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RESEARCH ARTICLE

EFFECT OF CULTIVATION CONDITIONS AND *GFBA* GENE ON FORMATION OF BIOFILM BY *STREPTOCOCCUS DYSGALACTIAE*

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ABSTRACT

The pathogenesis of *Streptococcus dysgalactiae* is ascribed to a group of extracellular variables and characteristics such as biofilm formation and adherence. This study evaluated various parameters, additives and compounds of bovine milk for their effect on the biofilm formation of *S. dysgalactiae*, as well as the existence of *gjba* gene using PCR. Extracellular DNA and *DNasel* influence were then evaluated in the produced biofilms. Optimal formation of biofilm occurred at 37ºC when the pH value was modified to 7.0, whereas glucose and lactose additives minimized this formation with the testing of bovine milk compounds. The PCR examination exhibited the *gfba* gene was yielded by 78% of the isolates. The presence of extrachromosomal DNA in the supernatants of cell free proposes spontaneous DNA release to the medium. These outcomes shed light on important variables during biofilm formation of *S. dysgalactiae* related with mastitis for supporting the development of new therapeutic approaches for dealing with this pathogen.

Key words: Pathogenesis. Mastitis. *Streptococcus dysgalactiae*. Biofilm. Bovine. *gfba* gene.

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INTRODUCTION

Some genera of bacteria are capable to produce biofilm during their growth via a natural process (Hall-Stoodley, 2005). Most of bacterial infections be composed of biofilm (around 60%), which it is a huge number of the bacterial cells, and this the biofilm play important role in resistance of antibiotics (Costerton, 1999). Microbiota of human and animal contains naturally various species of *Streptococcus*. Nevertheless some members of this genus are important pathogens with the capability to attain a high density due to biofilm formations. *Streptococcus dysgalactiae* is a widespread bacterium could be found mainly in the dairy farms, and it has ability to infect the mammary gland the cattle (Leigh, 1999). Infections involving *S. dysgalactiae* pose a significant threat to the cattle that depend on pasturage. Huge economic damages as a result of shortage in milk yields can arise from mastitis infections due to a bacterium pathogen like *S. dysgalactiae*, and the frequent mastitis infections can cause problems in the remedy due to the capability of pathogens in forming biofilms (Melchior, 2006). Antimicrobial sensitivity tests are presently adopted for treating this disease according to the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2013). Standard remedy for bacteria isolates are usually used at the planktonic status, although, the antimicrobial resistance and the pathogenic profile differ completely due to the biofilms, which reduce the success rate of treatments. Moreover, the ability of biofilm formation is critical for pathogenicity to the animal, and for industries of milk, as the pathogen can adhere to food processing structures and is capable of persisting during biofilm formation in adverse circumstances. Emergence of the biofilm based on the pathogen's ability to bind to epithelial cells of bovine mammary. There are many virulence agents of *S. dysgalactiae*, such as their ability to adhere to the surface of the host cell by the GFBA protein during the adherence process (Almeida, 2006). Biofilm formation of *S. dysgalactiae* isolated from mastitis in cows has been previously reported (Genteluci *et al*., 2015). The potential role of biofilms in *S. dysgalactiae* based mastitis infections and the potential negative economic impact in global milk production provide the impetus to investigate the circumstances that may contribute to biofilm formation, which could help in the development of approaches for more efficient treatments. Environmental circumstances tend to affect the ability of biofilm formation in many bacterial species (Kaur *et al*., 2009; Rosini, 2015; Kim, 2016). However, there are scant studies on the effect of additives in the biofilm formation of *S. dysgalactiae*(Abureema, 2013; Varhimo, 2011), and different agents have not previously been examined for their potential effect on *S. dysgalactiae*. This study attempted to investigate the effect of various agants on formation of biofilm by *S. dysgalactiae* with existence of the *gfba* gene by PCR. The agents examined were pH value of the medium, temperature, time, glucose and lactose as additives,

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bovine milk compounds as skimmed milk, casein hydrolysate, α-casein, and bovine serum albumin. In addition, investigations were made of extracellular DNA and the influence of *DNaseI* in formation of biofilm by *S. dysgalactiae*.

