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Drug Resistance and Epigenetic Modulatory Potential of Epigallocatechin-3-Gallate Against *Staphylococcus aureus*

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Abstract

Antimicrobial resistance of human pathogens, such as methicillin-resistant *Staphylococcus aureus*, is described by the World Health Organization as a health global challenge and efforts must be made for the discovery of new effective and safe compounds. This work aims to evaluate epigallocatechin-3-gallate (EGCG) epigenetic and modulatory drug potential against *S. aureus* in vitro and in vivo. *S. aureus* strains were isolated from commensal flora of healthy volunteers. Antibiotic susceptibility and synergistic assay were assessed through disk diffusion accordingly to EUCAST guidelines with and without co-exposure to EGCG at final concentrations of 250 µg/ml, 100 µg/ml, 50 µg/ml, and 25 µg/ml. Transcriptional expression of *orfx*, *spdC*, and *WalKR* was performed through qRT-PCR. A 90-day interventional study was performed with daily consumption of 225 mg of EGCG. Obtained data revealed a high prevalence of *S. aureus* colonization in healthcare workers and clearly demonstrated the antimicrobial and synergistic potential of EGCG as well as divergent resistant phenotypes associated with altered transcriptional expression of epigenetic and drug response modulators genes. Here, we demonstrate the potential of EGCG for antimicrobial treatment and/or therapeutic adjuvant against antibiotic-resistant microorganisms and report divergent patterns of epigenetic modulators expression associated with phenotypic resistance profiles.

Abbreviations

EGCG	Epigalocatenin-3-gallate
HA-MRSA	Healthcare-associated *methicillin-resistant Staphylococcus aureus* infections
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
RT-qPCR	Real-time quantitative reverse transcription
WHO	World Health Organization
SCC*mec*	*Staphylococcal* cassette chromosome *mec* complex

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Introduction

The World Health Organization (WHO) describes antimicrobial resistance in human pathogens as a global health challenge [1]. For the past decades, with the global escalation in the development of antibiotic-resistant microorganisms [2], both social and scientific concerns have emerged regarding the intensive prescription of antibiotics and the development of pathogens with resistant phenotypes [3].

Airborne microorganisms, such as *Staphylococcus aureus*, are one of these pathogens. Some *S. aureus* strains are resistant to multiple antibiotics, which makes difficult the treatment of infections caused by this pathogen, and great attention has been given to methicillin-resistant *S. aureus* (MRSA). During its evolution, *S. aureus* acquired, via horizontal genetic transfer, the *Staphylococcal cassette chromosome mec* complex (SCC*mec*), in which is inserted the *mecA* gene encoding the modified penicillin-binding protein 2 (PBP2), responsible for the resistance to β-lactam antibiotics (oxacillin, streptomycin, tetracycline, erythromycin, among

others), by decreasing the affinity of the binding of antibiotics to their receptor in the bacterium [4–6]. MRSA is one of the most important human pathogens and a major cause of infections worldwide [7] associated with extremely high mortality rates for invasive blood-stream and pneumonic infections [8]. Additionally, the ability to become part of the human commensal flora is also a potentially relevant hazard since human MRSA carriers have increased risk for subsequent occurrence of clinical disease (up to tenfold) and become a bacterial reservoir with associated high risk to transfer the infection to others or contaminate foods and food surfaces during handling [9]. Currently, it is estimated that *S. aureus* colonizes transiently or persistently approximately 30% of the human population asymptomatically, in which the highly variable nasal microbiota plays a key role [10]. Moreover, high nasal colonization by MRSA among health professionals is considered a major issue in order to prevent nosocomial infections [11]. Although most of the studies performed regarding *S. aureus* epidemiology have been focused on the emergence and dissemination of MRSA strains in healthcare settings, currently much attention is also given for dissemination in the community [12].

Considering the relevance of this pathogen for clinical setting and the community and general as well as the development of new resistant strains, the identification of new compounds with antimicrobiological potential and resistance reversion is crucial. Relevantly, reversal of the resistance phenotype of this microorganism has been suggested in some in vitro studies associated with exposure to epigallocatechin-3-gallate (EGCG), the largest constituent of green tea [13]. Synergism between EGCG and β-lactams has been focused on *S. aureus*, particularly MRSA, and showed that EGCG could damage the bacterial cell wall, compromising its integrity as it binds to peptidoglycan [14, 15]. Roccaro et al. proved that catechins interact synergistically with tetracycline against *S. aureus* [15] and different studies have also demonstrated a synergistic effect with penicillin, oxacillin, ampicillin/sublactam, and imipenem in MRSA [13, 15–17].

Epigallocatechin-3-gallate (EGCG) is able to affect human plasma profile [18] (and associated health benefits have been related to their epigenetic effects as these compounds are able to target both histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulate acetylation of histones and non-histone chromatin proteins, and affect DNA methylation [19, 20]. In studies related to carcinogenesis, EGCG ability to induce epigenetic reactivation of silenced genes or epigenetic inhibition of oncogenes has been associated with the inhibition of DNMTs or the activity of HDACs [20]. In *Staphylococci*, the *orfx* gene encodes a conserved *Staphylococcal* ribosomal methyltransferase, produced constitutively during bacterial growth, inserted in the SCC*mec* complex, which contains the *mecA* gene, responsible for resistance to β-lactams in MRSA [21]. Moreover, the *WalKR* system is a two-component system, which upregulates *spdC* gene expression, considers a new virulence factor in *S. aureus* [22], known to be crucial for the rapid adaptation of *S. aureus* to a wide range of environmental conditions, and modulates drug resistance [22–25].

