

Deletion of *codY* impairs *Staphylococcus epidermidis* biofilm formation, generation of viable but non-culturable cells and stimulates cytokine production in human macrophages

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Abstract

Introduction. *Staphylococcus epidermidis* biofilms are one of the major causes of bloodstream infections related to the use of medical devices. The diagnosis of these infections is challenging, delaying their treatment and resulting in increased morbidity and mortality rates. As such, it is urgent to characterize the mechanisms employed by this bacterium to endure antibiotic treatments and the response of the host immune system, to develop more effective therapeutic strategies. In several bacterial species, the gene *codY* was shown to encode a protein that regulates the expression of genes involved in biofilm formation and immune evasion. Additionally, in a previous study, our group generated evidence indicating that *codY* is involved in the emergence of viable but non-culturable (VBNC) cells in *S. epidermidis*.

Gap statement/Hypothesis. As such, we hypothesized that the gene *codY* has have an important role in this bacterium virulence.

Aim. This study aimed to assess, for the first time, the impact of the deletion of the gene *codY* in *S. epidermidis* virulence, namely, in antibiotic susceptibility, biofilm formation, VBNC state emergence and *in vitro* host immune system response.

Methodology. Using an allelic replacement strategy, we constructed and then characterized an *S. epidermidis* strain lacking *codY*, in regards to biofilm and VBNC cell formation, susceptibility to antibiotics as well as their role in the interaction with human blood and plasma. Additionally, we investigate whether the *codY* gene can impact the activation of innate immune cells by evaluating the production of both pro- and anti-inflammatory cytokines by THP-1 macrophages.

Results. We demonstrated that the deletion of the gene *codY* resulted in biofilms with less c.f.u. counts and fewer VBNC cells. Furthermore, we show that although WT and mutant cells were similarly internalized *in vitro* by human macrophages, a stronger cytokine response was elicited by the mutant in a toll-like receptor 4-dependent manner.

Conclusion. Our results indicate that *codY* contributes to *S. epidermidis* virulence, which in turn may have an impact on our ability to manage the biofilm-associated infections caused by this bacterium.

INTRODUCTION

Staphylococcus epidermidis is considered one of the main causes of bloodstream infections due to the ability to form biofilms on the surface of medical devices such as central line catheters [1]. Biofilms are composed of cells with varying metabolic states, including viable but non-culturable (VBNC) cells [2]. VBNC cells are characterized by low metabolic activity, high tolerance to antibiotics and loss of culturability on standard culture media [3, 4], which make the treatment and the diagnosis of *S. epidermidis*

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Abbreviations: AMP, ampicillin; ATC, anhydrotetracycline hydrochloride; CLSM, confocal laser scanning microscopy; CM, chloramphenicol; cRPMI, complete RPMI; DAPI, 4',6-diamidino-2-phenylindole; EUCAST, european commission on antibiotic susceptibility testing; IND, induced; MOI, multiplicities of infection; OD, optical density; PMA, phorbol 12-myristate 13-acetate; PRE, prevented; PSC, peak serum concentration; qPCR, quantitative PCR; TLR, toll-like receptor; TSA, tryptic soy agar; TSB, tryptic soy broth; VBNC, viable but non-culturable; WGA, wheat germ agglutinin. Two supplementary figures and three supplementary tables are available with the online version of this article.

biofilm-associated infections troublesome [5, 6]. Given the impact of VBNC cells in diagnosis and infection persistence and recurrence, an *in vitro* model in which the proportion of VBNC cells in *S. epidermidis* biofilms could be modulated was established, allowing the investigation of the mechanisms underlying the emergence of the VBNC state [7]. Using this model, transcriptomic and proteomic analyses of biofilms formed under VBNC-inducing conditions uncovered alterations in the expression of an array of genes as well as in the immunoreactivity of a set of proteins [8, 9].

CodY, a global regulator that has been associated with biofilm phenotype in several bacterial species [10–13], was among the proteins showing high reactivity to human serum when cells were grown under VBNC-inducing conditions [9]. Recently, our group demonstrated that *codY* expression was upregulated in biofilms with higher proportions of VBNC cells [14], substantiating the potential involvement of this global regulator in *S. epidermidis* virulence. In *Staphylococcus aureus*, in response to nutrient-limited conditions, CodY seems to regulate several genes involved in biofilm formation, including the *agr* [15] and *ica* loci [16], *hla* [17], and genes involved in cell metabolism [18, 19]. Furthermore, CodY was shown to regulate the expression of genes encoding proteins involved in the evasion of the host immune response in *S. aureus* [16], *Streptococcus suis* serotype 2 [20] and *Listeria monocytogenes* [21]. However, the role of CodY in *S. epidermidis* virulence is still unknown.

As such, herein, the role of the gene *codY* in *S. epidermidis* biofilm formation ability, VBNC cell emergence and susceptibility to antibiotics was explored. Moreover, we investigated the role of *codY* in bacterial survival in human blood and plasma and in the interaction with human macrophages.

METHODS

Strains and growth conditions

The bacterial strains used in this study are described in Table S1, available in the online version of this article. Both *Escherichia coli* and *S. epidermidis* strains were cultured on trypticase soy broth (TSB, Merck, Darmstadt, Germany) at 37°C, 120 r.p.m. Solid media (trypticase soy agar, TSA) were prepared by adding 1.5% (w/v) agar (Liofilchem, Roseto degli Abruzzi, Italy) to the broth culture medium. When appropriate, antibiotics were added to the medium at the following concentrations: 10 µg ml⁻¹ chloramphenicol (CM₁₀) (Sigma-Aldrich, Darmstadt, Germany), 100 µg ml⁻¹ of ampicillin (AMP₁₀₀) (NZYtech, Lisbon, Portugal) or 1 µg ml⁻¹ of anhydrotetracycline hydrochloride (ATC₁) (Sigma-Aldrich). Before each experiment, overnight cultures were sonicated at 33% amplitude for 10 s (Ultrasonic Processor Model CP750, Cole-Parmer, IL, USA) to disrupt cell clusters. Of note, this sonication cycle does not influence cell viability as previously determined by propidium iodide staining [22]. Then, a suspension with an optical density at 640 nm (OD_{640nm}) of 0.25 ± 0.05, which corresponds to ~2×10⁸ c.f.u. ml⁻¹, was prepared and diluted further to obtain the required cell concentration.

