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## Characterisation of the manganese superoxide dismutase of *Enterococcus faecium*

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1 **Title**

2 **Characterisation of the Manganese Superoxide Dismutase of *Enterococcus faecium***

3

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15

16 **Abstract**

17           The manganese superoxide dismutase (SodA) of *E. faecium* strain AUS0004 has been  
18 characterised. It is most closely related to *E. hirae*, *E. durans*, *E. villorium*, and *E. mundtii*  
19 with 100%, 91,55%, 90,85%, and 90,58% homology, respectively, but more distant from  
20 SodA of *E. faecalis* (81.68%). A *sodA* deletion mutant has been constructed. Compared to the  
21 parental strain, the  $\Delta$ *sodA* mutant was affected in aerobic growth and more sensitive to  
22 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), cumene hydroperoxide (CuOOH), and the superoxide anion (O<sub>2</sub><sup>•-</sup>)  
23 generator menadione. The *E. faecium* strain AUS0004 is part of those bacteria accumulating  
24 H<sub>2</sub>O<sub>2</sub> to high concentrations (around 5 mM) starting from late exponential growth phase.  
25 Accumulation of the peroxide was around 25 % less in the mutant suggesting that this part of  
26 H<sub>2</sub>O<sub>2</sub> is due to the dismutation of O<sub>2</sub><sup>•-</sup> by SodA. The *sodA* gene of *E. faecium* AUS0004 was  
27 induced by oxygen, peroxides and menadione but the corresponding regulator remains  
28 hitherto unknown. Finally, we showed that SodA activity is important for virulence in the  
29 *Galleria mellonella* model.

30

31 **Keywords**

32 *Enterococcus faecium*; oxidative stress; superoxide dismutase; virulence

## 33 1. Introduction

34 Enterococci are Gram-positive lactic acid bacteria (LAB) and are widely distributed in  
35 nature (plants, soils and water) but their main habitat is the gastrointestinal tract of humans  
36 and animals [1,2]. These otherwise innocuous commensals have been recognized as important  
37 opportunistic pathogens mainly for hospitalized patients. The infections they cause are  
38 difficult to treat due to intrinsic and acquired antibiotic resistances [3]. The vast majority of  
39 these are due to two species, *Enterococcus faecalis* and *Enterococcus faecium*, but those  
40 caused by *E. faecium* are more problematic because of higher prevalence of antibiotic-  
41 resistance, especially to the last line, glycopeptide vancomycin [4–6].

42 To control host infections and kill invading pathogens, the immune system exposes  
43 bacteria to many stresses, including oxidative stress [7,8]. Once activated, macrophages and  
44 neutrophils actively produce superoxide anions ( $O_2^{\bullet-}$ ) via the membrane bound NADPH  
45 oxidase [9]. Due to its negative charge,  $O_2^{\bullet-}$  cannot penetrate bacterial membranes but  
46 spontaneously dismutates to hydrogen peroxide ( $H_2O_2$ ) which diffuses readily into the  
47 bacteria but also outwards the phagosome limiting its active concentration against the  
48 engulfed pathogen [10]. Although there is no doubt about the importance of these reactive  
49 oxygen species (ROS) as antimicrobial weapons, the mechanism of killing and the targets of  
50 these phagosome-generated ROS are still incompletely understood [10,11].

51 Bacteria have evolved antioxidative defences against  $O_2^{\bullet-}$  and  $H_2O_2$ . Superoxide  
52 dismutases (SOD) catalyse the dismutation of two  $O_2^{\bullet-}$  into  $O_2$  and  $H_2O_2$  and  
53 catalases/peroxidases reduce the peroxide to water.  $O_2^{\bullet-}$  does not react with biomolecules  
54 *in vitro* but its toxicity seems to be indirect by damaging [Fe-S] clusters present in certain  
55 enzymes mainly part of the TCA cycle [12].  $Fe^{2+}$  is liberated during this process and can react  
56 with  $H_2O_2$  to generate the highly reactive and toxic hydroxyl radical ( $HO^{\bullet}$ ) [13,14]. LAB,  
57 including enterococci, lack the TCA cycle and hence most of the oxidant-sensitive enzymes

58 [10]. Nevertheless,  $O_2^{\bullet -}$  seems also to be problematic for enterococci since they harbour a  
59 *sodA* gene encoding a manganese-cofactored SOD (Mn-SOD). A *sodA* mutant of *E. faecalis*  
60 was more sensitive to different oxidants such as the redox cycling agent menadione and  $H_2O_2$   
61 and its survival was highly affected inside peritoneal macrophages [15]. Furthermore, the Mn-  
62 SOD is of prime importance for the tolerance of *E. faecalis* and *E. faecium* to bactericidal  
63 antibiotics especially penicillin and vancomycin [16,17]. Except one communication  
64 concerning an oxidative stress-sensing regulator negatively regulating the expression of genes  
65 encoding two peroxidases and a catalase [18], nothing is known about the physiological roles  
66 of anti-oxidative defence activities in *E. faecium*. Here, we present a characterization of the  
67 role of the Mn-SOD of the clinical *E. faecium* isolate AUS0004 previously sequenced [19].

## 68 2. Materials and methods

### 69 *Bacterial strains, plasmids and growth conditions*

70 Bacterial strains and plasmids used in this study are presented in Supplementary  
71 **Table S1**. *E. faecium* wild-type (WT) strain and its isogenic mutant were routinely grown at  
72 37°C in Todd Hewitt Broth (THB; Condalab, Madrid, Spain) [20]. Over-night, static and  
73 aerobic growths were performed in 30 mL tubes and in 100 mL Erlenmeyer flasks,  
74 respectively. Aerobic cultures were carried out at 60 rpm or 180 rpm. For the study of  
75 virulence, *E. faecium* strains were grown at 37°C in Brain Heart Infusion (BHI; Biokar  
76 Diagnostics, Allonne, France) broth. If necessary, spectinomycin (500 mg.L<sup>-1</sup>) was added.

77 *Escherichia coli* EC1000 was grown at 37°C in Lysogeny Broth (LB) with moderate  
78 agitation (120 rpm) [21]. When appropriate, 100 mg.L<sup>-1</sup> spectinomycin (Santa Cruz  
79 Biotechnology, Dallas, TX) was added to the cultures.

80

### 81 *2.1. Construction of the ΔsodA mutant strain*

82 The *sodA* gene was deleted by double homologous recombination. Briefly, sequences  
83 upstream and downstream of *sodA* (approximately 1 kb each) were amplified using primers  
84 presented in Supplementary **Table S2**. An overlapping PCR allowed to merge up and down  
85 fragments and to keep only the initiation and stop codons of the *sodA* gene. This fragment  
86 was then cloned into the pWS3 plasmid [22] and the recombinant plasmid was transformed  
87 into *E. coli* EC1000 [23]. The construction was verified by sequencing using primers  
88 pWS3\_F1 and pWS3\_R1 (Supplementary **Table S2**). The resulting plasmid was named  
89 pWS3Δ*sodA* and used to transform *E. faecium* AUS0004 strain. Allelic exchange of the WT  
90 *sodA* gene with the deleted copy was carried out as described previously [22]. *sodA* deletion  
91 and absence of point mutations in *E. faecium* AUS0004 Δ*sodA* mutant were verified by whole

92 genome sequencing and sequence analysis was performed with the software “CLC Genomics  
93 Workbench 10 version” (CLC Bio, Waltham, MA).

