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Article

Different modulatory effects of four methicillin-resistant *Staphylococcus aureus* clones on MG-63 osteoblast-like cells

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Abstract: *Staphylococcus aureus* is a Gram-positive bacterium causing a range of mild to life-threatening infections including bone infections such as osteomyelitis. *S. aureus* is able to invade and persist within non-professional phagocytic cells such as osteoblasts. In the present study four different *S. aureus* strains, 2SA-ST239-III, 5SA-ST5-II, 10SA-ST228-I, and 14SA-ST22-IVh were tested for their ability to modulate cell viability in MG-63 osteoblast-like cells following a successful invasion and persistence. Methicillin-sensitive *S. aureus* (MSSA) ATCC-12598-ST30 was used as control. Despite the demonstrated similar abilities of internalization and persistence of ATCC-12598-ST30, 2SA-ST239-III, and 14SA-ST22-IVh strains in MG-63 osteoblast-like cells under our experimental conditions, we demonstrated that the decrease in cell viability was due to the different behavior of the considered strains, with the number of intracellular bacteria playing a limited role. We focused our attention on different cellular biochemical functions related to inflammation, cell metabolism, and oxidative stress during osteoblast infections. We were able to show that: 1) ATCC-12598-ST30 and 2SA-ST239-III were the only two clones able to persist and maintain their number into the cellular hostile environment during the entire period of infection; 2) 2SA-ST239-III was the only clone able to significantly increase the gene expression (3 and 24 h) and protein secretion (24 h) of both interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in MG-63 osteoblast-like cells; 3) the same clone determined a significant up-regulation of transforming growth factor-β1 (TGF-β1) and the metabolic marker glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs at 24 h post infection; 3) neither the MSSA nor the four MRSA strains induced oxidative stress phenomena in MG-63 cells, although a very different expression pattern towards nuclear factor E2-related factor 2 (Nrf2) and its downstream gene heme oxygenase 1 (HO-1) activation was observed among the different clones. Our results can open a new way of considering therapies, going in the direction of an individualized therapeutic strategy that should take into account the difference existing between MSSA and MRSA as well as the distinctive features of the different clones. Not only, therefore, a different antibiotic approach but also a starting point for considering different host factors, i.e. the modulation of specific cytokines such as IL-6, TNF-α, and TGF-β1.

Keywords: *Staphylococcus aureus*; osteoblast-like cells; internalization; inflammation; immune system; host-pathogen interaction; cytokines.

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1. Introduction

Some pathogens are able to internalize and establish persistent and lifelong infections. Several of these pathogens manage to evade the host immune system and cause disease by replicating inside the host cells [1,2]. The main characteristic of bacterial strains, including those with the ability to internalize, is "to adapt the metabolism" so as to escape the defenses of the cell for a longer or shorter period. However, this system is not able to completely eliminate the eukaryotic cell response that differs according to the type of offense [1].

Over the past fifty years, *Staphylococcus aureus*, representing one of the main adaptable human pathogens responsible for community and nosocomial infections, has been able to acquire numerous resistance and virulence genes [3]. *S. aureus* has the ability to invade and persist within non-professional phagocytic cells such as osteoblasts, to remain alive in the intracellular environment, and to evade the immune system (the so called "phagosomal escape") [4]. This intracellular persistence was often associated with a metabolic variant, named small colony variant (SCV), which became able to persist inside human cells and was refractory to antibiotic therapy [5].

Orthopedic infections, such as osteomyelitis and prosthetic joint infections, are recurrent and chronic infections that often require prolonged antimicrobial therapies and surgical interventions due to the challenges in eradicating the bacteria from osteoblasts [6,7]. The prevalence of methicillin-sensitive *S. aureus* (MSSA) in this infection is higher than methicillin-resistant *S. aureus* (MRSA), but these pathogens are often associated with antimicrobial resistance, with high rates of patient's hospitalization and mortality [8]. A recent study reported that some clones belonging to ST239, ST642 and ST107 are more associated with orthopedic infections [9]. Among the clones of healthcare-associated MRSA (HA-MRSA) most circulating in Italy we find ST239-SCC*mec*III, ST5-SCC*mec*II, ST228-SCC*mec*I, and ST22-SCC*mec*IV [10]. One of the most diffused HA-MRSA clones is ST5-SCC*mec*II or New York/Japan. This clone is particularly diffused in USA, Japan, Canada, South Korea, Australia, and Europe [11,12]. For more of 10 years the most diffused clone in Italy was ST228-SCC*mec*I, known as German or Italian Clone. This clone was particularly associated to bacteremia, endocarditis, and low respiratory infection [10,13]. The gentamicin-susceptible clone ST22-SCC*mec*IV has been recognized as the most successful and rapidly disseminating HA-MRSA clone first in England in 1990, then throughout Europe. This clone it was able to cause outbreaks also in the community, and replaced other clones in New Zealand, Australia, and India. In Italy it replaced the most diffused clone ST228-SCC*mec*I [12,14].

The activation of the immune system and the inflammatory process are relevant factors in many pathologies such as diabetes [15], cancer [16], systemic [17], and neurodegenerative diseases [18,19]. Furthermore, inflammation, and more specifically pro-inflammatory cytokines play a critical role during the onset and progression of bacterial infection [20,21]. In particular, it has been shown that infection induced by *S. aureus* determines a deregulated production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) as well as of several chemokines [22,23]. The dysregulated production of these cytokines is also correlated to oxidative stress, a condition characterized by an imbalance between pro-oxidant (excess) and antioxidant (deficiency) species [24]. Cells are able to activate the antioxidant machinery to maintain a status of homeostasis, including the activation of nuclear factor E2-related factor 2 (Nrf2), regulating several hundred genes involved in the antioxidant defense response [25]. Among the different types of cells that are infected by *S. aureus*, osteoblasts represent one of the most studied. These cells, following the infection with *S. aureus*, take part in both the initiation and the maintenance of the inflammatory process through the production of cytokines and the recruitment of immune cells to the inflammation site [6]. Recently, Horn *et al.* discussed the ability of *S. aureus* to internalize in phagocytic (macrophages) and

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non-phagocytic cells (osteoblasts), highlighting how bacterial persistence within these cells is connected to immune evasion phenomena and chronic infection [26].

In the present study, the intracellular persistence and toxicity of four genetically different strains of MRSA on MG-63 osteoblast-like cells was investigated. In order to shed more light on the different behavior observed in these strains, we also determined their influence on the gene expression of IL-6, TNF-α, TGF-β1, and GAPDH as well as Nrf2 and its downstream effector heme oxygenase 1 (HO-1). The human MG-63 osteoblast-like cell line was selected not only because it represents a validated model to study the pro-inflammatory response to bacterial infection [27-29], but also because shows a number of features typical of an undifferentiated osteoblast phenotype including the synthesis of collagen types I and III, a low basal expression of alkaline phosphatase which is increased following 1,25-dihydroxyvitamin D (1,25(OH)2D) administration, and the production of osteocalcin in the presence of 1,25(OH)2D [30-32].

2. Materials and Methods

2.1. Materials and reagents

Materials and reagents were all of analytical grade and were purchased from Sigma (St. Louis, MO, USA) or Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA) unless specified otherwise. *Staphylococcus aureus* subsp. aureus Rosenbach (ATCC® 12598™) (Cowan ST30-t076) and the human osteosarcoma cell line MG-63 (ATCC® CRL-1427™) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). C-Chip disposable hemocytometers were obtained from Bulldog Bio, Inc. (Portsmouth, NH, USA). QuantiTect SYBR Green PCR Kit, RNA extraction kit (RNeasy Mini Kit), RNase-free DNase Set, QuantiTect Primers, and Custom Multi-Analyte ELISArray Kit were all purchased from Qiagen (Hilden, Germany). Eppendorf LoBind Microcentrifuge Tubes PCR Clean (1.5 ml) and PCR tubes were supplied by Eppendorf (Hamburg, Germany).