Construction of *codY*-mutant and complemented strains

The construction of the *codY*-mutant strain was achieved following previously published protocols [23, 24]. Briefly, the temperature-sensitive shuttle vector pIMAY was cut open with SmaI (NEB, Ipswich, MA, USA) and ligated with *codY*-gene-flanking regions, previously amplified with Phusion High Fidelity DNA Polymerase (NEB), using the T4 DNA ligase enzyme (NEB). pIMAY plasmid was transformed into *E. coli* DC10B by chemical transformation, and the obtained clones were selected with TSA_{CM10} plates. The presence of the insert was confirmed by restriction enzyme digestion of the plasmid, with SalI and NotI (NEB) and DNA sequencing using primers for the pIMAY multiple cloning site [23]. Then, plasmid DNA from the selected clones was purified using the QIAGEN Plasmid Midiprep Kit (Hilden, Germany) and transformed into *S. epidermidis* 1457 strain by electroporation, as described before [23]. Positive clones were selected with TSA_{CM10} plates. To confirm the presence of the knockout (KO) plasmid, plasmid purification was performed using a QIAGEN Plasmid Midiprep Kit. Noteworthy, to lyse the cells, 1 mg ml⁻¹ of lysostaphin (Sigma-Aldrich) was added to the resuspension buffer provided in the kit and incubated for ~30 min at 37°C. To perform gene deletion, an allelic replacement procedure was completed, and the selection of the KO clones was performed by counter-selection with ATC₁ (Sigma-Aldrich), as described before [23]. The deletion of the gene was confirmed by PCR.

Regarding the construction of the complemented strain, the plasmid pRB473 was used [25]. In brief, the sequence of the target gene, together with its promotor region, was cloned into pRB473 previously cut open with BamHI and SalI (NEB) and transformed into *E. coli*, by chemical transformation. The obtained clones were selected with TSA_{AMP100} plates and confirmed by PCR using primers for the pRB473 multiple cloning site. The plasmid was purified with a QIAGEN Plasmid Midiprep Kit and then transformed into the $\Delta codY$ strain through electroporation and the clones selected in TSA_{CM10}. To ensure successful complementation, the expression of the target gene was confirmed by quantitative PCR (qPCR) (Fig. S1). The primers used for gene deletion and qPCR were designed with the support of Snapgene software using *S. epidermidis* 1457 complete genome as a template (NCBI accession no. CP020463.1) (Table S1). The specificity of the sequences of the designed primer was evaluated by BLASTn.

Antibiotic susceptibility

The MIC of six antibiotics, with three different mechanisms of action, was determined using the microdilution method, according to the European Commission on antibiotic susceptibility testing (EUCAST) guidelines. Briefly, serial dilutions of the antibiotics

were performed in TSB, in a 96-well plate, to obtain the range of concentrations indicated in Table S2. Then, suspensions of all *S. epidermidis* strains with $\sim 1 \times 10^6$ c.f.u. ml⁻¹ were prepared, in TSB, and 100 µl of these suspensions was inoculated into 100 µl of the previously prepared antibiotic dilutions. Thereafter, the plates were incubated at 37°C for 24 h. The lowest concentration of antibiotic that visually inhibited bacterial growth was considered the MIC value. Additionally, the OD_{620nm} was measured to aid in the MIC value determination. Of note, the strain *S. aureus* ATCC 29213 was used for quality control.

Furthermore, a time-kill curve was performed using the peak serum concentration (PSC) of the same antibiotics (Table S2). Briefly, in 14-ml glass tubes, 7 ml of a suspension with $\sim 1 \times 10^7$ c.f.u. ml⁻¹, with the respective concentration of antibiotic, was prepared and incubated at 37°C with shaking at 120 r.p.m. Bacteria without antibiotics and medium without bacteria were used as positive and negative controls, respectively. After 2, 4 and 6 h, 1 ml of each suspension was collected and centrifuged at 16 000 g for 10 min at 4°C. The pellet was washed once and then suspended in 1 ml of 0.9% NaCl. Bacteria susceptibility was assessed by c.f.u. counting. Briefly, 10-fold dilutions were performed in 0.9% NaCl, and 5 µl of each dilution was plated, in duplicates, on TSA or TSA_{CM10}. The plates were then incubated for at least 16 h at 37°C. The antimicrobial effect was estimated by calculating the difference between the number of c.f.u. obtained before (T0 h) and after (T2, 4 and 6 h) incubation with antibiotics ($\log_{10} T0 h - \log_{10} T2/4/6 h$).

Survival in human blood and plasma

S. epidermidis survival in human blood and plasma was assessed following a previously optimized human *ex vivo* model [26–29]. Human blood was collected from six healthy volunteers (three females and three males) into tubes spray coated with lithium heparin (Vacuette, Kremsmünster, Austria). Plasma was separated from whole human blood by centrifugation at 4400 g for 20 min at 4°C. Then, in a 2 ml tube, 50 µl of bacterial suspension ($\sim 10^6$ c.f.u. ml⁻¹) was incubated with 450 µl of either TSB plus heparin (TSB_{heparin}, to discard the effect of the anticoagulant), plasma or human blood. The tubes were incubated at 37°C at 80 r.p.m. for up to 4 h of incubation. After 2 and 4 h of incubation, 50 µl of each sample was collected for c.f.u. counting. Bacteria survival was estimated using initial bacterial load (T0 h) as a control.

Biofilm and VBNC cell formation

For the evaluation of biofilm formation, 10 µl of a suspension with $\sim 10^8$ c.f.u. ml⁻¹ was inoculated into 1 ml of TSB supplemented with 0.4% of glucose (TSB_{0.4%G}) already distributed in the wells of a 24-well plate (Orange Scientific, Braine-l'Alleud, Belgium). Thereafter, the plates were incubated at 37°C at 120 r.p.m. for up to 48 h, with medium replacement after 24 h of growth. To assess the formation of VBNC cells, biofilms were prepared as previously described [30]. In short, biofilms were first formed with either TSB_{0.4%G} or TSB_{0.4%G} supplemented with 20 mM of MgCl₂ (TSB_{0.4%G+MgCl2}). After 24 h of growth at 37°C and 120 r.p.m., the spent medium was replaced with TSB supplemented with either 1% of glucose (TSB_{1%G}), for the induction of the VBNC state, or TSB_{1%G+MgCl2}, for the prevention of the VBNC state and incubated for further 24 h under the same temperature and shaking conditions.

Next, in both types of biofilm assays, biofilms were washed with 1 ml of 0.9% NaCl, carefully scraped from the bottom of the well and suspended in the same volume of 0.9% NaCl. The suspensions were sonicated as detailed above. The total biofilm biomass was then estimated by OD_{640nm}, and the number of culturable cells was assessed by c.f.u. counting. The proportions of VBNC cells were estimated by the ratio (%) between induced and prevented VBNC conditions (IND/PRE), for both total biomass and culturability analysis.

