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Sheep mastitis *Staphylococcus epidermidis* biofilm effects on cell adhesion and inflammatory changes

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Highlights

- *Staphylococcus epidermidis* biofilm does not interfere with adhesion to mammary cells
- Biofilm does not affect inflammatory degree in infected ovine mammary gland
- *S. epidermidis adhesion to mammary cells isn't related with inflammation intensity*

ABSTRACT

Mastitis in sheep is an important disease as it causes a decrease in milk yield and quality. *Staphylococcus epidermidis* is recognized as the main cause of mastitis in sheep. Its ability to produce biofilm is believed to contribute to mammary gland pathology. The aim of this study was to assess the influence of *S. epidermidis* biofilm production on cell adhesion and on the inflammatory response of the mammary gland. A total of 109 *S. epidermidis* isolates from mastitic sheep milk samples were analyzed for *in vitro* slime production, assessed by Congo red agar test (CRA), and for biofilm formation, evaluated by tissue culture plate assay (TCP). The influence of biofilm production was estimated on the adherence of bacteria to mammary cells and on the mammary gland inflammatory reaction.

Only 7.3% isolates produced slime on CRA and 8% produced biofilm according to TCP. The relation between slime production in CRA and biofilm production analyzed by TCP was highly significant ($P < 0.001$). The differences between biofilm production and different recorded inflammatory situations were non-significant ($P \geq 0.05$). Adherence to mammary epithelium cells ranged from 0.05% to 1.23%. Biofilm production was not related to cell adhesion. Moreover, *S. epidermidis* isolates that induced different degrees of inflammation did not express differences in their ability to adhere to mammary epithelium. The present study suggests that biofilm production by sheep mastitis *S. epidermidis* is unlikely to affect either cell adhesion or mammary gland inflammatory response.

Keywords: Mastitis; *Staphylococcus epidermidis*; mammary inflammation; biofilm; cell adhesion.

1. Introduction

Sheep mastitis is accountable for the death and early culling of animals (Arsenault et al., 2008), decreased milk yield (Rovai et al., 2015), reduced lamb growth and survival (Fthenakis and Jones, 1990) and lower milk quality (Leitner et al., 2004).

Staphylococcus epidermidis is the main cause of subclinical mastitis in sheep, although it can occasionally cause clinical mastitis (Bergonier et al., 2005; Cuccuru et al., 2011; Gelasakis et al., 2016; Kiossis et al., 2007; Marogna et al., 2010; Queiroga, 2017).

The production of biofilm is considered a major virulent factor, increasing the ability to colonize inert materials and accounts for the high incidence of infections caused by *S. epidermidis.* Its major components are an exopolysaccharide matrix (slime), proteins and environmental DNA (eDNA) along with the bacterial cells (Büttner et al., 2015).

In the mammary gland, biofilms increase microbial survival and contribute to pathogens' persistence in the farm (Gomes et al., 2016). Biofilm production has been considered responsible for antimicrobial resistance and for persistent mastitis (Cucarella et al., 2004; Fontaine and Smith, 2006; Melchior et al., 2006; Tremblay et al., 2013; Vanderhaeghen et al., 2014; Veh et al., 2015). Nevertheless, study results on biofilm effect on successful bacterial adhesion to the mammary gland epithelium have not been consensual. Valle et al. (2012) found that adhesion of *Staphylococcus aureus* to bovine epithelial cells *in vitro* is promoted by the biofilm associated protein (Bap). Moreover *S. aureus* adhesion to sheep mammary cells *in vitro* significantly increased when mediated by slime, and adhesion was independent of eukaryotic membrane proteins (Aguilar et al., 2001). Likewise, sheep intramammary inoculation of biofilm producing *S. aureus* strains revealed a significantly higher ability to colonize the mammary gland than non-producing strains (Baselga et al., 1993). However, other studies suggest that the production of biofilm may hinder bacterial adhesion molecules and decrease cell adhesion ability (Baldassarri et al., 1997).

Several reports are available on biofilm producing *S.epidermidis* recovered from bovine mastitis (Oliveira et al., 2006; Simojoki et al., 2012; Tremblay et al., 2013; Watts et al., 1990) and ovine mastitis (Tormo et al., 2005). However, there is no relevant data related to *S. epidermidis* biofilm production and its ability to adhere to mammary epithelium cells.

