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# Oxidative stress response in reference and clinical *Staphylococcus aureus* strains under Linezolid exposure

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ABSTRACT

*Background:* Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are some of the most widespread pathogens with multi-resistant to antimicrobial agents (AA). AA provoke several changes inside bacteria, which cannot be solely explained by the main mechanisms of action reported.
*Objective:* The role of oxidative stress in bacteria exposed to bacteriostatic AA has not been widely studied; hence, the aim of our work was to investigate the effect of linezolid (LZD) on *S. aureus* strains.
*Methods:* Oxidative stress markers, such as superoxide dismutase (SOD) enzyme activity, the global antioxidant response, advanced oxidation protein products (AOPP) and basal levels of glutathione in 28 clinical and 2 reference strains were measured.
*Results and conclusions:* We identified 10 of 30 strains showing a slight increase in reactive species under LZD treatment with respect to the untreated control (between 22% and 56%). Higher generation was detected in clinical strains compared with the reference strains; however, the impact on the antioxidant response was not significant, and the oxidized protein levels were almost undetectable. The strains exposed to this oxazolidinone did not suffer acute oxidative stress. This is the first work reporting the behaviour of clinical and reference strains of *S. aureus* exposed to LZD, showing negligible oxidative stress.


## 1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are widespread in communities and hospitals around the world. The ability of pathogens to acquire resistance to available antimicrobial agents (AA) has therefore become a global problem to be solved [1]. Moreover, in several countries, despite a combination of new AA and hospital infection control, community strains of MRSA continue to proliferate [2].

The complex mechanisms related to AA action have been under increasing scientific study to understand their global effects on cellular metabolism. Lobritz et al. identified cellular respiration to be a main subprocess after the antibiotic reaches the cellular target [3]. Numerous studies have also demonstrated that bacterial death caused by AA cannot be fully ascribed to their interaction with AA within the reported target. The concomitant occurrence of changes in cellular metabolism and the induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been described, and play a significant role in modulating antibiotic susceptibility [4–9]. Our previous studies have also demonstrated the oxidative damage provoked under ROS influence, with these species being found to induce oxidized macromolecules defined as stress markers (e.g. advanced oxidation protein products, AOPP) in bacteria [10,11]. Moreover, ROS promote the antioxidant defence system to maintain cell homeostasis, for instance glutathione (GSH) and superoxide dismutase (SOD) [12]. However, most of these studies were performed with bactericidal antibiotics.

The oxazolidinone linezolid (LZD) was launched on to the market in 2000 to treat infections caused by MRSA [13]. It is a bacteriostatic antibiotic which binds to the 50S subunit of the prokaryotic ribosome, thereby preventing it from complexing with the 30S subunit, mRNA, initiation factors and formyl methionyl-tRNA [14,15].

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LZD is a relatively safe antibiotic when given for short periods; however, prolonged LZD therapy can cause many side effects. Vivekanandan et al. reported that LZD showed a significant increase in lipid peroxidation in MRSA-infected rats and the concomitant administration of an antioxidant compound reduced LZD-induced hepatotoxicity and oxidative stress [16]. Wang et al. noted the oxidative stress generated in blood cells exposed to LZD and linked it to the resulting haematological toxicity [17,18].

The role of oxidative stress in *S. aureus* strains under LZD treatment has not been studied. Thus, our central goal was to study if LZD produces an oxidative and nitrosative stress imbalance by determining different markers such as ROS levels by reducing nitroblue tetrazolium (NBT) assay, fluorescence microscopy and spectrofluorometry with specific probes for ROS and RNS; also, the RNS levels were tested using the Griess method. AOPPs, the enzymatic antioxidant systems (SOD), the total antioxidant capacity (ferric reducing antioxidant power [FRAP] assay) and GSH basal levels were also quantified in 28 genetically characterized *S. aureus* clinical isolates and two reference strains, in order to find a relationship between the mechanism of action of LZD and the oxidative stress.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

