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1 Dialogue between *Staphylococcus aureus* SA15 and *Lactococcus garvieae* strains
2 experiencing oxidative stress.
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11

12 **Abstract**

13 Background

14 *Staphylococcus aureus* is an important foodborne pathogen. *Lactococcus garvieae* is a
15 lactic acid bacterium found in dairy products; some of its strains are able to inhibit *S. aureus*
16 growth by producing H₂O₂. Three strains of *L. garvieae* from different origins were tested for
17 their ability to inhibit *S. aureus* SA15 growth. Two conditions were tested, one in which
18 H₂O₂ was produced (high aeration) and another one in which it was not detected (low
19 aeration). Several *S. aureus* genes related to stress, H₂O₂-response and virulence were
20 examined in order to compare their level of expression depending on the inoculated *L.*
21 *garvieae* strain. Simultaneous *L. garvieae* H₂O₂ metabolism gene expression was followed.

22 Results

23 The results showed that under high aeration condition, *L. garvieae* strains producing
24 H₂O₂ (N201 and CL-1183) inhibited *S. aureus* SA15 growth and impaired its ability to deal
25 with hydrogen peroxide by repressing H₂O₂-degrading genes. *L. garvieae* strains induced

26 overexpression of *S. aureus* stress-response genes while cell division genes and virulence
27 genes were repressed. A catalase treatment partially or completely restored the SA15 growth.
28 In addition, the H₂O₂ non-producing *L. garvieae* strain (Lg2) did not cause any growth
29 inhibition. The SA15 stress-response genes were down-regulated and cell division genes
30 expression was not affected. Under low aeration condition, while none of the strains tested
31 exhibited H₂O₂-production, the 3 *L. garvieae* strains inhibited *S. aureus* SA15 growth, but to
32 a lesser extent than under high aeration condition.

33 Conclusion

34 Taken together, these results suggest a *L. garvieae* strain-specific anti-staphylococcal
35 mechanism and an H₂O₂ involvement in at least two of the tested *L. garvieae* strains.

36

37 **Keywords**

38 *Lactococcus garvieae* – *Staphylococcus aureus* – antimicrobial – hydrogen peroxide – gene
39 expression

40

41 **Abbreviations**

42 BHI: Brain Heart Infusion

43 GOI: Gene Of Interest

44 LAB: Lactic Acid Bacteria

45 ROS: Reactive Oxygen Species

46

47 **Background**

48 *Staphylococcus aureus* is an opportunistic human pathogen that can be responsible for
49 food poisoning [1]. Its pathogenic activity is due to the production of various enzymes and
50 toxins. It can be found in different environments including milk and dairy products [2]. In

51 cheese, its level should not exceed 10^5 CFU.g⁻¹ (European Community Regulation No. 852-
52 853/2004).

53 *Lactococcus garvieae* is an ubiquitous LAB (Lactic Acid Bacteria) that can be found
54 in various fermented foods including dairy products [3,4], in fish, ruminant or human
55 microbiota [5] and can be associated with pathologies such as fish lactococcoses [6,7]. LABs
56 such as *Lactococcus lactis* or *Lactococcus garvieae* are able to inhibit the proliferation of
57 pathogens in cheese by the production of hydrogen peroxide [8], bacteriocins [9], by
58 competition for nutrients [10,11] or by acidification of the medium [12,14]. Depending on its
59 concentration, hydrogen peroxide has a bactericide or a bacteriostatic effect on *S. aureus*
60 [15]. *L. garvieae* raises our interest because a specific dairy strain of this LAB, N201 strain,
61 can inhibit *S. aureus* growth by the production of H₂O₂ [2,16,17]. Moreover, it has a strong
62 technological potential as a ferment for cheese production [18] and has almost no effect on
63 acidification of the medium, compared to other LABs [2]. The inhibitive properties of *L.*
64 *garvieae* N201 were confirmed on 2 strains of *S. aureus*: a human pathogenic strain, MW2
65 [19] and a non-pathogenic dairy strain, SA15, isolated from Saint-Nectaire cheese [17].
66 Delpech *et al.* [20] showed that *S. aureus* had no effect on *L. garvieae* growth and H₂O₂-
67 related gene expression. On the contrary, *L. garvieae* N201 impaired the capacity of both
68 strains of *S. aureus* to deal with the presence of H₂O₂ which led to growth deficiency
69 [2,16,17].

70 In order to investigate the interaction between *S. aureus* and *L. garvieae* in oxidative
71 stress-inducing culture conditions, genes involved in H₂O₂ metabolism were first choice
72 targets, whose expression has been monitored by Delpech *et al.* [17, 20] in co-cultures of *L.*
73 *garvieae* N201 and *S. aureus* SA15 or MW2.

74 In *L. garvieae*, superoxide dismutase *sodA* [21,22] and pyruvate oxidase *poxB* genes
75 [23,24] are involved in H₂O₂ synthesis. As they do not have any catalase, LAB generally

76 degrade H₂O₂ using alkyl hydroperoxidase (Ahp) [25] or glutathione peroxidase (Gpx)
77 [26]. Thioredoxine reductase (Trx) are involved in response to Reactive Oxygen Species
78 (ROS) [27,28].

79 In *S. aureus*, Catalase (KatA) and Alkyl hydroperoxidase (Ahp) play a role in H₂O₂
80 degradation [29,30]. Ahp leads to a dual function in oxidative-stress resistance,
81 environmental persistence and host-pathogen interaction [29]. Amongst the targeted genes,
82 *dnaK* is known to be involved in H₂O₂-resistance [31,32], *clpC* has an important role in
83 oxidative stress regulation, and *ctsR* is a transcriptional repressor of stress-genes [33].
84 Moreover, the 2 latter genes, belonging to the *dcw* cluster involved in cellular division, might
85 be modulated by H₂O₂-stress, leading to *S. aureus* growth impairment [34]. So, if LAB can
86 modify this genes cluster expression, it could have an inhibiting effect on cellular division.