This work aims to assess *S. aureus* methicillin-resistant (MRSA) and susceptible (MSSA) strains colonization prevalence in healthcare workers, potential epigenetic, and drug response transcriptional modulator effects and to evaluate the potential of EGCG in reversing MRSA phenotype in vivo. This study is intended to demonstrate in vitro and in vivo EGCG potential for antimicrobial treatment and/or therapeutic adjuvant against resistant microorganisms.

Materials and Methods

Study Population and Design

For in vitro assay, *S. aureus* strains were isolated from occupationally exposed healthcare workers ($n = 38$). For the in vivo human assay, we performed an interventional, uncontrolled, prospective, longitudinal, and of individual analysis study, which included 30 healthy individuals (with no previously diagnosed pathologies). Inclusion criteria considered were adult voluntaries (ages superior to 18 years old and less than 65 years old) with no acknowledged previously diagnosed pathology of any type. Exclusion criteria applied included viral infections, consumption of tea, and forgotten capsules on consecutive days during the study. Data were analyzed under blind conditions.

Supplementation and Questionnaires

Human clinical trials demonstrated that 400 mg and 800 mg of EGCG intake [26] result in peak serum concentrations in the range of 100–400 ng/ml [27] with no reported severe secondary effects. Commercial capsules of green tea extract with 225 mg EGCG/capsule (My Protein®) were provided for all the participants in the study with the instructions to take one capsule daily. EGCG dosage was selected considering that 400 mg and 800 mg of EGCG intake, which are considered safe doses previously used in human clinical trials. Thus, the selected dosage is considered safe. The participants filled up two questionnaires, the first in the moment of the first specimen collection (T0; June) with questions regarding demographic data, such as age, gender, weight (cm), height (kg), and smoking habits. In the second questionnaire, after 90 days (T90; September) of the interventional study, the participants were asked to report adverse effects through the intervention, namely alterations in the nervous system (headaches, migraines, mood swings); gastrointestinal system (heartburn, reflux, diarrhea, cramps,

weight loss/gain); cardiac and respiratory effects; and information regarding potential missing capsules intake.

Biological Samples

For isolation of *S. aureus* from commensal flora, biological samples were obtained through nasopharyngeal swab procedure using transport swabs with Stuart media and immediately transported to the laboratory.

For in vivo assessment of EGCG exposure effect, biological samples were collected at time 0 (T0) followed by 90 days of ingestion of commercial capsules of green tea extract (225 mg EGCG/capsule) daily. After 90 days of exposure, a second biological sampling through a nasopharyngeal swab was performed (T90).

S. aureus Identification

For *S. aureus* identification, biological samples were inoculated in Columbia Agar with 5% sheep blood non-selective media as well as in selective media CHROMID® MRSA and incubated for 24 h and 48 h at 37 °C. After incubation, *S. aureus* suspicious colonies were isolated and incubated for 24 h at 37 °C. Identification of *S. aureus* was performed through catalase test and Slidex Staph-Kit (Biomerieux ref #73115). MRSA strains were identified through Slidex MRSA detection Test Kit (Biomerieux ref #73117). In this work positive (*S. aureus* MRSA laboratory collection) and negative (*S. aureus* ATCC 25923) control strains were included as positive and negative controls.

Antimicrobial Assay

S. aureus strains isolated from commensal flora were inoculated in 1 ml of Mueller Hinton at 0.5 McFarland turbidity. Strains initial antibiotic susceptibility (with no EGCG) was assessed through disk diffusion method seeded in Mueller Hinton Agar with commercial discs of amoxicillin (25 µg), tetracycline (30 µg), gentamicin (30 µg), and imipenem (10 µg). After incubation of 18 h, 24 h, and 48 h at 37 °C, antibiotic disk zone of inhibition was measured, and susceptibility was assessed using EUCAST Clinical Breakpoint Tables v. 10.0, valid from 01-01-2020 [28] data summarized in Table 1.

Synergy Evaluation

To assess the synergistic potential of EGCG (E4143 Sigma), *S. aureus* strains isolated from commensal flora and infections were inoculated in 1 ml of Mueller Hinton Broth with EGCG at final concentrations of 250 µg/ml, 100 µg/ml, 50 µg/ml, and 25 µg/ml at 0.5 McFarland turbidity. Antibiotic susceptibility was assessed through disk diffusion method seeded in Mueller Hinton Agar with commercial discs of amoxicillin (25 µg), tetracycline (30 µg), gentamicin (30 µg), and imipenem (10 µg). After incubation of 18 h, 24 h, and 48 h at 37 °C, antibiotic disk zone of inhibition was measured and susceptibility assessed using EUCAST Clinical Breakpoint Tables v. 10.0, valid from 01-01-2020 [28]. The significant differences between the different times of exposure, MSSA, and MRSA strains were assessed using Student's *t* test. *P*-values <0.02 were considered significant for EGCG exposure per se and *P*-values <0.01 were considered significant for EGCG co-exposure with imipenem, tetracycline, gentamycin, and amoxicillin.

Table 1 Inhibition zone intervals to determine sensitivity or resistance to antibiotics, according to values in the EUCAST v10.0 table valid from 01-01-2020 [28]

Antibiotics	Zone of inhibition (mm)	
	Susceptible	Resistant
Imipenem 10 µg[a]	≥22	<22
Tetracycline 30 µg	≥22	<19
Gentamicin 30 µg	≥18	<18
Amoxicillin 25 µg[a]	≥22	<22

[a]The values of the measurement intervals of the zones of inhibition for imipenem and amoxicillin were inferred from the values of cefoxitin, according to the indications in the EUCAST v10.0 table valid from 01-01-2020

Transcriptional Expression Analysis

For the transcriptional analysis study, MSSA and MRSA strains with divergent resistance profiles identified at 24 h of EGCG exposure were selected.