94

## 95 ***2.2. Protein extraction and zymogram profiling of the superoxide dismutase activity***

96 *E. faecium* AUS0004 WT and isogenic  $\Delta$ sodA mutant were grown in THB under weak  
97 agitation (60 rpm). Then, cells were harvested by centrifugation when the optical density at  
98 600 nm (OD<sub>600nm</sub>) reached 0.5. They were next washed twice in 50 mM TRIS, 100 mM  
99  $\beta$ -Mercaptoethanol and 2 mM EDTA (pH 7) and resuspended in 50 mM TRIS, 100 mM  
100  $\beta$ -Mercaptoethanol, 2 mM EDTA, 100 mM NaCl and 140 mM PMSF (pH 7). Cells were  
101 disrupted using a FastPrep-24™ 5G instrument (MP Biomedicals, Illkirch, France). The  
102 solution was centrifuged for 10 min at 2000 g and supernatants containing the proteins were  
103 stored at -80°C. Protein concentration was determined using the Quick Start Bradford Protein  
104 Assay kit (Biorad, Hercules, CA).

105 1  $\mu$ g of proteins from *E. faecium* WT or  $\Delta$ sodA mutant was separated by non-  
106 denaturing polyacrylamide gel electrophoresis in duplicate. One gel was then coloured with  
107 Coomassie brilliant blue in order to verify the protein concentration homogeneity between  
108 samples and a second gel was used to reveal the superoxide dismutase activity according to  
109 the protocol of Beauchamp and Fridovich [24].

110

## 111 ***2.3. Oxidative challenges***

112 After a static overnight growth (16 hours), the bacterial cultures were diluted in THB  
113 to obtain an initial OD<sub>600nm</sub> of ~ 0.02. When OD<sub>600nm</sub> 0.5 was reached after a growth at 37°C  
114 under weak agitation (60 rpm), cells were harvested by centrifugation. The pellets were  
115 resuspended in 10 mL of fresh THB medium containing the oxidizing agent (10 to 300 mM  
116 methyl-viologen, 25 mM menadione  $\pm$  2 mM deferoxamine, 50 to 150 mM tert-butyl

117 hydroperoxide (t-BOOH), 10 mM cumene hydroperoxide (CuOOH) or 2 mM of H<sub>2</sub>O<sub>2</sub>.  
118 Methyl viologen, menadione, deferoxamine, t-BOOH, CuOOH and H<sub>2</sub>O<sub>2</sub> were purchased  
119 from Sigma-Aldrich (Saint-Louis, MO). These cultures were incubated at 37°C with weak or  
120 vigorous (60 or 180 rpm) agitation. Duration of treatments was adapted according to  
121 sensitivity of WT to oxidants. Survival was determined by plate counting. Percentage of  
122 survival corresponds to the ratio of colony-forming units (CFU) of treated to initial CFU.  
123 Experiments were repeated one to five times and a control performed without oxidant was  
124 included each time.

125

#### 126 **2.4. Total RNA extraction**

127 For total RNA extraction, *E. faecium* wild-type cells cultured to OD<sub>600nm</sub> 0.5 were  
128 exposed to non-lethal concentrations of oxidants (0.1 to 2.5 mM of menadione, 10 mM  
129 CuOOH, 150 mM t-BOOH or 1 mM H<sub>2</sub>O<sub>2</sub>) for 30 or 60 min. After each challenge, cells were  
130 harvested by centrifugation at 6000 g for 10 min at 4°C and stored at -80°C in acetone/ethanol  
131 (1:1) until RNAs were extracted. Three biological replicates using one concentration were  
132 performed for CuOOH, t-BOOH and H<sub>2</sub>O<sub>2</sub> challenges and one biological replicate using three  
133 different concentrations was performed for the menadione treatment.

134 After resuspension of the pellets in 1 mL of TE (10 mM TRIS, 1 mM EDTA, pH 8),  
135 the cells were lysed using the FastPrep-24™ 5G instrument (MP Biomedicals). Two volumes  
136 of TRIzol Reagent (Invitrogen, Carlsbad, CA) were then added to the lysate. The mixture was  
137 incubated 5 min at room temperature and then centrifuged 2 min at 12,000 g. One volume of  
138 chloroform/isoamyl alcohol was added to the aqueous phase. After another centrifugation,  
139 one volume of ethanol was added to the supernatant and RNA was purified using Direct-Zol  
140 RNA Miniprep kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions.

141 Quality and quantity of RNA were determined by agarose gel electrophoresis and  
142 spectrophotometry using NanoDrop™ 2000 (Thermofisher, Waltham, MA).

### 143 **2.5. RT-qPCR**

144 After RNA extraction, DNase treatment and reverse transcription were carried out  
145 with 1 µg of RNA using Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany)  
146 according to the manufacturer's recommendations.

147 The qPCR analyses were carried out using GoTaq qPCR master mix (Promega,  
148 Madison, WI) and the primers listed in Supplementary **Table S2**. Reactions were performed  
149 with a CFX96 Real-Time PCR detection system (Biorad) using the following program: 95°C  
150 for 3 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s. The transcription level of  
151 *sodA* gene was normalized to that of housekeeping gene *gyrA*. Genomic DNA from  
152 *E. faecium* AUS0004 WT was used to calculate the PCR efficiency. Fold change ratios are  
153 defined as the normalized expression levels in oxidants treated versus non-treated WT strain.

154

### 155 **2.6. H<sub>2</sub>O<sub>2</sub> quantification**

156 100 µL supernatants were taken from aerobically growing (60 rpm) cultures of  
157 *E. faecium* WT and  $\Delta$ *sodA* mutant and stored at -80°C until H<sub>2</sub>O<sub>2</sub> quantification. The amount  
158 of H<sub>2</sub>O<sub>2</sub> was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit  
159 (Invitrogen, Carlsbad, CA) accordingly to the manufacturer's recommendations.

160

### 161 **2.7. Galleria mellonella infection model**

162 *Galleria mellonella* was used as an insect model to evaluate *in vivo* virulence of  
163 *E. faecium* AUS0004 WT and  $\Delta$ *sodA* mutant.  $7.63 \times 10^6 \pm 3.07 \times 10^6$  CFU and  $9.40 \times 10^6 \pm$   
164  $3.80 \times 10^6$  CFU of WT and  $\Delta$ *sodA* mutant in 10 µL of sterile saline solution (0.9 % NaCl),  
165 respectively, were injected subcutaneously into the last abdominal segment of the insect

166 larvae using a 26 G needle (Terumo, Tokyo, Japan) and a syringe pump (KD Scientific,  
167 Holliston, MA). The infected caterpillars were incubated at 37°C in Petri dishes and their  
168 survival were monitored every two hours between 20 and 28 h and between 44 and 48 h post-  
169 infection. Three independent infection experiments were carried out. For each experiment, 20  
170 larvae were infected with *E. faecium* AUS0004 WT, 20 others with the  $\Delta sodA$  mutant and 5  
171 larvae received 10  $\mu$ L of saline solution (control group).

