Veterinary Microbiology 235 (2019) 93–100

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

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

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ARTICLE INFO

Keywords:
Biofilm
Staphylococcus aureus
Alpha-1-acid glycoprotein
Ica

ABSTRACT

Staphylococcus aureus biofilm-related infections are of clinical concern due to the capability of bacterial colonies to adapt to a hostile environment. The present study investigated the capability of the acute phase protein alpha $_1$-acid glycoprotein (AGP) to a) disrupt already established *S. aureus* biofilm and b) interfere with the biofilm *de novo* production by using Microtiter Plate assay (MtP) on field strains isolated from infected quarters by assessing. The present study also investigated whether AGP could interfere with the expression of bacterial genes related to biofilm formation (*icaA, icaD, icaB,* and *icaC*) and adhesive virulence determinants (*fnbA, fnbB, clfA, clfB, fib, ebps, eno*) by quantitative real-time PCR (qPCR). The results provided the evidence that AGP could disrupt the biofilm structure only when it was already developed, but could not prevent the *de novo* biofilm formation. Moreover, AGP could interfere with the expression levels of genes involved in biofilm formation in a dose- and strain-dependent way, by upregulating, or downregulating, *icaABC* genes and *fnbB,* respectively. The results presented in this study provide new insights about the direct antibacterial activity of AGP in bovine milk. It remains to be demonstrated the molecular bases of AGP mechanism of action, in particular for what concerns the scarce capability to interact with the *de novo* formation of biofilm.

1. Introduction

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

Alpha$_1$-acid glycoprotein (AGP - Orosomucoid) is an acute phase protein and is as such it belongs to a group of structurally un-related proteins whose serum concentration changes during systemic reaction of inflammation (Ceciliani and Lecchi, 2019). AGP fulfils at least two major sets of functions: on the one hand, it is one of the most important serum binding proteins for small hydrophobic molecules. On the other hand, AGP is an immunomodulatory protein, reducing the collateral

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https://doi.org/10.1016/j.vetmic.2019.06.007
Received 25 April 2019; Received in revised form 6 June 2019; Accepted 10 June 2019

Table 1
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).

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

damages on tissues involved in the inflammatory process (Lecchi et al., 2008, 2013; Rinaldi et al., 2008). Although the main source of AGP, as most of Acute Phase Proteins (APPs), is the liver. AGP expression is ubiquitous, and its coding mRNA was found in most of the bovine tissues and fluids where it was searched for (Lecchi et al., 2009), including milk. AGP is produced in the mammary gland (Ceciliani et al., 2005) and/or carried into the milk by neutrophils (Ceciliani et al., 2007a), where it is stored in secondary granules and it is exocytosed after pro-inflammatory challenge (Rahman et al., 2008). Beside immunomodulatory activities, AGP is able to provide a direct antibacterial role, being active against *Mycoplasma* (Athamna et al., 1996), *Bacillus anthracis* (Shemyakin et al., 2005) and *Klebsiella* (Hochepied et al., 2000). To the best of the knowledge of the authors, the activity of AGP on biofilm formation was not explored so far. The aim of this study was to cover this gap by exploring whether AGP can interfere with *S. aureus* biofilm.

2. Materials and methods

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

Four field strains of *S. aureus* (identified as #39, #40, #41, #42) were isolated from raw milk collected from quarters of Holstein Friesians cows with intramammary infection (IMI) between March and May 2017 from dairy farms located in northern Italy. For the isolation of *S. aureus* strains, milk sample analysis was carried out following the National Mastitis Council guidelines (Adkins et al., 2017). Each milk sample, stored for a maximum of 24 h at +4 °C, was thawed at room temperature and 10 µL were spread on Tryptic Soy agar + 5% sheep blood (Microbiol, Italy) for primary isolation and incubated aerobically at 37 °C overnight. Candidate staphylococcal colonies were subcultured on Mannitol salt agar (Microbiol, Italy) for genus identification. Conventional biochemical tests including catalase, coagulase and oxidase activity confirmed the *S. aureus* isolation. After the isolation, the strains were stored in glycerol at −20 °C until the use. Biofilm producing capability was determined by a quantitative method (MtP assay) as previously described (Stepanović et al., 2007).