Cell lysates were used for extraction of bacteria total RNA using the NZY Total RNA Isolation kit (Nzytech), according to the manufacturer's protocol. RNA samples concentration was determined using the Qubit™ RNA HS Assay Kit in Qubit™ 3.0 Fluorometer (Invitrogen) and 1 µg of total RNA was reverse transcribed to cDNA by means of random hexamers from the RevertAid RT Kit (Thermo Scientific). Quantitative real-time PCR (qRT-PCR) was performed on a CFX Connect™ Real-Time PCR Detection System (Bio-rad) using specific primers, listed in Table 2, for the genes *orfx*, *spdC*, and *WalKR* and *16S rRNA* was used as a reference gene for data normalization. Reactions were performed in triplicate using the iQ SYBR Green Supermix (Bio-rad) in a final volume of 20 µl. Additionally, control PCRs were performed for all primer combinations without template. The utilized cycling conditions were as follows: initial activation of 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s. Potential primer-dimer formation and contaminations were also

Table 2 Primer sequences, accession numbers, and product lengths for qRT-PCR analysis

Genes	Forward primer (5'→3')	Reverse primer (3'→5')	Reference
Orfx	GGGCAAAGCGACTTTGTATT	TGGGAATGTCATTTTGCTGA	AAW81344.1[a]
SpdC	GCAGTAGGATACATTGGTT	CAGCCTCAGTATGATTAGTT	22
WalKR	GTGTACTGTGCATACGATGGTAATGATGC	CGTTACATAGTCATCTGCACCTAGTTCTA	22
16S rRNA	ACGTGGATAACCTACCTATAAGACTGGGAT	TACCTTACCAACTAGCTAATGCAGCG	22

[a]GenBank Accession Numbers (National Center for Biotechnology)

assessed and excluded. Relative quantification was undertaken by normalizing threshold cycles (Ct) of the target genes with the mean Ct of 16S rRNA. Transcription levels were assessed by calculating ΔΔCt [ΔΔCt = ΔCt resistant phenotypes − mean ΔCt most resistant strains (control)].

Statistical Analysis

All statistical calculations were performed using IBM SPSS statistics 22 software. The significant differences between different groups were analyzed by a Student's *t* test (comparison for two groups) and $P < 0.01$ was considered statistically significant. Results are presented as mean ± standard deviation.

Results

Healthcare Workers Presented High Prevalence Levels of *S. aureus* Colonization with Associated Divergent Resistant Phenotypes and EGCG Effects

The prevalence of *S. aureus* in the population studied in samples collected from healthcare workers ($n = 38$) was 42.1% (16/38), of which 18.4% (7/38) were MSSA and 23.7% (9/38) MRSA.

S. *aureus* (MSSA and MRSA) Commensal Strains Presented Divergent Resistant Phenotypes Against Imipenem, Tetracycline, Gentamycin, and Amoxicillin

Phenotyping of the antibiotic resistance profile of strains isolated from samples collected from volunteers with healthcare occupational exposure was performed regarding sensitivity to imipenem 10 µg, tetracycline 30 µg, gentamicin 30 µg, and amoxicillin 25 µg in order to identify divergent profiles. After incubation (24 h at 37 °C), the antibiotic inhibition zones were measured for each strain (Fig. 1), and the susceptible (S) and resistant (R) characterization were performed according to the values established by EUCAST and the results obtained are recorded in Table 3. Data showed that all strains are susceptible to tetracycline and gentamicin. MSSA strains encoded as CC4, MB1, MB6, and MB10 and MRSA strains encoded with MB2, MB4, VFXB14, VFXB15, and VFXB16 were resistant to amoxicillin. The coded strain MB12 was resistant to both imipenem and amoxicillin.

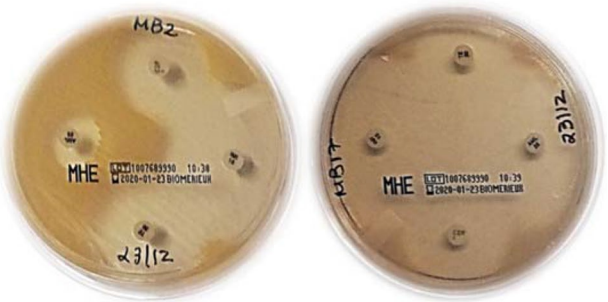

Fig. 1 Representative plates of divergent antibiotic resistance profile of strains isolated from samples collected from volunteers with healthcare occupational exposure after 24 h of EGCG exposure with imipenem (10 µg), tetracycline (30 µg), gentamycin (30 µg), and amoxicillin (25 µg)

Table 3 Resistance profile divergence of isolated MSSA and MRSA strains to imipenem (10 µg), tetracycline (30 µg), gentamycin (30 µg), and amoxicillin (25 µg)

	Code	IMIP 10	TETRA 30	GENTA 30	AMOX 25
	CC3	S	S	S	S
M	***CC4***	S	S	S	**R**
S	***MB1***	S	S	S	**R**
S	***MB6***	S	S	S	**R**
A	***MB10***	S	S	S	**R**
	MB17	S	S	S	S
	5-7S	S	S	S	S
	MB2	S	S	S	**R**
	MB4	S	S	S	**R**
M	*MB5*	S	S	S	S
R	***MB12***	**R**	S	S	**R**
S	*1-2S*	S	S	S	S
A	*VFXB7*	S	S	S	S
	VFXB14	S	S	S	**R**
	VFXB15	S	S	S	**R**
	VFXB16	S	S	S	**R**

Resistant phenotype is indicated as R (bold) and sensible as S

Exposure to EGCG Induces Differential Synergistic Effects in MSSA- and MRSA-Resistant Phenotypes

In order to assess potential synergism divergence between EGCG and the different antibiotics tested, isolated strains were inoculated at different concentrations of EGCG (250 µg/ml; 100 µg/ml; 50 µg/ml; and 25 µg/ml) and antibiotic resistance tests were performed.