2.2. Bacterial strains

For this study, four different bacterial strains, belonging to two diverse STs, namely 2SA-ST239-III, 5SA-ST5-II, 10SA-ST228-I, and 14SA-ST22-IVh were used [12]. These strains had already been studied for their ability to internalize and persist in MG-63 osteoblast-like cells [33]. The MSSA ATCC-12598-ST30 (Cowan ST30-t076), an invasive isolate, was used as the control strain for invasion and persistence assays, imaging flow cytometry, as well as gene and protein expression analysis [34]. **Table 1** reports the phenotypical and molecular characteristics of the bacteria included in this study.

Table 1. Phenotypical and molecular characteristics of the five different *S. aureus* strains.

LAB CODE	ST-SCC*mec*-spa type	sourse	FOX	CN	DA	E	CIP	TE	SXT	K	RD	BPR
ATCC-12598	30-MSSA-III-t976	-	-	-	-	-	-	-	-	-	-	-
2SA	239-III-t037	wound	R	R	Ri	R	R	R	R	R	2	2
5SA	5-II-t2154	blood	R	R	S	R	R	R	S	R	>32	2
10SA	228-I-t041	blood	R	R	R	R	R	S	S	R	>32	2
14SA	22-IVh-t032	blood	R	S	Ri	R	R	S	S	S	0.008	1

LAB CODE	ST-SCC*mec*-spa type	sourse	DAL	CPT	LNZ	DPT	TGC	FU	VA	TC	GRD
ATCC-12598	30-MSSA-III-t976	-	-	-	-	-	-	-	-	-	-
2SA	239-III-t037	wound	0.125	2	2	0.5	0.25	>256	1	2	VSSA
5SA	5-II-t2154	blood	0.012	4	32	0.5	0.5	>256	1	0.5	hVISA
10SA	228-I-t041	blood	0.125	1	8	1	0.125	0.125	1	1	VSSA
14SA	22-IVh-t032	blood	0.064	1	1	1	0.125	0.064	0.5	0.25	VSSA

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FOX: cefoxitin; CN: gentamicin; DA: clindamycin; E: erythromycin; CIP: ciprofloxacin; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole; K: kanamycin; RD: rifampin; BPR: ceftobiprole; DAL: dalbavancin; CPT: ceftaroline; LNZ: linezolid; DPT: daptomycin; TGC: tigecycline; FU: fusidic acid; VA: vancomycin; TC: teicoplanin; GRD: MIC test strip glycopeptide-resistance detection; Ri: Inducible clindamycin resistance; clone characterization by means of: ST - Sequence Type; SCC*mec* - Staphylococcal Cassette Chromosome *mec*; spa type - staphylococcal protein A.

2.3. Eukaryotic cell culture preparation

Infection experiments were performed on the human osteosarcoma cell line MG-63. During the expansion period, the cells were grown in 75 cm^2 flasks with modified Eagle's medium (MEM), 1X GlutaMAX™ supplemented with 10% FBS (fetal bovine serum) and 100 U/ml of penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2/95% air and the medium was changed twice a week. Twenty-four hours prior to infection, cells were harvested, counted with a C-Chip disposable hemocytometer, and seeded (full medium without penicillin/streptomycin) in 6-, or 96-well plates at the appropriate density.

2.4. Infection of MG-63 cells

Intracellular frequency of the three different bacterial strains was evaluated in MG-63 osteoblast-like cells at a multiplicity of infection (MOI) of 100:1. This MOI was selected based on previous esperiments in which MG-63 cells were infected with the ATCC-12598-ST30 clone, our control strain, at increasing MOI (12, 50, 100, and 200), observing that with a MOI of 12 or 50 the ability of *S. aureus* to internalize in MG-63 cells was very low, while 200 was quite cytotoxic. Our selection is also supported by many publications where the MOI of 100:1 was reported as the standard for osteoblasts' infections [35-39]. Bacterial isolates were grown in Brain Heart Infusion broth (BHI) at 37 °C overnight. The bacterial concentration was evaluated by optical density at 600 nm by using the GENESYS™ 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial suspensions were prepared using MEM supplemented with 1X GlutaMAX™ and 10% FBS in the absence of penicillin/streptomycin. The same medium composition was also used to grow MG-63 cells that were infected for 3 and 24 h in antibiotic-free conditions. Extracellular bacterial lysis was carried out for 1 h at 37 °C with 100 mg/ml lysostaphin [33].

2.5. Evaluation of the frequency of internalization and intracellular persistence by colony-forming unit (CFU) and spots counting

The amount of intracellular bacteria 3 and 24 h after bacterial infection was estimated by CFU/ml counting as previously described [36]. When estimating the percentage of bacteria internalized into MG-63 cells the following factors were considered: 1) MOI; 2) number of cells; 3) number of CFUs counted after cell lysis.

The number of spots (bacteria) 24 h after bacterial infection was measured by using imaging flow cytometry as previously described [36]. A representative sample of 10,000 events was acquired for each experimental conditions.

2.6. Evaluation of cell viability by MTT assay

To evaluate the effect of *S. aureus* internalization and persistence on the viability of MG-63 cells plated in 96-well plates (2.5×10^3 cells/well) under our different experimental conditions, the MTT (([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay was performed as previously described [40,41], with slight modifications. Two h post infection the extracellular bacterial lysis was carried by using 100 mg/ml lysostaphin. After an incubation of 1 h, the medium containing lysostaphin and lysed bacteria was discarded from each of the 96 wells, the cells were washed twice with sterile phosphate-buffered saline

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(PBS) 0.01 M, and 100 µL of MEM medium supplemented with 1X GlutaMAX™ and 10% fetal bovine serum (FBS) were added to each well. The MTT solution (20 µL at the concentration of 5 mg/mL) was added to each well 3 or 24 h after bacterial infection followed by incubation (2 h) at 37 °C. At the end of the incubation step, the medium was removed and the formed formazan crystals were melted by adding 200 µL/well of anhydrous dimethyl sulfoxide (DMSO) and gentle stirring the plate in a gyratory shaker for 10 min. Next, the absorbance at 569 nm was read using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek, Shoreline, WA, USA).

2.7. Gene expression analysis by quantitative Real-Time PCR (qRT-PCR)

The concentration of total RNA, recovered from 3.5 × 10^5 not infected MG-63 cells (indicated as No Bacteria) or cells infected for 3 and 24 h with ATCC-12598-ST30, 2SA-ST239-III, 5SA-ST5-II, 10SA-ST228-I, or 14SA-ST22-IVh, was determined by measuring the absorbance at 260 nm with a NanoDrop® ND-1000 (Thermo Fisher Scientific). The RNA quality was tested by Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). For reverse transcription, sample amplification, fluorescence data collection and sample quantification, the same protocol as previously described was used [42,43]. The information of QuantiTect Primer Assays used for the gene expression analysis is reported in **Table 2**.

Table 2. The list of primers used for quantitative real-time PCR (qRT-PCR).

Official name[#]	Official symbol	Alternative titles/symbols	Detected transcript	Amplicon Length	Cat. No.[§]
nitric oxide synthase 2, inducible	NOS2	NOS; INOS; NOS2A; HEP-NOS	NM_000625 NM_153292	92 bp	QT00068740
cytochrome b-245 beta chain	CYBB	CGD; NOX2; IMD34; AMCBX2; GP91-1; GP91PHOX; p91-PHOX; GP91-PHOX	NM_000397	124 bp	QT00029533
transforming growth factor beta 1	TGFB1	CED; LAP; DPD1; TGFB; IBDIMDE; TGFbeta; TGF-beta1	NM_000660	108 bp	QT00000728
interleukin 6	IL6	CDF; HGF; HSF; BSF2; IL-6; BSF-2; IFNB2; IFN-beta-2	NM_000600 XM_005249745	107 bp	QT00083720
tumor necrosis factor	TNF	DIF; TNFA; TNFSF2; TNLG1F; TNF-alpha	NM_000594	98 bp	QT00029162
glyceraldehyde-3-phosphate dehydrogenase	GAPDH	G3PD; GAPD; HEL-S-162eP	NM_001256799 NM_002046 NM_001289745	95 bp	QT00079247

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			NM_001289746		
nuclear factor, erythroid 2 like 2	NFE2L2	NRF2; HEBP1; Nrf-2; IMDDHH	NM_006164	153 bp	QT00027384
heme oxygenase 1	HMOX1	HO-1; HSP32; HMOX1D; bK286B10	NM_002133	99 bp	QT00092645
actin beta	ACTB	BRWS1; PS1TP5BP1	NM_001101	146 bp	QT00095431

[#]https://www.ncbi.nlm.nih.gov/gene/

[§]https://www.qiagen.com/it/shop/pcr/real-time-pcr-enzymes-and-kits/two-step-qrt-pcr/quantitect-primer-assays/

2.8. Cytokine secretion analysis by ELISA

The quantification of IL-6, TNF-α, and TGF-β1 in cell culture supernatants from not infected MG-63 cells or cells infected for 24 h with ATCC-12598-ST30, 2SA-ST239-III, or 10SA-ST228-I was carried out by using a Custom Multi-Analyte ELISArray Kit according to manufacturer's instructions [44].