The reference strains of *S. aureus,* American Type Culture Collection (ATCC) 29213 and MRSA ATCC 43300, and 28 genetically characterized clinical isolates were selected for the present work (see Supplementary Table S1). The *S. aureus* clinical strains were selected from those recovered in different surveillance projects of molecular epidemiology of MRSA infections in Córdoba, Argentina during 2001–2014 [19,20]. In addition, the *S. aureus* strain LGA251-XI was also analysed. This strain is a livestock-associated MRSA (LA-MRSA) with SCCmec XI, and was kindly provided by Mark A. Holmes (Dept. of Veterinary Medicine, University of Cambridge, UK). The samples were conserved in tryptic soy broth (TSB-Britania) and glycerol 10% v/v at −80 °C. Bacterial strains were cultured in tryptic soy agar (TSA-Britania) for 18 h at 35 °C and finally, one single isolated colony was collected to culture once again in the appropriate media depending on the assay conditions.

### 2.2. Minimum inhibitory concentration determination

The minimum inhibitory concentrations (MICs) were determined according to the guidelines of the Clinical Laboratories Standards Institute [21] in all the strains to define the appropriate antibiotic concentrations in order to perform the oxidative stress experiments. Overnight cultures obtained in TSA were diluted to $10^6$ CFU/mL in Mueller Hinton broth (Britania). Then, a stock solution of LZD (Sigma-Aldrich) (10 mL, 32 μg/mL) was prepared in phosphate-buffered saline (PBS) pH 7, and 500 μL of bacterial suspensions were inoculated with 500 μL of twofold diluted solutions of LZD in the concentration range between 16–0.15 μg/mL. Bacteria growth was observed at 24 h of incubation, according to Clinical and Laboratory Standards Institute (CLSI) indications. The MIC was defined as the lowest dilution of drug that inhibited visible growth (without turbidity) after 24 h of incubation at 35 °C.

### 2.3. Superoxide anion ($O_2^{\cdot-}$) generation determined by NBT

Overnight cultures of methicillin-sensitive *S. aureus* (MSSA) ATCC 29213 and MRSA ATCC 43300 in TSB were prepared, after which each bacterial suspension (OD ∼1) was centrifuged for 15 min at 4000 rpm and rinsed twice with PBS. Then, 50 μL of bacterial suspension was incubated with 50 μL of LZD at supra-MIC, MIC and sub-MIC concentrations for each strain or PBS (control condition) for 4 h followed by addition of 50 μL of NBT (Sigma-Aldrich, 1 mg/mL) and incubation for 30 min at 35 °C in a dark environment. Next, 10 μL of HCl (Cicarelli, 0.1 M) was added, and the samples were centrifuged at 4000 rpm for 10 min. The pellets were treated with 40 μL dimethyl sulfoxide (DMSO) (Anedra) and finally, the optical density was assessed at 540 nm [22].

### 2.4. Reactive nitrogen species measurements

Bacterial suspensions were grown in TSB for 18 h at 35 °C (10 mL of bacterial sample at the following concentration, OD 600 nm ∼1). They, were centrifuged for 10 min at 4000 rpm and the pellets were suspended in PBS. Suspensions of MSSA ATCC 29213 and MRSA ATCC 43300 (200 μL) were incubated for 4 h with an equal volume of LZD (4, 2 and 1 μg/mL) or PBS (control without antibiotic), after which, the samples were centrifuged at 4000 rpm for 10 min, washed twice with PBS and suspended in PBS. Then, 100 μL of the supernatants were mixed with 100 μL of Griess reagent (1:1) and incubated in the dark for 30 min at 35 °C. A stock solution of Griess reagent (Cicarelli), sulfanilamide 2% (w/v) (Sigma-Aldrich) in HCl (5% v/v) and *N*-1-naphthyl ethylenediamine dihydrochloride (Sigma-Aldrich) 0.1% (w/v) in sterile distilled water was prepared. A standard curve with sodium nitrite in concentrations from 0.8 to 400 μM in PBS was constructed. The spectrophotometry was performed at 540 nm [23].