The Tryptic Soy Broth (TSB) was supplemented with the type and concentration of carbohydrates identified in preliminary studies to promote *in vitro* biofilm formation. Two concentrations of glucose (1% and 2.5%) and the physiological concentration of lactose found in dairy cow milk (4.7%) were tested in order to ascertain whether different types and concentration of carbohydrates could influence biofilm formation, identifying glucose 1% as the ideal supplementation for optimal biofilm formation, as presented in further details in the "Results" section.

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

2.2. Molecular typing of S. aureus strains

In order to confirm at molecular level the capability of the selected strains to produce biofilm, Repetitive Sequence-Polymerase Chain Reaction (RS-PCR) and *agr* typing were carried out. Genomic DNA was isolated as previously described (Adwan, 2014). Its concentration and homogeneity was determined at 260 nm and by A260/A280 ratio (Eppendorf BioPhotometer 6131). The RS-PCR amplifies the 16S-23S rRNA intergenic spacers (Graber, 2016). The primers used and amplification conditions were as previously reported (Fournier et al., 2008). To study the *agr* locus two duplex PCRs were carried out for determination of *agr* type (I-IV) as previously reported (Pereyra et al., 2016). Primers and amplifying conditions (Shopsin et al., 2003) are reported in Table 1.

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

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

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

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

2.3.1. Measurement of AGP capability to disrupt established biofilm

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

2.3.2. Measurement of AGP capability to interact with de novo biofilm formation

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

2.4. RNA extraction and qPCR for detection and quantification of genes related to adhesion and biofilm formation

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

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

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

2.5. Statistical analysis

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

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

3. Results

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

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

The strains number #39, #40, #41 and #42 displayed different biofilm producing abilities as reported in Supplemental Fig. 2. The cut-

off point, calculated on negative controls, was set at OD_{570}: 0.22. The strains #40 and #42 resulted strongly biofilm producers with OD_{570} values of 1 and 1.4, respectively. On the contrary, strains #39 and #41 were weak and moderate biofilm producer with OD_{570} values of 0.3 and 0.51, respectively. Following this second set of preliminary experiments, strains #40 and #42 were selected and included in the following experiments.

3.2. Molecular typing of biofilm producing strains

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

3.3. AGP interaction with biofilm

Based on preliminary experiments (Supplemental Fig. 3), two concentration of AGP, namely 1μg/ml and 50μg/ml, were selected. The capability of AGP to disrupt biofilm, or inhibit its formation, is presented in Fig. 1a. AGP caused the partial disruption of already formed biofilm structures of *S. aureus* strains #40 and #42 in a strain-related mode. In details, the AGP co-incubation of strain #40 induced a disruption, which is dose-dependent, and were significant when cultures were incubated with 50 μg/mL of AGP, as compared with controls or cultures that were co-incubated with AGP at 1 μg/mL. Strain #42 behaved differently. The incubation with 1 μg/mL induced a significant increase of biofilm formation, whereas with 50 μg/mL induced a disruption. The disruptive activity of AGP on biofilm formation was found to be specific, since albumin, used at the same concentration, was not. The biofilm formation was not inhibited by AGP (Fig. 1b).

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

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

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

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

3.5. Heatmap analysis

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

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

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

4. Discussion

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

The study was carried out on *S. aureus* field strains that were isolated from milk of mastitis affected dairy cows. The molecular typing demonstrated that the two strains that were selected for their capability to produce biofilm belonged to B genotype (Graber, 2016). Both strains #40 and #42 revealed the presence of *agr*-I group, which features high biofilm forming ability in TSB medium and are penicillin-resistance (Melchior et al., 2009). The genetic characterisation of the *agr* locus (Melchior et al., 2009) confirmed at a molecular level the ability of *agr*-I strains to produce biofilm in TSBg medium in a strain-to-strain dependant way.