87 In addition to *S. aureus* growth modulation, *L. garvieae* may also have an effect on its
88 virulence. Enterotoxins are the main toxins responsible for *S. aureus* food poisoning. Ninety-
89 four per cent of *S. aureus* isolated from cow milk have at least one enterotoxin-encoding gene
90 [35]. Amongst the different enterotoxins, enterotoxin C, encoded by *sec4* gene, is the most
91 frequently involved in food poisoning [36–39]. Cretenet *et al.* [40] showed that this
92 virulence-related gene expression can be modified by *L. lactis* in cheese. *S. aureus* virulence
93 is under control of the *agr* system which is involved in regulating many stress response and
94 virulence genes [41,43]. *agrA* is a response regulator [44] and is able to induce *hld*, a δ -lysin
95 gene [43], and enterotoxin C encoding gene *sec4* [45]. The *agr* system itself is controlled by
96 several molecular intermediates such as SaeRS and SrrAB. SaeRS is a two-component
97 system involved in response to environmental stress which could inhibit *agr* system [46] and
98 also control virulence genes [47,50]. SrrAB is also a two-component system activated in an
99 anaerobic environment [51]. It would be involved in virulence gene control [52] in response
100 to H₂O₂. Indeed, in the presence of H₂O₂, *srrA* is repressed in *S. aureus* [53]. Under

101 anaerobic conditions, SrrA represses *agrA* and *hld* expression[54,55]. CodY, a regulatory
102 protein involved in repressing virulence gene expression in *S. aureus*, is also involved in
103 controlling *agr* system and virulence genes [56].

104 The level of aeration can change the level of H₂O₂-production according to the LAB
105 strain. Indeed, it has been demonstrated that the transcriptomes of *L. lactis* and *L. garvieae*
106 are significantly modified by the aeration level [20,57]. *Lactobacillus crispatus* can produce
107 H₂O₂ in high aeration conditions but not in static conditions [58]. Contrariwise, *Lactobacillus*
108 *delbrueckii* subsp. *bulgaricus* can also produce H₂O₂ in static conditions, even though the
109 amount produced is lower than in high aeration conditions [59].

110 The aim of the present study was to compare the transcriptional response involved in
111 the antagonistic interaction between *S. aureus* and different strains of *L. garvieae*. In addition
112 to N201 strain isolated from raw milk Saint-Nectaire cheese and already well described
113 [2,16], two other *L. garvieae* strains were selected for their different capacities to produce
114 H₂O₂: a H₂O₂ -producing strain, CL-1183 (VIVASET, Veterinary Faculty, Complutense
115 University from Madrid) isolated in Brazil from the milk from buffalo cows affected by
116 subclinical mastitis [5] as well as a H₂O₂-non producing strain, Lg2, a fish pathogenic strain
117 isolated in Japan [60]. The expression of *S. aureus* genes related to oxygen metabolism,
118 response to stress, cell division and virulence was measured as well as the expression of *L.*
119 *garvieae* genes related to oxygen metabolism.

120

121 **Methods**

122 **Strains and culture conditions**

123 Strains *Lactococcus garvieae* N201, CL-1183 and Lg2 and *Staphylococcus aureus*
124 SA15 were cultivated in Brain-Heart Infusion buffered to pH 7 using Potassium Phosphate
125 (BHI, Biokar Diagnostic, Pantin, France) at 30°C and 37°C, respectively, under static

126 condition. After 20 hours, cells were harvested at 3500 g during 15 min. Cell pellets were
127 resuspended in 2 ml of BHI and syringed 5 times. Cell concentration was then determined
128 using a Petroff-Hausser counting chamber. Fifty ml of buffered BHI, containing or not 4000
129 IU ml⁻¹ of catalase from bovine liver (ref. C100, Sigma), were then co-inoculated at ~10⁷
130 cells per ml for *L. garvieae* and ~10⁶ cells per ml for *S. aureus*, or just with *S. aureus* at the
131 same concentration in 250 ml Erlenmeyer flasks or in 50 ml Falcon tubes, as described
132 previously [2,17].

133 Then, Erlenmeyer flasks were incubated for 6 h, 9 h or 24 h at 30°C under shaking
134 condition (150 rpm), corresponding to the high level of aeration condition. The low level of
135 aeration condition corresponded to static fully filled 50 ml Falcon tubes. At 0 h, 6 h, 9 h and
136 24 h, 1 ml of the cultures was removed to be syringed and serially diluted in Ringer's
137 solution. After adequate mixing, 100 µl of each dilution were plated onto solid Baird Parker
138 for *S. aureus* and BHI agar for *L. garvieae* for numeration. Colony-forming units were
139 counted after overnight incubation at 37°C for *S. aureus* and 30°C for *L. garvieae*. Also, 1 ml
140 was removed from the cultures for H₂O₂ content analysis and 40 ml for RNA extraction. Cell
141 pellets from 6 h, 9 h and 24 h cultures were resuspended in 200 µl of cold Tris-EDTA buffer
142 and then frozen at -80°C. The experiment was carried out in triplicate within each of three
143 independent biological replicates, for a total of 9 samples per time point. pH measurements
144 were made with the rest of the cultures.

145 **Quantitative analysis of H₂O₂ in *L. garvieae* cultures supernatant**

146 Hydrogen peroxide concentration was determined according to Batdorj *et al.* protocol
147 [61] with slight modifications. Cells were harvested by centrifugation at 11500 g, 15°C for
148 10 min. One hundred µl of the sample supernatant were mixed with 100 µl of 4-
149 aminoantipyrine 4 mg.ml⁻¹ (Sigma-Aldrich, St. Louis, Missouri, USA), 20 µl of water-
150 saturated phenol (Sigma-Aldrich, St. Louis, Missouri, USA), 750 µl of phosphate buffer

151 $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ 0.1 M (pH 7) and 30 μl of horseradish peroxidase type VI-A (500 $\text{U}\cdot\text{ml}^{-1}$
152 in sodium phosphate buffer pH 6, Sigma-Aldrich, St. Louis, Missouri, USA). Sample was
153 mixed by inverting the tube and OD was measured at 505 nm. Blank was done by replacing
154 sample by sterile medium. H_2O_2 concentration was determined using a standard curve
155 performed with concentrations ranging from 0 to 3 mM. The minimal concentration that
156 could be detected was 0.5 mM.

157

158 **RNA extraction and DNase treatments**

159 Cells in Tris-EDTA buffer were thawed out and 25 μl of 20% SDS, 500 μl of phenol
160 (pH 4), 3.5 μl of β -mercaptoethanol and 600 mg of Zirconium beads were added. The cells
161 were broken twice in a tissue homogenizer (Precellys[®] 24, Bertin Technologies, Montigny-le-
162 Bretonneux, France). Then, 200 μl of chloroform were added and mixed with the solution
163 and sample was centrifuged at 11400 g for 20 min at 4°C. RNA extraction was performed
164 using NucleoSpin RNA Midi kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany)
165 following the supplier's instructions. The RNA extracts were treated twice with DNase I
166 using an Ambion DNA-free kit following the supplier's instructions (Ambion, Inc., Austin,
167 Texas, USA). The RNA extracts were quantified using a Nanodrop[™] 2000C (Thermo Fisher
168 Scientific Inc., Waltham, Massachusetts, USA).