### 2.5. Reactive oxygen species levels by fluorescence microscopy

Suspensions of MSSA ATCC 29213 and MRSA ATCC 43300 in TSB (10 mL of $\sim 1.10^9$ CFU/mL) were centrifuged at 4000 rpm for 10 min and the pellets were suspended in PBS (5 mL). The suspensions obtained were incubated with 100 μL of LZD at the MIC of each strain for 4 h at 35 °C or PBS (control without antibiotic) and centrifuged again at 4000 rpm for 10 min and washed with PBS. Then, 100 μL of the samples were diluted in 1.8 mL of PBS, and 20 μL of the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA, Sigma-Aldrich), dissolved in DMSO was added. The solution was incubated for 30 min in a dark environment at 35 °C. Finally, an aliquot of 20 μL of 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) (5 μg/mL in milliQ water) was poured to stain the DNA as a cell marker. Fluorescence intensity was measured using a Nikon TE-2000U microscope (excitation, 490 nm; emission, 519 nm for $DCFH_2$-DA; and excitation, 358 nm; emission, 461 nm in the case of DAPI staining) [24].

### 2.6. Well plate reader measurements: ROS and RNS production detected with the fluorescent probe dihydrorhodamine 123

The fluorescent probe dihydrorhodamine 123 (DHR; Sigma-Aldrich) is oxidized to its fluorescent cationic and lipophilic state upon reaction with ROS and RNS [25]; due to this ability, we applied this dye for an oxidative and nitrosative stress screening of the 28 genetically characterized and 2 reference strains of *S. aureus* after LZD treatment. Suspensions of MSSA ATCC 29213 and MRSA ATCC 43300 strains were grown aerobically in TSB (OD 600 nm ∼1). The inocula (10 mL) were centrifuged for 10 min at 4000 rpm, and the pellets were rinsed and suspended in PBS. Stock solutions of 10 mL of LZD were prepared (1–4 μg/mL); 100 μL of bacterial suspensions were incubated with 100 μL of LZD for 4 h or PBS (control without antibiotic). Then, 80 μL of PBS plus 20 μL DHR (final concentration 1 μM, solubilized in 100 μL of DMSO and diluted in PBS) were added to each well and incubated for 20 min in dark conditions at 35 °C. Fluorescent intensity was recorded using a Synergy BioTeK multi-mode microplate reader (excitation, 488 nm; emission, 520 nm) [26].

### 2.7. Advanced oxidation protein products assay

Overnight cultures (10 mL of bacterial sample at the following concentration OD 600 nm ~1) of four MSSA (MSSA ATCC 29213, MSSA 15, MSSA 16 and MSSA 22), and four MRSA (MRSA ATCC 43300, MRSA 5, MRSA 9 and MRSA 28, all strains are described in Supplementary Table S1) were centrifuged (4000 rpm) for 10 min, and the pellet was suspended in PBS. In parallel, a stock solution of LZD was prepared in PBS (80 μg/mL), and 900 μL of bacterial suspension was mixed with 100 μL of LZD (2 and 4 μg/mL) or PBS (control without antibiotic) and incubated for different periods (2, 4 and 6 h). Then, 200 μL of each sample treated with LZD was collected and 10 μL KI (Cicarelli), (1.16 M) and 20 μL of glacial acetic acid were added. Immediately after the addition of these reagents, the spectrophotometric reading was performed at 340 nm. AOPP concentrations were expressed as milliequivalents of chloramine T per milligram of protein (mEq chloramine T/mg protein) [27].