172

### 173 **2.8. Bioinformatics and statistical analysis**

174 Oligonucleotides used in this study were designed using “Primer3web”  
175 (<https://primer3.ut.ee>). The theoretical molecular weight of SodA were calculated with  
176 “Compute pI/Mw” ([https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/)) [25]. The prediction of  
177 transmembrane helices in proteins was performed with “TMHMM server”  
178 (<http://www.cbs.dtu.dk/services/TMHMM/>) [26]. The presence of signal peptide and the  
179 cellular localization were predicted with web-servers “SignalP-5.0”  
180 (<http://www.cbs.dtu.dk/services/SignalP/>) and “BUSCA” (<http://busca.biocomp.unibo.it>) [27],  
181 respectively.

182 The genetic environment of the *sodA* gene was investigated with the DNA sequences  
183 of several strains of *E. faecium*. Phylogenetic tree based on available SOD protein sequences  
184 of *Enterococcus* species was constructed using the software “CLC Genomics Workbench 10  
185 version” ((CLC Bio, Waltham, MA) and the neighbour-joining algorithm (GenBank accession  
186 numbers of protein sequences used in this study are listed in Supplementary **Table S3**).  
187 Robustness of nodes was assessed by bootstrap analysis with 1000 replicates.

188 Manipulations were performed one to five times. Statistical analyses were carried out  
189 with the software “R” (<https://www.R-project.org/>, [28]) using Student’s unpaired *t*-test with  
190 Welch’s correction when homogeneity of variances was not verified. For the virulence test

191 data in the *G. mellonella* infection model represented as Kaplan-Meier curves, a log-rank test  
192 and a Bonferroni adjustment were applied. Statistical significance was defined as  $p < 0.05$ .

193 **2.9. Data availability**

194 Whole genome sequence accession numbers: *E. faecium* AUS0004, NCBI:  
195 SAMN19467926; *E. faecium* AUS0004  $\Delta$ *sodA*, NCBI: SAMN19467927.

196

### 197 3. Results and discussion

#### 198 3.1. Genomic context and protein characteristics of superoxide dismutase of 199 *Enterococcus faecium* AUS0004.

200 Analysis of the genome sequence of *E. faecium* strain AUS0004 revealed the presence  
201 of a unique gene (EFAU004\_01348) annotated to encode a Mn-SOD hereafter referred-to as  
202 SodA [SodA; EC:1.15.1.1]. The protein is composed of 202 amino acids and has a theoretical  
203 molecular mass of 22.9 kDa. No transmembrane domain or signal peptide has been identified  
204 arguing for a cytoplasmic localization although SodA of *Listeria monocytogenes* which also  
205 lacks a signal peptide has been shown to be secreted likely by the SecA2 auxiliary secretion  
206 system [29]. The genetic environment of the *sodA* locus seems to be conserved among the  
207 *E. faecium* clade since in all strains analysed (AUS0004; AUS0085; C68; COM15; D344SRF;  
208 DO; E1071; E1162; E1636; E1679; NRRL B-2354; T110; TC6; TX0082; TX0133; U0317),  
209 *sodA* is flanked upstream by two genes encoding a GtrA family protein and a cation diffusion  
210 facilitator, and downstream by two genes encoding a cold shock protein and a LytR-CpsA-Psr  
211 family protein (LCP enzyme). Two transcriptional start sites (TSS) have been mapped  
212 upstream of the *sodA* gene [30]. The main TSS is located 39 bp and the second TSS, less  
213 frequently used, 44 bp upstream of the ATG start codon, respectively. No transcriptional  
214 terminator was identified downstream of *sodA* [30].

215 In 2000, Poyart *et al.* demonstrated that *sodA* gene can be used for rapid species  
216 identification of *Enterococci*. They showed that *E. faecium* *sodA* was closest to *E. hirae*,  
217 *E. durans* and *E. mundtii* [31]. Our study includes new species associated to the genus  
218 *Enterococcus* and confirms and completes the former study. Indeed, among this genus  
219 currently composed of more than 50 species [3], we showed that SodA of *E. faecium* is most  
220 closely related to *E. hirae*, *E. durans*, *E. villorium*, and *E. mundtii* with 100%, 91.55%,  
221 90.85%, and 90.58% homology at the protein level, respectively (**Fig. 1**). Another

222 phylogenetic reconstruction based on whole genome of most enterococcal species [32] also  
223 clustered *E. faecium* with *E. hirae*, *E. durans*, *E. villorium*, and *E. mundtii*. These findings  
224 confirm that SodA sequence can be used to identify enterococci and also to reconstruct this  
225 genus phylogeny. Of note, *E. faecium* SodA only shares 81.68% homology with SodA of the  
226 other major pathogen of the genus, *E. faecalis*. The two species do not cluster together on the  
227 phylogenetic tree (**Fig. 1**), a phenomenon also observed by Lebreton *et al.* [32], highlighting  
228 that the two species diverged early in the course of evolution and thus justifying  
229 characterisation of SodA in *E. faecium*.

230

### 231 **3.2. Physiological roles of SodA during aerobic growth**

232 To define the cellular role of SodA of *E. faecium*, we first constructed a  $\Delta sodA$  mutant  
233 by in frame deletion of all the EFAU004\_01348 gene except the start and stop codons. The  
234  $\Delta sodA$  mutant was verified by whole genome sequencing which confirmed the correct  
235 deletion and absence of other mutations elsewhere in the genome of *E. faecium* AUS0004.  
236 Absence of any residual SodA activity was confirmed by zymogram (Supplementary **Fig. S1**).

237 We started the characterization of the  $\Delta sodA$  mutant by analysing its growth under  
238 different aeration conditions. The growth rate of the mutant was slightly lower than the  
239 parental strain when cultured without agitation but final OD<sub>600nm</sub> was comparable (**Fig. 2A**).  
240 The growth defect of the  $\Delta sodA$  mutant compared to the WT strain increased when grown  
241 with weak (60 rpm) or strong (180 rpm) oxygenation (**Fig. 2B**). This shows that aerobic  
242 metabolism leads to the generation of O<sub>2</sub><sup>•-</sup> or H<sub>2</sub>O<sub>2</sub> in *E. faecium* which introduces some kind  
243 of damages in the  $\Delta sodA$  mutant reducing its fitness. In contrast to *E. faecalis* which is well  
244 known to produce huge amounts of extracellular O<sub>2</sub><sup>•-</sup> *via* autooxidation of  
245 demethylmenaquinone (DMK), a compound of its respiratory chain [33], *E. faecium* should  
246 not have this capacity because of absence of a respiratory chain [34]. However, this does not