MATERIALS AND METHODS

Bacterial isolates: 29 species of *S. dysgalactiae* were isolated from samples of milk in this study, obtained from cows suffer of mastitis at 6 dairy farms in Taif city in the Makka Province of southwest Saudi Arabia. The bacterial isolates were identified previously phenotypically (Reinoso *et al*., 2010), and further identified genotypically by using restriction fragment length polymorphism (RFLP) of 16S rDNA, as reported by Jayarao *et al*. (1992), and Khan *et al*. (2003). The species investigated in this study were reported previously as biofilm producers (Genteluci *et al*., 2015), and *Staphylococcus epidermidis* MLAMC 395, a biofilm producing isolate characterized previously, was utilized as a positive control. This isolate was obtained from the Microbiology Laboratory, King Abdul-Aziz Medical City - Makkah - Saudi Arabia.

Microtiter plate assay: The experiment of microtiter plate was conducted to estimate the impact of various agents and additives. The influence of pH of the medium at 5.0 and 9.0, temperature (30ºC, 35ºC and 37ºC), time (2, 5, 24, 48, 72 hours), addition of glucose (5% p/v), lactose (0.5%, 5% p/v) and bovine milk compounds as skimmed milk (0.1%, 0.5% p/v), casein hydrolysate (3 mg/mL), α-casein (3 mg/mL) and bovine serum albumin (BSA) (5 mg/mL) (Sigma-Aldrich-USA) were estimated according to Christensen *et al*. (1985). The growth of bacterial isolates were examined already under various circumstances during the planktonic state, and each isolate was then examined further for biofilm formation in triplicate. Each plate with four wells contained uninoculated Tripticase Soy Broth (TSB) media (Sigma-Aldrich, USA), which were applied as blanks, furthermore, each plate contained media inoculated by *S. epidermidis* MLAMC 395 was utilized as biofilm positive control. Categorization of the bacterial isolates was done using a scale of average optical density of blank wells, and the mean of three times the standard deviation. If the optical density of the isolate was found to be lower than the cutoff value, it was considered as negative.

PCR amplification: Isolation of genomic DNA was done as detailed by Jayarao *et al*. (1992). Examination of PCR was conducted by 25 ng of DNA per reaction. Detection of the *gfba* gene of *S. dysgalactiae* was done using the specific oligonucleotide primers 5′ ACGACAGGTGTCCAAGTGAT 3′ and 5′ TAGACAAGGACTTCGTACAG 3′ designed with PRIMER3 software (Reinoso *et al*., 2011). Processes of amplifications were done in 25 mL of buffer solution containing 3 mM oligonucleotide primers, 200 mM of each deoxynucleoside triphosphate (Promega, Madison, WI, USA), 3.5 mM $MgCl_2$ and 2.5U DNA *Taq polymerase* (Promega, Madison, WI, USA).

Analysis of extrachromosomal DNA: The existence of extrachromosomal DNA (eDNA) was detected by analyzing cell supernatants. The cell supernatants were resuspended in isopropanol (300 µL) and centrifuged for 5 min. at 12,000 rpm, and DNA was precipitated with 300 µL of ethanol (100%). The yield DNA was then resuspended in TE buffer (10 mM Tris – 1 mM EDTA at pH 7.5), and the eDNAs were stored till utilize at –10 ºC. Specific oligonucleotide primers were utilized in performing PCR examinations for detecting *hasA* (5′ GAAAGGTC TGATGCTGAT 30 and 50 TCATCC CCTATG CTTACAG 30), *hasB* (5′ TCTAGACGC CGATCAAGC 30 and 50 TGAATTCCTAT GCGTCGATC 30) (Ward, 2001) and *gapC* (5′ GCTC CTGGTGGAGATGATGT 3′ and 5′ GTCACCAGT GTAAGCGTGGA 3′) (Fontaine, 2002) genes of *S. dysgalactiae*. *DNaseI* (2U Promega, Madison, WI, USA) was added in the culture medium devoted to produce biofilm in order to investigate the impact of the eDNA on the formation of biofilm for the incubation period of 24 h. and 48 h. *DNAsaeI* concentration was examined, and did not impact the growth of planktonic bacteria.

Statistical analysis: SPSS (Version 11.5, SPSS Inc. USA)was used to perform the statistical analyses. The data recorded were estimated by one way analysis of variance (ANOVA) and the Tukey's multiple comparison tests were also used. When the P values were less than 0.05, they were considered to be significant.