### 2.8. Superoxide dismutase activity

Overnight cultures of MRSA and MSSA were obtained in TSB after overnight culture (10 mL of bacterial sample at the following concentration OD 600 nm ~1). Then, suspensions were centrifuged for 10 min (4000 rpm) and rinsed with PBS pH 7. An aliquot of 500 μL of bacterial suspension was incubated with an equal volume of LZD at the concentration of 1, 2 or 4 μg/mL or PBS (control without antibiotic) for 4 h at 35 °C, after which, samples were again centrifuged for 10 min (4000 rpm). The pellet was solubilized in 250 μL of PBS, and then, 250 μL of DMSO was added to alter the cell permeability. Finally, the following volumes: 300 μL of methionine (Sigma-Aldrich) (39 mM); 300 μL of EDTA (300 nM); 100 μL of NBT (Sigma-Aldrich) (750 μM) and 300 μL of riboflavin (Sigma-Aldrich) (6 μM) were mixed with 100 μL of the bacterial suspensions. The samples were exposed to fluorescent light (20 W) for 6 min to trigger the riboflavin reaction. Positive controls with 30% v/v $H_2O_2$ were run in order to define the number of SOD units corresponding to the percentage reduction of NBT by SOD. The final absorbance was read at 595 nm [28].

### 2.9. Ferric reducing ability as a measure of antioxidant power assay

Overnight cultures in TSB (10 mL of ~$10^9$ CFU/mL) of four MRSA (MRSA ATCC 43300, MRSA 5, MRSA 9, MRSA 28) and four MSSA (MSSA ATCC 29213, MSSA 15, MSSA 16, and MSSA 22) were centrifuged for 10 min at 4000 rpm. The pellets were suspended in PBS, and LZD solutions (1–4 μg/mL) were prepared in PBS. An equal volume of the bacterial sample was incubated with LZD solutions or PBS (control without antibiotic) at 35 °C for 4 h. An aliquot of 10 μL was added to 300 μL of the FRAP reagent mixture, which was prepared with 10 parts 300 mM acetate buffer (pH 3.6), one part of 10 mM 2,4,6-*Tris*(2-pyridyl)-*s*-triazine (TPTZ, Sigma Aldrich) solution in 40 mM HCl 1 N, and one part of 20 mM $FeCl_3.6H_2O$ (Cicarelli). Aqueous solutions of $FeSO_4$ .7 $H_2O$ (Cicarelli), were used to create a calibration standard curve and the absorbances were determined in a microplate reader (Bio-Rad) at 595 nm. Results were expressed as μM $FeSO_4$/mg of protein [29].

### 2.10. Basal levels of glutathione

Overnight suspensions (10 mL of ~$10^9$ CFU/mL) of four MSSA (MSSA 29213, MSSA 15, MSSA 16, MSSA 22) and four MRSA strains (MRSA 43300, MRSA 5, MRSA 9, MRSA 28) were centrifuged (10 min at 4000 rpm) and rinsed with PBS (Basal). Then, 300 μL of 10% trichloroacetic acid in EDTA was added and neutralized with NaOH (1 M) to 300 μL of bacterial suspension. The sample was centrifuged for 10 min at 4000 rpm and incubated with 20 μL of glutathione reductase (6 U/mL, Sigma), 50 μL of NADPH (4 mg/mL, Sigma) and 20 μL of 5,5-dithiobis(2-nitrobenzoic acid) (1.5 mg/mL, Sigma) dissolved in 0.5% $NaHCO_3$. The absorbance was measured at 412 nm, and GSH levels were expressed in mM GSH/mg of protein [30].

### 2.11. Data and statistical analysis

All experiments were performed on three independent days. The results were represented as means of quadruplicates ± SD from the three experiments. One-way ANOVA from OriginPro 8.5 was applied to indicate statistical significance, $P < 0.05$. The reactive species induction percentage was calculated relative to the control (condition without antibiotic) using the following equation:

$$\%\ \mathrm{ROS} = \frac{au_t^{520} - au_c^{520}}{au_c^{520}} \times 100\%$$

where $au_t^{520}$ is the fluorescence recorded at 520 nm for a specific strain exposed for 4 h to a specific LZD concentration and $au_c^{520}$ is the fluorescence outcome at 520 nm from the strain without treatment.

## 3. Results

### 3.1. Minimum inhibitory concentration

The MIC of each strain was determined to determine the antibiotic concentration to use in the oxidative stress assays. The 2 reference strains (MSSA ATCC 29213 and MRSA ATCC 43300) and the 28 selected clinical strains for the study were sensitive to LZD, according to the CLSI breakpoints, with MICs ranging from 1 to 4 μg/mL (see Supplementary Table S1). The CLSI breakpoints were considered for interpretation of the MICs (sensitive ≤4 μg/mL and resistant ≥8 μg/mL).