Biofilm production by bacteria causing intramammary infection (IMI) may influence somatic cell count (SCC). In cows with *S. aureus* mastitis, SCC was higher in animals infected with biofilm producing strains than in animals infected with non-producers (Gomes et al., 2016; Tormo et al., 2005).

The aim of this study was to assess the influence of *S. epidermidis* biofilm production both on the ability of bacteria to adhere to mammary cells and the inflammatory response in the mammary gland.

2. Materials and methods

2.1. Bacterial isolates

A total of 109 *S. epidermidis* isolates from milk samples were collected from 90 sheep (five with clinical and 104 subclinical mastitis) over a six months period. These animals belonged to 14 different flocks, extensively bred in the Alentejo region, Portugal. Milk samples (approximately 50 mL) were aseptically collected and immediately refrigerated until processed, within no more than 12 hours. Bacteriological analysis were undertaken according to the National Mastitis Council methodology (2004) and isolates were identified as *S. epidermidis* using API-Staph (Biomérieux) and by internally transcribed spacer-PCR (ITS-PCR) (Pereira et al., 2003).

All isolates were analyzed for their ability to produce slime *in vitro* and to form biofilm. To evaluate biofilm production influence on the ability of bacteria to adhere to ruminant mammary epithelium cells, seven biofilm producing isolates, from six animals (from three different farms), and five non-producing isolates, from five animals (from five different farms), were co-cultured with a bovine mammary gland epithelial cells line (BME). As controls, *S. epidermidis* ATCC 35984 was used as a biofilm producer and *S. epidermidis* ATCC 12228 as a non-producer.

The association between biofilm formation of all 109 isolates and its effect on mammary inflammation was assessed.

2.2. Mammary gland inflammation

Mammary gland inflammatory response was determined according to milk somatic cell discharge using California mastitis test (CMT) (González-Rodríguez and Cármenes, 1996), for animals with subclinical

mastitis, and clinical mastitis evidence. CMT result was recorded as negative, “traces” (T), 1+, 2+ and 3+. Clinical mastitis (CM) was considered the highest grade of inflammation.

2.3. Slime production

Slime production was evaluated by a qualitative method based on characteristic colonies phenotypic expression on Congo red agar (CRA) as described by Freeman et al. (1989). Each *S. epidermidis* isolate was inoculated in brain heart infusion broth (BHI, Oxoid, CM225) and incubated at 37°C for approximately 18h. These cultures were plated in CRA and incubated at 37°C for 24h, followed by 24h incubation at room temperature. Black colonies were considered slime producing and red colonies non-producing.

2.4. Biofilm assay

Quantitative biofilm production was assessed by the tissue culture plate (TCP) method with slight changes (Christensen et al., 1985; Cucarella et al., 2002). Each *S. epidermidis* isolate was cultured in tryptone soya broth (TSB, Oxoid, CM129) and incubated for 18h at 37°C in normal atmosphere. After dilution 1:40 in TSB supplemented with 0.25% glucose (TSB-G), 200 µl of each culture was distributed, in triplicate, in 96-well flat bottom polystyrene microtiter plates. Control strains were processed identically. A blank control consisted of non-inoculated TSB-G. After incubation at 37°C, 18h, the wells were emptied and washed 3 times with 200 µl of phosphate buffered saline (PBS) pH 7. The plates were dried upside down at 60ºC for 1h. Produced biofilm was stained with 200 µl of 0.25% crystal violet for 1 min, after which the dye was removed and plates washed 3 times with running water and dried in an incubator at 37°C, for 2h.

Optical densities were read at 565 nm in an ELISA plate reader. For each isolate, results were calculated as the optical densities (OD) average value of the triplicates, subtracting the average value for the negative control (NC) in the same microplate according to the following formula: (∑ OD1, OD2, OD3)/3 - (∑ NC1, NC2, NC3)/3. This test was done in triplicate on three separate days. Biofilm production was

considered negative when at least two of the three results were below zero, and positive when at least two of the three results were above zero. The test was performed following randomized block design (RBD), using the day of determination as block (1, 2 and 3).

