

1 The bovine acute phase protein α_1 -acid glycoprotein (AGP) can disrupt *Staphylococcus aureus*
2 biofilm.

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8

9 Abstract

10

11 *Staphylococcus aureus* biofilm-related infections are of clinical concern due to the capability of
12 bacterial colonies to adapt to a hostile environment. The present study investigated the capability of
13 the acute phase protein alpha₁-acid glycoprotein (AGP) to a) disrupt already established *S. aureus*
14 biofilm and b) interfere with the biofilm *de novo* production by using Microtiter Plate assay (MtP) on
15 field strains isolated from infected quarters by assessing. The present study also investigated whether
16 AGP could interfere with the expression of bacterial genes related to biofilm formation (*icaA*, *icaD*,
17 *icaB*, and *icaC*) and adhesive virulence determinants (*fnbA*, *fnbB*, *clfA*, *clfB*, *fib*, *ebps*, *eno*) by
18 quantitative real-time PCR (qPCR). The results provided the evidence that AGP could disrupt the
19 biofilm structure only when it was already developed, but could not prevent the *de novo* biofilm
20 formation. Moreover, AGP could interfere with the expression levels of genes involved in biofilm
21 formation in a dose- and strain-dependent way, by upregulating, or downregulating, *icaABC* genes
22 and *fnbB*, respectively. The results presented in this study provide new insights about the direct
23 antibacterial activity of AGP in bovine milk. It remains to be demonstrated the molecular bases of
24 AGP mechanism of action, in particular for what concerns the scarce capability to interact with the
25 *de novo* formation of biofilm.

26

27 Keywords

28 Biofilm; *Staphylococcus aureus*; Alpha-1-acid glycoprotein; *ica*;

29

30 **1. Introduction**

31 Mastitis has a deep impact on bovine dairy farm economy due to production losses, culling and
32 treatment costs (Hogeveen and Van Der Voort, 2017). The main cause of mastitis is the development
33 of an intramammary infection caused by bacteria, among which *Staphylococcus (S.) aureus* is one of
34 most frequently isolated pathogens (Rainard et al., 2018). *S. aureus* is contagious, persistent in the
35 bovine mammary gland, has a high colonization attitude of skin and mammary gland mucosal
36 epithelia and is often low responsive to antibiotic therapy (Rainard et al., 2018). A significant risk
37 factor in *S. aureus* infections is its ability to form a syntrophic consortium called biofilm within a
38 self-produced matrix of extracellular polymeric substances (EPS). The production of biofilm is a
39 strictly coordinated process by which planktonic bacteria switch from free-floating forms to sessile
40 anchored cells embedded in self-produced EPS (Fox et al., 2005). Biofilm development is related to
41 environmental signals and communication systems, that reflects on specific gene expression (Hall
42 and Mah, 2017). In *S. aureus*, the switch from planktonic to sessile forms is controlled by *quorum-*
43 *sensing* proteins encoded by the *agrABCD* operon, whose abundance is related to virulence and
44 pathogenicity (Antunes et al., 2010). Biofilm development further requires the expression of the gene
45 cluster *icaADBC*, that produce polysaccharide intercellular adhesin (PIA), composed of β -1,6-linked
46 *N*-acetylglucosamine with partially deacetylated residues (Arciola et al., 2005). Of pivotal importance
47 for biofilm formation is the adhesion/colonization of biotic/abiotic surfaces related to the expression
48 of microbial surface proteins that recognize adhesive matrix molecules (MSCRAMMs) such as
49 laminin (*eno*), fibronectin A and B (*fnbA*, *fnbB*), collagen (*cna*), fibrinogen (*fib*) and clumping factor
50 (*clfA*, *clfB*). Most of *S. aureus* isolated from subclinical mastitis are biofilm producers (Rainard et al.,
51 2018), and a recent study provided the evidence that *S. aureus* biofilms can develop in bovine udder
52 (Schönborn and Krömker, 2016).

53 Alpha₁-acid glycoprotein (AGP - Orosomuroid) is an acute phase protein and is as such it belongs to
54 a group of structurally un-related proteins whose serum concentration changes during systemic

55 reaction of inflammation (Ceciliani and Lecchi, 2019). AGP fulfils at least two major sets of
56 functions: on the one hand, it is one of the most important serum binding proteins for small
57 hydrophobic molecules. On the other hand, AGP is an immunomodulatory protein, reducing the
58 collateral damages on tissues involved in the inflammatory process (Lecchi et al., 2008, 2013; Rinaldi
59 et al., 2008). Although the main source of AGP, as most of Acute Phase Proteins (APPs), is the liver.
60 AGP expression is ubiquitous, and its coding mRNA was found in most of the bovine tissues and
61 fluids where it was searched for (Lecchi et al., 2009), including milk. AGP is produced in the
62 mammary gland (Ceciliani et al., 2005) and/or carried into the milk by neutrophils (Ceciliani et al.,
63 2007a), where it is stored in secondary granules and it is exocytosed after pro-inflammatory challenge
64 (Rahman et al., 2008). Beside immunomodulatory activities, AGP is able to provide a direct
65 antibacterial role, being active against *Mycoplasma* (Athamna et al., 1996), *Bacillus anthracis*
66 (Shemyakin et al., 2005) and *Klebsiella* (Hochepped et al., 2000). To the best of the knowledge of the
67 authors, the activity of AGP on biofilm formation was not explored so far. The aim of this study was
68 to cover this gap by exploring whether AGP can interfere with *S. aureus* biofilm.