2.9. Statistical analysis

Statistical analysis was performed and the relative graphs prepared using version 8 of Graphpad Prism software (Graphpad software, San Diego, CA, USA). The within group comparison was carried out by the one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons *post hoc* test. Only two-tailed p-values less than 0.05 were considered statistically significant. All experiments were performed at least in triplicate.

3. Results

3.1. The intracellular persistence and the number of bacteria into single MG-63 osteoblast-like cells vary significantly among the different strains

Figure 1A shows the amount of intracellular bacteria for the five different *S. aureus* strains, calculated as CFU/ml, 3 and 24 h after infection.

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A

S. aureus strain	CFU 3 h p.i.	CFU 24 h p.i.	Difference
ATCC-12598-ST30	2.6 x 10^6	2.5 x 10^6	-0.1 x 10^6
2SA-ST239-III	2.8 x 10^6	2.4 x 10^6	-0.4 x 10^6
5SA-ST5-II	4.2 x 10^6	8.9 x 10^5	-3.3 x 10^6
10SA-ST228-I	3.7 x 10^6	2 x 10^6	-1.7 x 10^6
14SA-ST22-IVh	3 x 10^6	1.2 x 10^6	-1.8 x 10^6

p.i. = post infection

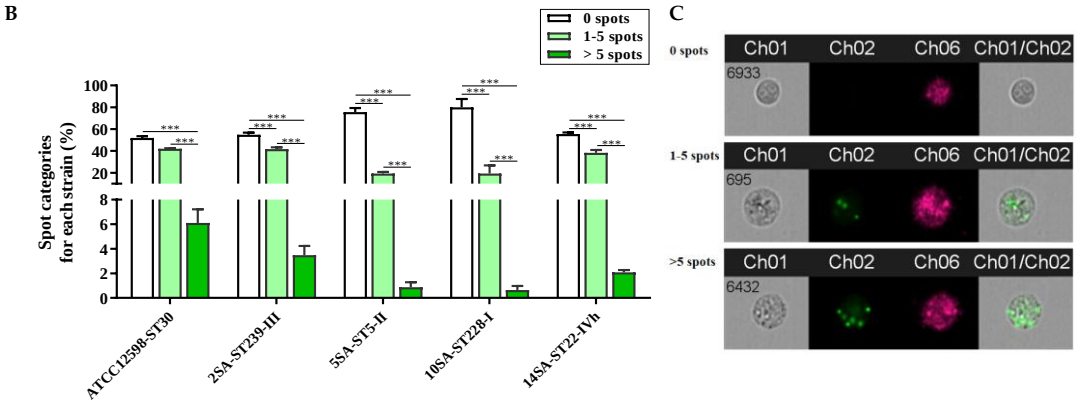

Figure 1. A) Measured CFU/ml counting following cell lysis for the five different *S. aureus* strains 3 and 24 h after infection. B) Percentage of spot categories (0 spots, 1 to 5, and over 6 spots) for each strain 24 h after bacterial infection. Values are reported as means ± SD of three independent experiments. Significant comparisons between each category are indicated by lines. ***Significantly different, $p < 0.001$. C) Representative image showing MG-63 without spots inside the cell (0 spots), a single MG-63 cell with three spots inside (1-5 spots), and a single MG-63 cell with 6 spots (>5 spots). CH1 = brightfield; CH2 = 505–560 nm; CH6 = side scatter (SSC) at 785 nm.

The measured CFU/ml 3 h post infection was quite similar for ATCC-12598-ST30, 2SA-ST239-III, and 14SA-ST22-IVh, ranging from 2.6 to 3 x 10^6 CFU/ml, while was higher for 5SA-ST5-II (4.2 x 10^6) and 10SA-ST228-I (3.7 x 10^6) strains. The variation of the number of bacteria during the following 21 hours, then 24 h post infection, was very different among the five different *S. aureus* strains, as clearly showed by the calculated difference in CFU/ml between 3 and 24 h post infection. In fact, while in the case of ATCC-12598-ST30 and 2SA-ST239-III the measured CFU/ml was pretty similar to that observed at 3 h post infection, the quantity of bacteria measured at 24 h post infection strongly decreased in the case of 5SA-ST5-II, 10SA-ST228-I, and 14SA-ST22-IVh.

To better examine the different ability to infect and persist among the five invasive *S. aureus* strains, especially between the four MRSA clones, the spot categories (0 spots, 1-5 spots, >5 spots) for each strain (%) were calculated (**Figure 1B**). Based on the average spots number per cell, a similar distribution of the three spot categories, %0 spots > %1-5 spots > %>5 spots ($p < 0.001$), was observed for all the *S. aureus* strains considered, except in the case of ATCC-12598-ST30 where %0 spots and %1-5 spots were comparable. **Table 3** reports the existing interstrain differences obtained by comparing the % of the same spot category.

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Table 3. Additional statistics regarding the comparison of spots counting measured under all our experimental conditions. Only % belonging to the same spot category were compared.

Bonferroni's multiple comparisons test	Significant?	Summary	*p*-value
ATCC12598-ST30:0 spots vs. 2SA-ST239-III:0 spots	No	ns	>0,9999
ATCC12598-ST30:0 spots vs. 5SA-ST5-II:0 spots	Yes	****	<0,0001
ATCC12598-ST30:0 spots vs. 10SA-ST228-I:0 spots	Yes	****	<0,0001
ATCC12598-ST30:0 spots vs. 14SA-ST22-IVh:0 spots	No	ns	>0,9999
ATCC12598-ST30:1-5 spots vs. 2SA-ST239-III:1-5 spots	No	ns	>0,9999
ATCC12598-ST30:1-5 spots vs. 5SA-ST5-II:1-5 spots	Yes	****	<0,0001
ATCC12598-ST30:1-5 spots vs. 10SA-ST228-I:1-5 spots	Yes	****	<0,0001
ATCC12598-ST30:1-5 spots vs. 14SA-ST22-IVh:1-5 spots	No	ns	>0,9999
ATCC12598-ST30:> 5 spots vs. 2SA-ST239-III:> 5 spots	No	ns	>0,9999
ATCC12598-ST30:> 5 spots vs. 5SA-ST5-II:> 5 spots	No	ns	>0,9999
ATCC12598-ST30:> 5 spots vs. 10SA-ST228-I:> 5 spots	No	ns	>0,9999
ATCC12598-ST30:> 5 spots vs. 14SA-ST22-IVh:> 5 spots	No	ns	>0,9999
2SA-ST239-III:0 spots vs. 5SA-ST5-II:0 spots	Yes	****	<0,0001
2SA-ST239-III:0 spots vs. 10SA-ST228-I:0 spots	Yes	****	<0,0001
2SA-ST239-III:0 spots vs. 14SA-ST22-IVh:0 spots	No	ns	>0,9999
2SA-ST239-III:1-5 spots vs. 5SA-ST5-II:1-5 spots	Yes	****	<0,0001
2SA-ST239-III:1-5 spots vs. 10SA-ST228-I:1-5 spots	Yes	****	<0,0001
2SA-ST239-III:1-5 spots vs. 14SA-ST22-IVh:1-5 spots	No	ns	>0,9999
2SA-ST239-III:> 5 spots vs. 5SA-ST5-II:> 5 spots	No	ns	>0,9999
2SA-ST239-III:> 5 spots vs. 10SA-ST228-I:> 5 spots	No	ns	>0,9999
2SA-ST239-III:> 5 spots vs. 14SA-ST22-IVh:> 5 spots	No	ns	>0,9999
5SA-ST5-II:0 spots vs. 10SA-ST228-I:0 spots	No	ns	>0,9999
5SA-ST5-II:0 spots vs. 14SA-ST22-IVh:0 spots	Yes	****	<0,0001
5SA-ST5-II:1-5 spots vs. 10SA-ST228-I:1-5 spots	No	ns	>0,9999
5SA-ST5-II:1-5 spots vs. 14SA-ST22-IVh:1-5 spots	Yes	****	<0,0001
5SA-ST5-II:> 5 spots vs. 10SA-ST228-I:> 5 spots	No	ns	>0,9999
5SA-ST5-II:> 5 spots vs. 14SA-ST22-IVh:> 5 spots	No	ns	>0,9999
10SA-ST228-I:0 spots vs. 14SA-ST22-IVh:0 spots	Yes	****	<0,0001
10SA-ST228-I:1-5 spots vs. 14SA-ST22-IVh:1-5 spots	Yes	****	<0,0001
10SA-ST228-I:> 5 spots vs. 14SA-ST22-IVh:> 5 spots	No	ns	>0,9999