Biofilm structure analysis by confocal laser scanning microscopy

Biofilms for confocal laser scanning microscopy (CLSM) analysis were prepared as described above for biofilm formation evaluation but with Nunc Thermanox coverslips inside the wells (Thermo Fisher Scientific). After 24 h of incubation, biofilms were washed with 0.9% NaCl, and the coverslips were carefully removed and transferred onto a glass slide for staining. Biofilms were then incubated, for 5 min, with 100 µl of DAPI (4',6-diamidino-2-phenylindole, 100 µg ml⁻¹), which stains nucleic acids, followed by a 10-min incubation with 100 µl wheat germ agglutinin (WGA-TRITC, 10 µg ml⁻¹) that binds to polysaccharides. In addition, to assess cell viability, biofilms were stained with the BacLight Bacterial Viability Kit (Thermo Fisher Scientific), which consists of a mixture of SYTO 9 and propidium iodide. A control of dead cells was prepared by incubating biofilms, for 15 min, with 1 ml of methanol. All stainings were performed at room temperature, in the dark and duplicates. Olympus FluoView FV1000 CLSM (Olympus, Lisbon, Portugal) microscope was used for imaging. Images were acquired using a 10× and a 40× water-immersion objective and with 640×640 resolution. The analysis of the acquired images was made using the FV10-ASW 4.2 Viewer Software (Olympus).

Cell lines

The human monocytic cell line THP-1 was purchased from ATCC (TIB-202) and was cultured in T-25 flasks in complete RPMI (cRPMI), which is composed of RPMI 1640 medium (VWR) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France), 4 mM L-glutamine (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 1% (v/v) penicillin/

streptomycin (PEN/STREP, Sigma-Aldrich) and 0.05 mM β -mercaptoethanol (Sigma-Aldrich). The THP-1-Dual KO-TLR4 cell line (KO-TLR4), generated from the human THP-1-Dual monocytic cell line, was purchased from InvivoGen (San Diego, CA, USA) and cultured in T-25 flasks in cRPMI supplemented with 100 $\mu\text{g ml}^{-1}$ of Normocin (InvivoGen) for initial propagation. For further maintenance, cells were kept and subcultured in cRPMI media supplemented with 100 $\mu\text{g ml}^{-1}$ of Normocin and with selective antibiotics, namely 100 $\mu\text{g ml}^{-1}$ of Zeocin and 10 $\mu\text{g ml}^{-1}$ of blasticidin, which were provided with the KO-TLR4 cell line. Both cell lines were subcultured, for no more than 10 passages, and maintained at 37°C in a humidified atmosphere of 5% CO_2 .

To differentiate monocytes into macrophages, both THP-1 and KO-TLR4 cells were seeded, in flat 96-well plates, at the concentration of 5×10^5 cells ml^{-1} in cRPMI media containing 50 nM of phorbol 12-myristate 13-acetate (PMA) (InvivoGen) and maintained under the same temperature and atmospheric conditions for 24 h. Then, the culture medium was replaced with fresh PMA-free medium and incubated for a further 24 h before infection assays.

Infection of THP-1 and KO-TLR4 macrophages

Bacterial cultures for the infection of human macrophages were grown overnight, in TSB, at 37°C and 120 r.p.m. Thereafter, cells were washed twice with PBS and suspended in either 1 ml of PBS to obtain a suspension with live cells or 1 ml of methanol to obtain dead cells. The latter was incubated for 15 min on ice and then washed twice and suspended in 1 ml of PBS. Bacteria concentration was then determined by flow cytometry using SYTO BC (1:10 000, Thermo Fisher Scientific)/propidium iodide (5 $\mu\text{g ml}^{-1}$, Sigma-Aldrich) staining in a BD FACSCanto II cytometer using the BD FACSDiva Software (BD Biosciences, Franklin Lakes, NJ, USA). The inoculum was prepared in cRPMI without penicillin/streptomycin (cRPMI^{-PEN/STREP}) to obtain multiplicities of infection (MOI) of 1 bacterium to 1 macrophage or 1 bacterium to 3 macrophages, in the case of live bacteria, and 10 bacteria to 1 macrophage, in the case of dead bacteria. Noteworthy, CM₁₀ was added to the complemented strain. A negative control, with cRPMI^{-PEN/STREP} only (with and without CM₁₀), and a positive control with LPS from *E. coli* O127:B8 (0.1 $\mu\text{g ml}^{-1}$, Sigma-Aldrich) were also prepared.

Inhibition of the TLR2

Macrophages differentiated from both cell lines were prepared as mentioned above, and before being challenged with *S. epidermidis* strains, the culture medium was removed, and the cells were incubated for 3 h with either cRPMI^{-PEN/STREP} media supplemented with 50 μM of TL2-C29 inhibitor (C29, InvivoGen) or with cRPMI^{-PEN/STREP} medium alone. Then, bacterial cultures were added to obtain an MOI of one bacterium to one macrophage, and the plate was incubated for an additional 24 h at 37°C and 5% CO_2 . Besides the LPS O127:B8, an ultrapure LPS from *E. coli* K12 (LPS-EK Sigma-Aldrich) was also used to discard possible contaminants. Pam3CSK4 (TLR2 agonist, InvivoGen) at 10 ng ml^{-1} was used as a control for the TL2-C29 inhibitor. Medium alone was used as a negative control.

Bacterial survival within human macrophages

S. epidermidis suspensions were prepared with the aid of flow cytometry, as described above. Afterwards, THP-1-derived macrophages (5×10^5 cells ml^{-1}), previously seeded in flat 96-well plates, were infected at an MOI of 10 or 5 bacteria to 1 macrophage. Plates were incubated at 37°C and 5% CO_2 for 30 min to allow bacteria internalization. Then, culture supernatants were discarded, and RPMI plus 50 $\mu\text{g ml}^{-1}$ gentamicin (Biowest) was added for 60 min to eliminate extracellular bacteria. After that period, the supernatant was removed, and macrophages were washed with warm PBS. To assess the number of intracellular bacteria, macrophages were lysed by incubating with PBS supplemented with 0.01% of saponin (Sigma-Aldrich) for 10 min and the suspension used for c.f.u. counting.

Cytokine quantification

After 24 h of THP-1- and KO-TLR4-derived macrophage infection with *S. epidermidis* strains, culture supernatants were collected and frozen at -20°C until further use. The concentration of the pro-inflammatory cytokines IL-6, TNF- α and IL-8, and the anti-inflammatory cytokine IL-10, was measured by ELISA using DuoSet ELISA (R and D system, USA) following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9 (Trial version, San Diego CA, USA) with either one- or two-way ANOVA, with Tukey's or Šidák's multiple comparisons tests or mixed-effects model. A $P < 0.05$ was considered significant. At least three independent experiments were performed for each assay detailed.