69

70 **2. Materials and methods**

71 *2.1. S. aureus isolation and selection of biofilm producing strains*

72 Four field strains of *S. aureus* (identified as #39, #40, #41, #42) were isolated from raw milk collected
73 from quarters of Holstein Friesians cows with intramammary infection (IMI) between March and
74 May 2017 from dairy farms located in northern Italy. For the isolation of *S. aureus* strains, milk
75 sample analysis was carried out following the National Mastitis Council guidelines (Adkins et al.,
76 2017). Each milk sample, stored for a maximum of 24 h at +4°C, was thawed at room temperature
77 and 10 µL were spread on Tryptic Soy agar+5% sheep blood (Microbiol, Italy) for primary isolation
78 and incubated aerobically at 37°C overnight. Candidate staphylococcal colonies were subcultured on
79 Mannitol salt agar (Microbiol, Italy) for genus identification. Conventional biochemical tests

80 including catalase, coagulase and oxidase activity confirmed the *S. aureus* isolation. After the
81 isolation, the strains were stored in glycerol at -20°C until the use. Biofilm producing capability was
82 determined by a quantitative method (MtP assay) as previously described (Stepanović et al., 2007).
83 The Tryptic Soy Broth (TSB) was supplemented with the type and concentration of carbohydrates
84 identified in preliminary studies to promote *in vitro* biofilm formation. Two concentrations of glucose
85 (1% and 2.5%) and the physiological concentration of lactose found in dairy cow milk (4.7%) were
86 tested in order to ascertain whether different types and concentration of carbohydrates could influence
87 biofilm formation, identifying glucose 1% as the ideal supplementation for optimal biofilm formation,
88 as presented in further details in the “Results” section.

89 The biofilm forming ability of each of the four field strains was measured by applying Mtp assay
90 (Stepanović et al., 2007). As negative control, TSB + 1% glucose (TSB_g) without bacteria was used.
91 Each strain was tested in triplicate on three independent plates. Briefly, fresh overnight subcultures
92 of the strains were 1:100 fold diluted in TSB_g and 200 µL were plated. After 24 hours at 37°C, the
93 supernatant was gently removed and the biofilm was washed with sterile phosphate buffered saline,
94 fixed with methanol and stained with 2% crystal violet. The absorbance of negative controls was used
95 to set the optical density cut-off (OD_c) as three standard deviations above the mean OD of negative
96 control. Strains were classified as follow: non-adherent $OD \leq OD_c$; weakly adherent $OD_c < OD \leq 2 \times$
97 OD_c ; moderately adherent $OD_c < OD \leq 4 \times OD_c$; strongly adherent $OD > 4 \times OD_c$.

98

99 2.2. Molecular typing of *S. aureus* strains

100 In order to confirm at molecular level the capability of the selected strains to produce biofilm,
101 Repetitive Sequence-Polymerase Chain Reaction (RS-PCR) and *agr* typing were carried out.
102 Genomic DNA was isolated as previously described (Adwan, 2014). Its concentration and
103 homogeneity was determined at 260nm and by A260/A280 ratio (Eppendorf BioPhotometer 6131).
104 The RS-PCR amplifies the 16S-23S rRNA intergenic spacers (Graber, 2016). The primers used and

105 amplification conditions were as previously reported (Fournier et al., 2008). To study the *agr* locus
106 two duplex PCRs were carried out for determination of *agr* type (I-IV) as previously reported (Pereyra
107 et al., 2016). Primers and amplifying conditions (Shopsin et al., 2003) are reported in Table 1.

108

109 *2.3 Measurement of AGP capability to regulate biofilm disruption and de novo formation*

110 To determine the capability of AGP to disrupt already established staphylococcal biofilm, a MtP
111 assay using a volume of 190 μ L of bacteria culture (after 1:100 fold final dilution) and 10 μ L of AGP
112 at different concentrations was tested in 96-well flat-bottom plates (Sigma-Aldrich, Italy).

113 Given the background that the concentration of AGP in milk during acute mastitis is unknown, a
114 preliminary set of experiments was carried out to determine the working (effective) concentrations of
115 AGP (Sigma Aldrich, Italy), which was dissolved in sterile PBS (Sigma Aldrich, Italy), starting from
116 the physiological concentration of AGP in milk (1 μ g/mL) (Ceciliani et al., 2005). The homogeneity
117 of commercial AGP was confirmed by Sodium Dodecyl Sulphate-PolyAcrylamide Gel
118 Electrophoresis (SDS-PAGE) and western-blotting on nitrocellulose membrane using an anti-AGP
119 antibody specific for bovine AGP (Ceciliani et al., 2007b). The following AGP concentration were
120 used: 1, 10, 50, 100, 300, 900 μ g/ml. Equivalent concentrations of bovine serum albumin (Sigma-
121 Aldrich - Italy) were used as specificity control, to rule out that the effects were related to non specific
122 interaction of the protein with biofilm. Following the results of these preliminary experiments, that
123 are reported in details in the “results” section, two concentrations of 1 and 50 μ g/mL of AGP were
124 used.

125 Two independent Mtp experiments were carried out to measure a) the disruptive ability of AGP
126 against already established biofilm and b) the capability of AGP to interfere with biofilm formation.
127 Negative control included TSBg only, while non-treated groups (NT) were bacteria cultivated without
128 AGP and BSA. The experiments were replicated ten times for each AGP concentration.

129 *a) Measurement of AGP capability to disrupt established biofilm*

130 Biofilm was grown as previously described (Stepanović et al., 2007) for 24 h at 37°C, after which a
131 final concentration of 1 µg/mL and 50 µg/mL of AGP was added. Plates were further incubated
132 aerobically at 37°C for another 24 h, after which the effects of AGP were identified by staining the
133 biofilm biomass with crystal violet and spectrophotometric reading (Labsystem Multiscan Plus). Each
134 experiment was replicated three times per strain and three independent plates were used.

135 b) *Measurement of AGP capability to interact with de novo biofilm formation*

136 The MtP assay was applied to study the ability of AGP to inhibit the formation of biofilm, by using
137 the same concentrations of AGP and albumin as those used for biofilm disruption assay. AGP was
138 added immediately after bacterial dilution, plates were incubated aerobically at 37°C for 24 h, after
139 which the AGP effect was quantified by staining with crystal violet and spectrophotometric
140 reading (Labsystem Multiscan Plus). Each experiment was replicated three times per strain and three
141 independent plates were used.

142

143 2.3. *RNA extraction and qPCR for detection and quantification of genes related to adhesion and*
144 *biofilm formation*

145 The capability of AGP to modulate the expression of genes related to *S. aureus* adhesion and biofilm
146 formation was assessed. Each well was washed three times with 200 µL of fresh sterile PBS and
147 scraped with sterile tips to allow biofilm detachment. The replicates were pooled and transferred in a
148 sterile 1.5 mL Eppendorf tube, stored at -20°C until RNA extraction. Samples were thawed on ice
149 and then centrifuged at 10'000g at 4 °C for 3 min; the pellet was used for further procedures. FastRNA
150 SPIN Kit for Microbes (MP Biomedicals, France) was used to extract total bacterial RNA following
151 the manufacturer's instructions. RNA quantity and purity were checked using NanoDrop 1000
152 Spectrophotometer (Thermo Scientific - Italy) through 260/208 and 260/230 absorbance ratio.