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

At molecular level, staphylococcal biofilms are encased in an extracellular matrix composed of extracellular DNA and polysaccharides, the last ones synthesized by enzymes encoded by the *icaABCD* operon, whose products include PIA (Arciola et al., 2005), and proteins

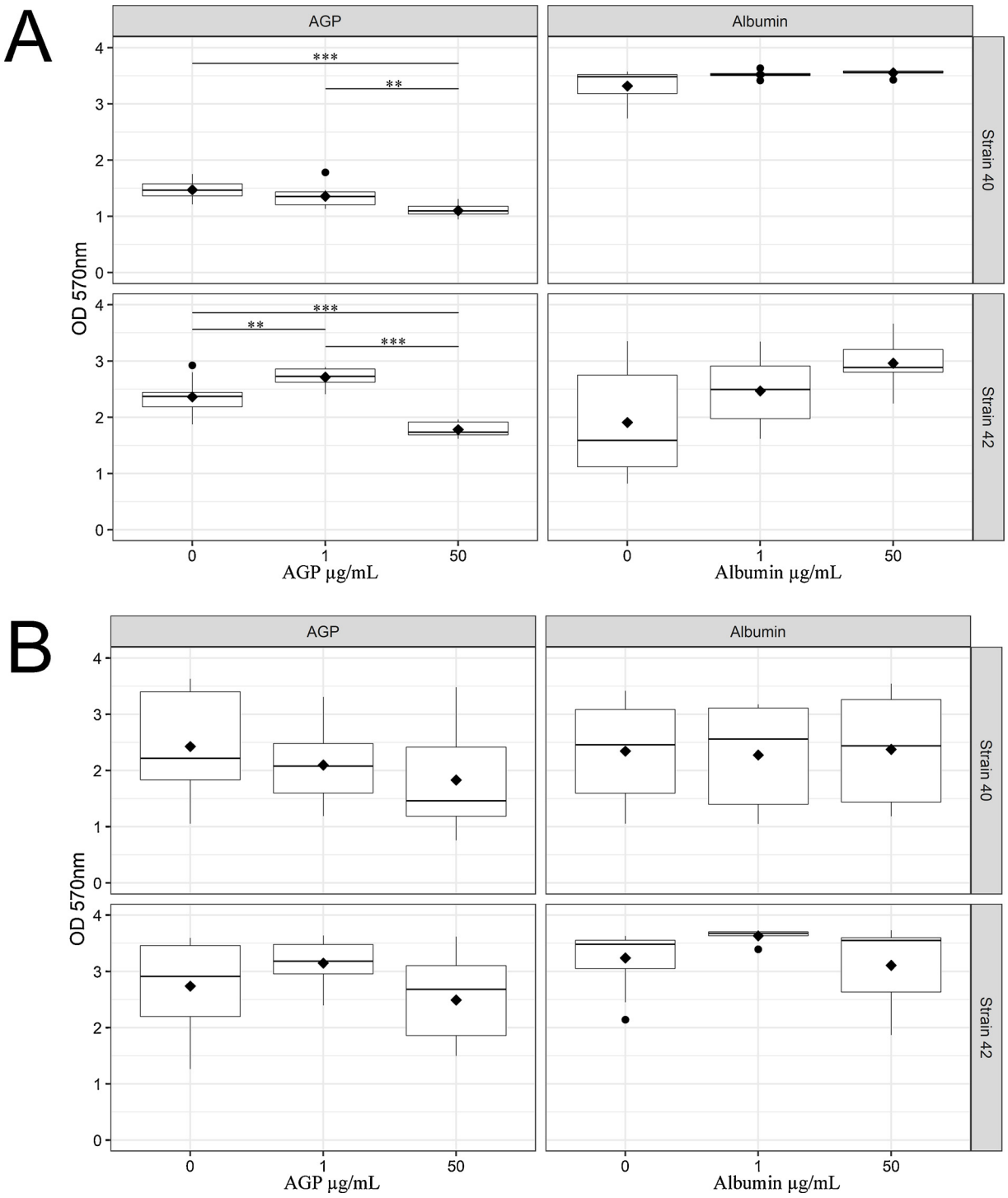

Fig. 1. the interaction between AGP and staphylococcal biofilm. A: quantitative effects of AGP against already formed biofilm. The one-way ANOVA statistical analysis revealed a significant differences between strain 40 and 42 (*p value* ≤ 0.001). t-student's test was used to compare treatments of each strain (*: *p value* ≤ 0.05 and 0.01; **: p value ≤ 0.01; ***: *p value* ≤ 0.001) B: quantitate effects of AGP on *de novo* of biofilm.
No statistical differences were found at both concentrations tested