3.2. Infection with ATCC-12598-ST30, 2SA-ST239-III, 5SA-ST5-II, 10SA-ST228-I, and 14SA-ST22-IVh strains differently affected MG-63 osteoblast-like cells viability

Figure 2 reports the changes in MG-63 cell viability after infection with the five different *S. aureus* strains at 3 and 24 h.

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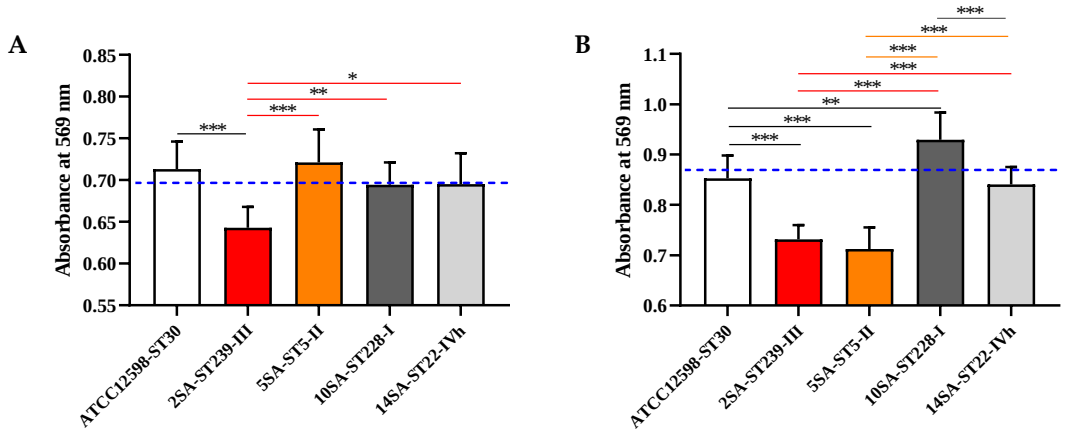

Figure 2. Changes in cell viability of MG-63 osteoblast-like cells infected at MOI of 100:1 with five different *S. aureus* strains detected after A) 3 h and B) 24 h. Values are reported as means ± SD of at least five independent experiments. Values are reported as absorbance measured at 569 nm. Blue dotted line indicates the absorbance measured at 569 nm for uninfected MG-63 cells. Significant comparisons between each experimental condition are indicated by lines. *Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$; ***Significantly different, $p < 0.001$.

The viability of MG-63 cells infected with the MRSA 2SA-ST239-III strain, measured as absorbance at 569 nm, was significantly lower ($p < 0.001$) compared to that of cells infected with the reference control strain ATCC-12598-ST30 as well as to the other MRSA strains ($p < 0.001$ vs. 5SA-ST5-II; $p < 0.01$ vs. 10SA-ST228-I; $p < 0.05$ vs. 14SA-ST22-IVh) at 3 h post infection (**Figure 2A**). The absorbance values measured in the case of MG-63 cells infected with all the other strains were very similar to the reference control strain at 3 h. A quite different situation was observed 24 h post infection. In this case, both 2SA-ST239-III and 5SA-ST5-II strains were able to significantly decrease the cell viability compared to ATCC-12598-ST30-infected cells ($p < 0.001$ for both of them) and to 10SA-ST228-I and 14SA-ST22-IVh ($p < 0.001$) MRSA strains (**Figure 2B**). The infection of MG-63 cells with the other two strains led to viability values comparable (14SA-ST22-IVh) or even higher (10SA-ST228-I; $p < 0.01$) than those observed for cells infected with the reference strain (**Figure 2B**).

Overall, the results reported in **Figure 2,** along with previous studies [33], show that the ability of *S. aureus* strains to infect and persist in MG-63 osteoblast-like cells is not always proportionally correlated with changes in cell viability. Therefore, the subsequent experiments were finalized to determine whether the bacteria internalization and the related changes in cell viability were connected to any variation of different cellular biochemical functions related to inflammatory phenomena and the modulation of the antioxidant machinery.

3.3. The increase of inflammation mediators 2SA-ST239-III-induced in MG-63 osteoblast-like cells is accompanied by an up-regulation of TGF-β1 and GAPDH

In the case of gene expression analysis, we compared the values obtained for each *S. aureus* strain with each other, normalizing them in comparison to the values obtained for cells in the absence of bacteria, indicated as No Bacteria. **Figure 3** shows the different mRNA expression levels of two well-known pro-inflammatory cytokines, namely IL-6 and TNF-α, following infection for 3 and 24 h with the five different bacterial strains.

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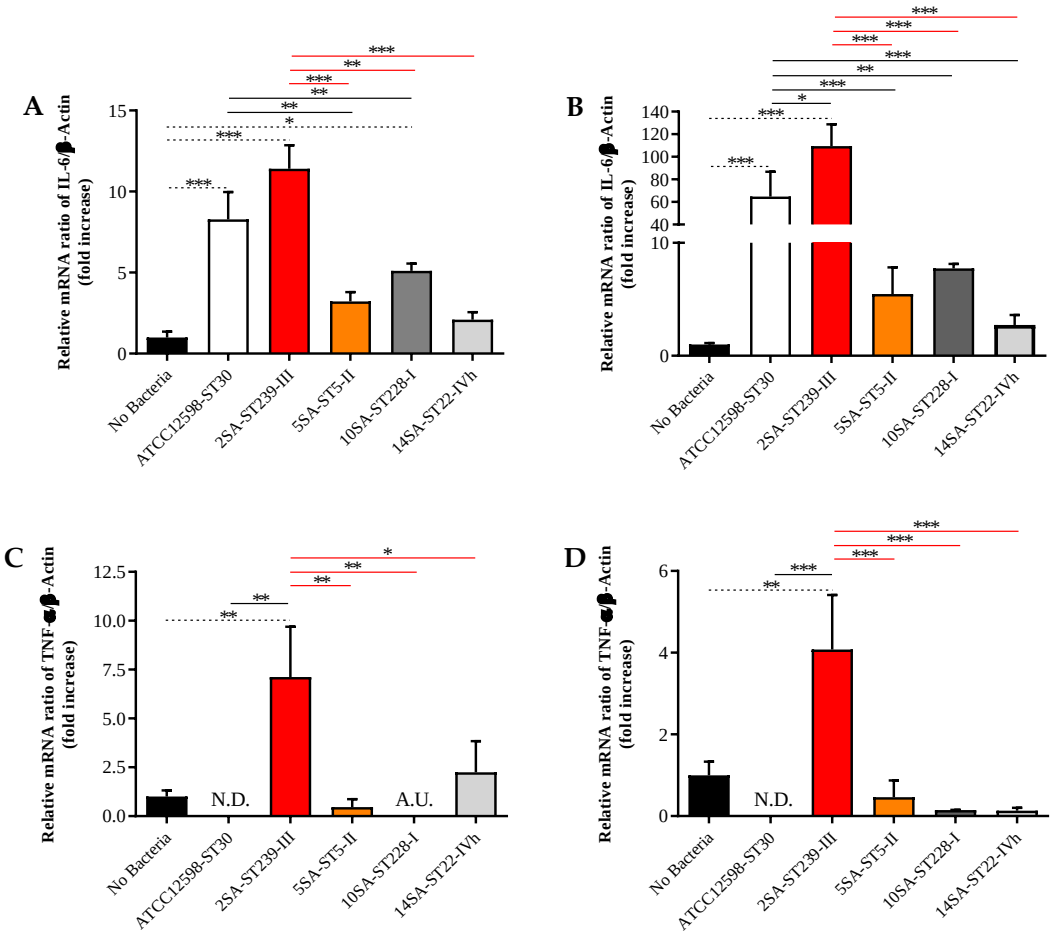