153 In order to remove the genomic DNA, 1 µg of RNA was treated with DNase I, RNase-free (Thermo
154 Scientific, Italy).

155 cDNA was obtained after RT-PCR using iScript cDNA Synthesis Kit (Biorad, Italy), following the
156 manufacture's procedure. The expression abundance of genes involved in biofilm formation (*icaA*,
157 *icaD*, *icaB*, and *icaC*) and adhesive virulence determinants (*fnbA*, *fnbB*, *clfA*, *clfB*, *fib*, *ebps*, *eno*) was
158 measured by qPCR. The qPCR was performed on CFX Connect Real-Time PCR Detection System
159 (Biorad, Italy) in 15 µl total volume, using EvanGreen mix (Biorad, Italy). The sequence and
160 concentration of primers (Federman et al., 2016) are listed in Table 1. Each sample was tested in
161 duplicate. No-RT controls and no template controls were performed. Thermal profile was the same
162 for all targets: 95 °C for 5min, 40 cycle at 95 °C for 10s, 60 °C for 30s and a final step from 55 °C to
163 95 °C with an increase of 5 °C every 5 seconds for the melting curve. After data normalization with
164 the reference gene (*gyrB*), quantification was performed using the Bio-Rad CFX Maestro Software
165 using the $2^{-\Delta\Delta Cq}$ method. The MIQE guidelines (Bustin et al., 2009) were followed.

166

167 2.5 Statistical analysis

168 Statistical significance in optical densities between groups, on MtP data, were determined by analysis
169 of variance (ANOVA) using GraphPad Prism v6 (GraphPad Software[®], USA), and *p-values* ≤ 0.050
170 were considered significant. The statistical analysis on mRNA abundance data were performed using
171 XLStat for Windows (Addinsoft, USA). Statistical significance was accepted at *p value* ≤ 0.050 .

172 Data were tested for normality using the Shapiro-Wilk, Anderson-Darling, Lilliefors and Jarque-Bera
173 test. Because the data were not normally distributed, nonparametric statistical tests (Kruskall-Wallis
174 and Dunn test) were applied. Additionally, a heatmap analysis and clustering were performed on
175 XLStat (Addinsoft, USA), using the average of qPCR data.

176

177 3. Results

178 3.1. Identification of biofilm producing field strains and biofilm quantitative assay

179 The effects of glucose at 1% and 2.5% and lactose at 4.7% were tested to determine the experimental
180 conditions to test *S. aureus* biofilm forming capability, as reported in Supplemental Fig. 1. The
181 analysis of variance and the T-student's test showed statistical differences (p value ≤ 0.050) between
182 the two concentrations of glucose and lactose. Although lactose is the most abundant in milk, it was
183 not able to induce an adequate biofilm production as compared to glucose, which strongly affected
184 the ability of strains to produce biofilm. Based on these results, 1% of glucose was used as
185 supplementation ratio for the following *in vitro* studies.

186 The strains number #39, #40, #41 and #42 displayed different biofilm producing abilities as reported
187 in Supplemental Fig. 2. The cut-off point, calculated on negative controls, was set at OD₅₇₀: 0.22. The
188 strains #40 and #42 resulted strongly biofilm producers with OD₅₇₀ values of 1 and 1.4, respectively.
189 On the contrary, strains #39 and #41 were weak and moderate biofilm producer with OD₅₇₀ values of
190 0.3 and 0.51, respectively. Following this second set of preliminary experiments, strains #40 and #42
191 were selected and included in the following experiments.

192

193 3.2. *Molecular typing of biofilm producing strains*

194 The two strains #40 and #42 were classified following amplification of 16S-23S rRNA intergenic
195 spacers and clustered in the genotype B group. The multiplex PCRs used to characterize the *agr* locus
196 provided the evidence that both #40 and #42 strains belong to *agr*-I group. These results confirmed
197 at molecular level that the two strains #40 and #42 were able to produce biofilm.

198

199 3.3 *AGP interaction with biofilm.*

200 Based on preliminary experiments (Supplemental Fig. 3), two concentration of AGP, namely 1 $\mu\text{g/ml}$
201 and 50 $\mu\text{g/ml}$, were selected. The capability of AGP to disrupt biofilm, or inhibit its formation, is
202 presented in Fig. 1a. AGP caused the partial disruption of already formed biofilm structures of *S.*
203 *aureus* strains #40 and #42 in a strain-related mode. In details, the AGP co-incubation of strain #40

204 induced a disruption, which is dose-dependent, and were significant when cultures were incubated
205 with 50 µg/mL of AGP, as compared with controls or cultures that were co-incubated with AGP at 1
206 µg/mL. Strain #42 behaved differently. The incubation with 1 µg/mL induced a significant increase
207 of biofilm formation, whereas with 50 µg/mL induced a disruption. The disruptive activity of AGP
208 on biofilm formation was found to be specific, since albumin, used at the same concentration, was
209 not. The biofilm formation was not inhibited by AGP (Fig. 1b).

210

211 *3.4. Modulation of the expression of intracellular adhesion cluster (ica) genes*

212 In order to investigate whether the activity of AGP on *S. aureus* culture also reflected on the
213 expression of genes related to biofilm formation, a list of genes involved in bacterial adherence and
214 polysaccharide production was identified, and their abundance was measured by means of a qPCR
215 approach. The selected targets were detected in all samples. Results are presented in Fig. 2.

216 AGP differently modulates the expression of target mRNA of strain #40 and #42. In details, an up
217 regulation of *icaA* ($p = 0.045$, ratio $AGP_{[50\mu\text{g/mL}]} / NT = 3.5$), *icaB* ($p=0.036$ ratio $AGP_{[50\mu\text{g/mL}]} / NT = 2.9$),
218 *icaC* ($p=0.05$, ratio $AGP_{[50\mu\text{g/mL}]} / NT = 2.7$) and *fnbB* ($p=0.027$ ratio $AGP_{[50\mu\text{g/mL}]} / NT = 2.1$) was
219 observed in strain #40; *icaD* abundance followed the same trend but the data was not statistically
220 significant.