247 exclude intracellular sources of  $O_2^{\bullet-}$  production in this species. On the other hand, *E. faecium*  
248 has been shown to generate huge amounts of  $H_2O_2$  able to kill *Caenorhabditis elegans* [35].  
249 Since one reaction product of SodA is  $H_2O_2$ , we wondered if  $H_2O_2$  production was altered in  
250 the  $\Delta sodA$  mutant. During exponential aerated growth (60 rpm),  $H_2O_2$  levels were very low  
251 for both strains whereas accumulation of this ROS increased rapidly in the WT during  
252 stationary phase to reach 2.8 and 5.25 mM after 10 and 16 hours, respectively (**Fig. 3A**). On  
253 the other hand, the  $H_2O_2$  concentration was under the detection limit after 10 hours and 1.85  
254 mM after 16 hours of culture for the  $\Delta sodA$  mutant (**Fig. 3A**). We determined viable cell  
255 counts for both strains after 10 and 16 hours of culture which showed that there was around 2  
256 times more CFU for the parental strain at both time points (**Fig. 3B**). We then normalized  
257  $H_2O_2$  accumulation for the 16-hour cultures and found 1.31 mM / $10^8$  CFU  $\pm$  0.13 and 0.86  
258 mM / $10^8$  CFU  $\pm$  0.18 for the WT and the  $\Delta sodA$  mutant ( $p = 0,024$ ), respectively. We  
259 concluded that around one quarter of total  $H_2O_2$  accumulated during stationary phase by *E.*  
260 *faecium* AUS0004 seems to be due to the dismutation of  $O_2^{\bullet-}$  by SodA.

261

### 262 **3.3. sodA is induced under aerobic conditions and by oxidants**

263 In order to determine if the *sodA* gene is inducible by oxidative stress conditions in  
264 *E. faecium*, we analysed its expression by RT-qPCR in cells grown under various conditions.  
265 Compared to cultures grown without agitation, *sodA* expression was  $2.37 \pm 0.15$  and  $6.17$   
266  $\pm 3.96$  times higher in mid exponential and early stationary phase aerated cultures (60 rpm),  
267 respectively. We then exposed cells to menadione, a strong oxidant which induces ROS  
268 generation *via* redox cycling [36] and to three peroxides;  $H_2O_2$ , CuOOH and t-BOOH.  
269 Expression of *sodA* was also induced by all peroxides tested (1 mM  $H_2O_2$ , 150 mM t-BOOH  
270 and 10 mM CuOOH) as well as by menadione (0.01, 0.5 and 2.5 mM) with induction factors  
271 comprised between 2- to 5 (**Table 1**). Strong induction of *sodA* by t-BOOH and methyl

272 viologen has been also reported for *S. aureus* whereas H<sub>2</sub>O<sub>2</sub> and CuOOH did not induce its  
273 expression in this species [37,38]. Our results showed that *sodA* expression in *E. faecium* is  
274 induced by superoxide and peroxides and we therefore screened the genome sequence for the  
275 presence of genes encoding transcription factors known to regulate *sodA* expression. Indeed,  
276 expression of the *sodA* gene is tightly regulated in *E. coli* and is under the control of the  
277 SoxRS transcriptional regulators under oxidative stress conditions [39]. Only few information  
278 about transcriptional regulators directly involved in control of expression of *sodA* are  
279 available for Gram-positive bacteria. In *B. subtilis*, expression of the *sodA* gene is dependent  
280 on the stress sigma factor SigB [40]. *S. aureus* has two genes, *sodA* and *sodM*, both encoding  
281 Mn-SODs and expression of both genes are under the direct control of the transcriptional  
282 repressor SarA [38]. However, no homolog for *soxRS*, *sigB* or *sarA* are present in the genome  
283 of *E. faecium* AUS0004.

284

### 285 ***3.4. Involvement of SodA in superoxide and peroxide stress resistance***

286 To determine the implication of SodA in defence against oxidative stress, we first  
287 challenged the  $\Delta$ *sodA* mutant with methyl viologen (paraquat), a redox cycling compound  
288 commonly used to increase intracellular levels of O<sub>2</sub><sup>•-</sup> [41]. However, regardless the  
289 concentration used (10 to 300 mM), no lethality was observed for the WT strain and the  
290  $\Delta$ *sodA* mutant was only marginally killed with the highest concentrations used  
291 (Supplementary **Fig. S2**). This is most likely due to the absence of a methyl viologen  
292 transporter in *E. faecium* AUS0004 as suggested for other bacteria [15, 38–40]. To further  
293 investigate how  $\Delta$ *sodA* mutant copes with O<sub>2</sub><sup>•-</sup> stress, we next exposed cells to menadione.  
294 Whereas menadione was bacteriostatic for the *E. faecium* AUS0004 WT strain, the  $\Delta$ *sodA*  
295 mutant was increasingly sensitive to growing concentrations of this agent and survival  
296 dropped by 99.99% after 2 hours of treatment with 25 mM menadione (**Fig. 4**). A  $\Delta$ *sodA*

297 mutant of *E. faecalis* was also more susceptible to this oxidant [15]. The current model of  $O_2^{\bullet-}$   
298 toxicity suggests the injury of [Fe-S] clusters of enzymes thereby liberating  $Fe^{2+}$  which can  
299 drive the Fenton reaction leading to the generation of  $HO^{\bullet}$  [12]. Consequently, the  
300 intracellular  $Fe^{2+}$  content should be higher in the  $\Delta sodA$  mutant treated with menadione  
301 leading to more  $HO^{\bullet}$  radical formation and lethal damage. We tested this statement using  
302 deferoxamine, a cell-penetrable iron chelator [45]. Treatment with 2 mM deferoxamine and  
303 25 mM menadione had a bacteriostatic effect on the parental strain but killing of the  $\Delta sodA$   
304 mutant was reduced by nearly two orders of magnitude (**Fig. 4**). We concluded that free  
305 intracellular iron seems to contribute to the killing of  $\Delta sodA$  mutant in *E. faecium* likely  
306 acting as a substrate in the Fenton reaction. Since the second reaction partner of the Fenton  
307 reaction is  $H_2O_2$ , we wondered if the *E. faecium*  $\Delta sodA$  mutant would be more sensitive to  
308 peroxides than the parental strain. This was indeed the case for treatments with  $H_2O_2$  (**Fig.**  
309 **5A**) and  $CuOOH$  (**Fig. 5B**). However, no killing of both strains was observed with another  
310 organic hydroperoxide, t-BOOH, up to a concentration of 150 mM (data not shown), although  
311 *sodA* was highly overexpressed during t-BOOH treatment (**Table 1**). The higher toxicity of  
312  $CuOOH$  relative to t-BOOH may be due to the 10-fold higher lipophilicity of the former  
313 compound.

