphylococcus aureus*

Antibiotic resistance in *S. aureus* has mostly been associated with gene mutations or the acquisition of resistance genes from other bacteria. One of the most studied antibiotic resistance in *S. aureus* is methicillin resistance, endorsed by gene 13 - *mecA*, which codes for alterations of the β-lactam receptor[35]. These alterations originate a PBP with low affinity for the antibiotic, resulting in bacteria resistance to the treatment[35]. Thus, some *S. aureus* strains are resistant to methicillin (Methicillin-Resistant *Staphylococcus aureus* [MRSA]), but also to a large range of other antibiotics, making it hard to treat, and the cause of several dangerous infections both in hospitals and in the community, associated with high morbidity and mortality rates[2,36-37]. Despite the fact that methicillin is not currently utilized in clinical practice or even produced commercially, the label MRSA has persevered. Moreover, with the exception of the latest cephalosporin-lactams generation, methicillin resistance emerges as resistance to mostly all lactam molecules, which results in remarkable difficulties regarding infection treatments[38]. This antibiotic resistance encloses vancomycin[39-40], considered one of the last treatment options for severe MRSA infections[41], and relatively new agents such as linezolid and daptomycin[39]. These resistant *S. aurei* also referred to as VRSA (Vancomycin-resistant *Staphylococcus aureus* [VRSA])[26], are also currently considered a significant challenge for clinical practice regarding the treatment and control of infections[24]. In this context, for the past years, the term *superbug* has been applied when referring to such strains of divergent and different antibiotic-resistant bacteria (not just for MRSA)[42-47]. Moreover, *S. aureus's* ability to acquire genes that confer antibiotic resistance, either by mutations or by the acquisition of resistance genes from other bacteria of the same species in association with host gene mutation has endorsed the development and evolution of multi-resistant pathogenic microorganisms for which current treatment options may be highly limited[48].

Currently, the scientific community defines MRSA into three different strains namely: HA-MRSA, associated with healthcare and hospital infections, CA-MRSA community--associated and LA-MRSA associated with animal husbandry (livestock)[2,49-50].

HA-MRSA and CA-MRSA are described as the most prevalent and clinically relevant strains.

HA-MRSA strains are known to affect particularly patients admitted to hospitals or nursing homes, the elderly, and newborns. Associated risk factors for the emergence of HA-MRSA infections are the prolonged use of antibiotics, long hospitalizations, surgery, and medical devices (including catheters, etc.). Transmission is essentially achieved through person-to-person contact (this includes health personnel, patients, visitors, and handled hospital equipment)[2,48,50]. The most common mode of transmission is poor hand hygiene[50]. Furthermore, prolonged exposure to bioaerosols, particularly in the workplace, is associated with potential health risks and may endorse the development of opportunistic infectious diseases both for workers and patients as well as the dissemination of these microorganisms in the community[2,30]. In healthcare, bioaerosols and hand contact are two of the most relevant transmission mechanisms for MRSA to spread. Additionally, the colonization risk is also very significant, as clinical personnel and public health workers are in constant direct contact with colonized and or infected patients, particularly during the collection of biological samples[30].

Furthermore, CA-MRSA has emerged, for the past years, as a pathogenic microorganism with high clinical relevance, associated with several infections, particularly in young healthy, asymptomatic individuals. These MRSA strains are able to combine methicillin resistance with increased virulence, causing highly invasive, progressive, and usually fatal diseases and thus are becoming a public health concern associated with the community and public health[30]. CA-MRSA strains are particularly associated with skin and soft tissue infections, ranging from boils to necrotizing fasciitis. As for identified risk factors for transmission of these microorganisms, sharing personal items, neglected skin lesions, and poor hygiene have been the most relevant[36,51-52].

Interestingly, CA-MRSA and HA-MRSA can coexist and are associated with divergent antibiotic resistance profiles. HA-MRSA strains are usually characterized by a broad resistance spectrum to several antibiotics, while CA-MRSA strains enclose high sensitivity to various antibiotics including gentamicin, clindamycin, sulfamethoxazole/trimethoprim, ciprofloxacin, and vancomycin[53].

Staphylococcus aureus antimicrobial resistance factors

Antimicrobial resistance has been associated with the development of virulence factors. Virulence factors, as antimicrobial resistance factors, are thought to have evolved and spread through gene transfer mediated by mobile genomic islands, bacteriophages, plasmids, transposons, and insertion sequences[54]. One of the most relevant virulence regulator genes is *Agr* (accessory gene regulator)[55-56]. The incredible success of *S. aureus* as a human pathogen has been largely associated with its ability to adapt to divergent environments by modulating the expression of a wide range of virulence factors[57].