RESULTS

Evaluation of *codY* deletion in *S. epidermidis* susceptibility to antibiotics

Considering the implication of antibiotic resistance/tolerance in the recurrence of *S. epidermidis* infections [31], we were interested in evaluating the susceptibility of the mutant strain to clinically relevant antibiotics. Regarding MIC values, according to the EUCAST clinical breakpoints, all strains were susceptible to the antibiotics tested, except for ciprofloxacin, in which the MIC range indicates an intermediate susceptibility. However, no differences were detected between the WT and the constructed strains (Table S3). Additionally, time-kill curve assays were performed, and, as can be seen in Fig. 1, only when using rifampicin (Fig. 1d), a significative difference in antimicrobial susceptibility was found between the mutant and WT strains.

Effect of *codY* deletion in *S. epidermidis* interaction with human blood and plasma

Since bloodstream infections are one of the most frequent complications of *S. epidermidis* biofilm formation on medical devices, the analysis of the interplay between *codY*-lacking bacteria and human blood is important to help elucidate its role in this bacterium virulence. For this purpose, the survival of the WT and the constructed strains was investigated when exposed to human blood and plasma (Fig. 2).

When evaluating the growth of the strains in human plasma in comparison with the TSB_{heparin} control, some inhibitory effect was observed; however, bacteria were still able to grow in plasma. In contrast, when in contact with human blood, a reduction of between ~55%(2 h) and ~87%(4 h) was observed. These differences between plasma and human blood suggest that the growth

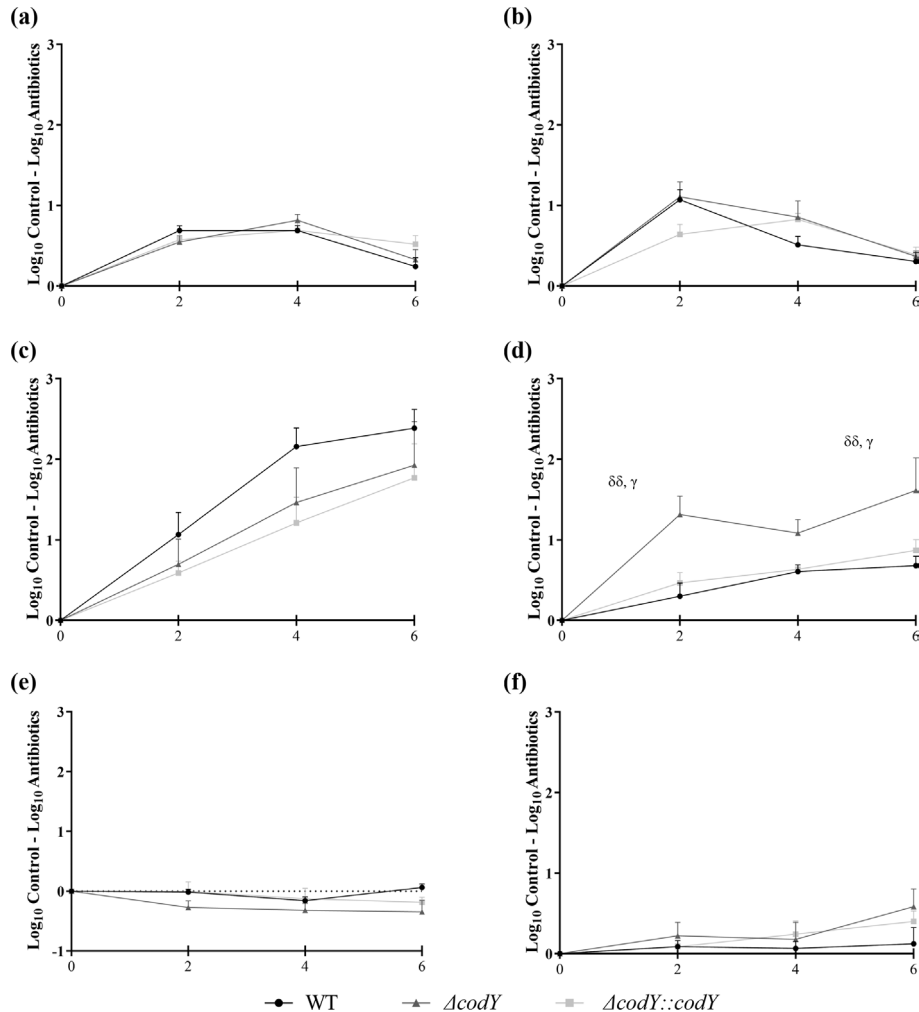


Fig. 1. Susceptibility of *S. epidermidis* strains upon 2, 4 and 6 h of incubation with PSC of vancomycin (a), dicloxacillin (b), ciprofloxacin (c), rifampicin (d), linezolid (e) and tetracycline (f). Data represent the mean plus SEM of at least three independent assays. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons tests. δ $\Delta codY$ vs WT. γ $\Delta codY$ vs $\Delta::codY$. $^{\gamma} P < 0.05$; $^{\delta\delta} P < 0.01$.