### 3.2. Screening of reactive oxygen and nitrogen species production

An initial screening of reactive species induction in the reference strain was carried out. The superoxide anion production obtained was 18% with respect to the control without antibiotic in MSSA ATCC 29213, treated with 1 μg/mL of LZD and 14% for MRSA ATCC 43300 with 2 μg/mL of LZD (Supplementary Fig. S1), according to the NBT assay. Additionally, the RNS normalized profile increased twofold with respect to the control without antibiotic in MSSA ATCC 29213 at MIC (2 μg/mL) and three-fold with respect to the control for MRSA ATCC 43300 at 1 μg/mL (Supplementary Fig. S2). To investigate

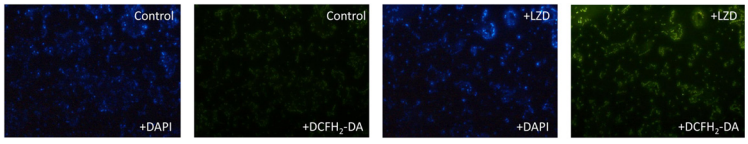


**Fig. 1.** Enhancement of intracellular ROS production in MSSA ATCC 29213 shown by the fluorescent probe $DCFH_2$-DA (green channel) and DAPI (blue channel) staining as the bacteria control. From left to right: MSSA control, blue channel; MSSA control, green channel; MSSA treated with LZD (MIC 2 μg/mL, blue channel) and MSSA treated with LZD (MIC 2 μg/mL, green channel).

further the general production of reactive species, fluorescence microscopy was applied with the fluorogenic reporter $DCFH_2$-DA being sensitive to ROS. Similarly, the enhanced fluorescence intensity was visualized, which demonstrated the intracellular production of ROS in MSSA ATCC 29213 by LZD (Fig. 1). This behaviour was different to that registered in MRSA ATCC 43300, which showed a slight fluorescence (data not shown), indicating that this strain suffered less stress.

To explore ROS and RNS induction in *S. aureus* triggered by LZD treatment, we also tested 28 genetically characterized clinical strains. The increase in reactive species over time was found for 15 clinical strains and 1 LA-MRSA, with 7 of these showing a percentage increase of between 22% and 56% with respect to the control without antibiotic after 4 h of exposure (Supplementary Fig. S3). Likewise, 3 of the 12 MSSA strains showed higher ROS production compared with their respective untreated controls (Supplementary Fig. S4). The fluorescence intensity obtained for MRSA 5, MRSA 9 and MRSA 28 is shown in Fig. 2. The clinical strains revealed a stress induction between 44% and 55% higher compared with the control condition for each (Fig. 2A, inset) and Fig. 2B (inset) shows the fluorescent intensity in which the strains presented an increase of between 25% and 35% in MSSA 15, 29 and 31% in MSSA16 and 29% and 35% in MSSA 22. Moreover, it is noteworthy to mention that LZD induced almost a fourfold ROS increase in the clinical strains compared with the references strains, MRSA ATCC 43300 and MSSA ATCC 29213.

To obtain a better understanding of the oxidative behaviour presented by the strains with the highest reactive species production (between 22% and 56% higher than the control condition), AOPP, FRAP, SOD, and GSH determinations were performed.

### 3.3. Exploring macromolecular damage by advanced oxidation protein products, a biomarker of oxidative stress

The oxidative stress biomarker AOPP was evaluated to investigate the damage generated by ROS and RNS in the stressed strains, and the oxidative lesion over time was monitored. The AOPP levels showed a particular pattern in each strain, with results ranging between 200 and 900 mEq chloramine T/mg protein when MSSA and MRSA were exposed to 2 and 4 μg/mL LZD. However, the increase in the AOPP levels in each strain after 6 h of incubation with LZD was not significant compared with the untreated sample (Supplementary Fig. S5). Meanwhile, the AOPP values has a slight decrease compared with the initial time (0 h) in MRSA ATCC 43300, MRSA 5, MRSA 9, MSSA ATCC 29213 and MSSA 22 after 6 h of treatment.