2.5. Adhesion assay

Seven isolates expressing biofilm producing ability (200E, 232D, 239D3, 256E, 280D, 287D and 287E), five non-producing (192D, 256D, 280E2, 304D and 320E), and controls, were used in this assay. All isolates were plated on Blood Agar Base no 2 (Oxoid, CM271), supplemented with 5% defibrinated ovine blood, and incubated for 18h at 37°C. For each isolate, a 10^6 cells/mL bacterial suspension in cell culture medium was prepared, using optical reading according to a previously drawn regression line that correlates the optical density with the number of colony forming units (CFU). The number of viable microorganisms per mL of each suspension was accurately determined using the Conventional Plate Count Method (Maturin and Peeler, 2001) on plate count agar (PCA).

Bovine mammary epithelial cells, courtesy by Professor Christian Burvenich (Ghent University, Belgium), were grown in appropriate culture medium [Ham's F-12 (Invitrogen)–40%, RPMI1640 (Invitrogen)–30%, NCNT 135 (Invitrogen)–20%, fetal calf serum (Invitrogen)–10%, Lactose (Sigma)–0,1%, Lactalbumin hydrolysate (Sigma)–0,1%, GSH (Sigma)–1.2 mM, L-ascorbic acid (Sigma)–1 µg/mL, Hydrocortisone (Sigma)–5 µg/mL, Insulin (Sigma)– 1 µg/mL, Antibiotic/ antimycotic mix (Sigma A5955)–10 mL] in 24 wells plates at 37°C, under 5% CO_2 atmosphere. Cell seeding density of $2X10^5$ cells per plate was expanded for six days until a confluent cell monolayer was formed. Culture medium was changed every two or three days and non-adherent cells were removed.

Adhesion assay was performed in triplicate. After removing antibiotic containing culture medium, cells were washed three times with PBS, with incubation periods of 10 min at 37°C, under 5% CO_2 atmosphere between washes. Co-cultures were then prepared in triplicate, with 1 mL of each bacterial suspension. Positive and negative controls were also co-cultured in triplicate and three wells were left

with cell culture medium alone. Bacterial suspensions were also grown in plates with no cells to check bacterial adherence to polystyrene. All the plates were incubated at 37°C, under 5% CO_2 atmosphere for 35 min, after which bacterial suspensions were removed and plates washed three times with PBS. Cells and bacteria were then harvested with trypsin-EDTA 0.1%/0.04% (Invitrogen 25300-054). For bacterial count, the number of CFU was determined according to the standardized method for viable microorganisms counting (Maturin and Peeler, 2001). The number of cell adherent bacteria and the percentage of inoculated bacteria adhering to cells were calculated considering the total plate well area, the bacterial percentage adhering to polystyrene plate and corrected to the area occupied by cells (7.065 cm^2).

2.6. Statistical Analysis

The relation between slime production in CRA and biofilm production in TCP was assessed by ANOVA using Proc GLM (SAS) and the following template:

$$Y_{ijk}=\mu+\alpha_i+\beta_j+\varepsilon_{ijk}$$

Y_{ijk}-biofilm production

μ-average

α_i- effect of slime production in CRA i

β_j-effect of the block (day of determination) j

ε_{ijk}-error

Biofilm production differences between classes of CMT/CM were also analyzed by ANOVA using Proc GLM (SAS):

$$Y_{ij}=\mu+\alpha_i+\varepsilon_{ij}$$

Y_{ij} -biofilm production

μ-average

α_i-effect of CMT/CM (1+, 2+, 3+, CM, T) i

ε_{ij}-error

To evaluate whether the ability to adhere *in vitro* to mammary cells differed between biofilm producing and non-producing isolates and the relationship with the intensity of the inflammatory reaction produced in the mammary gland, data were studied by ANOVA using Proc GLM (SAS):

$Y_{ijk}=\mu+B_i+CMT_j+(B_i*CMT_j)+e_{ijk}$

Y–adhesion proportion of biofilm producing isolate B_i that caused inflammatory reaction intensity CMT_j

μ–average

B_i–effect of biofilm production B_i

CMT_j–effect of inflammatory reaction intensity CMT_j

B_i*CMT_j–effect of the interaction between the production of biofilm $_i$ and inflammatory reaction intensity $_j$

e_{ijk}–error

3. Results

In the slime production assay, only eight (7.3%) of the 109 *S. epidermidis* isolates showed the ability to produce slime, according to the CRA analyses.

Concerning biofilm production, all CRA positive isolates issued results higher than zero on the TCP test. Only 12 out of 303 assays, corresponding to 101 negative CRA isolates, yielded higher than zero results in the TCP test. According to TCP test, the production of biofilm was 8% (26 out of 327 assays, corresponding to the 109 isolates). Ten isolates showed at least two of the three results above zero (Table 1).