Among the numerous *S. aureus* virulence factors, adherence factors; exoproteins, and toxins are of particular relevance.

Adherence factors (surface proteins and antigens). Numerous adhesins allow for *S. aureus* to adhere to the surface of the host cell. Proteins covalently linked to cellular peptidoglycan are one of the most common types of *S. aureus* adhesins. Key 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 comprise a group of exoproteins including exotoxins, surface proteins, and enzymes, such as nucleases, proteases, lipases, hyaluronidase, and collagenase, which are able to convert local host tissue into nutrients necessary for bacterial growth. These molecules' action results in the host cell breakdown and are thus classified as cytolytic: these cytolytic toxins induce the formation of pores/holes (β-barrel pores) in the plasma membrane, which leads to the leakage of the cell's content and consequently the lysis of the target cell.

Toxins. These include *S. aureus* α-haemolysin, β-haemolysin, γ-haemolysin, leucocidin, and Panton-Valentine leucocidin (PVL). These microorganisms also produce an additional group of extremely relevant toxins named the toxic shock syndrome toxin, secreted staphylococcal enterotoxins, and the exfoliative toxins A and B which are associated with toxic shock syndrome, food poisoning, and staphylococcal scalded skin syndrome[26,54,56-63]. Toxins such as α-haemolysin are encoded in the genome, but others, such as PVL, are encoded on mobile genetic elements such as prophage[64].

Epigallocatechin-3-gallate: a new therapeutic approach for antibiotic-resistant *Staphylococcus aureus*

The continuous and widespread emergence of multi-resistant microorganisms endorsed the WHO to encourage and support the development of new antimicrobial molecules, including natural compounds that may be utilized against this microorganism[3]. Numerous studies have focused on the analysis of new therapeutic approaches based on natural products or compounds with therapeutic potential[12,22,65]. One such approach has been the use of green tea catechins, and the study of its beneficial properties[13-14,16,18,21]. Green tea is mostly produced in Asian countries from the leaves of the *Camellia sinensis* plant and its chemical composition includes, among other components, polyphenols (flavonoids and catechins), which are reported to be responsible for its beneficial properties.

The four main catechins present in green tea are: epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin--3-gallate (ECG) and epigallocatechin (EGC)[22]. Interestingly, EGCG is the most abundant and therapeutically relevant catechin, associated with significant anti-inflammatory, antioxidant, and anti-carcinogenic potential, high antimicrobial effectiveness, and demonstrated synergism with numerous antibiotics[13,15,17-18,22-23,67-69]. Several studies have reported synergism between EGCG and different antibiotics[14,18,24,70-71] including β-lactams and focused their target on S. aureus, in particular MRSA. In fact, it has been demonstrated that EGCG endorses damage to the bacteria's cellular wall, compromising its integrity[83]. Other studies have also analysed catechins' synergistic interaction with tetracycline against *S. aureus*[14,67,72] and the synergistic effect with penicillin, oxacillin, ampicillin/sulbactam and imipenem on MRSA[14,21-22,67,72]. It has been shown that EGCG displays the strongest antibacterial activity of all green tea catechins[13,18,21,23,73], with associated disruption of the bacterial membrane[50,73], and epigenetic and drug resistance modulator capacity which has led to advances as a potential target for new therapeutic approaches[9,64,74]. Data from ongoing studies[10] 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 nasal colonization *in vitro*.

Transcriptional levels of *S. aureus* virulence factors are affected by EGCG

EGCG exposure effects in *S. aureus* transcriptional patterns have been described, associated with the upregulation of several genes, crucial for membrane transport (to recover membrane function); and downregulation of key genes linked to toxin production and stress response[9-10,65,75-76]. It has been shown that EGCG is able to affect *S. aureus* strains at a concentration of 500 mg/L, which is similar to the EGCG content of green tea (a cup of regular tea has about 800 mg/l of EGCG)[68]. EGCG transcriptional effects in genes implicated in toxin production and stress response were associated with lower transcription levels of *hlgA*, *hlgB*, and *hly* genes[76].