Figure 3. Gene expression of IL-6 (A, B) and TNF-α (C, D) in uninfected (No Bacteria) MG-63 osteoblast-like cells and in MG-63 cells infected at MOI of 100:1 with five different *S. aureus* strains detected at 3 and 24 h. N.D. = not detectable; A.U. = almost undetectable. The abundance of each mRNA is expressed relative to the abundance of β-Actin-mRNA. Values are means ± SD of three independent experiments. Significant comparisons between each experimental condition are indicated by lines. *Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$; ***Significantly different, $p < 0.001$.

ATCC-12598-ST30, 2SA-ST239-III, and 10SA-ST228-I strains were able to significantly up-regulate the expression of IL-6 mRNA at 3 h post infection compared to uninfected cells ($p < 0.001$ for ATCC-12598-ST30 and 2SA-ST239-III; $p < 0.05$ for 10SA-ST228-I), with maximal effect observed when MG-63 cells were infected with 2SA-ST239-III (~11-fold increase) (**Figure 3A**). The increase due to 2SA-ST239-III was also significantly higher than that of 5SA-ST5-II- ($p < 0.001$), 10SA-ST228-I- ($p < 0.01$), and 14SA-ST22-IVh-induced ($p < 0.001$). In the case of reference strain, its induction led to a significant up-regulation of IL-6 mRNA expression compared to 5SA-ST5-II and 10SA-ST228-I ($p < 0.01$ for both of them). The prolongation of the time of infection (up to 24 h) exacerbated bacterial pro-inflammatory effects, especially in the case of 2SA-ST239-III that gave IL-6 mRNA expression level values significantly higher than cells in the absence of bacteria ($p < 0.001$) as well as ATCC-12598-ST30 ($p < 0.05$) and the remaining strains 5SA-ST5-II, 10SA-ST228-I, and 14SA-ST22-IVh ($p < 0.001$ vs. all of them) (**Figure 3B**). A different trend was observed in the case of the induction of TNF-α. In fact, 2SA-ST239-III was the only strain able to significantly enhance TNF-α mRNA expression levels at both 3 (**Figures 3C**; $p < 0.01$ vs. No Bacteria, ATCC-12598-ST30, 5SA-ST5-II, and 10SA-ST228-I; $p < 0.05$ vs. 14SA-ST22-IVh) and 24 h (**Figures 3D**; $p < 0.01$ vs. No Bacteria and $p < 0.001$ vs. the other four strains at both time points) post infection.

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Figure 4 shows the effects of the five bacterial strains on mRNA expression levels of TGF-β1, a cytokine over-expressed in osteomyelitis [45], and the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a metabolic marker [46].

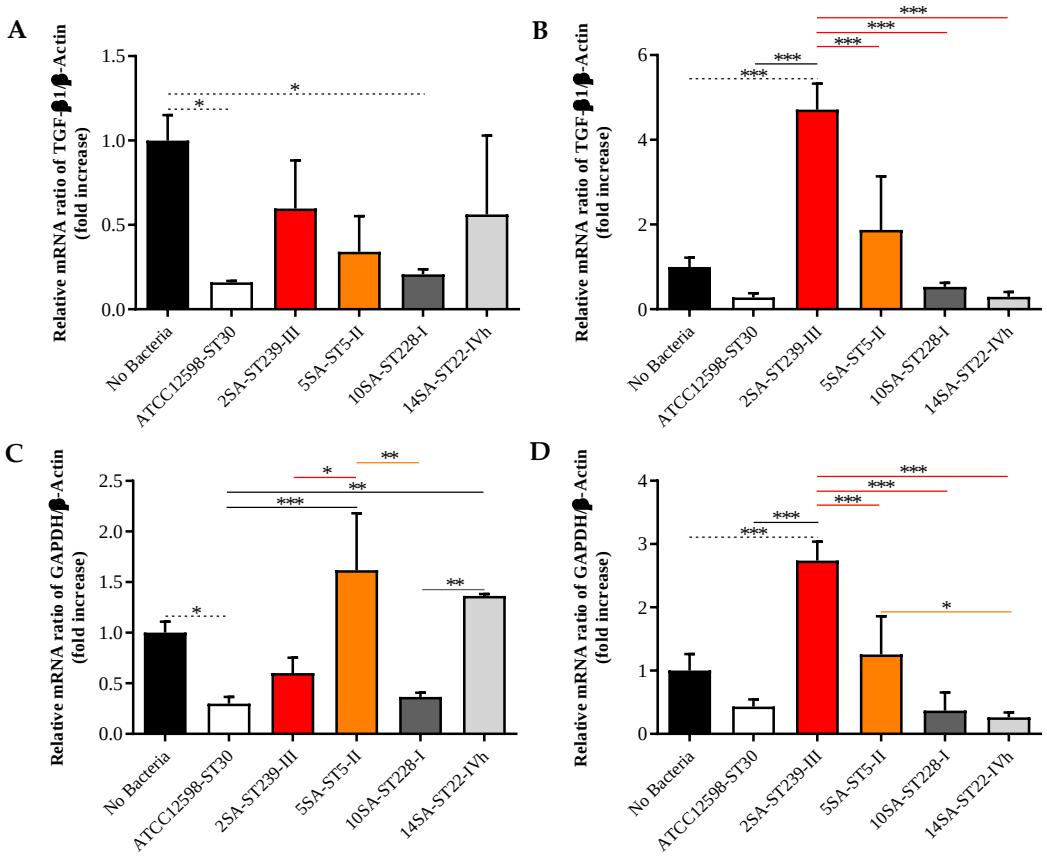

Figure 4. Gene expression of TFG-β1 (A, B) and GAPDH (C, D) in uninfected (No Bacteria) MG-63 osteoblast-like cells and in MG-63 cells infected at MOI of 100:1 with five different *S. aureus* strains detected, at 3 and 24 h. The abundance of each mRNA is expressed relative to the abundance of β-Actin-mRNA. Values are means ± SD of three independent experiments. Significant comparisons between each experimental condition are indicated by lines. *Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$; ***Significantly different, $p < 0.001$.