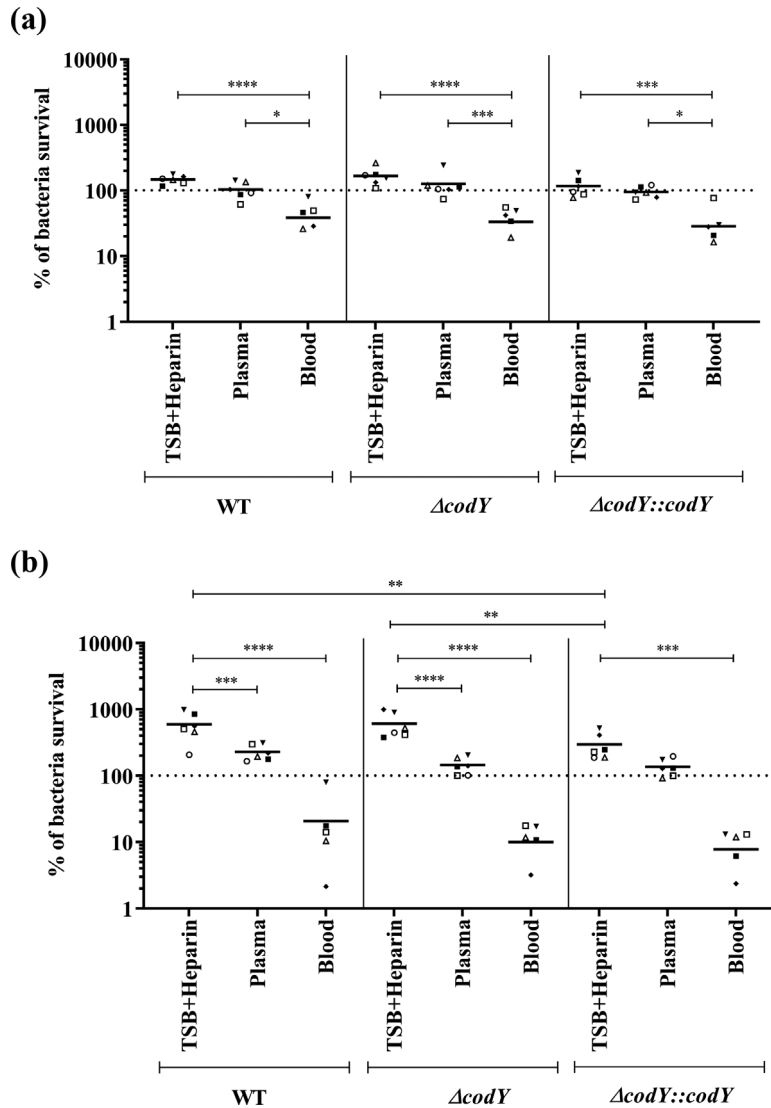


Fig. 2. *S. epidermidis* strains survival upon incubation with human plasma or blood for 2 (a) and 4 h (b). Blood was collected from three female (□, △ and ○) and three male (▼, ◆ and ■) donors. Data represent the percentage of survival compared with T0 h, and the horizontal solid lines represent the mean of six independent assays. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons tests. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

reduction effect observed in blood was due to blood cells rather than soluble immune factors. However, the deletion of *codY* does not seem to influence this bacterium's survival, as no significant differences were found in the number of c.f.u. of the WT and mutant strains upon 4 h of interaction with either blood or plasma.

Impact of *codY* deletion in *S. epidermidis* internalization and pro-inflammatory response by human macrophages

To assess the impact of *codY* in *S. epidermidis* interaction with host immune cells, the interplay between macrophages and the *S. epidermidis* strains under study was addressed using either THP-1- or KO-TLR4-derived macrophages and live or dead bacteria. As can be seen in Fig. 3a, the different strains were equally internalized, suggesting that the lack of *codY* did not affect the recognition nor internalization of the bacteria by macrophages.

Despite the role of macrophages in pathogen killing, they also have an important function in signalling the infection to other immune cells through the secretion of cytokines [32]. As such, to better characterize the impact of *codY* deletion, we quantified the levels of cytokines produced by macrophages challenged with either live or dead bacteria (Fig. 3b–g). THP-1-derived macrophages challenged with live cells showed a higher secretion of both pro- and anti-inflammatory cytokines when infected

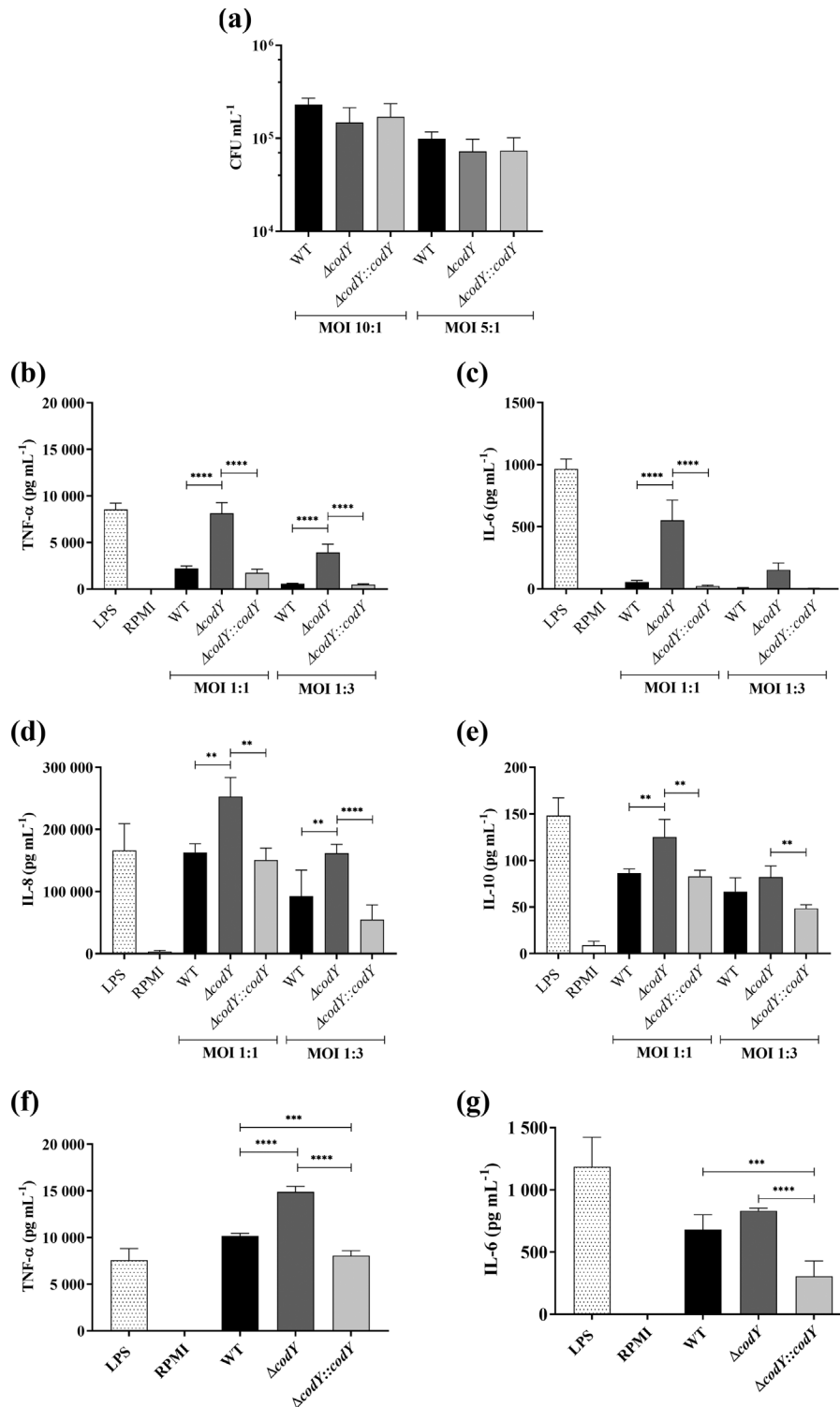


Fig. 3. THP-1-derived macrophages challenged with *S. epidermidis* strains. (a) The number of internalized bacteria by THP-1-derived macrophages, upon 30 min of incubation, with an initial load of 5×10^6 cells mL⁻¹ (MOI 10 bacteria to 1 macrophage) or 2.5×10^6 cells mL⁻¹ (MOI 5 bacteria to 1 macrophage). (a–g) Levels of pro- and anti-inflammatory cytokines detected in the supernatant upon 24 h of infection with (b, c, d and e) live bacteria (MOI 1 bacterium to 1 macrophage or 1 bacterium to 3 macrophages) and (f and g) dead bacteria (MOI 10 bacteria to 1 macrophage). Data represent the mean plus SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