221 The *icaC* and *icaD* mRNA abundance levels was also influenced, although with a negative
222 correlation, by AGP treatment in strain #42 ; in details, *icaC* ($p=0.050$, ratio $AGP_{[50\mu\text{g/mL}]} / AGP_{[1\mu\text{g/mL}]}$
223 $= 0.35$) and *icaD* ($p=0.046$, ratio $AGP_{[50\mu\text{g/mL}]} / AGP_{[1\mu\text{g/mL}]} = 0.34$) abundance decreased when
224 bacteria were incubated with 50µg/mL of AGP, as compared with 1µg/mL. No significant difference
225 in abundance of other target genes was detected.

226

227 *3.5. Heatmap analysis*

228 The mRNA abundance of eleven genes was determined and the results are presented as heat map in
229 Fig. 3; the major changes were detected only in the abundance of genes involved in the biofilm
230 formation (*ica* genes), while no relevant modifications were appreciable in genes related to bacterial
231 adhesion (*eno*, *ebps*, *clfa*, *fib*, *fnbA* and *fnbB* genes).

232 The two analyzed strains can be sorted in two major clusters; interestingly, control group (0) and
233 1µg/mL AGP group (1) formed a tighter cluster in both strains, demonstrating that the gene
234 expression profile was similar between groups 0 and 1 and that it differed from the 50 µg/mL AGP
235 group. However, the gene expression profile is different between the #40 and #42 strains without
236 AGP treatment, suggesting the presence of individual features of the strain.

237 The two strains showed a different response to AGP treatment. The major effects in genes expression
238 were detected in strain #40, which shown a stronger up regulation of *ica* and *fnbA*, genes, involved
239 in biofilm formation and bacteria adhesion, respectively. No relevant changes were observed in strain
240 #42 except for *fib*, whose expression increased after 50µg/mL AGP treatment.

241

242 **4. Discussion**

243 The present study investigated for the first time the capability of the acute phase protein AGP to
244 interact with biofilm of field strains of *S. aureus* isolated from infected quarters. The results provided
245 the evidence that AGP could induce the disruption of the biofilm in a dose dependent and in a strain-
246 dependent way even when used at the physiological concentration of AGP in milk (Ceciliani et al.,
247 2005). On the contrary, AGP was not able to interact with the *de novo* formation of biofilm. It was
248 also demonstrated that the incubation with AGP modifies the expression rate of genes involved in
249 biofilm formation in a dose- and a strain- dependent way.

250 The study was carried out on *S. aureus* field strains that were isolated from milk of mastitis affected
251 dairy cows. The molecular typing demonstrated that the two strains that were selected for their
252 capability to produce biofilm belonged to B genotype (Graber, 2016). Both strains #40 and #42

253 revealed the presence of *agr*-I group, which features high biofilm forming ability in TSB medium
254 and are penicillin-resistance (Melchior et al., 2009). The genetic characterisation of the *agr* locus
255 (Melchior et al., 2009) confirmed at a molecular level the ability of *agr*-I strains to produce biofilm
256 in TSBg medium in a strain-to-strain dependant way.

257 AGP is an immunomodulatory protein, whose function is mostly focused on decreasing collateral
258 damages related to uncontrolled inflammation (Ceciliani and Lecchi, 2019). In cow, AGP is regarded
259 as a minor acute phase protein: its serum concentration increases between two- to four-folds during
260 inflammation. Bovine healthy milk contains AGP, which is produced from mammary gland and likely
261 exudates from serum during inflammation, at a concentration of 1.3 $\mu\text{g/mL}$) (Ceciliani et al., 2005).
262 The amount of milk AGP during mastitis has not been measured so far. We demonstrated in this study
263 that AGP is effective in disrupting *S. aureus* biofilm. The molecular mechanism at the background of
264 AGP activity on biofilm disruption is unknown, but at least two features of AGP structure could be
265 potentially involved. Firstly, bovine AGP is a 37kDa polypeptide, with a very low pI of 2.7. Secondly,
266 AGP exposes on its surface a very complex carbohydrate moiety, accounting for more than 42% of
267 the protein weight, and including as terminal residue of each of the five glycan chain several sialic
268 acid molecules (Ceciliani and Pocacqua, 2007). The very low pI of AGP may provide a reasonable
269 background to explain the disruption of *S. aureus* biofilm, which has been shown to be dependent of
270 pH values, and it is reduced by acid pH (Zmantar et al., 2010). The high content of sialic acid terminal
271 residues over the surface of AGP may also be partially related to biofilm disruption. Sialylated
272 oligosaccharides have been shown to block the adhesion of *Helicobacter pylori* to epithelial cells
273 (Simon et al., 1997) and these findings have been recently confirmed in *S. aureus* as well, where it
274 was demonstrated that sialylated glycoconjugates could inhibit colonies' growth (Zeng et al., 2018).
275 It must also be said that no report about the sensitivity of already developed biofilm to changes of pH
276 has been reported so far.