RESULTS

Effect of time, temperature and pH on biofilm formation: The potential for biofilm formation by bacterial isolates was examined at varied times. The examination of microtiter plate showed all of the bacterial isolates examined to be capable of forming biofilms at varied times. The results of the statistical analysis in comparing the absorbance values for biofilms produced after 24, 48 and 72 h revealed variance in the production values at 2, 5, 24 and 48 h ($p < 0.05$) and between 48 and 72 h ($p < 0.05$). No statistical variance was evident for biofilm formation between the isolates subjected for 24 and 72 h (Fig. 1A). The results of the test of capability for biofilm formation by the bacterial isolates at three various temperatures confirmed this capability, but showed evidence of decreased biofilm production at 30ºC and 35ºC. Statistical variance in biofilm formation was noted at temperatures of 30ºC or 35ºC and 37ºC ($p < 0.05$) (Fig. 1B). Optimal results were achieved at 37ºC, and the effect of pH values were also examined. Optimal results of biofilm formation were achieved at starting pH value of 7.0, and was there evident statistical variance between pH 5 or pH 9 and pH 7 ($p < 0.05$) in biofilm formation (Fig. 1C).

Effect of glucose and lactose on biofilm formation: Examination of supplementing the media with glucose (5% p/v) and lactose (0.5% and 5% p/v) has been done. The additives both showed signs of decrease in biofilm formation. The Statistical variation in biofilm formation by the addition of glucose or lactose and the TSB medium ($p < 0.005$) was found to be significant, but the isolates of *S. dysgalactiae* showed no significant variation in the existence of lactose (0.5% and 5%) in growth medium. The behavior of each source of carbohydrate is presented in Fig. 2.

Effect of bovine milk compounds on biofilm formation: Skimmed milk, α-casein and BSA showed no significant influence on biofilm production when added to the medium (Fig. 3 A & B). While, addition of casein hydrolysate decreased the production of biofilm significantly ($p < 0.005$).

3.4. PCR amplification. Throughout of the experiments the outcomes showed that not all of the isolates produced the *gfba* gene, where, the PCR examination exhibited that the *gfba* gene was just yielded by 78% of the bacterial isolates.