169

170 **Reverse Transcription and quantitative PCR**

171 Reverse Transcription (RT) was performed using 0.5 μg of RNA twice treated with
172 DNase, 5 μl of 10X buffer, 2 μl of 25X dNTP, 5 μl of random primers and 2.5 μl of
173 retrotranscriptase (Applied Biosystems[®], Life Technologies, Foster City, California, USA).
174 The RT was done in a thermocycler (Techne[®] Prime, Bibby Scientific, Stone, Staffordshire,
175 UK) with the following parameters: 10 min at 25°C and 120 min at 37°C.

176 Genes Of Interest (GOI) Ct were determined in quantitative PCR (qPCR) assays using
177 2.5 µl of cDNA suspensions 10-fold diluted in RNase-free water and 10 µl of qPCR mix
178 containing 1.25 µl of each primer (10 mM, Table 1, Table 2), 6.25 µl of qPCR Rotor Gene
179 SybrGreen mix (Qiagen, Hilden, Germany) and 1.25 µl of RNase-free water. The qPCR was
180 performed according to the protocol on Rotor Gene Q (Qiagen, Hilden, Germany) with the
181 following parameters: 4 min at 94°C, then for 35 cycles, 30 s at 94°C, 30 s at 55°C, 60 s at
182 72°C. Each reaction was performed in triplicate within each of three independent biological
183 replicates, for a total of 9 reactions per time point.

184 Primers efficiencies were determined according to a 10-fold template dilution
185 standard and were all ranging from 1.90 (~95%) to 2.293 (~115%) according to the following
186 equation: Efficiency = $10^{(-1/\text{slope of calibration curve})}$ [62]. The primers used were
187 previously described by others [17]. One reference gene was used for *L. garvieae* data: *tufB*
188 encoding the elongation factor Tu. Considering that the lag in growth phase reflects the
189 inhibition of *S. aureus* by *L. garvieae*, we evaluated gene expression at identical time points
190 corresponding to different population levels. Two reference genes whose expression was
191 stable over time under the different conditions were used for *S. aureus* data: housekeeping
192 genes *recA*, encoding the recombinase A, and *hu*, encoding a DNA-binding protein. The GOI
193 expression was calculated according to the formula introduced by Hellemans *et al.* [63].

194

195 **Comparison of gene expression**

196 Differential gene expression was evaluated by comparing expression between two
197 conditions. First, the influence of aeration on gene expression in pure culture was evaluated
198 for *S. aureus*. Then, the influence of different strains of *L. garvieae* (N201, CL-1183 and
199 Lg2) on *S. aureus* SA15 gene expression was observed under high or low aeration condition.
200 Secondly, *L. garvieae* gene expression was compared between the different strains in co-

201 culture with *S. aureus* SA15 under both aeration conditions. A gene was considered as
202 differentially expressed when its expression was changed by at least a factor of 2.

203 **Statistical analyses**

204 Statistical analyses on microbial counts and H₂O₂ concentrations were performed using R
205 software [64] by one-way analysis of variance (ANOVA) followed by Least Significant
206 Difference (LSD) test. The gene expression statistical analyses were performed using R by
207 ANOVA with Newmann-Keuls post-hoc test. Significance was declared at P<0.05.

208

209 **Results**

210 In order to investigate the interaction between *S. aureus* and *L. garvieae* in oxidative stress-
211 inducing culture conditions, we followed the growth of the strains and measured the amount
212 of H₂O₂ in two culture conditions i.e. under high and low aeration levels. The genes whose
213 expression has been monitored by Delpech *et al.* [17, 20] were chosen to compare the
214 transcriptional response involved in the antagonistic interaction between *S. aureus* and
215 different strains of *L. garvieae*. The expression of *S. aureus* genes related to oxygen
216 metabolism, response to stress, cell division and virulence was measured as well as the
217 expression of *L. garvieae* genes related to oxygen metabolism (Table 3).

218

219 **Ability of *L. garvieae* strains to produce H₂O₂ and to inhibit *S. aureus*** 220 **growth**

221 The effect of aeration level on microbial growth was tested by determining cellular
222 concentrations of both microorganisms. In pure culture, *S. aureus* growth was almost
223 identical under both high and low aeration conditions. However, the population count reached
224 at 24h was 1.2 log CFU/ml lower under low aeration than under high aeration (Figure 1A).
225 The 3 different strains of *L. garvieae* grew as well under high aeration level as under low

226 aeration level independent of the presence of *S. aureus* (Additional file 1). pH values
227 remained stable (7.0 ± 0.2) in all cultures over the whole experiment (data not shown).

228 Detectable amounts of H_2O_2 were produced exclusively under high aeration level by
229 N201 and CL-1183 strains in co-culture with SA15. N201 strain produced from $1.327 \text{ mM} \pm$
230 0.09 to $1.517 \text{ mM} \pm 0.18$ of H_2O_2 . The strain CL-1183 produced slightly more H_2O_2 than the
231 strain N201, from $1.663 \text{ mM} \pm 0.23$ to $2.415 \text{ mM} \pm 0.34$. While N201 H_2O_2 -production peak
232 was at 9 h, CL-1183 H_2O_2 -production stayed stable after 9 h (Table 4).

233 Under high aeration, when co-cultivated with N201, SA15 growth was 2.1 log
234 [CFU.ml⁻¹] lower than in pure culture as early as 6 h with a maximal growth inhibition of 5.8
235 log [CFU.ml⁻¹] at 24 h. SA15 growth co-cultivated with CL-1183 was 1.7 log [CFU.ml⁻¹]
236 lower compared to pure culture as early as 6 h. The maximal growth inhibition was observed
237 after 24 h of co-culture (4.5 log [CFU.ml⁻¹] lower). SA15 co-cultivated with Lg2 did not
238 show any significant impairment in growth throughout the experiment compared to pure
239 culture (Figure 1A).

240 Under low aeration level and in co-culture with N201, CL-1183 or Lg2, SA15 growth
241 was lower than in pure culture as early as 6 h ($0.6 \text{ log [CFU.ml}^{-1}]$, $0.9 \text{ log [CFU.ml}^{-1}]$ or 0.6
242 $\text{ log [CFU.ml}^{-1}]$, with a maximal growth inhibition at 24 h ($2.5 \text{ log [CFU.ml}^{-1}]$, $3.5 \text{ log$
243 [CFU.ml^{-1} or $1.6 \text{ log [CFU.ml}^{-1}]$], respectively) (Figure 1A).