involved host cell adhesion, such as fibronectin binding proteins A and B (*fnbA* and *fnbB*), that allow cell interaction with α5β1 integrins. Therefore, the second step of the present study was to investigate whether the co-incubation of *S. aureus* strains reflected on bacterial genes involved in biofilm formation. The effects of AGP were dose- and strain- dependents, high concentrations of AGP inducing an upregulation of *icaA, icaB and icaC* in strain #40. The effects on Strain #42 are opposite. For Strain #42, the dysregulation of *icaC* and *icaD* genes parallels an increase in biofilm disruption. Moreover, an apparent upregulation of *icaABCD* genes, although not statistically significant, corresponds to a decrease in biofilm disruption. The regulatory mechanism of biofilm is complex and not yet fully understood. PIA production seems to undergo to strain-to-strain variation (Cue et al., 2012), as confirmed by the different sensitivity of strain #40 and strain #42 to the challenge with AGP. The abundance of genes described above is presented as a heatmap analysis in which is possible to appreciate the

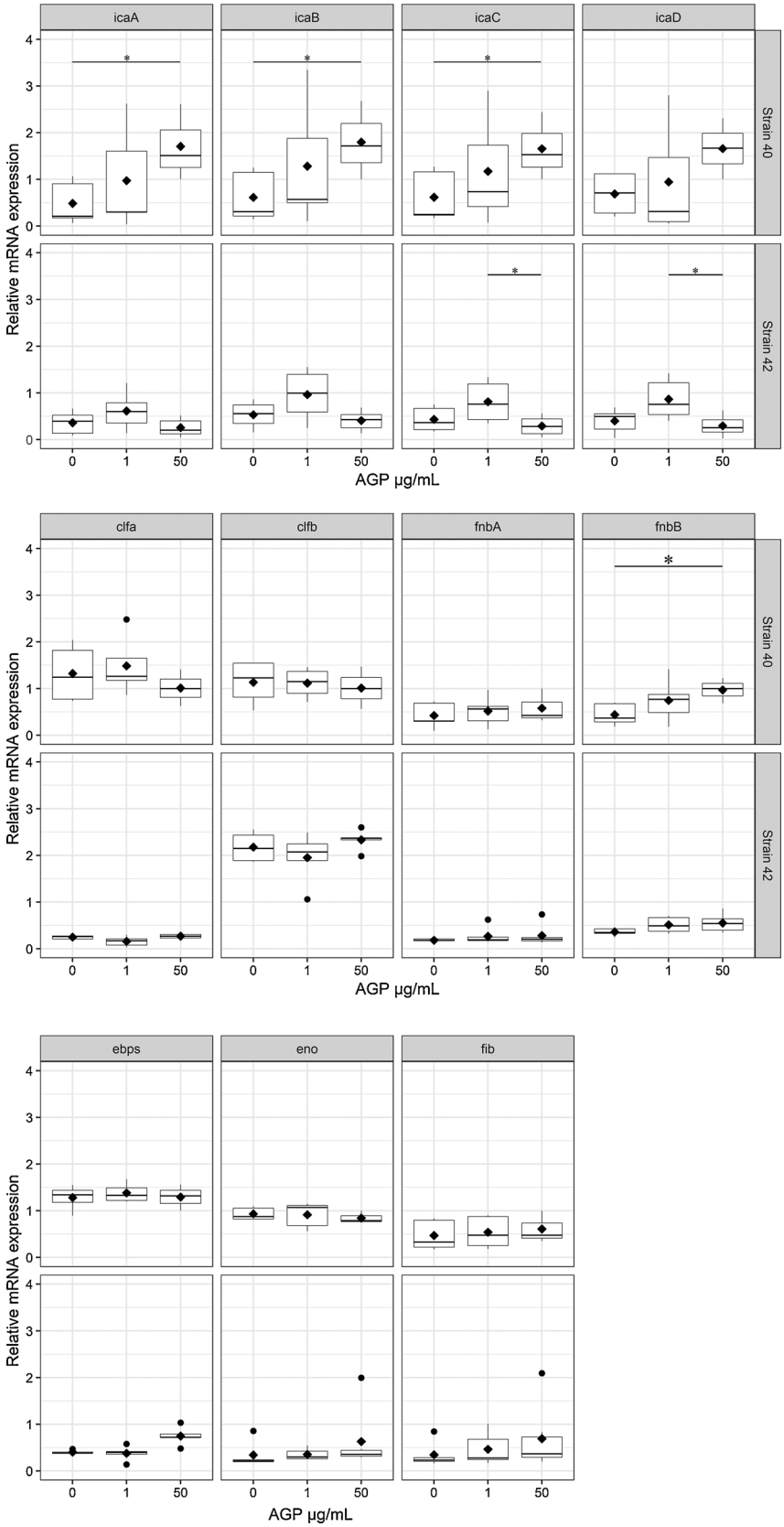

Fig. 2. Relative mRNA expression of ica locus and adhesion-related genes.
Relative mRNA abundance of *ica* genes and genes involved in bacterial adhesion in response to AGP treatments. The box plots shown the median (line into the box plot), the mean (rhombus shape) and the upper and lower quartiles (end of the box). The highest and lowest values are shown by the extreme lines. The statistically significance ($p\ value \leq 0.05$) is indicated by *.

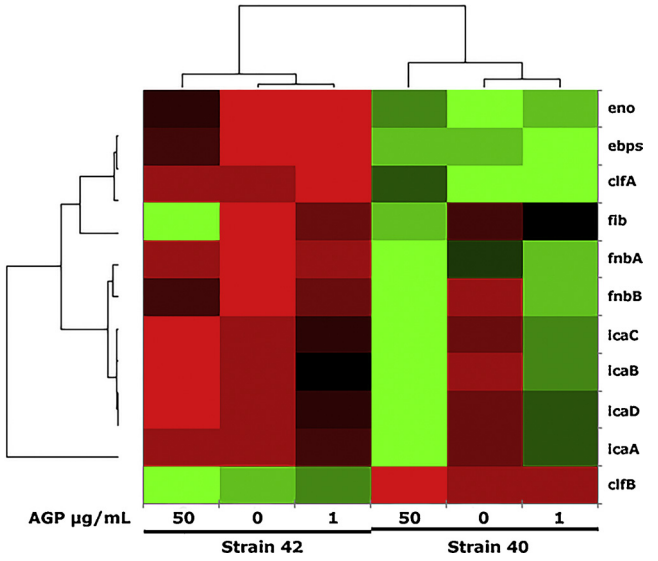

Fig. 3. Heatmap and clustering analysis.