### 3.4. Superoxide dismutase induction as an acute stress marker

SOD activity was determined to investigate the stress situation among the strains which showed a reactive species increase. Fig. 3 displays the enzymatic activity of each strain treated with increasing concentrations of the antibiotic. A slightly dose-dependent effect was identified for MRSA 5, MRSA 9, MRSA 28 and MSSA ATCC 29213 strains.

### 3.5. Ferric reducing ability as a measure of antioxidant power assay

After 4 h of exposure with increasing concentrations of LZD, the FRAP assay was performed in all the strains which showed an oxidative stress induction above 22% with respect to the control. No dose-dependent effect was observed and there was a marked

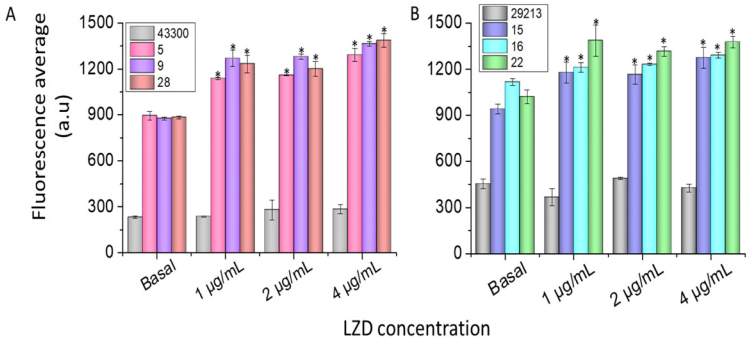


**Fig. 2.** The fluorescence intensity recorded (proportional to ROS production) as a function of increasing LZD concentration after 4 h of exposure. Inset A: performance of four MRSA strains (MRSA ATCC 43300, grey; MRSA 5, pink; MRSA 9, violet; MRSA 28, red). Inset B: MSSA strains (MSSA ATCC 29213, light grey; MSSA 15, blue; MSSA 16, cyan; MSSA 22, green). The data represent the mean ± SD of four replicates in four independent days. The stressed clinical strains show a statistically significance difference compared with basal levels ($^*P \leq 0.05$).

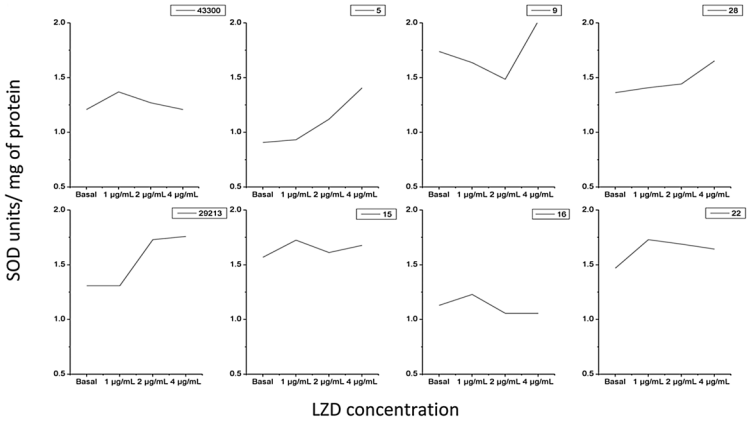


**Fig. 3.** Superoxide dismutase (SOD) activity in S. aureus strains treated with increasing concentrations of LZD. From left to right: bacteria control; bacteria + 1 μg/mL of LZD; bacteria + 2 μg/mL of LZD; and bacteria + 4 μg/mL of LZD. MRSA ATCC 43300, MRSA 5, MRSA 9 and MRSA 28 are presented on the top row of the plot (from left to right) and the MSSA strains ATCC 29213, MSSA 15, MSSA 16 and MSSA 22 on the bottom row (from left to right).