277 At molecular level, staphylococcal biofilms are encased in an extracellular matrix composed of
278 extracellular DNA and polysaccharides, the last ones synthesized by enzymes encoded by the
279 *icaABCD* operon, whose products include PIA (Arciola et al., 2005), and proteins involved host cell
280 adhesion, such as fibronectin binding proteins A and B (*fnbA* and *fnbB*), that allow cell interaction
281 with $\alpha5\beta1$ integrins. Therefore, the second step of the present study was to investigate whether the
282 co-incubation of *S. aureus* strains reflected on bacterial genes involved in biofilm formation. The
283 effects of AGP were dose- and strain- dependents, high concentrations of AGP inducing an
284 upregulation of *icaA*, *icaB* and *icaC* in strain #40 .The effects on Strain #42 are opposite. For Strain
285 #42, the dysregulation of *icaC* and *icaD* genes parallels an increase in biofilm disruption. Moreover,
286 an apparent upregulation of *icaABCD* genes, although not statistically significant, corresponds to a
287 decrease in biofilm disruption. The regulatory mechanism of biofilm is complex and not yet fully
288 understood. PIA production seems to undergo to strain-to-strain variation (Cue et al., 2012), as
289 confirmed by the different sensitivity of strain #40 and strain #42 to the challenge with AGP. The
290 abundance of genes described above is presented as a heatmap analysis in which is possible to
291 appreciate the substantial differences between the two strain and in terms of levels expression panels.
292 Although the formation of biofilm is strictly correlated to the production of PIA/PNAG, the present
293 results are consistent with what has been previously reported in *S. epidermidis*, e.g. that treatment
294 with linezolid, and antibiotic choice for anti-*Staphylococcus* therapy, increases the level of
295 transcription of gene involved in PIA/PNAG synthesis such as *icaA* gene in already established
296 staphylococcal biofilms (Reiter et al., 2014). The main limitation of the present study is the *in vitro*
297 experimental approach. *In vitro* models are necessarily simplistic as compared to *in vivo* conditions.
298 In most cases, biofilms include polymicrobial communities, and develop with the support and
299 presence of complex environment, including serum proteins, inflammatory mediators and adhesion
300 proteins and specific surfaces(Crawford et al., 2014). Given that the experimental results were
301 conducted *in vitro*, it is at least conceivable that genes linked to adhesive matrix molecules did not

302 show major changes in expression. It is reasonable to suppose that the expression levels of this genes
303 can also be modulated by the presence of host molecules which interact with the bacteria adhesion
304 proteins.

305

306 **5. Conclusions**

307 In conclusion, the present study demonstrated for the first time that AGP can disrupt already formed
308 *S. aureus* biofilm, but has no inhibitory capability on biofilm synthesis. As a consequence of AGP
309 activity, a dysregulation of genes involved in biofilm formation was also demonstrated, following a
310 dose- and strain-dependent pattern. Overall, results of this study provide new insights about AGP
311 direct antibacterial activity of AGP in bovine milk.

312 Remains to be demonstrated the molecular bases of AGP mechanism of action, in particular for what
313 concerns the limited capability to interact with the de novo formation of biofilm.

314

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318

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439 Figure captions

440 Fig. 1: the interaction between AGP and staphylococcal biofilm.

441 Fig. 1A: quantitative effects of AGP against already formed biofilm. The one-way ANOVA statistical
442 analysis revealed a significant differences between strain 40 and 42 (p value ≤ 0.001). t-student's test
443 was used to compare treatments of each strain (*: p value ≤ 0.05 and 0.01 ; **: p value ≤ 0.01 ; ***: p
444 value ≤ 0.001)

445 Fig. 1B: quantitate effects of AGP on *de novo* of biofilm.

446 No statistical differences were found at both concentrations tested

447

448 Fig. 2: Relative mRNA expression of *ica* locus and adhesion-related genes.

449 Relative mRNA abundance of *ica* genes and genes involved in bacterial adhesion in response to AGP
450 treatments. The box plots shown the median (line into the box plot), the mean (rhombus shape) and
451 the upper and lower quartiles (end of the box). The highest and lowest values are shown by the
452 extreme lines. The statistically significance (p value ≤ 0.05) is indicated by *.

453

454 Fig. 3: Heatmap and clustering analysis.

455 Heatmap and clustering analysis shows changes in gene expression profile of eleven genes of *S.*
456 *aureus* (Strain 40 and 42) in response to treatment with AGP protein. A bright red or green represent
457 a gene strongly down or up regulated respectively. The different shades represent intermediate
458 situation. The dendrogram, on the top and on the left part of the figure, shows the clusters among the
459 strains and the genes, respectively.

460

461 Supplemental Fig. 1 Setup of carbohydrate concentration to supplement TSB.

462 Effect of different carbohydrate concentrations on biofilm formation of four *S. aureus* isolates from
463 mastitic milk. Two concentrations of glucose (1 and 2.5%) and the physiological concentration of
464 lactose in milk were used (4.7%) to supplement TSB and identify the optimal concentration able to
465 induce strongest biofilm biomass formation. Analysis of variance revealed significant differences
466 between the three groups for each strain, T-student's test revealed the reported statistical differences
467 (*: *p* value between 0.05 and 0.01; **: *p* value < 0.01; ***: *p* value <0.001).

468

469 Supplemental Fig. 2: Biofilm forming ability of the four *S. aureus* strains.

470 Optical densities of #39, #40,#41 and #42 *S.aureus* strains as measured following the method
471 previously described (Stepanović et al., 2007). After MtP assay, strain 40 and strain 42 were classified
472 as strongly biofilm producers, while strain 39 and 41 resulted, respectively, weak and moderate
473 biofilm producers.

474

475 Supplemental Fig. 3 Setup of AGP concentration to assess its role against staphylococcal biofilm.

476 Effect of increasing concentrations of AGP on biofilm disruption in # 40 and # 42 *S.aureus* strains
477 included in the study (A,B). Albumin was used to exclude aspecific interactions (C,D).