Regarding the results obtained with imipenem, the MRSA strain encoded as MB12, which showed a resistant phenotype (Table 3), when exposed to different concentrations of EGCG, the phenotype went from resistant to susceptible. EGCG potentiated the action of imipenem and the phenotype reversal was observed after co-exposure with EGCG at final concentrations of 250 µg/ml, 100 µg/ml, 50 µg/ml, and 25 µg/ml.

On the other hand, amoxicillin MSSA- and MRSA-resistant strains, namely CC4, MB1, MB6, MB10, MB2, MB4, MB12, VFXB14, VFXB15 and VFXB16 after exposure with EGCG presented divergent resistance phenotypes, which are summarized in Table 4. Strains encoded as MB6, MB2, and MB4, when exposed to EGCG in different concentrations, the resistance phenotype reverted to susceptible, indicating that EGCG potentiated the action of amoxicillin in these strains. Strains encoded as MB1 and VFXB16 only demonstrated the reversion of the resistance to susceptible phenotype at 250 µg/ml and 100 µg/ml EGCG concentrations. Strains encoded as MB10, MB12, VFXB14, and VFXB15 maintained the resistance phenotype, that is, in these strains there was no synergism between EGCG and amoxicillin.

Imipenem, Tetracycline, Gentamycin, and Amoxicillin Inhibition Zones Values are Affected by EGCG Exposure

Regarding time exposure effects in EGCG co-exposure with the tested antibiotics, we observed significant divergence in MSSA with imipenem and amoxicillin between 18 and 24-h exposure ($P = 0.00190135$ and $P = 1.5058\text{E}{-}05$, respectively) and 24 h and 48 h ($P = 0.014651$ and $P = 0.000525$, respectively).

MRSA between 18 and 24-h exposure in imipenem and amoxicillin ($P = 3.27033\text{E}{-}05$ and 0.049234613, respectively) and 24–48 h only in amoxicillin ($P = 0.000159$). On the other hand, at the same exposure time, we also reported differences between MSSA and MRSA strains, namely after 18-h EGCG co-exposure with imipenem and amoxicillin ($P = 2.87778\text{E}{-}07$ and $P = 0.000201292$, respectively) and imipenem for 24-h and 48-h exposures ($P = 8.04449\text{E}{-}07$ and $6.5191\text{E}{-}06$, respectively).

Epigenetic and Drug Resistance Modulators Expression Patterns Differ in Divergent Resistance Phenotypes Strains After EGCG Exposure

Expression analysis of staphylococci methyltransferase (*orfx*) and drug resistance (*spdC* and *WalKR*) genes was performed in selected MSSA and MRSA strains with described divergent resistance phenotypes obtained after EGCG exposure. Results are summarized in Table 5. MSSA strain MB10 and MRSA strain VFXB14 were the most resistant strains and so utilized for transcriptional expression comparison in relation to the other selected strains.

Orfx, *spdC*, and *WalKR* Expression Levels are Affected by Co-exposure with EGCG in Selected MSSA and MRSA Strains

EGCG exposure particularly altered expression patterns of the analyzed genes with higher susceptible phenotypes when compared with the most resistant strains.

Regarding MSSA-selected strains (Fig. 2A), MB10 strain was considered the most resistant due to the fact that like the other strains was susceptible to imipenem, tetracycline, and gentamicin, but resistant to amoxicillin and, after EGCG exposure, no reversion was observed in the phenotype. Conversely, MB17 strain was susceptible to all

Table 4 Resistance profile of isolated MSSA and MRSA strains resistant to amoxicillin (25 µg) after co-exposure with EGCG at final concentrations of 250 µg/ml, 100 µg/ml, 50 µg/ml, and 25 µg/ml

	Code	AMOX	AMOX + 250 µg/ml EGCG	AMOX + 100 µg/ml EGCG	AMOX + 50 µg/ml EGCG	AMOX + 25 µg/ml EGCG
M	*CC4*	**R**	*S*	*S*	*S*	**R**
S	*MB1*	**R**	*S*	*S*	**R**	**R**
S	*MB6*	**R**	*S*	*S*	*S*	*S*
A	*MB10*	**R**	**R**	**R**	**R**	**R**
	MB2	**R**	*S*	*S*	*S*	*S*
M	*MB4*	**R**	*S*	*S*	*S*	*S*
R	*MB12*	**R**	**R**	**R**	**R**	**R**
S	*VFXB14*	**R**	**R**	**R**	**R**	**R**
A	*VFXB15*	**R**	**R**	**R**	**R**	**R**
	VFXB16	**R**	*S*	*S*	**R**	**R**

Resistant phenotype is indicated as R (bold) and sensible as S

Table 5 MSSA and MRSA-selected strains code and resistant phenotype regarding [imipenem (IMIP), tetracycline (TETRA), gentamycin (GENTA), and amoxicillin (AMOX)] selected for transcriptional analysis