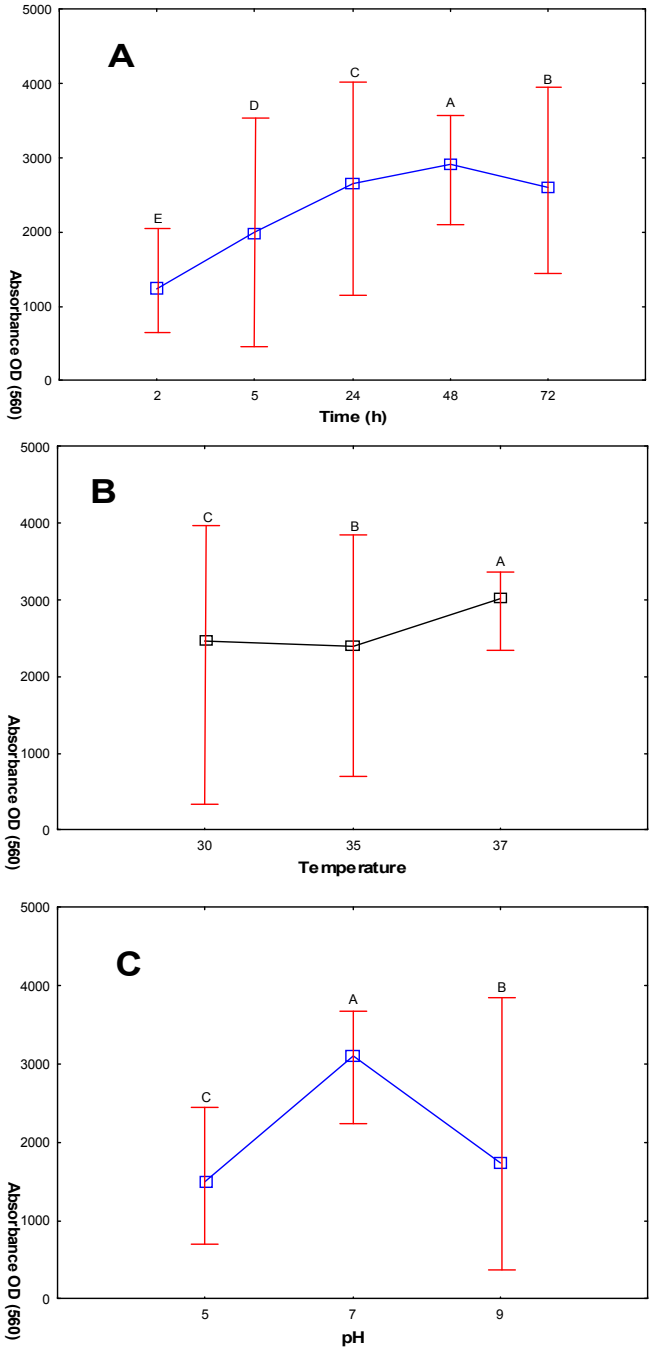

Fig. 1. Effect of variousagents on *S.dysgalactiae* biofilm formation. (A): Time; (B): Temperature; (C): pH. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$)

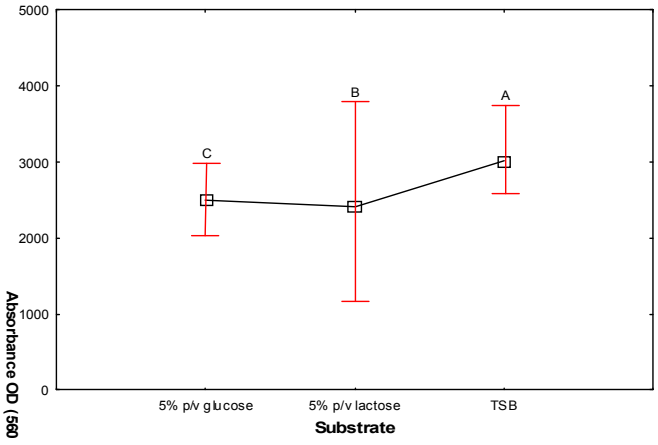

Fig. 2. Effect of glucose and lactose (5% p/v) on *S.dysgalactiae* biofilm formation. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$)

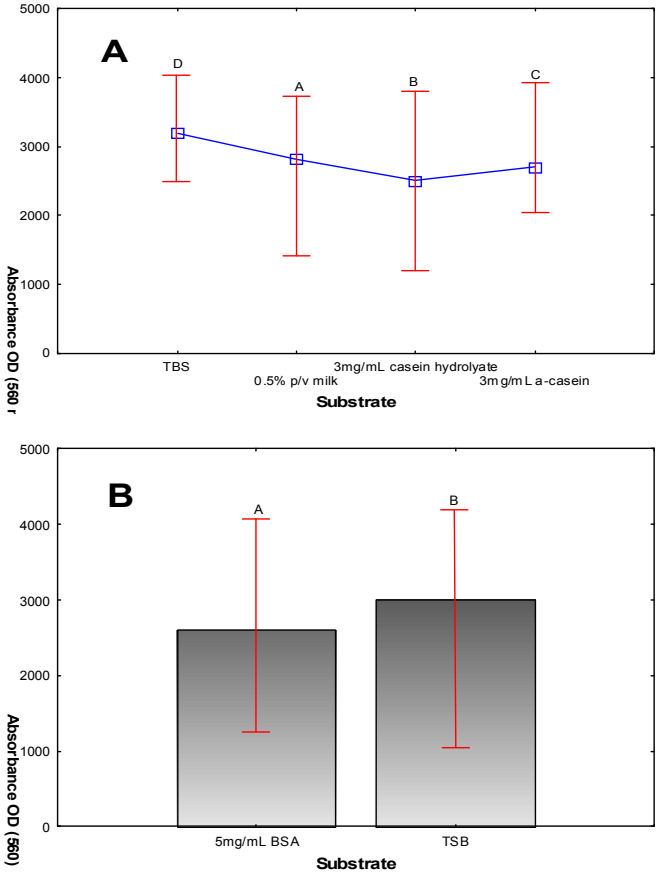

Fig. 3. Effect of bovine milk compounds on *S.dysgalactiae* biofilm formation. (A): Skimmed milk (0.5%); Casein hydrolizate (3 mg/ml); α-casein. (B): BSA. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$)