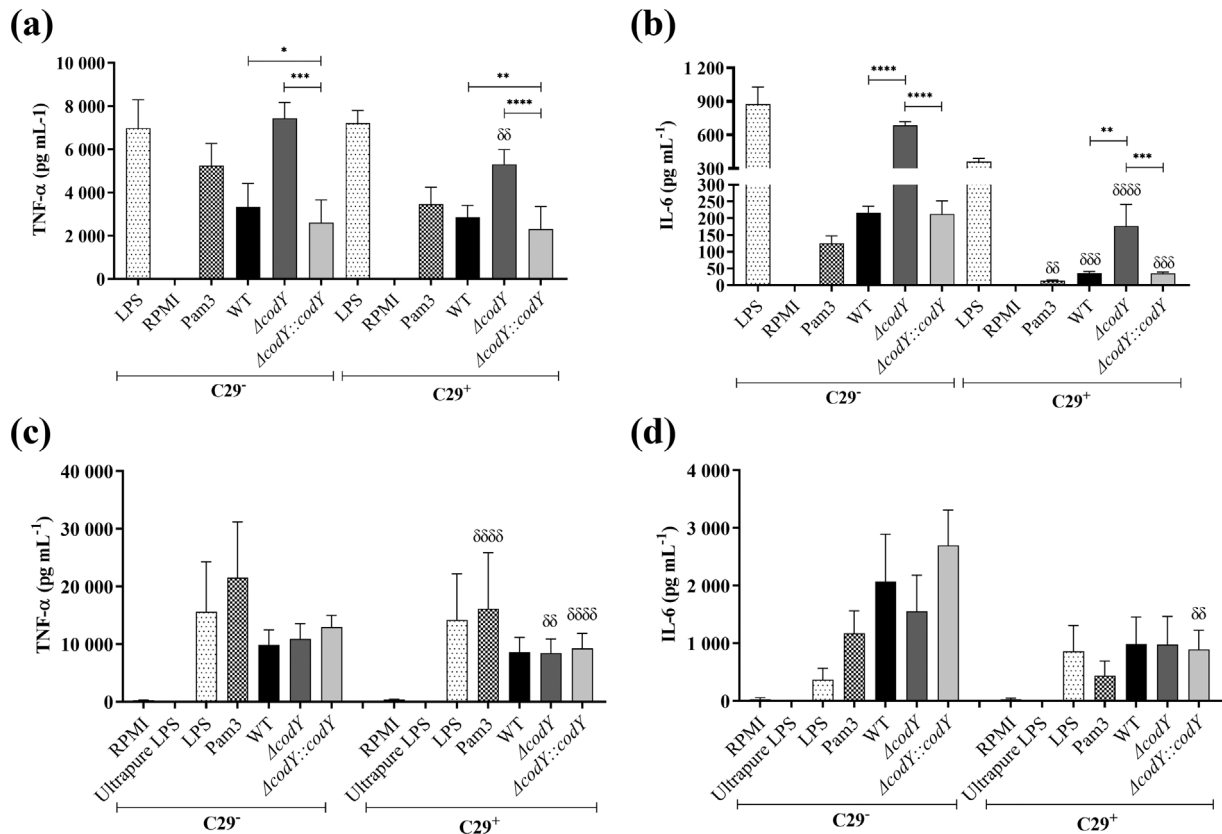


Fig. 4. Incubation of macrophage cells with (+) or without (–) the TLR2 inhibitor C29. Levels of pro-inflammatory cytokines were detected in the supernatant of THP-1- (a and b) and KO-TLR4-derived macrophages (c and d) infected with *S. epidermidis* strains at an MOI of one bacterium to one macrophage. Data represent the mean plus SEM of at least three independent experiments. Statistical analysis was performed using the unpaired two-tailed t-test with Welch's correction (a and b), two-way ANOVA (or mixed-effects model) (c and d) ([§] with vs without C29 inhibitor) or one-way ANOVA with Tukey's multiple comparisons test (* among strains). * $P < 0.05$; [§] $P < 0.01$; ^{§§§} $P < 0.001$; ^{§§§§} $P < 0.0001$.

with $\Delta codY$ mutant (Fig. 3b–e), regardless of the MOI used, being more marked for TNF-α (Fig. 3b) and IL-6 (Fig. 3c). Hence, the levels of these cytokines were also assessed upon stimulation with dead cells, and the same trend was observed (Fig. 3f, g).

Additionally, we hypothesized that the higher cytokine secretion induced by the mutant strain could be associated with differential recognition of *S. epidermidis* strains that would trigger distinct signalling pathways. TLR2 has been shown to mediate *S. epidermidis* recognition by human cells [33]. To assess whether the higher production of cytokines induced by $\Delta codY$ could be mediated by TLR2 in THP-1 cells, we inhibited TLR2 signalling using TLR2-C29 (Fig. 4).

The inhibition of TLR2 signalling in THP-1-derived macrophages resulted in lower levels of both TNF-α and IL-6 (Fig. 4a, b), with a more marked effect in IL-6. However, the secretion of IL-6 and TNF-α was still higher upon infection with $\Delta codY$. This indicates that other receptors could be involved. Therefore, we similarly stimulated KO-TLR4-derived macrophages. As shown in Fig. 4c, d, no differences were found in the levels of the assessed cytokines when TLR4-deficient THP-1 cells were used as responders, implicating TLR4 in the exacerbated effect on cytokine production induced by $\Delta codY$. The inhibition of TLR2 signalling in TLR4-deficient THP-1 cells did not translate into significantly lower production of the cytokines tested by any of the three bacterial strains (Fig. 4c, d). Altogether, these results show that *codY* expression exerts a down-modulatory effect in the production of pro-inflammatory cytokines induced by *S. epidermidis*. Our results also implicate TLR4 in host cell recognition of the *codY* mutant.