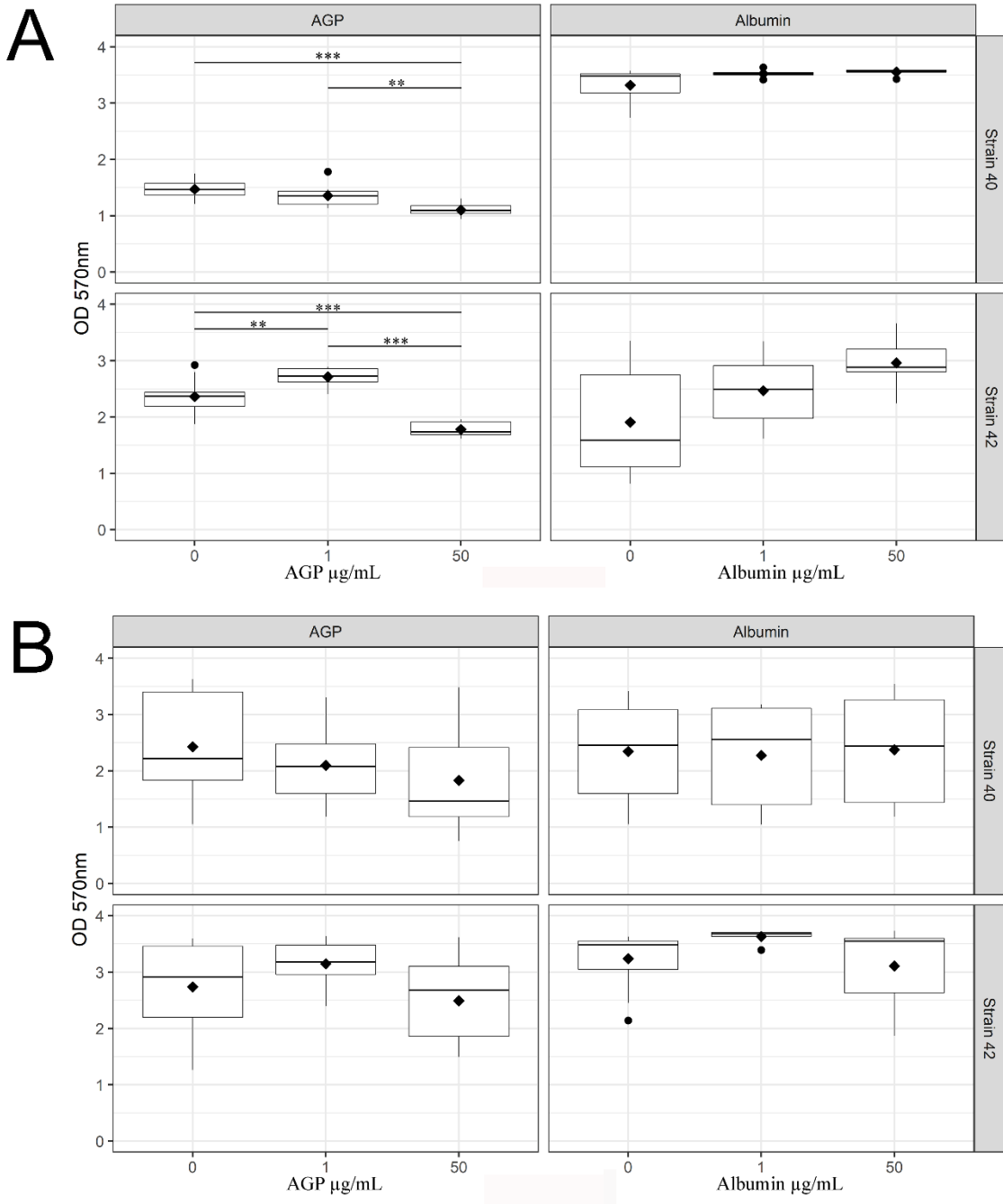
478 In the second preliminary experiment, starting from 0.1 µg/mL up to 100 µg/mL was created a scaling
479 down gradient of AGP (E, F) and same concentrations of albumin (G,H). One-way ANOVA test and
480 T-student's test were used to determine statistical differences (*: *p* value between 0.05 and 0.01; **:
481 *p* value < 0.01; ***: *p* value <0.001)

482

Table 1

	Gene	Sequence (5'-3')		Amplicon size (bp)	Primer Concentration (nM)
Adhesion genes	<i>clfA</i> (clumping factor A)	F	ACCCAGGTTTCAGATTCTGGCAGCG	165	350
		R	TCGCTGAGTCGGAATCGCTTGCT		
	<i>clfB</i> (clumping factor B)	F	AACTCCAGGGCCCGCGTTG	159	400
		R	CCTGAGTCGCTGTCTGAGCCTGAG		
	<i>ebps</i> (elastin binding protein)	F	GGTGCAGCTGGTGAATGGGTGT	191	300
		R	GCTGCGCTCCAGCCAAACCT		
	<i>fib</i> (fibrinogen binding protein)	F	CGTCAACAGCAGATGCGAGCG	239	350
		R	TGCATCAGTTTTCGCTGCTGGTTT		
<i>fnbA</i> (fibronectin binding protein A)	F	AAATTGGGAGCAGCATCAGT	121	300	
	R	GCAGCTGAATTCCCATTTC			
<i>fnbB</i> (fibronectin binding protein B)	F	ACGCTCAAGGCGACGGCAAAG	197	300	
	R	ACCTTCTGCATGACCTTCTGCACCT			
<i>eno</i> (laminin binding protein)	F	TGCCGTAGGTGACGAAGGTGGTT	195	350	
	R	GCACCGTGTTCGCCTTCGAACT			
Biofilm genes	<i>icaA</i> (intercellular adhesion gene)	F	GAGGTAAAGCCAACGCACTC	151	350
		R	CCTGTAACCGCACCAAGTTT		
	<i>icaD</i> (intercellular adhesion gene)	F	ACCCAACGCTAAAATCATCG	211	300
		R	GCGAAAATGCCCATAGTTTC		
<i>icaB</i> (intercellular adhesion gene)	F	ATACCGGCGACTGGGTTTAT	140	350	
	R	TTGCAAATCGTGGGTATGTGT			
<i>icaC</i> (intercellular adhesion gene)	F	CTTGGGTATTTGCACGCATT	209	350	
	R	GCAATATCATGCCGACACCT			
Reference gene	<i>gyrB</i> (gyrase subunit B)	F	CGAAGGGGACTCTGCCGGG	175	350
R		GTCGCCACCGATTCTGTACC			
quorum-sensing genes	<i>pan-agr</i> (accessory gene regulator)	F	ATGCACATGGTGCACATGC		
	<i>agr-I</i> (accessory gene regulator)	R	GTCACAAGTACTATAAGCTGCGAT	440	
	<i>agr-II</i> (accessory gene regulator)	R	GTATTACTAATTGAAAAGTGCCATAGC	573	
	<i>agr-III</i> (accessory gene regulator)	R	CTGTTGAAAAGTCAACTAAAAGCTC	406	
	<i>agr-IV</i> (accessory gene regulator)	R	CGATAATGCCGTAATACCCG	588	