Code	Resistance phenotype
CC4	IMIP S; TETRA S; GENTA S; AMOX R reversion up to 50 µg/ml EGCG
MB6	IMIP S; TETRA S; GENTA S; AMOX R total reversion with ECGC
MB10	IMIP S; TETRA S; GENTA S; AMOX R no reversion
MB17	IMIP S; TETRA S; GENTA S; AMOX S
MB2	IMIP S; TETRA S; GENTA S; AMOX R total reversion with ECGC
MB12	IMIP R total reversion; TETRA S; GENTA S; AMOX R no reversion
MB5	IMIP S; TETRA S; GENTA S; AMOX S
VFXB14	IMIP S; TETRA S; GENTA S; AMOX R no reversion
VFXB16	IMIP S; TETRA S; GENTA S; AMOX R reversion up to 100 µg/ml EGCG

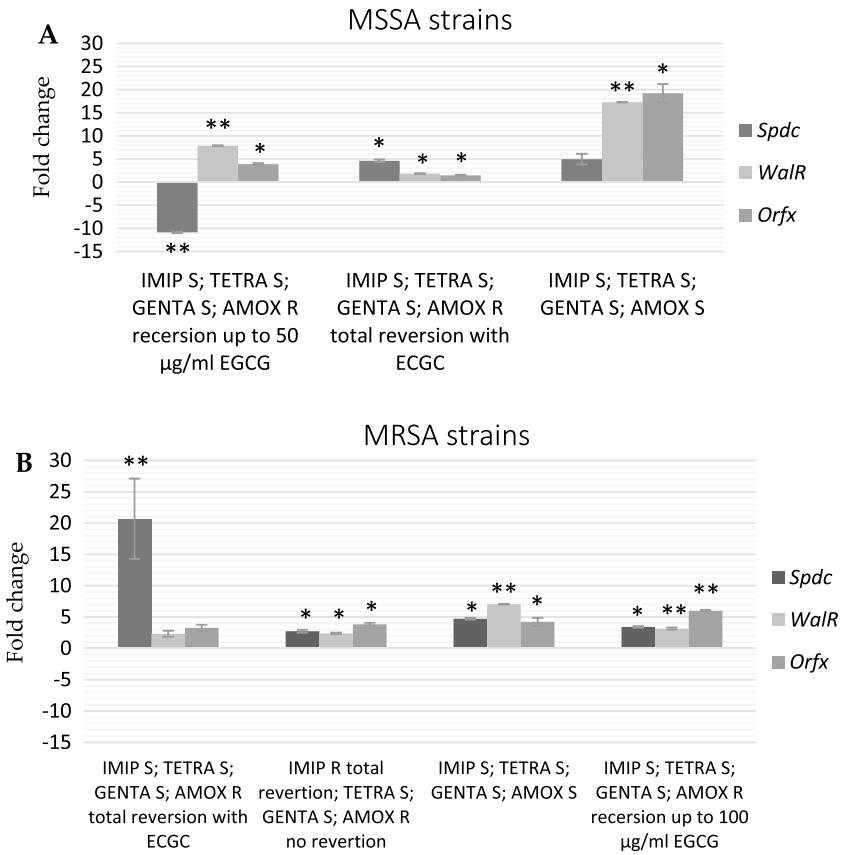

Fig. 2 Graphic representation of MSSA (**A**) and MRSA (**B**)-selected strains with divergent resistance phenotypes after EGCG exposure qRT-PCR results. Data represent the relative expression for the genes: *orfx*, *spdC*, and *WalKR*. *16S rRNA* was used for normalization. Error bars represent the standard deviation between independent treatments and qRT-PCR replicates. Significant statistical values, which were compared with the most resistant strains and calculated with Student's *t* test, are illustrated as: $*P<0.01$ and $**P<0.001$

assessed antibiotics and after EGCG exposure, we observed a significant increase in transcriptional expression of all analyzed genes, namely *spdC*, *WalR*, and *orfx* (4.98 ± 1.14; 17.29 ± 1.9; 19.21 ± 1.99; $P<0.01$, respectively). Also, MB6 with total reversion of amoxicillin resistance *spdC*, *WalR*, and *orfx* mRNA were upregulated (1.62 ± 0.26; 1.85 ± 0.1; 1.5 ± 0.1; $P<0.01$, respectively). On the other hand, after EGCG exposure CC4 strains, which presented a reversion of the resistant phenotype for amoxicillin up to 50 µg/ml, a downregulation of *spdC* expression was observed (-10.865 ± 0.16; $P<0.001$) and an increase in *WalR* and *orfx* mRNA levels (7.88 ± 0.1; $P<0.001$ and 3.9 ± 0.2; $P<0.01$, respectively).

In MRSA-selected strains, the same pattern was observed (Fig. 2B). For MRSA strains, VFXB14 was selected for comparison as it presented the same resistant phenotype as MB10, namely, susceptible to imipenem, tetracycline, and gentamicin, but maintained resistance to amoxicillin after EGCG exposure. In concordance with MSSA strains, MB5 strain, the most susceptible selected MRSA which was sensitive to all antibiotics. Transcriptional analysis demonstrated an increase in mRNA levels of *spdC* and *orfx* (4.74 ± 0.14; 4.23 ± 0.65; $P<0.01$, respectively) and more significantly *WalR* (6.8 ± 0.28; $P<0.01$).

On the other hand, in MB2 strains, which presented total reversion of amoxicillin resistance, only *spdC* was significantly upregulated (20.7 ± 6.4; $P < 0.001$).