The role of *codY* on *S. epidermidis* biofilm formation ability and VBNC cell formation

Several studies have reported distinct roles of CodY in biofilm formation by different bacterial species. While *Streptococcus pyogenes* [34] or *Bacillus cereus* [35] cells lacking *codY* revealed a reduced ability to form biofilms, in some *S. aureus* strains, the absence of *codY* enhanced biofilm formation [11]. In the case of *S. epidermidis*, to the best of our knowledge, no studies have so far addressed the role of *codY* in biofilm formation.

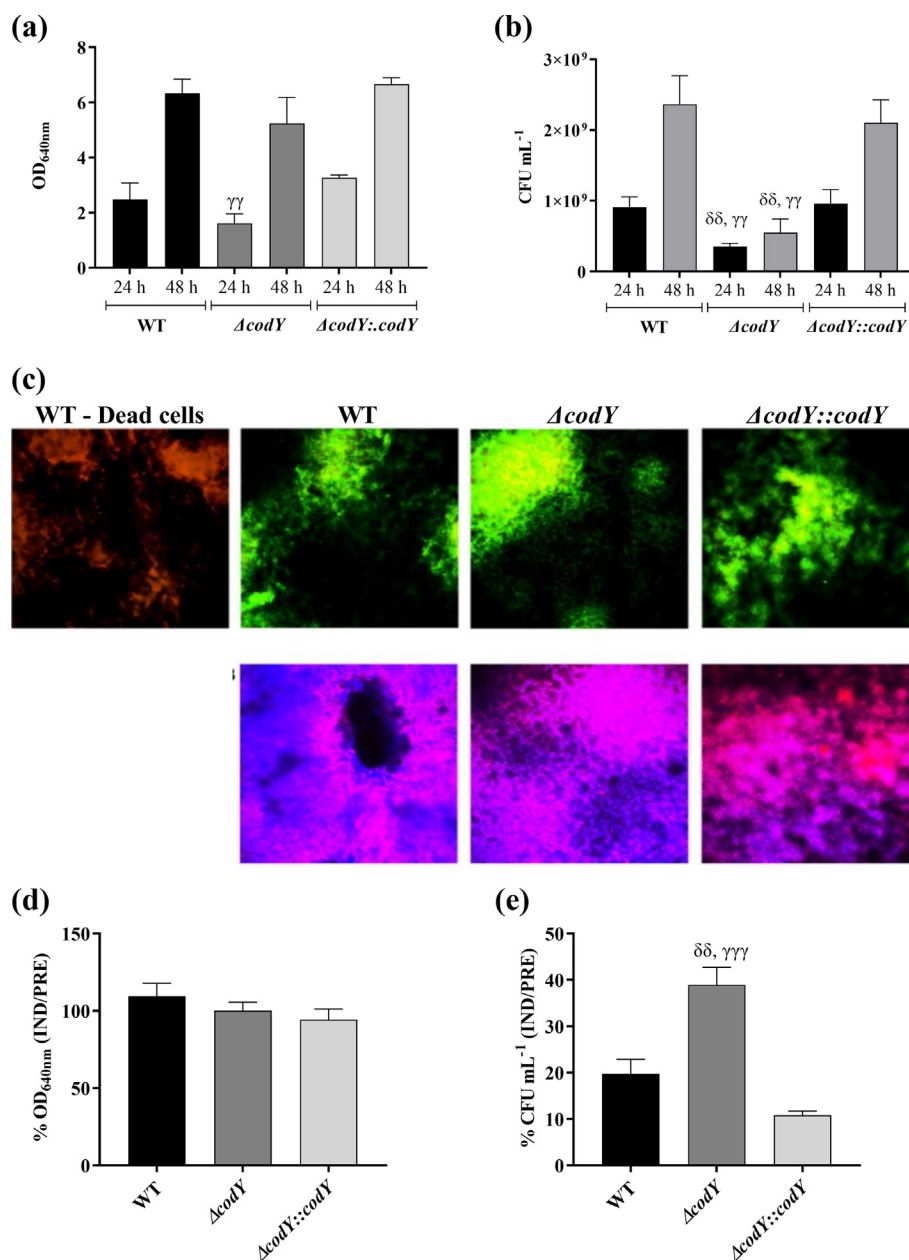


Fig. 5. Effect of the gene *codY* deletion on *S. epidermidis* biofilm formation capacity and VBNC cell formation. Total biofilm biomass (a) and c.f.u. mL⁻¹(b), after 24 and 48 h of growth in TSB. CLSM representative images of 24-h-old biofilm cells viability using BacLight Bacterial Viability Kit (c, top panel), where green indicates viable cells and red indicates dead cells, and analysis of biofilm structure with DAPI (nucleic acids, blue) and WGA (polysaccharides, red) staining (c, lower panel). Ratio (%) between induced and prevented VBNC conditions (IND/PRE), for both total biomass (d) and c.f.u. mL⁻¹ (e). The results represent the mean plus SEM of at least three independent experiments. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons tests. [§] $\Delta codY$ vs WT. [†] $\Delta codY$ vs $\Delta codY::codY$. ^{§§} $P < 0.01$; ^{†††} $P < 0.001$.

Our results show that although the deletion of *codY* did not cause significant alterations regarding biofilm biomass (Fig. 5a), it resulted in a significant reduction in the number of c.f.u. mL⁻¹ (Fig. 5b), implicating *codY* expression in biofilm formation. Given these results, the biofilm structure as well as biofilm cell viability was evaluated by CLSM. No marked differences were found among the strains concerning cell viability (Fig. 5c, top panel) or structure and polysaccharides distribution (Fig. 5c, lower panel). Thus, the reduction observed in the number of c.f.u. mL⁻¹ in the biofilms formed by the mutant does not seem to be related to cell death.

The substantial amount of VBNC cells in biofilms and their contribution to antibiotic failure and infection recalcitrance and persistence [2, 36] triggered the interest to explore the mechanisms underlying VBNC state induction. Hence, we evaluated the

level of VBNC cells in biofilms formed by $\Delta codY$ (Fig. 5d, e). In terms of biofilm biomass, the results obtained under VBNC-inducing or -preventing conditions were similar among all tested strains (Fig. 5d). In contrast, a higher IND/PRE ratio, i.e. lower number of VBNC cells, was observed in the biofilms formed by the mutant strain (Fig. 5e). Of note, we estimated the level of the VBNC state by comparing the total amount of cells (OD_{640nm}) and culturable cells (c.f.u.) since we previously demonstrated that using either the number of live cells, as assessed by flow cytometry through the live/dead staining, and OD measurements yielded similar results [14].