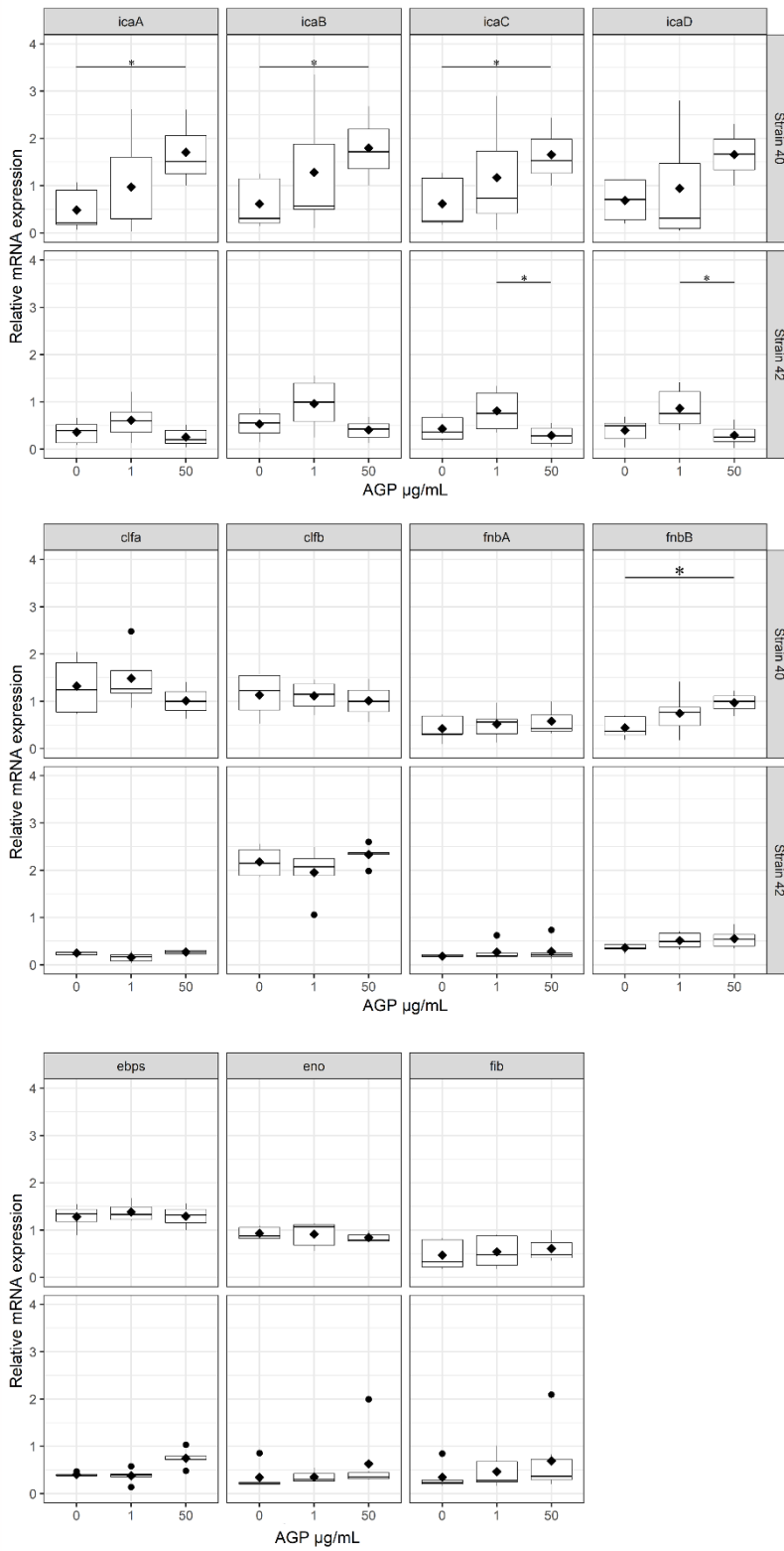
Primers for adhesion genes (*clfA*, *clfB*, *ebps*, *fib*, *fnbA*, *fnbB*, *eno*), biofilm genes (*icaA*, *icaD*, *icaB*, *icaC*) and reference gene (*gyrB*) were as previously reported (Federman et al., 2016). Primers for quorum sensing genes (*pan-agr*, *agr-I*, *agr-II*, *agr-III*, *agr-IV*) were from Shopsin et al., 2003.



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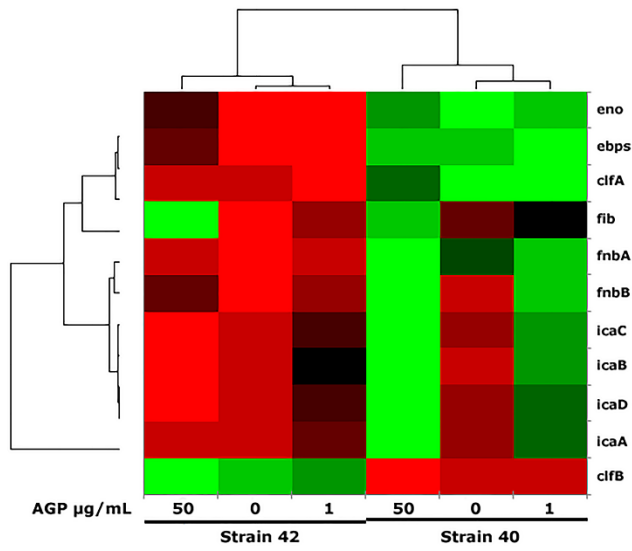
485 Figure 1

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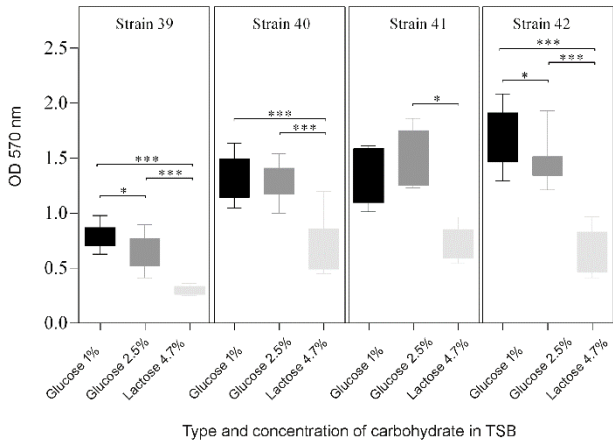
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488 Figure 2