According to least square means, the relation between slime production in CRA and biofilm production analyzed by TCP was highly significant ($P<0.001$). Changes that occurred in different days were not significant.

The results of the two methods used to evaluate slime production and biofilm formation and the association with mammary inflammation, expressed by the California mastitis test results, for subclinical mastitis, or clinical mastitis evidence (CMT/CM), are summarized in Table 1.

Biofilm production ability according to different inflammatory indicators in milk (CMT/CM) did not show significant differences ($P\geq0.05$).

Results of *in vitro* adhesion to mammary cells by biofilm producing isolates and non-producers are shown in Table 2. The degree of inflammation displayed by the mammary gland from which each isolate was collected is also shown in Table 2.

The number of bacteria that adhered to mammary epithelium cells in culture varied between 3.0 and 4.2 $\log_{10}$ per cell monolayer (7.065 cm^2). The percentage of adherent bacterial cells was between 0.05% and 1.23%. The number of adherent bacteria of biofilm producing isolates ranged between 3.0 and 4.0 $\log_{10}$, with adherence percentages between 0.18% and 1.23%. With regard to non-biofilm producing isolates, adherence to cells varied between 3.2 and 4.2 $\log_{10}$, corresponding to adherence percentages between 0.05% and 1.08% (Table 2).

The production of biofilm was found not to affect adhesion to mammary epithelium cells *in vitro*, nor was the degree of inflammation directly related to a different adhesion aptitude. Isolates with different biofilm production capacity did not express different abilities to adhere mammary epithelium cells. Similarly, isolates that induced different inflammation degrees did not demonstrate a different ability to adhere to mammary epithelium cells (Figure 1).

4. Discussion

Only eight (7.3%) *S. epidermidis* isolates expressed the capacity to produce slime on CRA and 8% produced biofilm in TCP, which is considerably lower than the percentage of biofilm producing isolates found by others in bovine milk: 15.4% from unclassified mastitis (Tremblay et al., 2013), 37.5% from subclinical mastitis (Oliveira et al., 2006), 40% from clinical and subclinical mastitis (Simojoki et al., 2012), 42.9% from unclassified mastitis (Watts et al., 1990). Studies aiming at the characterization of sheep milk staphylococci isolates are scarce. França et al. (2012) studied five isolates from subclinical mastitis, only one (20%) was positive on CRA. Biofilm formation is dependent on growth conditions (Chagnot et al., 2013) and culture medium (Fabres-Klein et al., 2015) and different studies might have mild divergences in procedures.

It has been previously suggested that the low percentage of biofilm producing isolates from sheep mastitis may be related to host defense mechanisms, which are more effective, fighting these strains more aggressively than non-producing ones (Heinzelmann et al., 1997). According to this hypothesis, producing strains would be more effectively eliminated from the host, which contradicts what has been described that mastitis-causing staphylococci biofilm formation may help to avoid immune response and cause persistent IMI. Although several authors mention a positive relationship between biofilm formation by mastitis bacteria and persistent IMI (Gomes et al., 2016; Vanderhaeghen et al., 2014), others concluded that biofilm production is not associated with the persistence of bovine IMI caused by coagulase-negative staphylococci (CNS) (Simojoki et al., 2012).

Tissue culture plate test confirmed the results of CRA test for positive isolates, showing that slime production may be accountable for biofilm accumulation, although not in total agreement if CRA negative isolates are considered. However, the relation between slime production in CRA and biofilm production analyzed by TCP was highly significant ($P<0.001$). Simojoki et al. (2012) compared slime production on CRA and biofilm formation in TCP for different CNS from bovine mastitis milk and observed a low association between these tests (kappa=0.18). Nevertheless, kappa test was different for distinct CNS species, with *S. epidermidis* showing the highest value. Oliveira et al. (2006) mentioned

a moderate agreement (kappa=0.467) for *S. epidermidis* and a fair agreement (kappa=0.259) for *S. aureus* of bovine mastitis origin. The fact that some biofilm producers in TCP were negative on CRA may be explained by the presence of a protein-based biofilm (Tremblay et al., 2013).