DISCUSSION

CodY has been reported as a global transcriptional regulator that predominantly represses the expression of hundreds of genes in different bacterial species under adverse conditions [13]. To date, aside from our initial study highlighting increased *codY* expression in *S. epidermidis* biofilms grown under VBNC conditions [14], which suggests its role in the formation of VBNC cells, no other studies have been reported on *S. epidermidis* regarding the role of *codY*. As such, given these observations and the clinical relevance of this bacterium, we constructed and characterized a *codY*-negative strain to elucidate the role of this gene in *S. epidermidis* virulence.

When assessing $\Delta codY$ susceptibility to antibiotics, a decrease in the number of c.f.u. ml^{-1} was only found with rifampicin. Since $\Delta codY$ formed smaller colonies than the WT counterpart (data not shown), this may explain the higher susceptibility to rifampicin as this antibiotic is effective against slow-growing staphylococcal species [37]. We hypothesized that the smaller colonies, as well as the lag of growth of $\Delta codY$ (Fig. S2) when compared with the WT strain, may result from the regulatory effect of CodY in the transcription of genes involved in the biosynthesis of the cell wall [11, 15, 16]. However, considering that this strain is pansusceptible to antibiotics [38], in the future, it would be valuable to explore antibiotic-resistant strains to delve deeper into the role of the gene *codY* in antibiotic susceptibility.

The deletion of *codY* did not interfere with bacterial survival in whole human blood or plasma. However, given the importance of macrophages in controlling staphylococcal infections [39], we characterized the interaction of *S. epidermidis* strains with human macrophages. Although we observed the presence of some cells within the macrophages, no statistically significant differences were found between the mutant and the WT or complemented strains. As non-opsonic phagocytic receptors mainly target bacterial cell wall components [40], this suggests that the deletion of *codY* did not significantly impact cell wall components that might be involved in phagocyte bacterial recognition.

After the recognition of pathogenic bacteria, macrophages produce pro-inflammatory cytokines such as TNF- α , IL-6 and IL-8 to initiate an immune response and recruit other immune cells to the sites of infection [41, 42]. Therefore, we have also evaluated the cytokine production profile of infected macrophages. *S. epidermidis* has been shown to induce high levels of pro-inflammatory cytokines secretion, both *in vitro* and *in vivo*. Namely, a marked increase in IL-6, IL-8 and TNF- α secretion was detected upon *in vitro* stimulation of THP-1-derived macrophages [43, 44] and human peripheral blood mononuclear cells [33, 45] with different *S. epidermidis* strains. Our research team has also shown this pro-inflammatory profile *in vivo*, in a murine model of bloodstream infection [28]. Similarly, herein, we observed increased release of IL-6 and TNF- α by THP-1-derived macrophages challenged with all strains but at higher levels when in the presence of the mutant. Therefore, our results show that the deletion of *codY* results in a stronger pro-inflammatory response, indicating that *codY* is involved in the modulation of the release of cytokines induced by *S. epidermidis*. The deletion of the gene *codY* in *S. aureus* resulted in increased potential to cause host necrotizing lesions [46], which might be in agreement with the higher pro-inflammatory effect of the *codY* mutant studied here.

To better understand the distinct pathways involved in *S. epidermidis* recognition, TLR2 receptor signalling was inhibited, significantly reducing IL-6 and TNF- α secretion. Nevertheless, the differences between the *codY* mutant and WT and complemented strains were maintained. This indicates that other receptors than TLR2, a major receptor associated with *S. epidermidis* recognition by human cells [33], mediate the effect observed when abrogating *codY* expression. As TLR4 has been shown to mediate *S. aureus* recognition in the host [47, 48], we have also assessed the role of this receptor by testing a KO-TLR4 cell line with or without TLR2 inhibition. Interestingly, the higher pro-inflammatory response induced by the mutant strain was abrogated in the absence of TLR4 expression, indicating that this receptor plays a major role in the recognition of the *codY* mutant. The down-modulatory effect of *codY* in the pro-inflammatory response elicited by *S. epidermidis* on THP-1 cells indicates that this gene may contribute to the immune evasion of this bacterium, being likely involved in the chronic nature of the *S. epidermidis* biofilm-associated infections and the difficulties associated with its treatment.

Finally, knowing that the ability of this bacterium to withstand antibiotics, as well as to evade the host immune response, is mostly associated with the capacity to form biofilms, we assessed the role of the gene *codY* in biofilm formation and the emergence of VBNC cells. The deletion of the gene *codY* resulted in biofilms with significantly lower c.f.u. counts, similar to what has been observed for *S. aureus*, though divergent results have been reported in this bacterium [34]. While biomass measurement provides insights into the overall quantity of the biofilm, encompassing cells and matrix components, c.f.u. assesses the number of cells within the biofilm. The observed decrease in c.f.u. count in the mutant suggests that the *codY* gene may play a role in supporting

bacterial survival and growth within the biofilm environment. This role may be associated with the maintenance of the biofilm structure by influencing adhesion, aggregation or dispersal dynamics. Moreover, these findings suggest that the absence of the *codY* gene does not seem to significantly impair the matrix of the biofilm. However, further experiments are needed to precisely elucidate the mechanisms underlying these observations. Similarly, fewer VBNC cells were detected in biofilms formed by the mutant strain, further substantiating previous results suggesting that the gene *codY* is involved in the formation of VBNC cells in *S. epidermidis* biofilms [14]. Considering the results obtained here and that was previously shown that murine bone marrow-derived macrophages challenged with *S. epidermidis* grown under VBNC-inducing conditions produced lower levels of IL-6 and TNF- α [30], one can speculate that during a biofilm-associated infection, *codY* may be involved in the lower cytokine stimulation by *S. epidermidis* biofilm cells. This involvement in VBNC cell formation and immune response modulation could impact and underestimate the clinical presentation of infections caused by *S. epidermidis*, potentially contributing to variable symptoms and treatment challenges. Yet, further studies are warranted to corroborate this hypothesis.

Overall, we demonstrated, for the first time, that *codY* contributes to *S. epidermidis* 1457 virulence, in particular, in biofilm formation ability, the emergence of VBNC cells and the stimulation of pro-inflammatory cytokine production. Given that *S. epidermidis* is a significant nosocomial pathogen linked to healthcare-associated infections, especially those involving biofilm formation on medical devices, these findings propose that the *codY* gene could be a promising candidate for the advancement of new prophylactic or therapeutic approaches against *S. epidermidis* infections, which are often associated with persistent symptoms and therapeutic difficulties.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Blood collection followed a protocol approved by the University of Minho Institutional Review Board [SECVS 002/2014 (ADENDA)], which is in strict accordance with the Declaration of Helsinki and Oviedo Convention. Moreover, all donors gave written informed consent before blood collection.

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