244 To determine the involvement of H_2O_2 produced by N201 and CL-1183 in growth
245 inhibition of SA15, co-cultures of SA15 with these 2 strains were performed under high
246 aeration level in presence of catalase (Figure 1B). No detectable amount of H_2O_2 was
247 observed at any time. *S. aureus* cell concentration was not significantly different in presence
248 of catalase compared to control (Figure 1A vs. Figure 1B) during both exponential and
249 stationary phases. Statistical analysis, using ANOVA, showed that in presence of catalase
250 there was no significant difference of SA15 count after 24 h in co-culture with CL-1183

251 compared to pure culture. In contrary, SA15 cell concentration in co-culture SA15/N201 was
252 still 0.8 log [CFU.ml⁻¹] lower than in pure culture. These results showed that the inhibition of
253 SA15 growth by N201 and CL-1183 was partially or completely suppressed by catalase.

254

255 **Effect of *L. garvieae* strains on *S. aureus* genes expression under high**
256 **aeration level (Additional file 2)**

257 Whatever the *L. garvieae* strain cultivated with SA15, none of the H₂O₂-response genes
258 tested was up-regulated. When SA15 was cultivated with *L. garvieae* N201 or CL-1183, *katA*
259 was down-regulated, at 9 h and 24 h with N201 (3.7 and 3.0-times, respectively), or at 6 h
260 and 9 h with CL-1183 (3.9 and 3.2-times, respectively). When cultivated with N201, *ahpF*
261 expression in SA15 was not modified in comparison with pure culture, whereas in co-cultures
262 SA15/CL-1183 and SA15/Lg2, it was down-regulated (2.1-times at 6 h and 2.9-times at 24 h,
263 6.9-times at 24 h, respectively). *sodA* was down-regulated at 6 h, 9 h and 24 h in co-cultures
264 with N201 (2.2, 4.4 and 3.9-times, respectively) or at 24 h (2.2-times) with CL-1183. No
265 change in expression of *katA* and *sodA* was observed with Lg2.

266 *L. garvieae* N201 induced an up-regulation of the three SA15 stress-response genes
267 tested at 9 h; *clpC*, *ctsR* and *dnaK* were 2.0-times, 3.7-times and 2.5-times more expressed
268 than in pure culture, respectively. When cultivated with CL-1183, only *dnaK* was 2.8-times
269 up-regulated at 6 h, while *clpC* and *ctsR* expressions were not affected. Conversely, in co-
270 culture SA15/Lg2, *clpC* and *dnaK* were 3.9-times and 19.8-times down-regulated
271 respectively, while *ctsR* did not show any significant difference in its expression.

272 In both SA15/N201 and SA15/CL-1183 co-cultures, *mraW* cell division gene was
273 strongly down-regulated at 6 h and 9 h (7.0-times and 20.3-times, and 20.0-times and 12.0-
274 times, respectively). In co-culture SA15/Lg2, *mraW* expression was not affected.

275 In co-culture, the five SA15 virulence-related regulator genes tested displayed
276 contrasted patterns of expression. At 9 h, in both SA15/N201 and SA15/CL-1183 co-cultures,
277 *agrA* was up-regulated (4.0-times with N201 and 3.4-times with CL-1183), while *srrA* was
278 down-regulated (7.1-times or 6.4-times with N201 or CL-1183 respectively). *codY*
279 expression was down-regulated 4.1-times at 24 h by N201 and 3.6-times at 6 h by CL-1183.
280 *saeS* expression was 2.4-times lower at 24 h in co-culture with N201 than in pure culture,
281 whereas it was not affected by CL-1183. *hld* expression remained stable until 24 h when
282 SA15 was in co-culture with N201 or CL-1183. In co-culture SA15/Lg2, at 24 h, *agrA*, *codY*
283 and *hld* expressions were all down-regulated (3.0-times, 2.6-times and 3.9-times,
284 respectively), while *saeS* and *srrA* expressions remained stable.

285 In co-cultures SA15/N201 and SA15/CL-1183, amongst the 2 enterotoxins-encoding
286 genes tested, only *sec4* expression was modified at 24 h, with a 4.1-times and a 9.0-times
287 down-regulation, respectively. *sel2* expression was not modified whatever the SA15 culture
288 conditions were. In co-culture SA15/Lg2, no enterotoxin gene expression was affected.

289

290 **Effect of *L. garvieae* strains on *S. aureus* genes expression under low**
291 **aeration level (Additional file 3)**

292 Under low aeration level, in presence of CL-1183, the H₂O₂-response gene *ahpF* was
293 4.2-times up-regulated at 9 h, whereas in presence of Lg2, *sodA* was 6.5-times down-
294 regulated at 24 h. All the stress-response genes tested were up-regulated at 9 h (3.4-times for
295 *clpC*, 4.0-times for *ctsR* and 2.1-times for *dnaK*) with Lg2. No change in stress-response
296 genes expression was observed when SA15 was cultivated with N201 or CL-1183.
297 Concerning the cell division gene *mraW*, its expression was not modified, whatever the strain
298 cultivated with SA15.

299 Amongst virulence-related regulator genes, *agrA* was up-regulated in all three co-
300 cultures at different stages of growth. It was the only virulence-related regulator gene
301 differentially regulated by N201 (6.0-times up-regulated at 9 h). In the same way, this gene
302 was 4.1-times up-regulated at 9 h by CL-1183, and 2.5-times up-regulated at 24 h by Lg2.
303 *codY* was 2.0-times down-regulated at 6 h but 2.0-times up-regulated at 24 h with CL-1183,
304 while *codY* and *srrA* were 2.1- and 2.7-times down-regulated at 9 h and 24 h, respectively
305 with Lg2.

306 The enterotoxin gene *sec4* was up-regulated in both N201/SA15 and CL-1183/SA15
307 co-cultures, (3.5- and 2.7-times, at 6 h and 9 h respectively, with N201, and 2.5-times at 9h
308 with CL-1183). *sel2* was 3.5-times up-regulated at 9 h only in the presence of CL-1183.