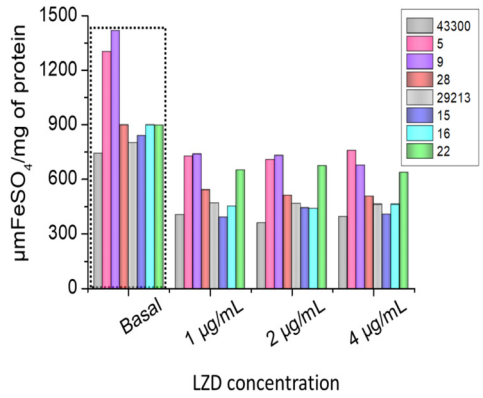


**Fig. 4.** Ferric reducing antioxidant power (FRAP) is illustrated in stressed strains due to LZD treatment. From left to right the bacteria control (in dotted rectangle) and bacteria with different LZD concentrations are shown (MRSA ATCC 43300, grey; MRSA 5, pink; MRSA 9, violet; MRSA 28, red; MSSA ATCC 29213, light grey; MSSA 15, blue; MSSA 16, cyan; MSSA 22, green).

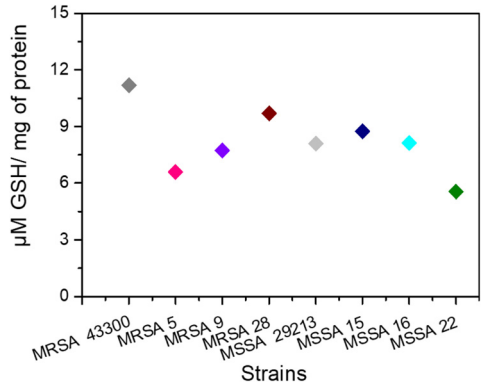


**Fig. 5.** Basal levels of GSH in MRSA and MSSA strains. The figure shows the quantity of GSH that each bacterium has available as a non-enzymatic defence system against oxidative stress before antimicrobial treatment.

decrease in FRAP for all conditions evaluated. Fig. 4 shows two reference strains (MRSA ATCC 43300 and MSSA ATCC 29213) and six clinical strains (MRSA 5, 9, 28 and MSSA 15, 16, 22) where a drop in reducing ability is highlighted with a dotted line showing the control condition values which are higher than all treated samples. The results showed that the total antioxidant capacity was not exacerbated by treatment with LZD, implying that the cell was not facing a stress condition.

### *3.6. Determining the basal levels of a non-enzymatic defence system in* S. aureus *strains: glutathione*

The levels of intracellular GSH were measured to identify any potential differences among the MRSA and MSSA strains in order to confirm the oxidative stress elicited. MRSA 28 and MRSA ATCC 43300 showed the highest GSH basal levels, while the remaining strains exhibited GSH basal values in the range between 7 and 9 µ M GSH/mg of protein (Fig. 5).

## 4. Discussion

The present study was designed to investigate the role of oxidative stress in the mechanism of action of LZD in reference and clinical strains of *S. aureus*.

Our results show the effects of LZD exposure on the *S. aureus* strains, which displayed diverse stress responses; we detected a dose-dependent ROS production, such as in the strains MRSA ATCC 43300, MRSA 5, MRSA 27 and MRSA 28 (Supplementary Fig. S3). Other strains showed almost the same levels of reactive species for the different LZD concentrations: MRSA 1, MRSA 9, MRSA 23, MRSA 24, MSSA 15, MSSA 16, MSSA 22, and MSSA ATCC 29213 (Supplementary Fig. S3 and S4), while the remaining strains revealed similar values between control and samples treated with LZD. An interesting result was obtained in the reference strains treated with LZD, where the RNS increase was significant ($P < 0.05$).