Interestingly, the shorter infection time (3 h) led to a significant decrease of TGF-β1 mRNA expression levels for ATCC-12598-ST30 ($p < 0.05$) and 10SA-ST228-I ($p < 0.05$) compared to uninfected MG-63 cells (**Figure 4A**), while no significant differences were observed for the other three strains. Of note, at prolonged infection time (24 h), the only clone able to significantly increase the gene expression of TGF-β1 was 2SA-ST239-III ($p < 0.001$ vs. all other experimental conditions) (**Figure 4B**). In the case of GAPDH mRNA expression levels, none of the bacteria considered was able to induce an up-regulation, and in the case of ATCC-12598-ST30 strain the measured expression was even lower than that observed for uninfected cells ($p < 0.05$) (**Figure 4C**). A great variability was instead found among the different bacterial strains, with 5SA-ST5-II giving an up-regulation significantly higher than that observed for cells infected with ATCC-12598-ST30 ($p < 0.001$), 2SA-ST239-III ($p < 0.05$), and 10SA-ST228-I ($p < 0.01$). Some of the bacterial strains behaved very differently after the longest infection time (24 h), especially 2SA-ST239-III strain. In fact, in the case of MG-63 cells infected with 2SA-ST239-III strain, a significant induction of TGF-β1 mRNA compared to all other experimental conditions ($p < 0.001$ vs. all) was observed (**Figures 4D**). Surprisingly, the effect of 5SA-ST5-II strain, evident at 3 h post

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infection compared to most of the other strains, was significant only if compared to 14SA-ST22-IVh ($p < 0.05$).

To further confirm the results obtained by carrying out qRT-PCR experiments, we performed additional experiments in which the cell culture supernatants from not infected MG-63 cells or cells infected for 24 h with ATCC-12598-ST30 (reference strain), 2SA-ST239-III (highest toxicity and internalization), and 10SA-ST228-I (lower toxicity and internalization). The analysis of cytokines in cell supernatants indicated a significant up-regulation of the pro-inflammatory cytokine IL-6 induced by the presence of ATCC-12598-ST30 and 2SA-ST239-III compared to uninfected MG-63 cells ($p < 0.001$) (**Figure 5A**), while 10SA-ST228-I did not lead to any significant modulation of IL-6 release.

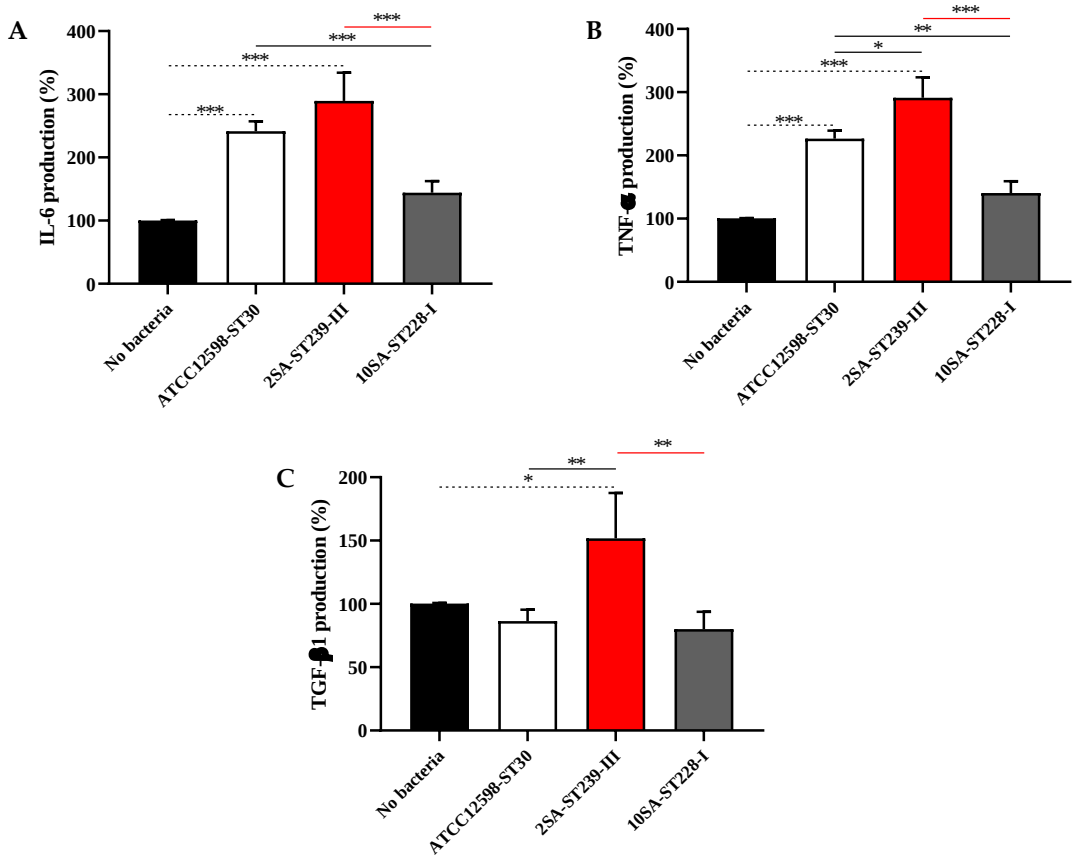

Figure 5. Modulation of IL-6, TNF-α, and TGF-β1 secretion by bacteria. Supernatants from uninfected (No Bacteria) MG-63 osteoblast-like cells and in MG-63 cells infected at MOI of 100:1 with three different *S. aureus* strains for 24 h were analyzed using a Custom Multi-Analyte ELISArray Kit. Each treatment was analyzed in at least in triplicate. The production of each cytokine is expressed as the percent variation with respect to the production recorded in uninfected (control) cells. A) IL-6, B) TNF-α, and C) TGF-β1. Values are means ± SD of three to four independent experiments. Significant comparisons between each experimental condition are indicated by lines. *Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$; ***Significantly different, $p < 0.001$.

A quite comparable release profile was observed in the case of TNF-α (**Figure 5B**); in fact, both ATCC-12598-ST30 and 2SA-ST239-III treatments led to a significant increase in the secretion of TNF-α protein compared to uninfected MG-63 cells ($p < 0.001$). Of note, in this case, the presence of 2SA-ST239-III led to an increased secretion even if compared to ATCC-12598-ST30 ($p < 0.05$). A different response to the considered bacteria was observed when measuring the release of TGF-β1. Neither ATCC-12598-ST30 nor 10SA-ST228-I was able to induce the release of TGF-β1 cytokine compared to uninfected MG-63 cells. As expected, 2SA-ST239-III led to a significant increase in the release of TFG-β1 compared to all

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the other experimental conditions considered ($p < 0.05$ vs. No Bacteria and $p < 0.01$ vs. the other two strains) (**Figure 5C**).

3.3. Nrf2 and its downstream gene HO-1 are more sensitive to the presence of 2SA-ST239-III than ATCC-12598-ST30, 5SA-ST5-II, 10SA-ST228-I, and 14SA-ST22-IVh

The infection of MG-63 cells for 3 h led to a significant down-regulation of Nrf2 mRNA expression levels for all the strains considered ($p < 0.01$ for all of them) compared to uninfected cells, with the exception of 2SA-ST239-III that gave values comparable with those of control cells (**Figures 6A**).