309

310 **Effect of aeration on *L. garvieae* strains (Additional file 4)**

311 To evaluate the strain-specific effect of aeration on *L. garvieae*, expression of 5
312 H₂O₂-related genes of them (*trxB1*, *ahpC*, *gpx*, *poxB* and *sodA*) was monitored and compared
313 between the 3 *L. garvieae* strains in co-culture with SA15. Overall, most differential
314 expressions concerned H₂O₂-degradation genes. Differences between H₂O₂-producing and
315 non-producing strains were essentially related to *ahpC*: both N201 and CL-1183
316 overexpressed this gene under low aeration (5.6-times at 24 h in N201, 4.0-times at 6 h and
317 6.5-times at 9 h in CL-1183). Conversely, Lg2 overexpressed *ahpC* (4.7-times) at 9 h under
318 high level of aeration. Both CL-1183 and Lg2 overexpressed *trxB1* under high level of
319 aeration (2.9-times at 6 h in CL-1183 and 2.1-times 24 h in Lg2). Amongst H₂O₂-synthesis
320 genes, only CL-1183 overexpressed *poxB* (4.1-times at 9 h under low aeration).

321

322 **Discussion**

323 The aim of the study was to compare the transcriptional response involved in the
324 antagonistic interaction between *S. aureus* and different strains of *L. garvieae*. Three strains
325 of *L. garvieae* from different origins were used: N201, a dairy-isolated strain known to be a
326 *S. aureus* inhibiting strain; CL-1183, a strain isolated from milk from buffalo cows suffering
327 from mastitis; and Lg2, a fish pathogenic strain. Under high aeration condition, two of them
328 were shown to be able to produce detectable amount of H₂O₂ (N201 and CL-1183), whereas
329 the third one (Lg2) was not.

330

331 ***L. garvieae* strains and aeration level effect on *S. aureus* SA15 growth and**
332 **on H₂O₂- and stress-responses**

333 Under high aeration level, only *L. garvieae* N201 and CL-1183 inhibited *S. aureus*
334 growth. CL-1183 down-regulated *S. aureus* H₂O₂-degradation genes (*ahpF* and *katA*) and
335 N201 and Lg2 only down-regulated *katA* or *ahpF* respectively. This could mean that SA15
336 lost its ability to deal with H₂O₂-stress and suggest that the H₂O₂ detoxification occurred
337 predominantly via KatA. These results are in accordance with those obtained by Cosgrove *et*
338 *al.* [29], showing that *S. aureus* *ahpC-katA* mutant was more sensitive to H₂O₂ than the *katA*
339 mutant. They also found out that, Ahp would have less affinity for H₂O₂ than KatA and that it
340 could be an alternative solution for H₂O₂-degradation when KatA was not functional. In our
341 study, despite N201 only down-regulated *katA* and not *ahpF*, AhpF did not play its
342 compensatory role in H₂O₂ detoxification. This result can be explained by the fact that KatA
343 was responsible for detoxifying high levels of H₂O₂, whereas AhpC was responsible for the
344 removal of low levels of H₂O₂ [29]. This could be correlated with the large amounts of H₂O₂
345 detected in the co-cultures SA15/N201. The down-regulation of *katA*, at 9 h and 24 h, can be
346 explained by the fact that *srrA* was also down regulated. Mashruwala and Boyd [65] found
347 that *srrAB* mutant strain had decreased transcription of genes encoding for H₂O₂ resistance

348 factors as *katA*. They also reported that SrrAB positively influenced H₂O₂ resistance during
349 periods of high O₂ dependent respiratory activity, but not when cellular respiration was
350 diminished as a result of lower O₂ availability. *S. aureus* SA15 genes involved in stress-
351 response (*clpC*, *ctsR*, *dnaK*) were all up-regulated by N201 and only *dnaK* was up-regulated
352 by CL-1183, whereas *clpC* and *dnaK* were down-regulated by Lg2. These results indicated
353 that in presence N201 and CL-1183, SA15 detected an oxidative stress as a result of H₂O₂
354 production by these two *L. garvieae* strains. However, the up-regulation of these genes did
355 not allow SA15 to fight against this stress, although precedent studies reported that *S. aureus*
356 *clpC* or *dnaK* mutants growth was impaired in the presence of H₂O₂ stress [32,66,67] and that
357 a basic level of expression of *dnaK* was sufficient in response to this stress [32]. All these
358 data highlighted the complexity of the stress response machinery and the important role of
359 *clpC*, *ctsR* and *dnaK* genes. Moreover, while *mraW* gene was not differentially expressed in
360 presence of Lg2, it was repressed at 6 h and 9 h by N201, confirming results obtained by
361 Delpech *et al.* [17], and by CL-1183 in our study, when *S. aureus* growth was inhibited. The
362 repression of *mraW* by N201 and CL-1183 was positively correlated with inhibition of SA15
363 growth. These results are supported by Cretenet *et al.* [40] showing that *L. lactis* was able to
364 inhibit *ftsH*, *ftsL* and *ftsZ* genes also involved in cellular division. Interestingly, several
365 studies reported that inhibition of cell division protein is a promising approach for anti-
366 staphylococcal therapy [68,69].

367 Catalase treatment partially reduced SA15 growth inhibition by N201, confirming the
368 result obtained by Delbes-Paus *et al.* [2], whereas the inhibition by CL-1183 was completely
369 suppressed. Oogai *et al.*[70] observed that in presence of catalase, the *S. aureus* MW2 growth
370 inhibition by *Streptococcus sanguinis* was completely suppressed. These observations
371 confirmed the role of H₂O₂ produced by LAB in growth inhibition of *S. aureus*. Our study
372 demonstrated that under high level of aeration, the inhibition of *S. aureus* by *L. garvieae*

373 involved strain specific mechanisms. Indeed, CL-1183 inhibited SA15 growth mainly by
374 H₂O₂ production whereas inhibition due to N201 may involve the combined action of H₂O₂
375 and other antagonistic mechanism.

376 Under low aeration condition, *S. aureus* growth was almost identical to that measured
377 under high aeration condition, except after 24 hours, when we observed a 1,2 log reduction in
378 the *S. aureus* population level that could be due to the depletion of oxygen. Ledala *et al.* [71]
379 showed that the growth rate of *S. aureus* was independent of oxygen limitation over 12 hours
380 although its metabolome was significantly affected. *S. aureus* growth was slightly inhibited
381 by the three *L. garvieae* strains, although none of them could produce detectable amounts of
382 H₂O₂ (Figure 1A). This suggests that there was another inhibitory mechanism involved.
383 These results match those obtained with N201 in the previous studies [2,17,20]. Delbes-Paus
384 *et al.* [2] have shown that in milk, even if H₂O₂ was not detected, *S. aureus* growth was
385 inhibited by *L. garvieae* N201. However, no clear hypothesis to explain the mechanism of the
386 anti-staphylococcal activity under low aeration could be drawn from the gene expression
387 data. However, the up-regulation of stress-response genes in the presence of Lg2 was
388 observed. Indeed, *clpC*, *ctsR* and *dnaK* genes, were all up-regulated at 6 h in co-culture with
389 Lg2, suggesting that Lg2 triggered a stress on SA15 which led to a growth inhibition.