In MB12 strain analysis, which was initially resistant to imipenem but the phenotype was reversed after EGCG exposure and no effect was reported for the amoxicillin resistance *spdC*, *WalR*, and *orfx* genes were upregulated (2.74 ± 0.24; 2.4 ± 0.12; 3.86 ± 0.22; $P < 0.01$, respectively). Additionally, VFXB16 strain which presented a reversion of the resistant phenotype for amoxicillin up to 100 µg/ml of EGCG exposure also presented increased mRNA levels of *spdC*, *WalR*, and *orfx* (3.43 ± 0.13; $P < 0.01$; 3.15 ± 0.17; 5.99 ± 0.14; $P < 0.001$, respectively).

Orfx, *spdC*, and *WalKR* Expression Patterns After 24-h Subculture of MSSA and MRSA Strains with Previous Co-exposure with EGCG

Transcriptional analysis performed in the selected strains after 24 h of subculture with no EGCG exposure demonstrated the overall maintenance of the expression patterns observed immediately after EGCG exposure (Fig. 3). CC4 strain maintained the downregulation of *spdC* expression (−10.69 ± 0.29; $P < 0.01$) and the *WalR* and *orfx* upregulation (0.73 ± 0.17; $P < 0.01$ and 3.9 ± 0.15; $P < 0.001$, respectively). However, in MB6, only *orfx* mRNA upregulation was maintained (1.77 ± 0.17; $P < 0.001$). Additionally, in MB17 strain the increase in transcriptional expression *WalR* and *orfx* was not altered (4.48 ± 2.55; 3.23 ± 0.35; $P < 0.01$, respectively) but a downregulation of *spdC* expression was reported (−7.35 ± 0.16; $P < 0.01$) (Fig. 3A).

Regarding MRSA strains transcriptional analysis, for MB2 strain we observed the stability of *spdC*, upregulation (13.1 ± 12; $P < 0.001$) and VFXB16 strain also maintained the previous pattern with associated upregulation of *spdC*, *WalR*, and *orfx* mRNA levels (2.11 ± 0.24; $P < 0.01$, 3.02 ± 0.12; $P < 0.01$ 5.99 ± 0.01; $P < 0.001$, respectively). Moreover, MB5, the most susceptible selected MRSA strain, also maintained the upregulation of *WalR* and *orfx* (1.06 ± 0.07; 4.47 ± 0.22; $P < 0.001$, respectively) (Fig. 3B).

Conversely in MB12 strain transcriptional analysis, *spdC*, *WalR*, and *orfx* genes were downregulated (−3.47 ± 0.1; $P < 0.01$ −3.35 ± 0.007; $P < 0.001$ −2.45 ± 0.15; $P < 0.01$, respectively).

EGCG Oral Intake Eliminated MRSA Phenotype in Nasal Colonization in the Community

Analyzed data from the performed interventional, uncontrolled, prospective, longitudinal, and of individual analysis study demonstrated that all samples presented normal commensal flora of gram-positive coccus *Staphylococcus* spp. and *Streptococcus* spp. We identified a prevalence of *S. aureus* 33.3% in which 70% were MSSA (23.3% total) and 30% MRSA (10% total) at the beginning of the study (Table 6). After 90 days of 225 mg EGCG oral exposure, *S. aureus* prevalence is maintained (36.6%). However, regarding MSSA strains, 50% of the colonized individuals

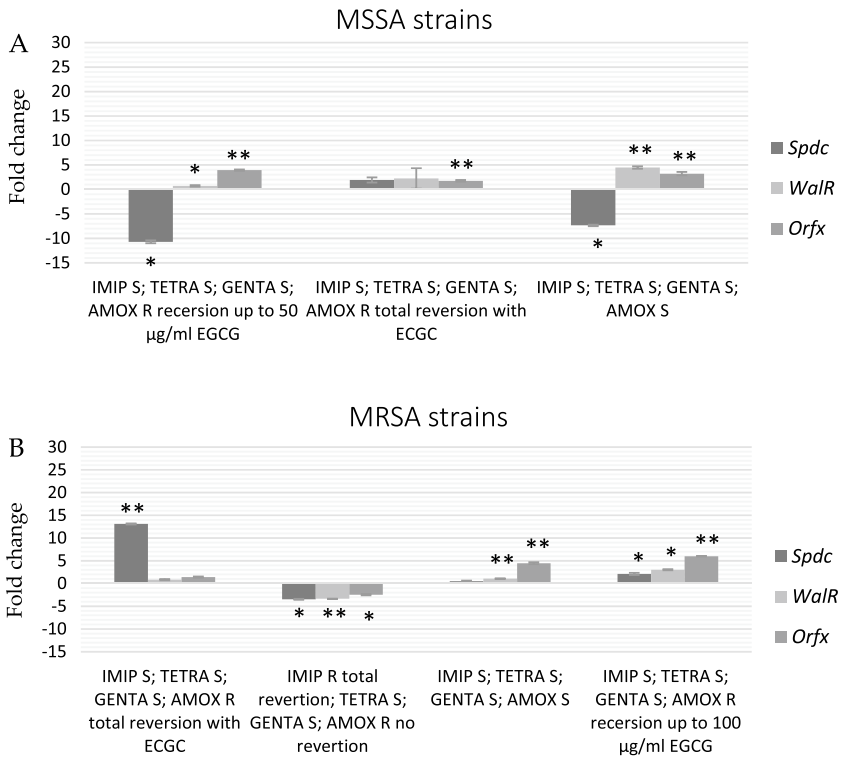

Fig. 3 Graphic representation of MSSA (**A**) and MRSA (**B**)-selected strains with divergent resistance phenotypes after EGCG exposure, qRT-PCR results after 24 h of subculture. Data represent the relative expression for the genes: *orfx*, *spdC*, and *WalKR*. *16S rRNA* was used for normalization. Error bars represent the standard deviation between independent treatments and qRT-PCR replicates. Significant statistical values, which were compared with the most resistant strains and calculated with Student's *t* test, are illustrated as: *$P < 0.01$ and **$P < 0.001$