These results indicate that the number of inflammatory cells that reach the mammary gland in response to infection is not affected by biofilm production, as proven by milk somatic cells discharge, according to California mastitis test and clinical mastitis. Biofilm production by bovine mastitis *S. aureus* seem to induce SCC discharge (Cucarella et al., 2004; Zuniga et al., 2015). Also, Gogoi-Tiwari et al. (2017) observed a neutrophil profuse infiltration in the mouse model mammary gland inoculated with a strong biofilm forming *S. aureus* strain and a significantly less severe reaction to a weak biofilm-forming strain. However, an association between biofilm production from bovine mastitis causing CNS with the intensity of inflammation was not detected by Simojoki et al. (2012).

In the present work, a mammary epithelium cell line, BME, was used to evaluate sheep mastitis *S. epidermidis* ability to adhere to mammary epithelium cells *in vitro*. The use of cell lines instead of primary cultures is known to increase repeatability (Van Belkum et al., 2002). Of the 12 *S. epidermidis* isolates from sheep intramammary infections co-cultured in BME monolayers, 0.05% to 1.23% of inoculated bacteria adhered to cells, after a 35 min incubation period. The percentage of bacteria that adhered to cells varied according to the isolate. Other authors referred the adherence capacity of bovine mastitis *S. epidermidis*, also using BME cell line, to range from 0.23% to 0.89%, after 30 min incubation (Hyvönen et al., 2009). A bovine mastitis *S. epidermidis* isolate showed 2.4% adherence to another mammary cell line, after a 30 min incubation (Almeida and Oliver, 2001). These authors used a mammary epithelium cell line distinct from the cell line employed here, which may justify the differences, since when primary cultures are used, the same isolate shows different adhesion capacity when tested in cells collected from different animals (Sutra and Poutrel, 1994).

Concerning the ability to produce biofilm and the capacity to adhere to mammary cells, the results suggest no association between these two aptitudes. Some authors mention that slime/biofilm

production enhances cell adhesion (Aguilar et al., 2001; Baselga et al., 1993; Valle et al., 2012), while others refer a decrease in this ability (Baldassarri et al., 1997). Here, slime/biofilm production did not influence adhesion to epithelial cells, as observed by others for bovine mastitis *S. aureus* (Cucarella et al., 2002, 2004). Our results are also in accordance with Simojoki et al. (2012), which found no association between biofilm formation or slime production with the adhesion ability of bovine mastitis CNS.

5. Conclusions

Our results suggest that biofilm production by sheep mastitis *S. epidermidis* does not affect cell adhesion and does not interfere with the inflammatory magnitude of the mammary gland. In addition, the ability to adhere to mammary cells *in vitro* was not associated with inflammation intensity. Biofilm formation is recognized as a major virulence factor, hampering the successful treatment and prophylactic measures of a series of bacterial infections of veterinary and human relevance. However, the present results regarding ovine mastitis suggest that biofilm production is neither related with colonization nor with the inflammatory process. Further studies are required to assess the validity of these results *in vivo*.

Conflict of interest

None.

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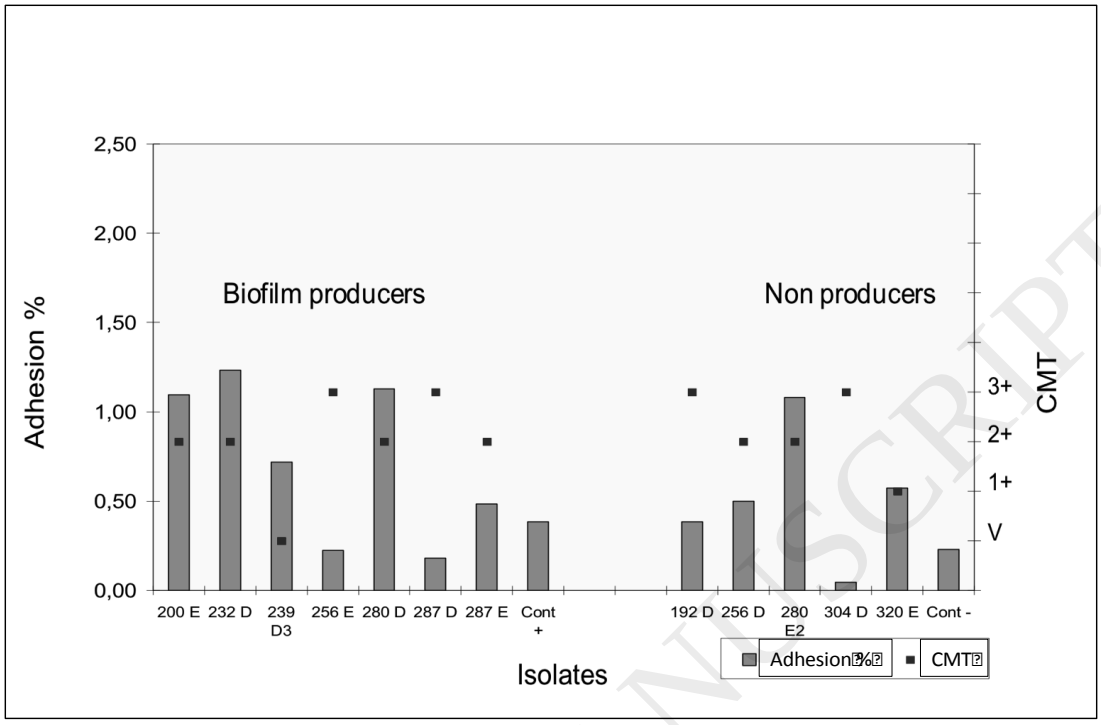
Figure captions