Previous experiments performed in vitro revealed induced oxidative stress with bactericidal and bacteriostatic antibiotics boosting ROS generation in the presence of heavy metal traces (Cu and Fe). Given this scenario, the oxidative stress observations were associated with the ability to induce Fenton chemistry [31]. Kohanski et al. demonstrated that bacteriostatic compounds such as chloramphenicol, tetracycline, erythromycin, and rifampicin did not induce hydroxyl radical formation as part of their actions. However, bactericidal compounds promote this formation, resulting in oxidative damage [32]. ROS disturb the tricarboxylic acid cycle causing NADH depletion and protein Fe-S cluster destabilization, thereby releasing $Fe^{2+}$ and stimulating the Fenton reaction. Other reports highlight that ROS production depends on the antibiotic and at which time it was applied against certain microorganisms [33].

Antibiotics affect several subcellular processes consuming cellular energy, with relevant consequences on bacterial metabolism. A study using metabolomics reported that bacteriostatic antibiotics, in particular, LZD, chloramphenicol, erythromycin and clindamycin in *S. aureus* ATCC 25923, can prompt sub-product accumulation from cellular inhibition. These authors showed that blocking protein synthesis leads to a decrease in the cellular respiration rates and an increase in amino acids and nucleotides, displaying a reduced incorporation of RNA chain and peptides [3]. Other metabolomic and proteomic signature analyses revealed that the central carbon metabolism is suppressed due to bacteriostatic treatment [34,35].

Our results for clinical *S. aureus* strains where ROS production was observed did not reveal any increase in antioxidant systems to counterbalance oxidative species to preserve cell homeostasis. Also, the response of SOD activity, an acute stress marker, was practically the same as for the control condition.

In terms of AOPP as a consequence of antibiotic exposure, ROS formation did not stimulate oxidative damage, which showed similar values over time for the control and the treated strains.

Bactericidal antibiotics increase the metabolic cellular rates, with the antibiotic efficacy being linked to the metabolic state of the bacterial sample during treatment [36]. Also, the bactericidal response affects the upregulation of the genes involved in the central carbon metabolism and cellular respiration, and several reports have suggested that antibiotics induce cellular death according to the oxidative stress levels provoked [37]. Related to this, it has been postulated that cellular death depends on the energetic cost required for the cell to turn on the cellular reparation machinery to deal with the damage [38,39].

In a previous work with a bacteriostatic antibiotic, Albesa et al. reported a dose-dependent reactive species formation in *S. aureus* strains with chloramphenicol, with a slight augmentation in ROS of 1.2 times when they were incubated with the antibiotic compared with the untreated strains (ROS with antibiotic/ROS without antibiotic) [40].

Recently, other authors contributed to revealing the role of ROS in *Streptococcus pneumoniae* clinical strains, under bactericidal and bacteriostatic antibiotics, with LZD being one of the studied antibiotics. They tested 13 clinical isolates under LZD, erythromycin and tetracycline (all 20 × MIC) exposure having minimally raised ROS levels [41].

Our results support the hypothesis that the inhibition of protein synthesis evokes a deceleration of cellular respiration that is crucial to metabolic activity. Moreover, our data showed a ROS induction which could depend on the metabolic situation of each

planktonic strain. The cells which did not exhibit oxidative stress may be in a dormant state, preventing the occurrence of ROS induction. The oxidative stress markers and the antioxidant global capacity did not show a marked increase, implying that the strains were not under an oxidative stress environment due to LZD exposure. Summing up, 10 strains (two reference and eight clinical strains) studied did reveal reactive species formation; however, this increase was insufficient to lead the cell to a state of acute oxidative stress. These results are in agreement to previous reports with bacteriostatic antibiotics [32].

In conclusion, the bacterial stress observed was associated with the metabolic state of each strain; although it was insufficient to induce oxidative damage. Thus, the effects mediated by ROS induction might not have played a significant role in the LZD inhibitory action compared with bactericidal antibiotics.

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## Competing interests

The authors declare no competing financial interest.

## Ethical approval

Not required

## Authors' contributions

SRM participated in the conception and design of the study, performed the assays and participated in the article preparation. VA, CS and MCB participated in the conception and design of the study, and in the article preparation. All authors have approved the final article.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.02.032.

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