390

391 ***L. garvieae* strains and aeration level effect on *S. aureus* SA15 virulence**

392 **gene expression**

393 Under high level of aeration, *L. garvieae* N201 reduced *S. aureus* virulence-related
394 genes expression confirming results obtained by Delpech *et al.* [17]. Cretenet *et al.* [40] and
395 Queck *et al.* [72] have shown that the *agr* system, involved in the regulation of genes linked
396 to *S. aureus* virulence, was repressed by *L. lactis* even if *L. lactis* does not produce H₂O₂ in
397 this condition. Molecular intermediaries SaeRS and SrrAB are involved in controlling the *agr*

398 system [46]. We showed *srrA* expression was repressed in co-culture with N201 and CL-
399 1183, when H₂O₂ was produced as already observed by Chang *et al.* [53]. Moreover,
400 Majerczyk *et al.* [56] have shown that a *codY* mutant could derepressed *agr* system. In our
401 conditions, *codY* was repressed by CL-1183 at 6 h explaining *agrA* up-regulation at 9 h,
402 whereas repression of *codY* at 24 h by Lg2 cannot explain *agrA* down-regulation suggesting
403 that another regulator might be involved. Moreover, at 24 h, despite SA15 growth was not
404 modified, Lg2 caused a down-regulation of three virulence-related regulator genes tested in
405 this study (*agrA*, *codY*, *hld*). *sec4* and *sel2* are two enterotoxin-encoding genes found in *S.*
406 *aureus* SA15 [17]. Although enterotoxin C-encoding gene *sec4* is under the control of the *agr*
407 system [45], *agrA* expression was only induced by N201 and CL-1183 and *sec4* was
408 repressed under high aeration level as shown by Delpech *et al.* [17]. Our data showed the
409 repression of virulence associated genes and of enterotoxin encoding genes as well as
410 simultaneous *S. aureus* growth inhibition in the presence of N201 or CL-1183, indicating that
411 these 2 strains not only inhibited SA15 growth but also potentially attenuated its virulence.

412 Under low aeration condition, *S. aureus* over-expressed *agrA* and enterotoxin-
413 encoding genes *sel2* and *sec4* in presence of N201 and CL-1183, whereas Lg2 did not induce
414 any modification. In presence of Lg2, *agrA* was up-regulated at 24 h. This result can be
415 explained by the fact that SA15 *srrA* gene was down-regulated, at the same time. The same
416 observation was reported by Yarwood *et al.* [55]. They showed that transcription of RNAIII
417 from the *agr* locus was inversely dependent on expression of *srrAB*. Inherently, despite
418 SA15 *agrA* up-regulation by Lg2, no modification in enterotoxin genes expression was
419 observed. Yarwood *et al.* [55] also reported that the SA15 *srrB* mutant growth was
420 significantly slower in anaerobic condition, which is in accordance with SA15 growth
421 inhibition in presence of Lg2.

422

423 ***L. garvieae* strain responses to low and high levels of aeration in co-culture**
424 **with *S. aureus* SA15**

425 It seemed that the three strains of *L. garvieae* differed essentially in their ability to
426 degrade H₂O₂. Presence of H₂O₂ would not depend only on H₂O₂-synthesis, but also on
427 H₂O₂-degradation by *L. garvieae* strains, which is an original mechanism, and in accordance
428 with the results obtained by Delpech *et al.* [20].

429 In high aeration level, Lg2 overexpressed two H₂O₂ degradation genes *ahpC* and *trxB*
430 at 9 h and 24 h respectively. The non-detection of H₂O₂ could be explained by the absence of
431 production of H₂O₂ or by its degradation by AhpC and TrxB. The latter mechanism could
432 explain why, under low aeration, despite CL-1183 overexpressed an H₂O₂-synthesis gene,
433 *poxB*, no detectable H₂O₂ was observed. Indeed, at the same time, this strain overexpressed
434 the H₂O₂ degradation gene *ahpC*. Our data showed that the importance of the inhibition
435 varies depending on the presence or absence of H₂O₂. Indeed, inhibition can occur while
436 H₂O₂ is not detected. As regards other potential explanations for the growth inhibition, the
437 only putative bacteriocin identified in the *L. garvieae* N201 genome was homologous to
438 garvieaecin Q (GarQ, data not shown), a class II d bacteriocin [73]. In view of the results of
439 previous experiments on *L. garvieae* N201 [20], the inhibition was probably caused neither
440 by garvieaecin Q nor by a protein, nor by a lipid, and nor by a polysaccharide. In the present
441 study, we evidenced *L. garvieae* strain-specific response in *S. aureus* gene expression which
442 suggests that molecular mechanisms involved in the inhibition could differ between *L.*
443 *garvieae* strains. Further investigations are needed to elucidate these mechanisms. A detailed
444 analysis of strains physiology is needed to evaluate the potential role of nutritional
445 competition in the inhibition. A global transcriptomic approach of the interaction would shed
446 some light on the metabolic dialogue between these strains, and more particularly the
447 potential involvement of quorum-sensing.

448

449

450 **Conclusions**

451 Our data evidenced an impact of the aeration condition on the interaction between
452 SA15 and *L. garvieae* strains.

453 Under high level of aeration, only N201 and CL-1183 produced detectable amounts of
454 H₂O₂ and consequently inhibited SA15 growth. Both *L. garvieae* strains activated SA15
455 stress-response genes and inhibited the expression of several genes involved in H₂O₂-
456 degradation , virulence and cellular division . A catalase treatment partially or completely
457 suppressed this inhibition and no H₂O₂ was detected. Lg2 strain did not produce any
458 detectable amount of H₂O₂ and did not inhibit SA15 growth. However, as N201 and CL-
459 1183, Lg2 inhibited SA15 virulence-related genes and stress-response related genes.

460 Under low aeration level, none of the *L. garvieae* strains produced any detectable
461 amount of H₂O₂. N201 and CL-1183 were still able to inhibit SA15 growth, as well as, Lg2.
462 While N201 and CL-1183 induced the up-regulation of the SA15 enterotoxin-encoding genes
463 and related regulator *agrA*, Lg2 caused up-regulation of SA15 stress-related genes and down-
464 regulated virulence-related regulators genes.