314

### 315 ***3.5. Role of SodA in the virulence of Enterococcus faecium AUS0004***

316 To test the role of SodA of *E. faecium* during infection of a host organism, we used the  
317 surrogate insect model based on larvae of the greater wax moth *Galleria mellonella*. The  
318 innate immune system of insects is closely related to the innate immune system of mammals  
319 [46]. Comparable to the primary immune response of mammals, insects have both cellular  
320 and humoral defences against microbial infections. Phagocytic immune cells called  
321 haemocytes are present in the haemolymph of the larvae which, after phagocytosis, kill

322 pathogens by mechanisms similar to neutrophils of mammals including superoxide production  
323 [47]. Furthermore, to efficiently fight bacterial infections the larvae synthesize numerous  
324 antimicrobial peptides [48,49]. To test virulence of the WT and  $\Delta sodA$  mutant, the larvae  
325 were infected with  $7.6 \pm 3.07 \times 10^6$  CFU and  $9.4 \pm 3.80 \times 10^6$  CFU, respectively. Kinetic of  
326 killing of the larvae by both strains was similar during the first day post-infection but the  
327  $\Delta sodA$  mutant was less virulent than the WT, with 53.3% and 20% larval survival at 48 hours  
328 post-infection, respectively (**Fig. 6**). This correlates well with our previous observation that a  
329 *sodA* mutant of another *E. faecium* strain was affected in survival in this insect model [50].  
330 We concluded that SodA of *E. faecium* seems to be important to withstand the innate immune  
331 response system of *G. mellonella*.

332

333 In conclusion, we showed in this study that SodA is an important antioxidant activity  
334 necessary for full virulence of *E. faecium* AUS0004. We evidenced that the *sodA* gene is  
335 inducible and it would be therefore interesting to identify the corresponding regulator of *sodA*  
336 expression. The regulators of *sodA* expression described in other Gram-positive bacteria are  
337 absent in *E. faecium* and the regulon of the oxidative stress response regulator AsrR  
338 previously characterised in *E. faecium* does not include *sodA* gene [18]. Regulation of  
339 expression of the *sodA* gene of *E. coli* by the ferric uptake regulator Fur has been reported  
340 [51,52]. In contrast, in *B. subtilis*, more closely related to *E. faecium* than *E. coli*, the *sodA*  
341 gene has not been reported to be part of the Fur regulon [53]. Since no Fur binding motif  
342 could be identified in the promoter region of *sodA*, we excluded Fur as a likely direct  
343 regulator of this gene in *E. faecium*. Furthermore, conflicting results have been obtained with  
344 the transcriptional activator HypR of *E. faecalis*. Northern blot analyses showed absence of  
345 implication of HypR in the regulation of *sodA* [54] whereas RT-qPCR suggested its  
346 implication [55]. A close homolog (EFAU004\_02675) of HypR is present in *E. faecium*

347 AUS0004 and might therefore be a prime candidate. Anyway, regulation of *sodA* of *E.*  
348 *faecium* remains hitherto unknown and deserves to be addressed in the near future.

349 **Conflict of interest**

350 The authors declare that they have no conflict of interest.

351

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519

520 **Legends to figures**

521 **Figure 1:** Phylogenetic tree based on available protein sequences of SodA of 47 species of  
522 the *Enterococcus* genus using the neighbour-joining algorithm. Protein sequence of SodA of  
523 *Lactococcus lactis* was used as an outgroup. Bootstrap values (1000 replicates) are indicated  
524 at nodes.

525

526 **Figure 2:** Effect of aeration conditions on growth of *Enterococcus faecium* AUS0004 WT  
527 and  $\Delta sodA$  mutant. Growth of *E. faecium* AUS0004 WT (closed symbols) and  $\Delta sodA$  mutant  
528 (open symbols) was carried out without (A) or with (B) low (60 rpm: triangles) and strong  
529 (180 rpm: circles) agitation. Mean values of three independent experiments and standard  
530 deviations are represented.

531

532 **Figure 3:** Production of hydrogen peroxide (A) and bacterial counts (B) during aerobic  
533 growth under low agitation (60 rpm) of *Enterococcus faecium* AUS0004 WT (grey bars) and  
534  $\Delta sodA$  mutant (white bars). Mean values of three independent experiments and standard  
535 deviations are represented. Asterisks indicate significant differences between WT strain and  
536 mutant. \*\*\*\*  $p < 0.0001$  (Student's *t*-test).

537

538 **Figure 4: Effect of menadione on survival of *Enterococcus faecium* AUS0004 WT and**  
539  **$\Delta sodA$  mutant.** Relative survival of *E. faecium* AUS0004 WT (grey bars)  
540 and  $\Delta sodA$  mutant (white bars) was determined in absence or in presence of different  
541 concentrations of menadione (0.05, 0.1, 0.25, 0.5, 1, 5 and 25 mM) or 25 mM of menadione +  
542 2 mM deferoxamine. Stress exposure was performed under weak agitation (60 rpm). Relative  
543 survival is defined as the ratio of CFU at different treatment exposure times to CFU at the  
544 beginning of treatment at OD<sub>600nm</sub> of 0.5. Mean values of three independent experiments are

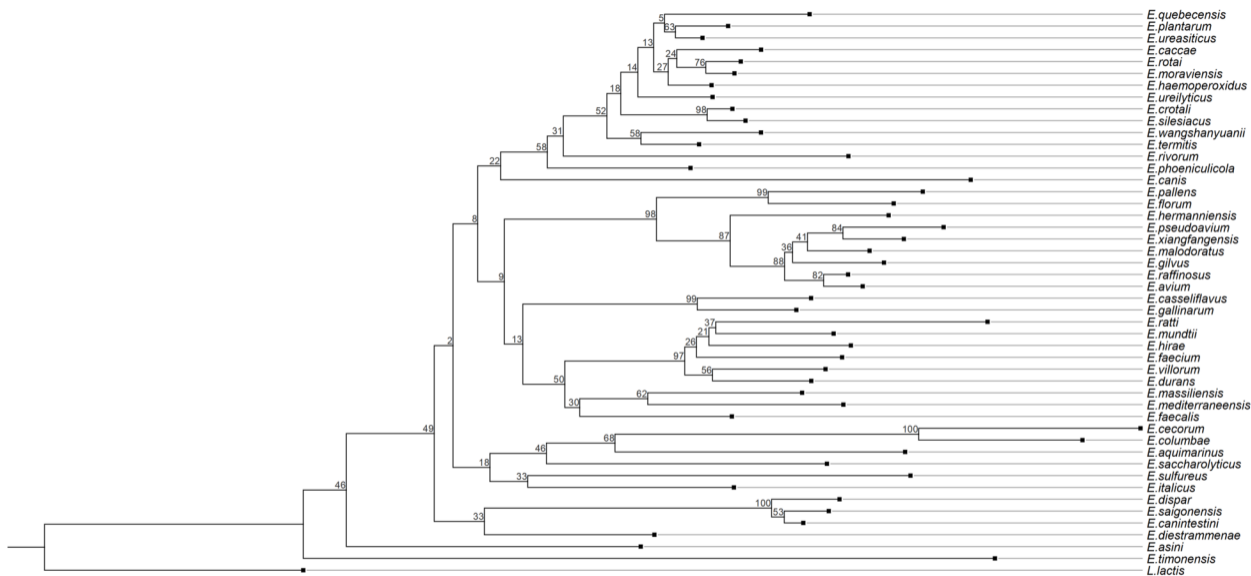
545 represented when standard deviations are shown. Asterisk indicates significant differences  
546 between menadione and menadione + deferoxamine treatments for mutant. \*\*\*\*  $p < 0.0001$   
547 (Student's *t*-test).