Table 6 MSSA and MRSA prevalence (%) in commensal flora of the participants enrolled in the study before (T0) and after (T90) 90 days of 225 mg EGCG oral exposure

ID	T0 prevalence	T90 prevalence
1	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
2	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
3	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
4	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
5	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
6	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
7	*Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
8	*Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
9	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
10	*Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
11	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
12	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
13	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
14	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
15	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
16	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
17	MRSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
18	MRSA; *Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
19	MRSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
20	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
21	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
22	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
23	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
24	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
25	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
26	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
27	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
28	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.	MSSA; *Staphylococcus* spp. and *Streptococcus* spp.
29	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.
30	*Staphylococcus* spp. and *Streptococcus* spp.	*Staphylococcus* spp. and *Streptococcus* spp.

maintained the colonization, 10% eliminated the colonization, and three new colonization's were observed, while in MRSA 33.3% of the colonized individuals eliminated the bacteria, while in 66.6% the resistance phenotype was reversed (Table 6).

Discussion

Over the past decade, there has been a concerning increase in resistance to antibiotics from microorganisms that are pathogenic to humans. This has become a global problem with hazardous health consequences, but also with implications for the economy, which has raised some concerns regarding the prescription and intensive use of antibiotics [2, 3, 29, 30]. Currently, infections caused by *S. aureus* are no longer an exclusive problem associated with hospital environments and are becoming an emerging problem in the community [31]. Thus, several studies in the scientific community have focused on the development of new and alternative therapeutic approaches based on the use of natural products or compounds with therapeutic properties and potential [16, 32, 33]. The use of catechin EGCG in green tea is one of these approaches, as well as the study of its beneficial properties [34–36]. It has been proven in several studies that EGCG has anti-infectious properties against Gram-negative and Gram-positive bacteria, in some fungi and viruses [16, 37, 38].

Here, we assessed the prevalence of *S. aureus* strains susceptible and resistant to methicillin in health and the potential of EGCG as a new compound with antimicrobial properties and synergistic potential with common antibiotics as well as its impact on epigenetic and resistance modulator genes in strains with divergent phenotypes of resistance.

Data analysis revealed that health care occupational exposure leads to a prevalence of 42.1% of *S. aureus* colonization (18.4% MSSA and 23.7% MRSA), which is concerningly high. Previous studies carried out in Portugal emphasize that the primary mode of transmission of MRSA is through the hands, with the absence of proper hand hygiene being the most common mode of transmission [39]. Since health professionals are exposed to patients who may be contaminated, non-compliance with hygiene rules can be a way of spreading MRSA inside and outside the hospital environment, but also in the community [40]. As previously mentioned, *S. aureus* has the ability to colonize different areas of the human body, with a preference for the nasopharynx [41] and the ability to spread as well as being transmitted by direct contact (mainly by hands) or indirect (contaminated surfaces) [42]. One of its fundamental biological characteristics is the ability to colonize the healthy population asymptomatically (asymptomatic carrier), thus assuming an essential role in spreading to other areas of the body, to other people, and even contaminating food and surfaces during handling [42]. This colonization is considered a risk factor for the onset of infections by *S. aureus*, often combined with methicillin resistance (MRSA), increasing the risk of clinical disease [42, 43]. Thus, our results sustain the prerogative that exposure to bioaerosols particularly at workplaces can represent a health hazard and potentially result in infectious disease [44] which is concerning either for workers and for the spread of these microorganisms in the community. Currently, there are several antibiotics with different modes of action, namely inhibition of cell wall synthesis; inhibition of protein synthesis; inhibition of nucleic acid synthesis; destruction of cell membrane function; and inhibition of metabolism available for therapeutic approaches. In this study, the strains were subjected to the action of four antibiotics: imipenem 10 µg, tetracycline 30 µg, gentamicin 30 µg, and amoxicillin 25 µg, with EGCG in different concentrations, in order to assess synergism and be able to define its resistance phenotype. B-lactams such as amoxicillin and imipenem (belonging to the carbapenem subgroup) are able to interfere with the bacterial cell wall synthesis leading to cell lysis [5, 14, 17, 45, 46].

In this study, we clearly demonstrated the synergism between EGCG and strains with resistance phenotype as well verified that there were changes in the phenotype from resistant to susceptible in some of the studied strains associated with imipenem and amoxicillin. Although only one isolated strain showed a phenotype of resistance to imipenem, it was reverted when exposed to different concentrations of EGCG. Regarding amoxicillin, of the strains tested, 10 had an initial resistance phenotype; however, when exposed to different concentrations of EGCG, three strains reversed the resistant to susceptible phenotype, one reversed the phenotype in concentrations of 250 µg/ml; 100 µg/ml; and 50 µg/ml, but at the concentration of 25 µg/ml the resistance phenotype remained. Additionally, in two strains, only at the concentrations of 250 µg/ml and 100 µg/ml of EGCG did a phenotype reversal occur. Although the number of strains collected from the community is reduced, we were able to verify that for antibiotics with a cell wall synthesis mechanism of action, there was a synergism with EGCG, which is in line with previous studies [13, 16, 38, 47]. Moreover, time exposure also seems to be critical for EGCG interaction as we observed that there are differences in MSSA and MRSA strains, between 18, 24, and 48 h of exposure for imipenem and amoxicillin.