Figure 1. Adhesion ability and inflammation intensity assessed by California mastitis test (CMT), for biofilm producers and non producers *S. epidermidis* isolates.

Figure 1



Cont + - *S. epidermidis* ATCC 35984 (RP 62A); Cont - - *S. epidermidis* ATCC 12228

Figure 1. Adhesion ability and inflammation intensity, assessed by California mastitis test (CMT), for biofilm producers and non producers *S. epidermidis* isolates.

Table 1. Mammary gland inflammation intensity and slime/biofilm production (N = 109)

Inflammation intensity[a] CMT/CM	Slime (CRA)	Biofilm (TCP)	N (%)
CM	-	≤ 0	6 (5.5)
	+	> 0	0 (0)
3+	-	≤ 0	34 (31.3)
	+	> 0	2 (1.8)
2+	-	≤ 0	31 (28.4)
		> 0	2 (1.8)
	+	> 0	4 (3.7)
1+	-	≤ 0	21 (19.3)
	+	> 0	1 (0.9)
T	-	≤ 0	7 (6.4)
	+	> 0	1 (0.9)

[a] Degree of inflammation displayed by the mammary gland from which each isolate was collected: CMT/CM – California mastitis test/ clinical mastitis; CM - clinical mastitis; T/ 1+/ 2+/ 3+ - CMT grades.

CRA – Congo red agar test; + - positive; - - negative; TCP – tissue culture plate test; ≤ 0 – result ≤ zero in at least two of the three assays; > 0 – result > zero in at least two of the three assays.

Table 2. Slime/biofilm production, mammary cell adhesion capacity and mammary gland inflammation intensity (N = 12)

Isolate	Slime	Biofilm	Cell adhesion capacity[b]		CMT[c]
	CRA	TCP	log_{10} CFU	%	
200E	+	> 0	4.0	1.10	2+
232D	+	> 0	3.7	1.23	2+
239D3	+	> 0	3.4	0.72	T
256E	+	> 0	3.6	0.22	3+
280D	+	> 0	3.9	1.13	2+
287D	+	> 0	3.0	0.18	3+
287E	+	> 0	3.6	0.48	2+
Cont +[d]	+	> 0	3.7	0.38	
192D	-	≤ 0	3.7	0.38	3+
256D	-	≤ 0	3.9	0.50	2+
280E2	-	≤ 0	4.2	1.08	2+
304D	-	≤ 0	3.2	0.05	3+
320E	-	≤ 0	3.2	0.57	1+
Cont -[d]	-	≤ 0	3.5	0.23	

CRA – Congo red agar test; + - positive; - - negative; TCP – tissue culture plate test; ≤ 0 – result ≤ zero in at least two of the three assays; > 0 – result > zero in at least two of the three assays.

[b] Cell adhesion capacity: $\log_{10}$ CFU - logarithmic number of bacterial cells that adhered to BME cell monolayer; % - percentage of bacteria contained in inoculum.

[c] CMT – California mastitis test, indicating the degree of inflammation displayed by the mammary gland from which each isolate was collected.

[d] Controls: Cont + - *S. epidermidis* ATCC 35984; Cont - - *S. epidermidis* ATCC 12228.