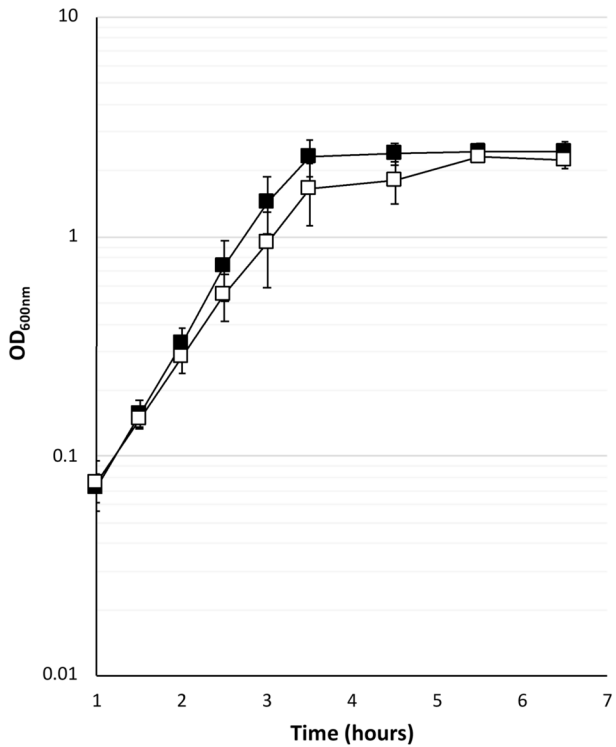
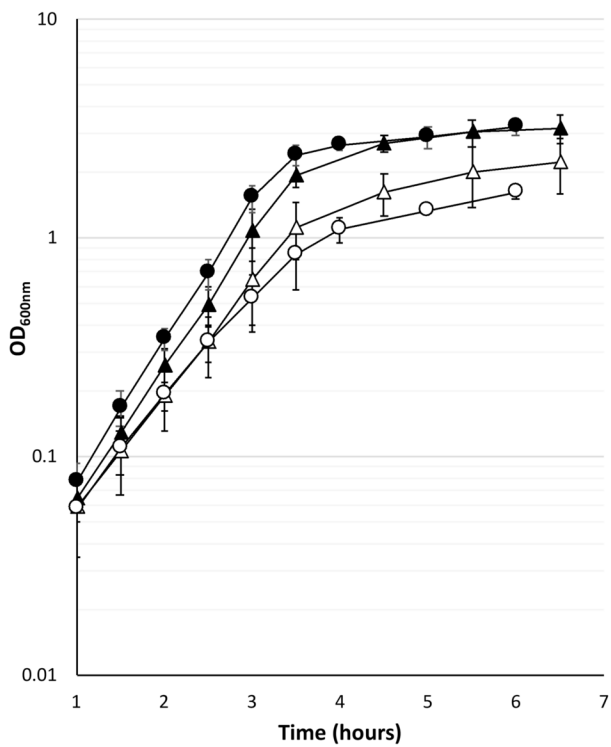
548

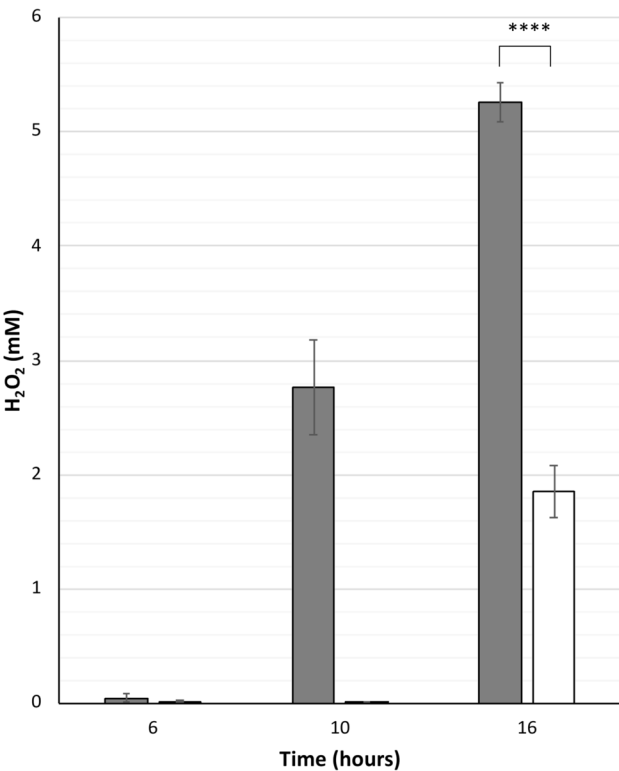
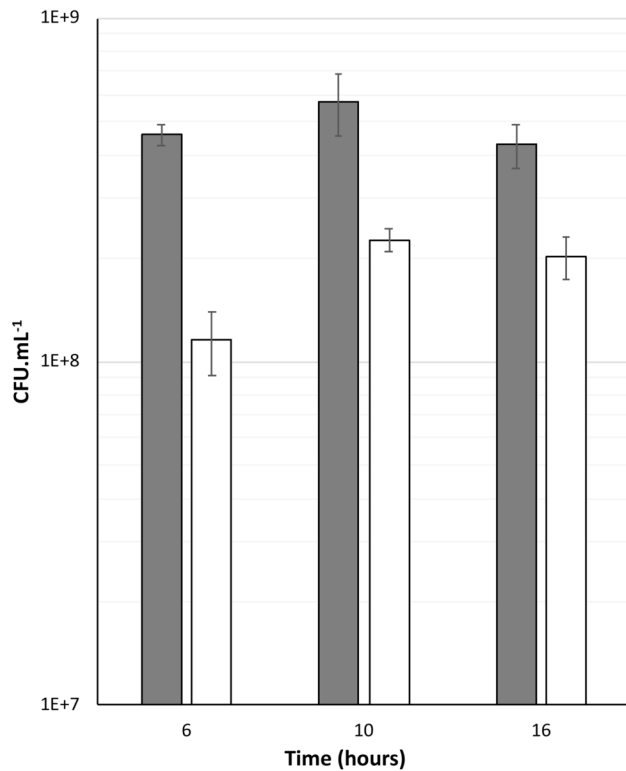
549 **Figure 5: Effect of hydrogen peroxide and cumene hydroperoxide on *Enterococcus***  
550 ***faecium* AUS0004 survival.** Relative survival of WT (grey bars) and  $\Delta sodA$  mutant (white  
551 bars) was determined after a 30-minute exposure to 1 or 2 mM of exogenous H<sub>2</sub>O<sub>2</sub> (A) or  
552 after 2, 4 or 6h exposure to 10 mM of CuOOH (B). Stress exposure was performed under  
553 weak agitation (60 rpm). Relative survival is defined as the ratio of CFU at different treatment  
554 exposure times to CFU at the beginning of treatment (corresponding to a culture with an  
555 OD<sub>600nm</sub> of 0.5). Mean values of three independent experiments and standard deviations are  
556 represented. Asterisks indicate significant differences between WT strain and mutant for a  
557 given time. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  (Student's *t*-test).