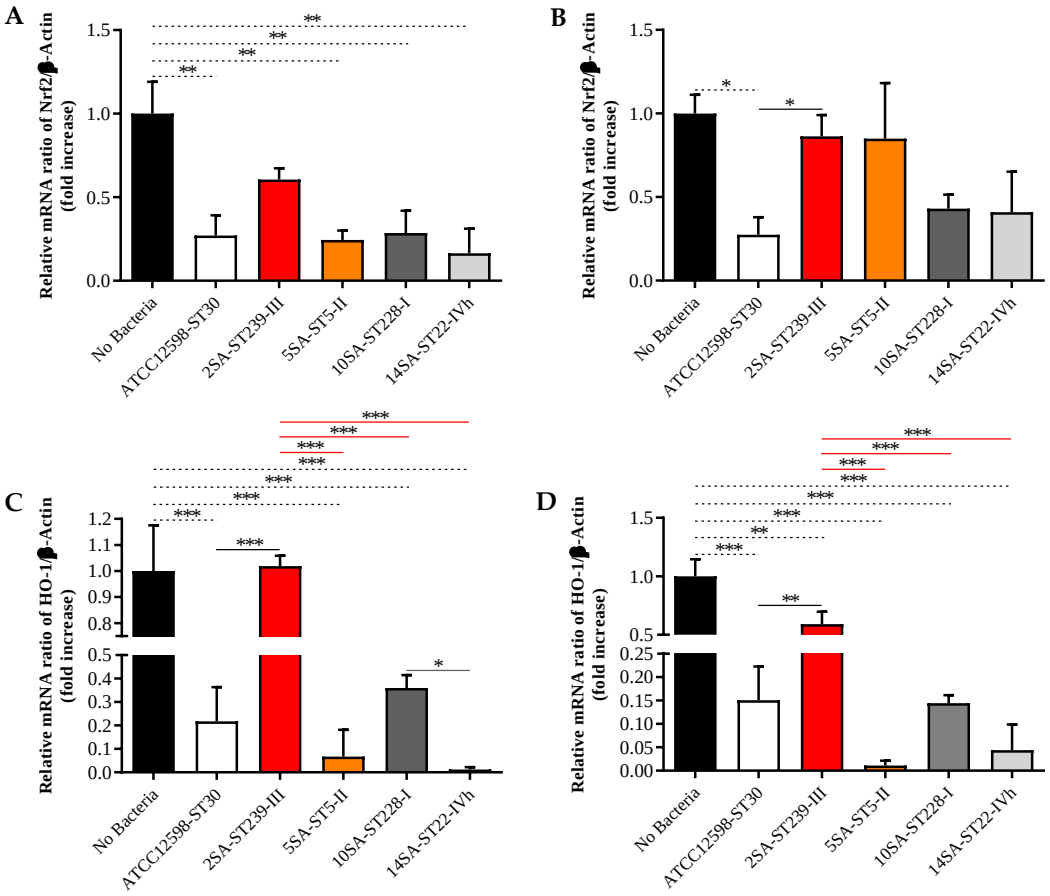

Figure 6. Gene expression of Nrf2 (A, B) and HO-1 (C, D) in uninfected (No Bacteria) MG-63 osteoblast-like cells and in MG-63 cells infected at MOI of 100:1 with five different *S. aureus* strains detected at 3 and 24 h. The abundance of each mRNA is expressed relative to the abundance of β-Actin-mRNA. Values are means ± SD of three independent experiments. Significant comparisons between each experimental condition are indicated by lines. *Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$; ***Significantly different, $p < 0.001$.

No significant differences were observed among the different bacterial strains at this time point. The prolongation of the time of infection (24 h) has leveled the exiting differences between uninfected and infected cells, except in the case of the reference strain ATCC-12598-ST30 that gave Nrf2 mRNA expression levels significantly lower compared to both uninfected and 2SA-ST239-III-infected cells ($p < 0.05$ for both of them) (**Figure 6B**). As observed in the case of Nrf2 at the shorter infection time, the treatment of MG-63 cells for 24 h led to a significant down-regulation of HO-1 mRNA expression levels for all the strains considered ($p < 0.001$ for all of them) compared to uninfected cells, with the exception of 2SA-ST239-III that gave values comparable with those of control cells and significantly increased compared to all the other strains ($p < 0.001$ vs. all) (**Figures 6C**). Very similar results were observed over the 24 h of infection, with the only difference regarding the

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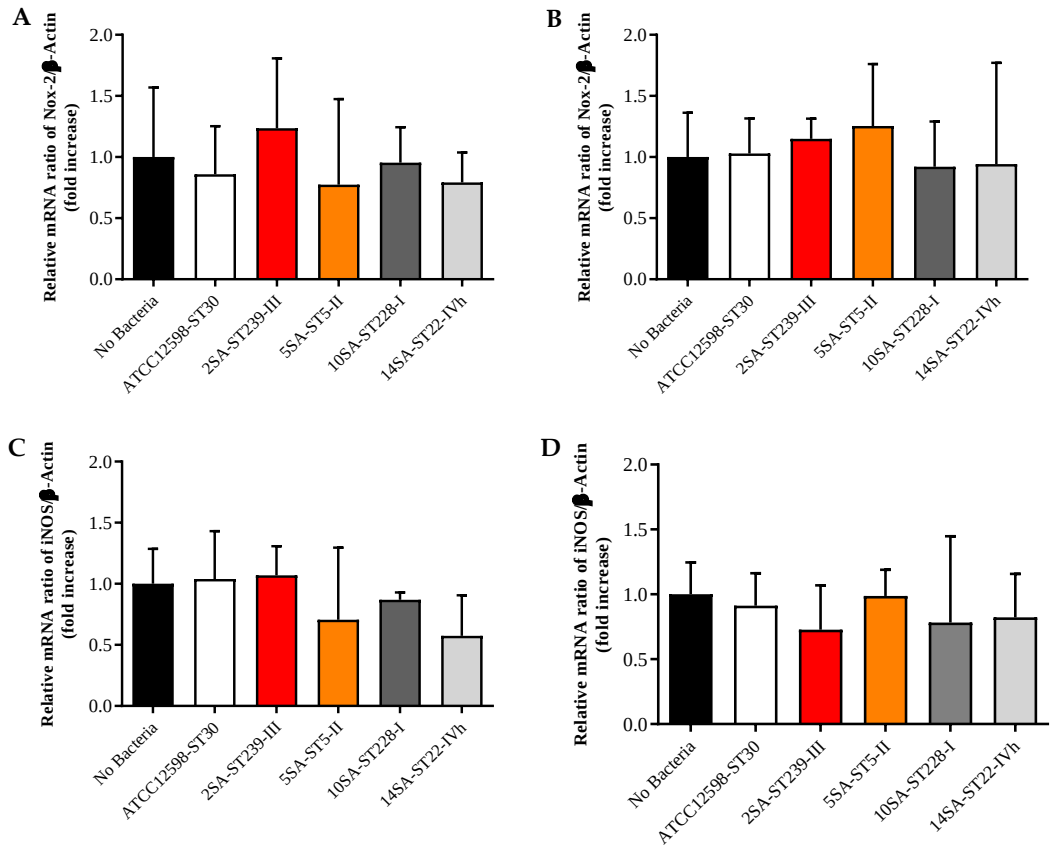
down-regulation of the HO-1 mRNA expression values for 2SA-ST239-III strain compared to uninfected cells ($p < 0.01$).

It is worth underlining that, despite the very clear differences observed between the various strains, none of the bacteria considered was able to induce Nrf2 and HO-1 gene expression values higher than those measured in uninfected cells.

As suggested by the above-mentioned results, oxidative stress should not contribute significantly to the bacteria-induced toxicity and pro-inflammatory phenomena. To further confirm this hypothesis, we carried out additional experiments in which the mRNA expression levels of inducible nitric oxide synthase (iNOS) and NADPH oxidase 2 (Nox-2), two enzymes whose activation is closely linked to oxidative stress events [33], were measured. As expected, the infection with the *S. aureus* strains in this study did not induce any significant increase in iNOS and Nox2 mRNA expression levels (**Supplementary Figure 1**).



Supplementary Figure 1. Gene expression of Nox-2 (A, B) and iNOS (C, D) in uninfected (No Bacteria) MG-63 osteoblast-like cells and in MG-63 cells infected at MOI of 100:1 with five different *S. aureus* strains detected at 3 and 24 h. The abundance of each mRNA is expressed relative to the abundance of β-Actin-mRNA. Values are means ± SD of three independent experiments.

4. Discussion

Staphylococci, in particular *S. aureus*, are the predominant cause of bone infections worldwide [47]. *S. aureus*, one of the major human pathogens, is responsible for the altered homeostasis between bone cells during infections through the induction of different responses from osteoblast cells [48].

Based on the above, by using human MG-63 osteoblast-like cells infected with four different clinically isolated *S. aureus* strains and one strain of MSSA as a control, we aimed to answer the following questions: i) Is the ability to infect and persist in MG-63 osteoblast-like cells of each strain directly related to changes in cell viability?; ii) Do different genetic

backgrounds lead to different pro-inflammatory and pro-oxidant responses in MG-63 osteoblast-like cells?