Heatmap and clustering analysis shows changes in gene expression profile of eleven genes of *S. aureus* (Strain 40 and 42) in response to treatment with AGP protein. A bright red or green represent a gene strongly down or up regulated respectively. The different shades represent intermediate situation. The dendrogram, on the top and on the left part of the figure, shows the clusters among the strains and the genes, respectively.

substantial differences between the two strain and in terms of levels expression panels.

Although the formation of biofilm is strictly correlated to the production of PIA/PNAG, the present results are consistent with what has been previously reported in *S. epidermidis*, *e.g.* that treatment with linezolid, and antibiotic choice for anti-*Staphylococcus* therapy, increases the level of transcription of gene involved in PIA/PNAG synthesis such as *icaA* gene in already established staphylococcal biofilms (Reiter et al., 2014). The main limitation of the present study is the *in vitro* experimental approach. *In vitro* models are necessarily simplistic as compared to *in vivo* conditions. In most cases, biofilms include polymicrobial communities, and develop with the support and presence of complex environment, including serum proteins, inflammatory mediators and adhesion proteins and specific surfaces (Crawford et al., 2014). Given that the experimental results were conducted *in vitro*, it is at least conceivable that genes linked to adhesive matrix molecules did not show major changes in expression. It is reasonable to suppose that the expression levels of this genes can also be modulated by the presence of host molecules which interact with the bacteria adhesion proteins.

5. Conclusions

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

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

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.007.

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