465 The antagonistic properties of *L. garvieae* against *S. aureus* were strain- and aeration
466 level-dependent. Under high aeration, they involved H₂O₂ production by two out of the three
467 *L. garvieae* tested strains. This study provides new insights into microbial interactions
468 mechanisms and shows the importance of investigating strain-specific effects.

469

470 **Declarations**

471 **Ethics approval and consent to participate**

472 Not applicable

473

474 **Consent to publish**

475 Not applicable

476

477 **Availability of data and materials**

478• All data generated or analysed during this study are included in this published article
479 [and its supplementary information files].

480

481 **Competing interests**

482 The authors declare that they have no competing interests.

483

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486

487 **Authors' Contributions**

488 CQ performed the RT-qPCR, analyzed genes expression, ran statistical analyses and
489 was a major contributor in writing the manuscript. MB contributed to cultivate the different
490 strains, performed the quantitative analysis of H₂O₂ in *L. garvieae* cultures, the RNA
491 extraction, DNase treatment and the RT-qPCR, and was also a contributor in writing the
492 manuscript. TS cultivated the different strains, performed the tests with added catalase, and
493 contributed in writing the manuscript. PD helped in RT-qPCR, genes expression analysis and
494 improvement of the manuscript. PV contributed to cultivate the different strains and to extract
495 the RNA. ER contributed to statistical analyses. CM contributed to the adjustment of the
496 method of quantitative analysis of H₂O₂. GG read and corrected the manuscript. SB and CD
497 conceived the study. CD contributed to genes expression analysis and to the structuring and

498 improvement of the manuscript. SB contributed to cultivate the different strains, performed
499 the RNA extraction and was also a contributor in writing the manuscript.

500

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505

506 **Additional materials**

507 • Additional file 1: Table 5. Gene expression changes in *S. aureus* SA15
508 co-culture with 3 *L. garvieae* strains under high aeration level

509 • Additional file 2: Table 6. Gene expression changes in *S. aureus* SA15
510 co-culture with 3 *L. garvieae* strains under low aeration level

511 • Additional file 3: Table 7. Aeration induced changes in *L. garvieae*
512 (N201, CL-1183 and Lg2 strains) H₂O₂-related gene expression in co-culture with *S. aureus*
513 SA15

514

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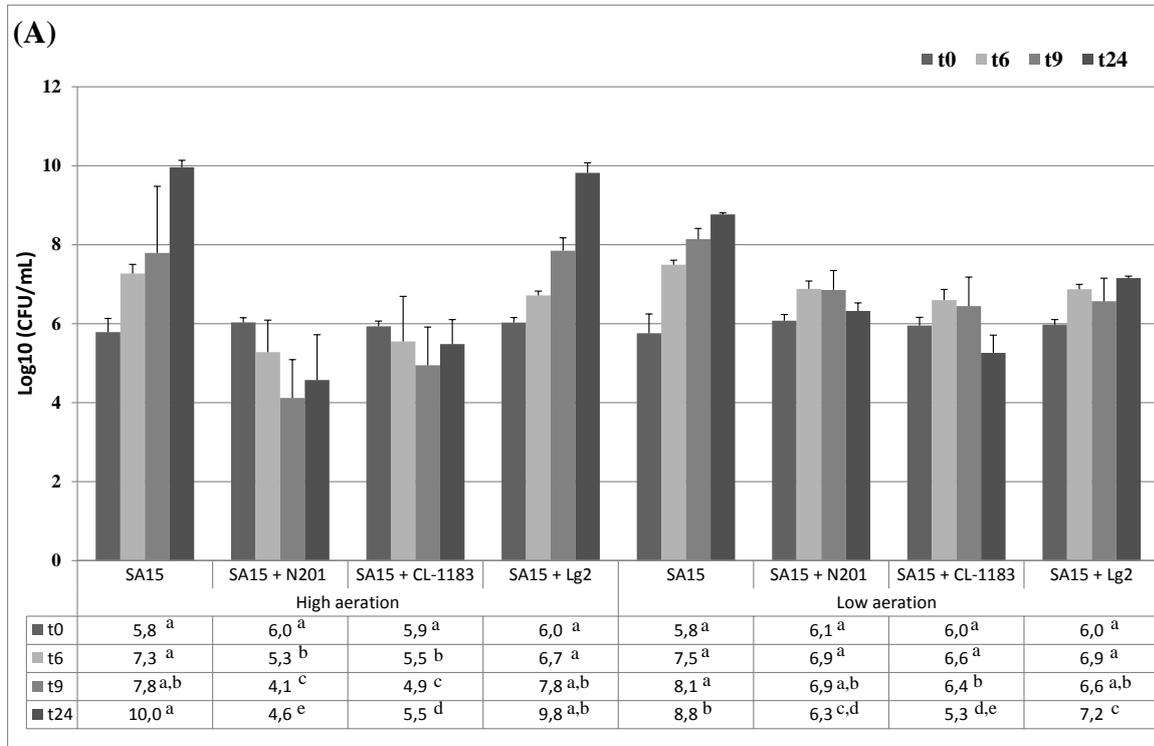
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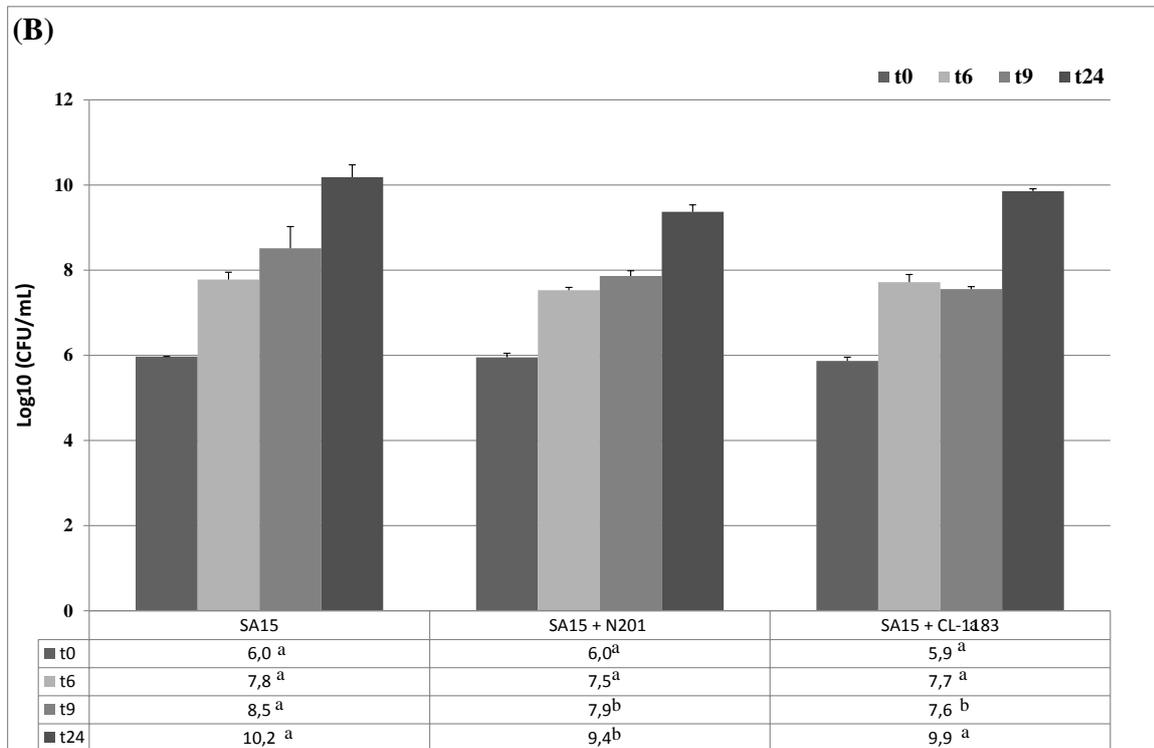
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729 Figure 1. Effect of *L. garvieae* strains on *S. aureus* SA15 growth. (A) Under different levels
 730 of aeration, (B) Under high aeration level in presence SA of catalase (4000 IU/ml).