Our results demonstrate that divergent resistant phenotypes are associated with different transcriptional expressions of epigenetic modulators, which were particularly noticeable in the most susceptible strains. Regarding *WalR*, it reaches its highest transcription levels in the most susceptible strains, obtaining 17.29 ± 1.9-fold change in MSSA and 6.8 ± 0.28-fold change for MRSA. The *WalKR* two-component system is essential for *S. aureus* viability, actively participating in the cell wall metabolism [23, 48]. *WalR* positively regulates autolysis, biofilm production, and alpha-hemolytic activity and positively regulates relevant virulence genes, including the *spdC* gene [22, 23]. Activation of *WalR* decreases *S. aureus* virulence, inducing an early triggering of the host's inflammatory response, including neutrophil recruitment and increased cytokine levels, thus leading to rapid bacterial clearance and lowered virulence [23]. In the present study, EGCG had a great influence in *WalR* transcription levels in the most susceptible strains, which may contribute to their lower virulence. On the other hand, *spdC* gene was the only one downregulated, namely in CC4, a MSSA strain, with amoxicillin resistance phenotype reverted with EGCG concentration up to 50 µg/ml. Reduced *spdC* transcription levels are associated with a lowered virulence and increased sensitivity to β-lactam antibiotics [22]. However, we could not find a significant association between the *spdC* gene transcription levels and the resistance phenotype. Moreover, in MSSA strains, we observed an increased expression of the *Staphylococcal* ribosomal methyltransferase, particularly in susceptible strains, reaching a 19.21 ± 1.99-fold change. Strains with resistant phenotype reversion in vitro also revealed a correlation between EGCG exposure and *orfx* transcription levels, from 1.5 ± 0.1 to 6 ± 0.14-fold change. This expression decreases after subculture in the absence of EGCG, which suggests a direct exposure effect, however maintaining the overall patterns previously observed. *Orfx* is conserved among all staphylococci, and it is constitutively produced during growth [21, 49]. Recently, this methyltransferase has been of particular interest due to its insertion site in the SCC*mec* mobile genomic island within its C terminus, in the attachment site (attB) [21, 50, 51]. Boundy et coworkers monitored *orfx* by Western blotting, showing that the

insertion of SCC*mec* into at site had no effect on gene expression or protein production [21, 51]. Thus, *S. aureus* susceptibility to oxacillin, mediated by the *mecA* gene in SCC*mec*, is not affected by the inactivation of *orfx*, which agrees with the results obtained for both MSSA and MRSA [50, 51]. On the other hand, *orfx* gene product has been assumed to play a key role in bacterial growth and survival, as it is present in all sequenced coagulase-positive or coagulase-negative *Staphylococcal* genomes [21, 49, 52]. *Orfx* methylate 70 S ribosomes, constituting a *Staphylococcal* ribosomal methyltransferase of RlmH type [49]. Relevantly, ribosome methylation in bacteria provides either moderate resistance to antibiotics or, on the contrary, determines susceptibility to antibiotics, thus affecting bacterial adaptation and resistance [21, 49, 52]. Our data suggest that *orfx*-mediated ribosomal methylation is affected by EGCG exposure, playing an important role in determining phenotype resistance reversion as an epigenetic modulator. In recent studies, Kitichalermkiat et co-workers conducted a microarray analysis on *S. aureus* treated with or without 500 mg/L of EGCG in which differentially expressed genes were identified and their changes at the transcription level were confirmed using real-time qPCR [53]. Similarly, to the present study, EGCG treatment resulted in increased transcription expression (75 genes) of genes particularly related to membrane transport and decreased transcription (72 genes) in genes involved in toxin production and stress response [53]. Additionally, cellular membrane potential assessment also concluded that EGCG markedly decreased membrane potential, which is suggestive of cell membrane damage [53].

Overall our results clearly demonstrate that EGCG exposure is able to alter expression patterns of key epigenetic and drug response genes in *S. aureus* with associated divergent resistant profiles and should be further investigated potentially as a natural antimicrobial agent and or a therapeutic adjuvant.

Conclusion

Overall, this study allowed to sustain the antimicrobial and synergistic potential of EGCG with antibiotics that inhibit cell wall synthesis (imipenem and amoxicillin) in strains of *S. aureus*, particularly MSSA, and the concerning high colonization prevalence of these pathogens.

This work also demonstrated that divergent resistant phenotypes of *S. aureus* strains are associated with differential expression of epigenetic and drug resistance modulator genes, which indicates a clear modulator effect induced by EGCG exposure and corroborates the potential of EGCG for antimicrobial and / or therapeutic adjuvant treatment against antibiotic-resistant microorganisms.

Author Contributions Conceptualization, ER; methodology, AM, MD, TP, and AZ; validation, ER and MB; formal analysis, ER; resources, ER and MB; writing—original draft preparation, AM, TP, and ER; writing—review and editing, ER; supervision, ER; project administration, ER; and funding acquisition, ER. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Statement This work is included in two projects from the Instituto Politécnico de Lisboa accepted in Escola Superior de Tecnologia da Saúde ethical council ref: CE-ESTeSL-No.18-2019 and CE-ESTeSL-No. 20-2020. All volunteers provided a signed written informed consent before enrollment in the study in accordance with the Helsinki Declaration and Oviedo Convention and in Agreement with the Portuguese law nº 58/2019 de 8 de agosto regarding data protection.

Institutional Review Board Statement The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Escola Superior de Tecnologia da Saúde ref: CE-ESTeSL-No.18-2019 and CE-ESTeSL-No. 20-2020.

Informed Consent Informed consent was obtained from all subjects involved in the study.

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