Bacteria haemolysins known as α, β, Δ, and gamma (PVL) haemolysins, primarily mediate *S. aureus* lysis of red blood cells[77]. Additionally, *S. aureus* also produces two types of two-component pore-forming toxin, g-haemolysin (Hlg) and Leucocidin, which consist of Hlg2 and LukF, and LukS

and LukF, respectively[60]. The genes that codify for Hlg/Luk components, *hlg2*, *lukS* and *lukF*, form a gene cluster in this order which is transcribed into hlg2 and lukS-F mRNAs[78]. "The *lukF/S-hlg*, *hlgA*, and *hla* genes encode for haemolysins 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"[79]. Alpha-toxin, or alpha-haemolysin (Hla), is the main cytotoxic agent produced by *S. aureus* and the first reported member of the pore-forming beta-barrel toxin family Alpha-toxin binds to the cell membrane of eukaryotic cells which results in the release of a low-molecular-weight molecule, leading to an eventual osmotic lysis[75,80]. Moreover, gamma-haemolysin is a bacterial toxin that also appears to act through pores formation in the cellular membrane[76,81] (or beta-barrel pore-forming toxins that are secreted from the bacteria as monomers[82], with both haemolytic and a leucotoxic activities[76,81].

Furthermore, the *hly* gene on the *S. aureus* chromosome is an alpha-haemolysin-precursor[81], which encodes for a 293-residue protein monomer, that forms heptameric units on the cellular membrane to create a complete beta-barrel pore that allows for the toxin to perform its major function, the formation of pores in the cellular membrane, eventually leading to cell death[78]. Also, β-toxin, or sphingomyelinase, is coded by the *hlb* gene and delta-haemolysin is a 26 amino acid peptide encoded by the *hld* gene[83]. Studies have reported that alpha-toxin is a key virulence factor in numerous infections[84], with associated lethality and tissue necrosis[85], and that *hly*+ mutant strains are more infectious when compared to *hly*- strains[86], which sustains the urge for more research focused on these genes in order to understand the infectious process.

In our previous studies, analysed data demonstrated a clear effect on S. *aureus hlgA, hlgB*, and *hly* transcriptional expression associated with EGCG exposure, with a reported decrease in transcriptional levels associated with divergent patterns in *hlgA* and *hlgB/hly,* suggesting a lower susceptibility of *hlgA's* expression to EGCG than *hlgB/hly*[87].

Overall, studies have demonstrated that EGCG may potentially act as a natural antibacterial agent, inhibiting *S. aureus* growth and toxin generation – which can be a response to the WHO concern regarding antibiotic resistance[1,3-4].

EGCG epigenetic modulator potential in *S. aureus*

For the past years, in our research group, we demonstrate that EGCG is able to affect human plasma molecular profile[88]. In addition to all previously described characteristics, EGCG health benefits have also been associated with its epigenetic effects, particularly by targeting both histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulating acetylation of histones and non-histone chromatin proteins and affect DNA methylation[89-90]. Data from our research, in addition, to clearly confirming the synergism between EGCG and *S. aureus* strains with associated changes in the phenotype from resistant to susceptible, also demonstrate that divergent *S. aureus* resistant phenotypes are associated with altered transcriptional expression patterns of epigenetic modulators, namely *orfx* a *staphylococci methyltransferase* and drug resistance genes s*pdC* and *WalKR* in strains isolated from commensal flora and from nosocomial infections[10,50]. Data suggested that *orfx*-mediated ribosomal methylation can be affected by EGCG exposure, which may play a key role in determining phenotype resistance reversion as an epigenetic modulator[10,50]. Regarding *WalR* transcription levels EGCG endorsed higher effects in the most susceptible strains, which could be associated with their lower virulence. Overall, results were more evident in the most susceptible strains when compared to more resistant MRSA strains[10,50]. Nevertheless, data supported the EGCG modulator effect and corroborated its potential as an antimicrobial and/or therapeutic adjuvant treatment against antibiotic-resistant microorganisms, including nosocomial-associated strains.

Even though information regarding epigenetic modulation and antimicrobial resistance development is still scarce, evidence of associations between epigenetics and antibiotic resistance including adaptive resistance and phenotypic heterogeneity has emerged[91]. Relevantly, it is acknowledged that the transient nature of epigenetic mechanisms potentiates their use for therapeutic purposes. Additionally, the reported associations between conserved DNA Methyltransferases and virulence, host colonization, and biofilm formation, among others, led to the hypothesis that DNA MTases are promising targets for the development of new therapeutic approaches for biomedical applications[92].

Concluding remarks

Antimicrobial resistance of human pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) is a global health concern, associated with high mortality and mobility rates. The emergence of multi-resistance strains has created the urge for the development of new valuable and effective therapeutic alternatives. Data from several research groups, including ours, have associated EGCG exposure with transcriptional regulation of key resistance and virulence genes as well as epigenetic modulation, in *S. aureus*. Overall data demonstrate the potential of EGCG as a natural compound to become a novel therapeutic option in the fight against antibiotic resistance.

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Conflito de interesses

As autoras declaram não possuir quaisquer conflitos de interesse.

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