Our research group has recently used MG-63 cells as a novel tool to study host-pathogens interaction in a complex set of experiments including 16 different bacterial strains able to differentially infect these cells. In particular, the ability of MSSA ATCC-12598-ST30 and the two MRSA 2SA-ST239-III and 14SA-ST22-IVh to internalize and persist in MG-63 osteoblast-like cells was very similar (50.49% ± 0.69, 50.33% ± 1.19, and 45.37% ± 1.31, respectively), while the other MRSA clones, 5SA-ST5-II and 10SA-ST228-I, behave very differently in terms of internalization ability (27.59% ± 2.50 and 20.74% ± 1.04, respectively) [33].

The answer to the first question was somewhat unexpected. In fact, despite the ability of the five strains to differentially internalize and persist in MG-63 cells (ST30, ST239, and ST22) demonstrated a very similar and high internalization rate with respect to ST5 and ST228), 2SA-ST239-III (at both 3 and 24 h) and 5SA-ST5-II (at 24 h) were the only strains able to significantly inhibit osteoblast viability. In particular, clone ST239, despite the same internalization rate than the MSSA strain control, was very toxic, while clone ST228, with a lower internalization rate compared to both of them, displayed a lower cytotoxic potential, even compared to the MSSA strain. Very interestingly, despite the similar decrease in cell viability induced at 24 h by 5SA-ST5-II and 2SA-ST239-III, the latter demonstrated greater adaptability to the intracellular hostile environment, being the only one able to maintain its number during the entire period of infection. ST239 is a well-studied and widespread clone presenting some genomic characteristic features [49] as well as virulence gene content [50]. Some studies performed on bacteremic patients demonstrated the development of a non uniform and unique immune response directed to different staphylococcal proteins [51]; furthermore, a recent paper on MRSA ST239 identified a specific pattern of genes, confirming the differences among this clone and the others [52].

With specific regard to the differences in the epidemiology and virulence observed between MSSA and MRSA, our findings are in line with different epidemiologic studies, including a meta-analysis, showing increased morbidity and/or mortality from MRSA compared with MSSA [53-56].

The different behavior observed for these strains was also accompanied by a different modulation of inflammatory phenomena, metabolism and antioxidant machinery.

In line with previously published data showing the inductive effects of *S. aureus* with regard to the expression of pro-inflammatory cytokines in different cell types [57,58] including osteoblasts [48], we found that the most persistent and at the same time toxic strain, 2SA-ST239-III, was able to strongly increase the expression levels of both IL-6 and TNF-α at both 3 and 24 h post infection compared with the other strains. This is relevant in the case of *S. aureus* infection; in fact, as recently suggested by Di Domenico *et al.* the production of pro-inflammatory cytokines can selectively promote *S. aureus* outgrowth, and, interestingly, there is an interplay between host pro-inflammatory cytokines and bacterial biofilm production [59]. In particular, the pro-inflammatory cytokines produced during *S. aureus* infection can promote its outgrowth subverting the composition of the healthy skin microbiome, while the production of biofilm plays a relevant role on the support of chronic colonization, providing an increased resistance to antimicrobial agents.

We then focused our attention on TGF-β1, a highly conserved anti-inflammatory cytokine, which acts as a key modulator of the microbiota and host immune cell cross-talk [60] involved in scar formation in osteomyelitis [45]. TGF-β1 plays a central role in immune suppression and repair after injury [61] and, most importantly, in the development and maintenance of bone competence cells such as osteoblasts and osteoclasts [62]. As has recently been demonstrated *in vivo* by Wang *et al.* employing a rat model mimicking the biological development of osteomyelitis obtained by injecting *S. aureus* into the medullary cavity, the expression of TGF-β1 gene as well as its receptors (TβRI and TβRII) is strongly up-regulated [45]. TβRI and TβRII are also expressed by the MG-63 osteoblast-like cells used

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in the present study [63]. TGF-β1 is a stimulator of type I collagen production [64], which is connected to the hypertrophic scarring of the soft tissue surrounding the infected bone in osteomyelitis [65]. Accordingly, 2SA-ST239-III, the clone that has so far been found to be more active in terms of invasiveness, toxicity, and inflammation, was the only one able to significantly enhance TGF-β1 gene and protein expression levels 24 h post infection compared to untreated cells as well as the other four strains. Moreover, we cannot exclude that our results might be partially explained also considering the origin of our cell model, MG-63 cells (a line derived from osteosarcoma), since it has been discussed in a review by Lamora *et al.*, TGF-β1 cytokine production plays a pivotal role in osteosarcoma progression through its pro-metastatic effects [66]. Nevertheless, the new data discussed in the present paper suggest a potential novel role of TGF-β1 in the pathophysiology of osteomyelitis as well as the relevance of TGF-β1 in determining the invasiveness and toxicity of different *S. aureus* strains.

According to this scenario, we hypothesize that TGF-β1 might initially increase in response to bacteria-related inflammation and augmented secretion of pro-inflammatory cytokines, thus gaining a pathogenic role promoting fibrosis in osteomyelitis, as demonstrated in animal models of osteomyelitis. Therefore, with regard to *S. aureus* infections, especially in the case of osteomyelitis, therapeutic approaches targeting TGF-β1 may represent a promising option to reduce the invasiveness of *S. aureus* and further studies should be conducted in animal models of osteomyelitis to assess whether selective inhibition of TGF-β1 signaling can also limit the negative effects of scar formation promoting functional muscle recovery.

As has been seen for TGF-β1, from the metabolic point of view, the presence of 2SA-ST239-III inside MG-63 osteoblast-like cells was the only condition able to significantly enhance cell metabolic status, evaluated through the measurement of GAPDH mRNA expression levels [46]. It is noteworthy that this metabolic enhancement was, as observed for TGF-β1, clearly evident at only 24 h post infection. The presence of GAPDH has been connected to the virulence and the adhesion of several pathogenic microorganisms [67], while its gene expression enhancement has been reported to participate in cell death phenomena [68]; both these findings support the highest MG-63 cell toxicity observed under our experimental conditions.

With the aim of verifying whether oxidative stress and the activation of antioxidant machinery were connected to the changes observed in MG-63 cells following bacterial infection, the expression levels of Nrf2 and its downstream gene HO-1 were measured under each of our experimental conditions. None of the bacteria, 2SA-ST239-III included, was able to provide Nrf2 and HO-1 gene expression values higher than those measured in uninfected cells; interestingly, there were clear and significant differences between the various strains, recalling the previously observed trends. The minor role played by oxidative stress was also emphasized by the absence of differences in iNOS and Nox2 gene expression at all the conditions considered. On the basis of these data we hypothesize that: 1) an infection time longer than 24 h is needed for the activation of both pro-oxidant enzymes and antioxidant machinery; 2) staphylococci are able to negatively modulate endogenous and exogenous oxidative and nitrosative stress [69].

5. Conclusions

Overall, our data suggest that there is extremely heterogeneous response after the infection of MG-63 cells with bacteria belonging to the same species. It seems indicative that a "general" approach should be replaced by a "clonal approach" for the treatment of *S. aureus* infections, paying particular attention to the importance of strain-specific behavior related to a specific host/infection. Our findings make the base for possible therapeutic strategies against staphylococcal-induced damage considering the use of molecules able to down-regulate the production of pro-inflammatory cytokines as well as to regulate gene

and protein expression of TGF-β1, whose activity has been related to invasiveness and toxicity of *S. aureus* and to scar formation in osteomyelitis.

Author Contributions: Conceptualization, N.M., G.C. and D.B.; Validation, N.M., G.C., D.B., M.G., and D.A.B.; Formal Analysis, N.M., G.C., D.B., M.G. and Fl.C.; Investigation, N.M., G.C., D.B., M.G., and D.A.B.; Resources, Fl.C., Fi.C. and S.S; Data Curation, N.M., G.C. and D.B.; Writing – Original Draft Preparation, N.M., G.C., D.B. and M.G.; Writing – Review & Editing, N.M., G.C., D.B., M.G., D.A.B., Fl.C., Fi.C. and S.S.; Visualization, N.M., G.C., D.B., M.G., and D.A.B.; Supervision, Fi.C. and S.S.; Project Administration, Fl.C., Fi.C. and S.S.; Funding Acquisition, Fl.C., Fi.C. and S.S..

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