731 a, b, c, d, e represent groups determined with LSD test, a same letter indicates values not
 732 significantly different (p-value < 0.05 by ANOVA1) through one Time.



733



734

735 Table 1. Targeted *S. aureus* genes.

Gene	Description	Category	Primers reference
<i>hu</i> ^a	DNA-binding protein	Housekeeping gene	[74]
<i>recA</i> ^a	Recombinase A	Housekeeping gene	[74]
<i>agrA</i>	Accessory gene regulator A	Virulence-related regulator	[40]
<i>ahpF</i>	Alkyl hydroperoxidase F	H ₂ O ₂ -response	[17]
<i>clpC</i>	Clp proteinase C	Stress-response	[40]
<i>ctsR</i>	Transcriptional repressor of stress-genes	Stress-response	[40]
<i>codY</i>	Transcriptional repressor	Virulence-related regulator	[17]
<i>dnaK</i>	Chaperone protein DnaK	Stress-response	[40]
<i>hld</i>	Deltahemolysin	Virulence-related regulator	[40]
<i>katA</i>	Catalase	H ₂ O ₂ -response	[40]
<i>mraW</i>	S-adenosyl methyltransferase	Cell division	[17]
<i>saeS</i>	Histidine protein kinase	Virulence-related regulator	[40]
<i>sec4</i>	Enterotoxin C	Enterotoxin	[40]
<i>sel2</i>	Enterotoxin L	Enterotoxin	[40]
<i>sodA</i>	Superoxide dismutase	H ₂ O ₂ -response	[40]
<i>srrA</i>	Staphylococcal respiratory response	Virulence-related regulator	[40]

^a Reference genes.

737 Table 2. Targeted *L. garvieae* genes.

Gene	Description	Category	Primers references
<i>tufB</i> ^a	Elongation factor Tu	Housekeeping gene	[20]
<i>ahpC</i>	Alkyl hydroxyperoxide reductase	H ₂ O ₂ -degradation	[20]
<i>gpx</i>	Glutathione peroxidase	H ₂ O ₂ -degradation	[20]
<i>poxB</i>	Pyruvate oxidase	H ₂ O ₂ -synthesis	[20]
<i>sodA</i>	Superoxide dismutase	H ₂ O ₂ -synthesis	[20]
<i>trxB1</i>	Thioredoxin reductase	H ₂ O ₂ -degradation	[20]

^a Reference gene.

738

739

740 Table 3. Differentially expressed *S. aureus* genes in co-culture with 3 *L. garvieae* strains
 741 (N201, CJ-1183 and Lg2), summary table.
 742 For detailed values, see additional files 2 and 3.
 743

High aeration	6 h			9 h			24 h		
Genes	N201	CL-1183	Lg2	N201	CL-1183	Lg2	N201	CL-1183	Lg2
Up-regulated	-	<i>dnaK</i>	-	<i>clpC</i> <i>cstR</i> <i>dnaK</i> <i>agrA</i>	<i>agrA</i>	-	-	-	-
Down-regulated	<i>sodA</i> <i>mraW</i>	<i>ahpF</i> <i>katA</i> <i>mraW</i> <i>codY</i>	-	<i>katA</i> <i>sodA</i> <i>mraW</i> <i>srrA</i>	<i>katA</i> <i>mraW</i> <i>srrA</i>	-	<i>katA</i> <i>sodA</i> <i>codY</i> <i>saeS</i> <i>sec4</i>	<i>ahpF</i> <i>sodA</i> <i>sec4</i>	<i>ahpF</i> <i>clpC</i> <i>dnaK</i> <i>agrA</i> <i>codY</i> <i>hld</i>

744

Low aeration	6 h			9 h			24 h		
Genes	N201	CL-1183	Lg2	N201	CL-1183	Lg2	N201	CL-1183	Lg2
Up-regulated	-	-	-	<i>agrA</i> <i>sec4</i>	<i>ahpF</i> <i>agrA</i> <i>sec4</i> <i>sel2</i>	<i>clpC</i> <i>cstR</i> <i>dnaK</i>	-	<i>codY</i>	<i>agrA</i>
Down-regulated	<i>sec4</i>	<i>codY</i>	-	-	-	<i>codY</i>	-	-	<i>sodA</i> <i>srrA</i>

745

746 Table 4. H₂O₂ concentration in *L. garvieae* co-culture with *S. aureus* SA15, under high
747 aeration

748 ^{a, b} represent groups determined with LSD test, a same letter indicates values not significantly
749 different (p-value < 0.05 by ANOVA1) through one column.

750 ND = not detected

751

Hydrogen peroxide concentration (mM)				
Time (h)	0	6	9	24
SA15	ND	ND	ND	ND
SA15 + N201	ND	1.343 ^a	1.517 ^a	1.327 ^a
SA15 + CL-1183	ND	1.663 ^a	2.415 ^b	2.409 ^b
SA15 + Lg2	ND	ND	ND	ND
N201	ND	1.455	1.549	1.105
CL-1183	ND	1.257	1.317	1.148
Lg2	ND	ND	ND	ND