558

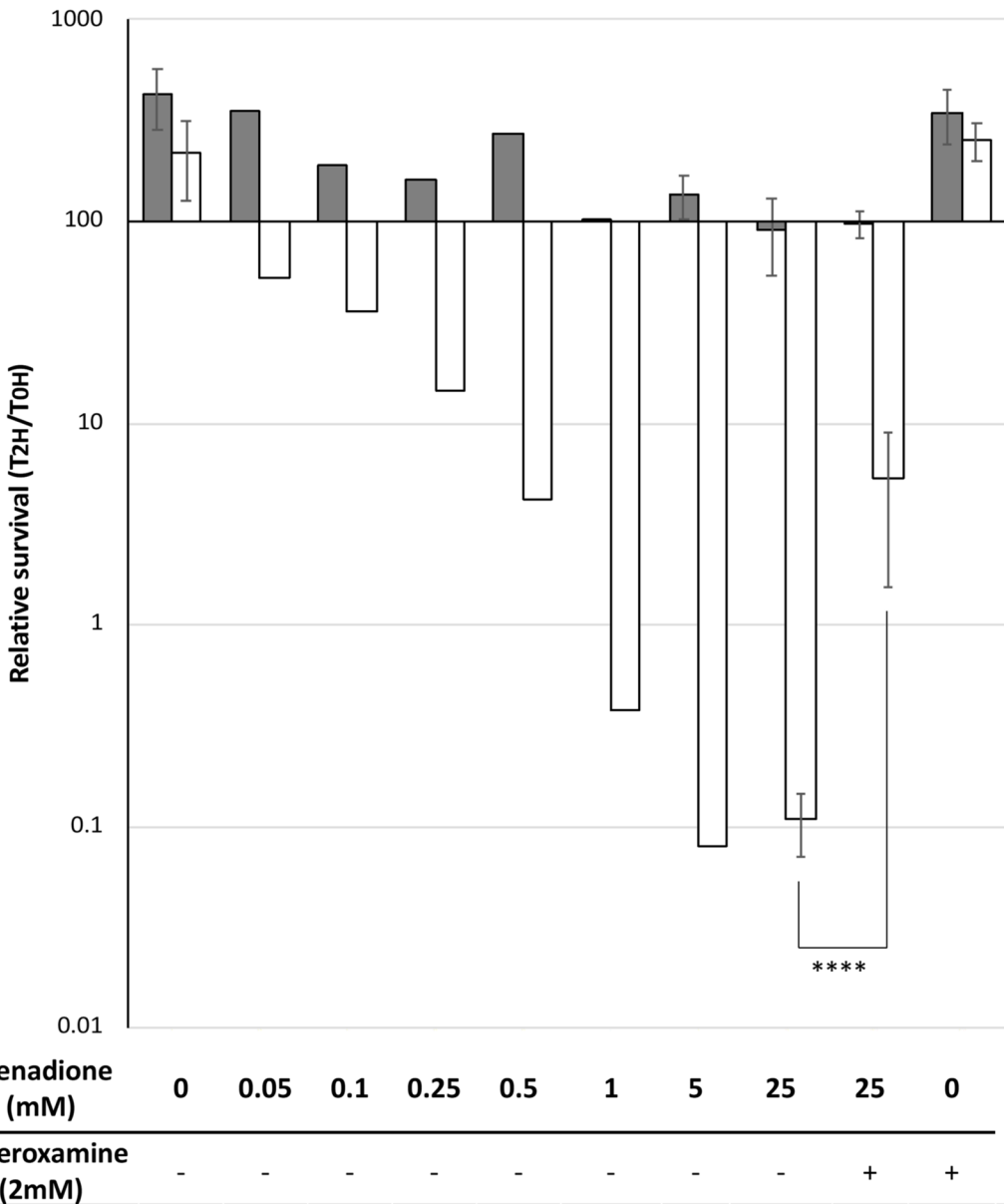
559 **Figure 6: Effect of *sodA* mutation on the virulence of *Enterococcus faecium* AUS0004.**  
560 The *Galleria mellonella* infection model was used to evaluate virulence of *E. faecium*  
561 AUS0004 WT (closed symbols) and  $\Delta sodA$  mutant (open symbols).  $7.63 \times 10^6 \pm 3.07 \times 10^6$   
562 CFU and  $9.40 \times 10^6 \pm 3.80 \times 10^6$  CFU of WT and  $\Delta sodA$  mutant, respectively, were injected  
563 per larvae (compiled data of 3 replicates of 20 larvae infected in each group).  $p = 0.0055$   
564 (Log-rank test with Bonferroni adjustment).

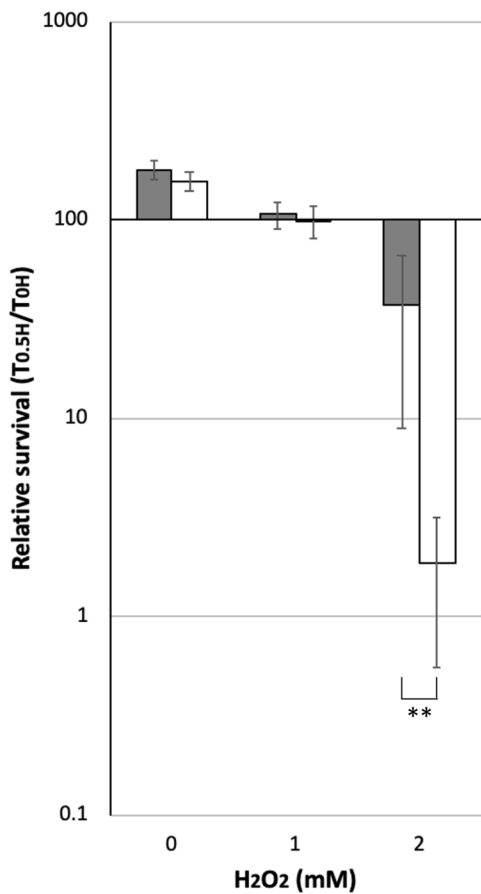
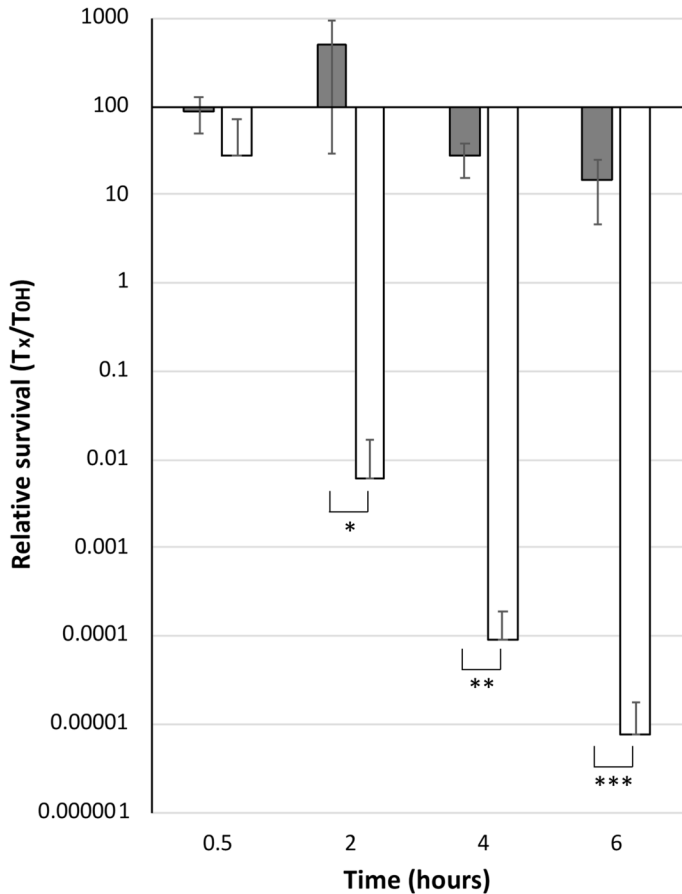