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490 Figure 3

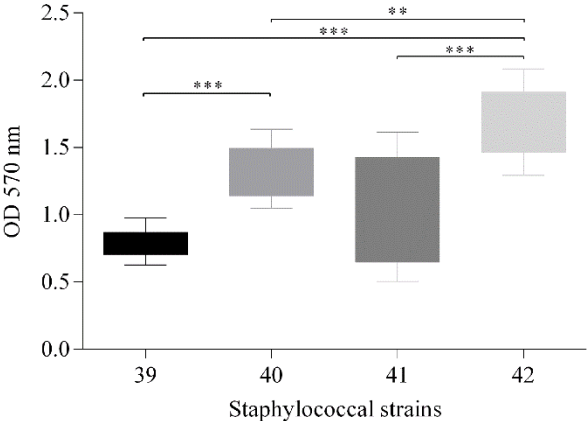


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492 Supplemental Figure 1

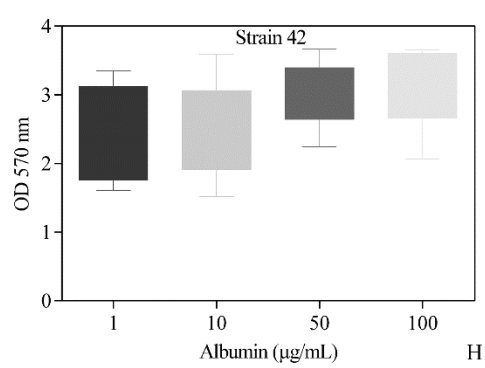
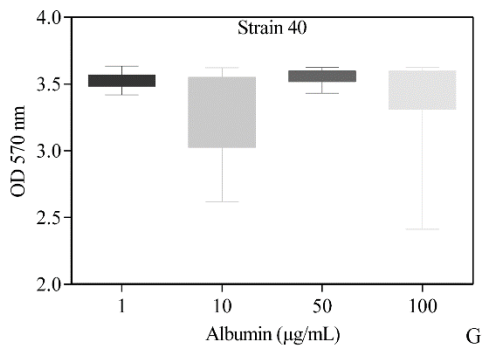
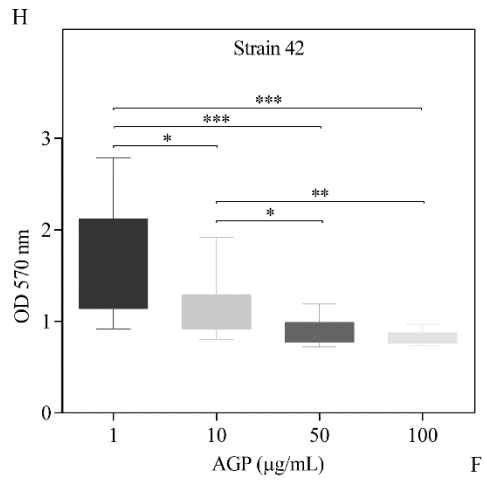
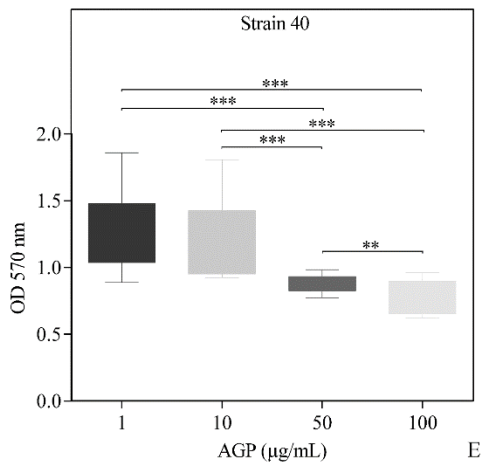
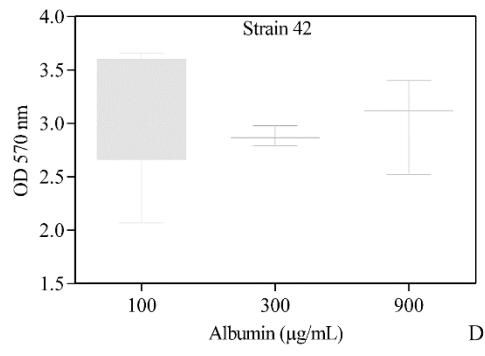
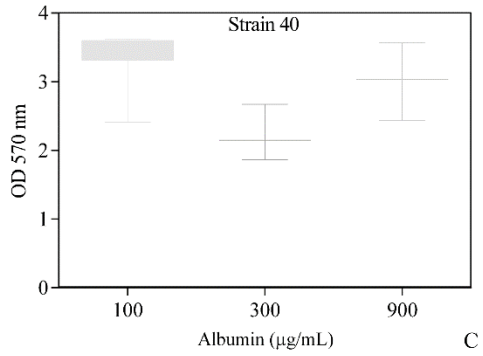
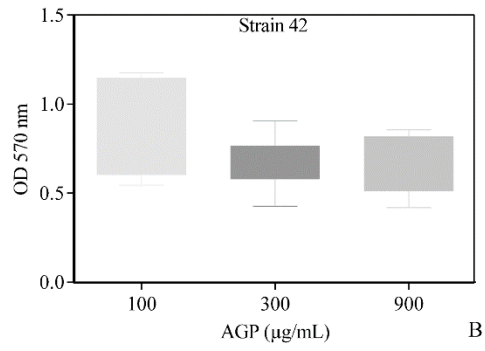
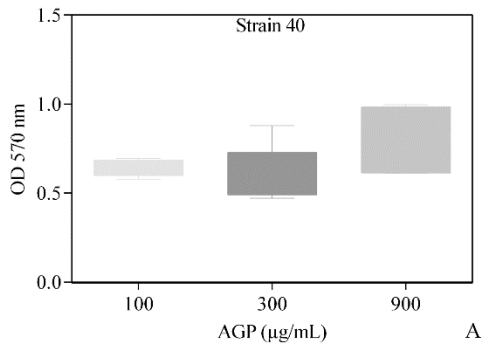
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496 Supplemental Figure 2



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