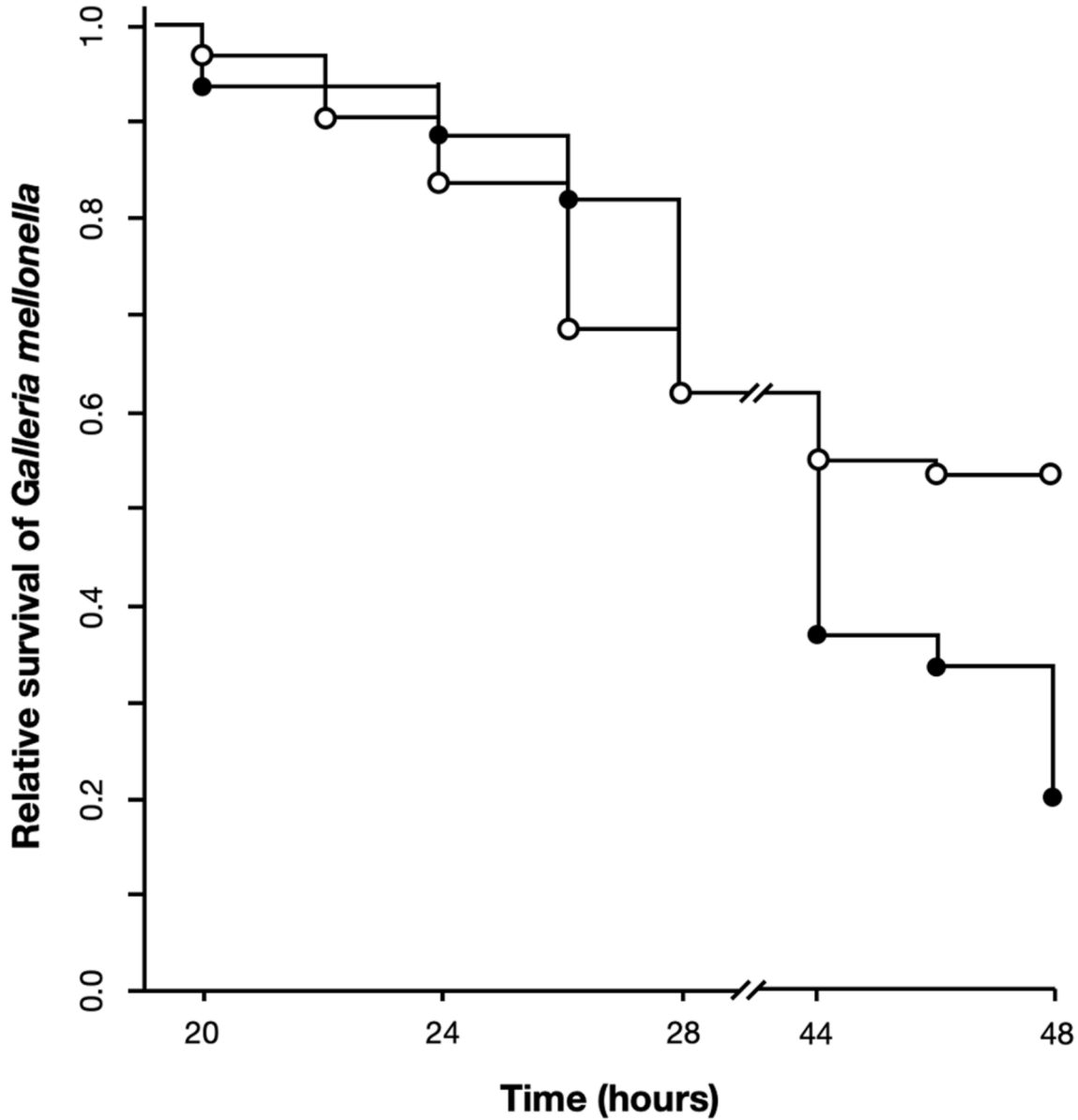
**A****B**

**A****B**

# Menadione



**A** $H_2O_2$ **B** $CuOOH$ 



**Table 1: Effect of menadione and peroxides stresses on *sodA* expression of *Enterococcus faecium* AUS0004.** Expression of the *sodA* gene was measured by RT-qPCR in *E. faecium* WT under no challenge or after exposition to menadione (0.1, 0.5 and 2.5 mM), H<sub>2</sub>O<sub>2</sub> (1 mM), CuOOH (10 mM) or t-BOOH (150 mM). Data were normalized using housekeeping gene *gyrA*. The relative expression ratios presented here are the normalized expression levels of the WT exposed or not to oxidative stresses during 30 min or 60 min relative to those of WT at the beginning of the experiment. Stress exposure was performed under weak agitation (60 rpm). Mean values of three independent experiments and standard deviations (SD) are

***sodA* gene expression after oxidative challenges**

	No oxidant (control)	H <sub>2</sub> O <sub>2</sub>	t-BOOH	CuOOH	Menadione		
		1 mM	150 mM	10 mM	0.1 mM	0.5 mM	2.5 mM
	Fold change ± SD	Fold change ± SD	Fold change ± SD	Fold change ± SD	Fold change ± SD	Fold change ± SD	Fold change ± SD
30 min	1.10 ± 0.23	4.95 <sup>b</sup> ± 1.91	3.53 <sup>a</sup> ± 1.76	2.30 <sup>a</sup> ± 0.60	1.47 ± 0.11	1.83 <sup>a</sup> ± 0.13	2.58 <sup>b</sup> ± 0.12
60 min	1.02 ± 0.22	4.27 <sup>b</sup> ± 1.51	4.32 <sup>c</sup> ± 0.63	2.34 <sup>a</sup> ± 0.43	2.67 <sup>a</sup> ± 0.25	2.79 <sup>a</sup> ± 0.24	2.08 <sup>b</sup> ± 0.13

represented.

<sup>a, b, c</sup> Significant differences between the control and stress conditions. <sup>a</sup>  $p < 0.05$ ; <sup>b</sup>  $p < 0.01$ ;

<sup>c</sup>  $p < 0.001$  (Student's *t*-test)