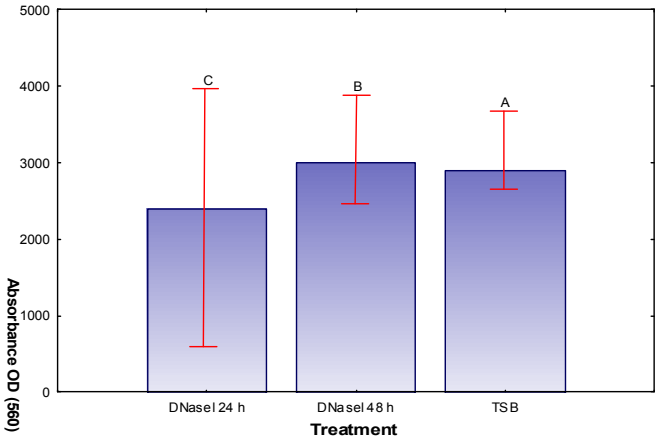

Fig. 4. Effect of *DNAsaeI* on *S.dysgalactiae* biofilm formation. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$)

Extra chromosomal DNA: The existence of eDNA was evident in cell supernatants. PCR examinations were performed to emphasize eDNA characteristics by utilizing primers corresponding to the *hasAB* and *gapC* genes. Amplification products were yielded by all the eDNA examined. Moreover, the capability to form biofilm in the existence of *DNaseI* was examined. The results of the examination of *DNaseI* in biofilm production exhibited its addition in the culture medium impacted the biofilm formation at 24 h, but no effect was observed on the biofilm at 48 h. The statistical variations were significant (Fig. 4) between biofilm formation at 24 h by adding *DNAsaeI* and the TSB control ($p < 0.05$), and 24 h and 48 h ($p < 0.05$).

DISCUSSION

The adhesion of bacteria to a surface with nutrients is the initial phase during biofilm formation, and the formation of biofilm can be affected by several environmental circumstances such as pH value, temperature (O'Toole, 2000). Examinations of biofilm were conducted to investigate the optimal circumstances in the production of biofilm of *S. dysgalactiae* under different conditions representing the mammary gland by first testing the capability of the bacterial isolates in biofilm formation at various times. Microtiter plate examination showed all the examined isolates to be capable of biofilm production at various times. The results revealed biofilm formation commencing between 2 h and 5 h of incubation, and the maximum production of biofilm was at 48 h, thereby it is suggest that the biofilms can arise early following the start of culture. However, mature biofilms tend to detach after 48 h. Testing of the capability of the isolates in biofilm formation at various temperatures showed variance in biofilm formation at temperatures of 30ºC or 35ºC and 37ºC, indicating *S. dysgalactiae* biofilm is produced under a cow conditions as a host. The outcomes of the present study propose pH value of the milk to be optimum for producing biofilm, thereby, indicating a propensity to form high scale of the biofilm of *S. dysgalactiae* in similar mammary gland circumstances. However, improvement of pH may be viable to utilize in progress of efficient approaches for current mastitis therapy due to the negative correlation of high pH with the formation of biofilm (Atulya, 2014).

On the other hand, glucose and lactose carbohydrates affect biofilm production negatively as it recorded in this work. Abureema (2003) mentioned the addition of glucose, fructose or sucrose in Tood-Hewitt broth as a source of carbohydrates, encourages the production of biofilm by *S. dysgalactiae*, but biofilm production is reduced significantly with the addition of lactose (Abureema, 2013). These results are supporting the findings obtained in the present study, which is contrary to the study of Xue *et al*. (2014) in which lactose increased the production of biofilm, mainly through stimulating the production of polysaccharide intercellular adherence in isolates of *Staphylococcus aureus* (Varhimo *et al*., 2011). These results contradicting those of Xue *et al*. (2014) suggest bacterial isolates may exhibit varied behavior based on the variation of the regions (Xue, 2014). Given that caseins are a prominent constituent of bovine milk, an evaluation was made of adding skimmed milk, casein hydrolyzed, α-casein and BSA. Biofilm formation was unaffected by any of the compounds, except for casein hydrolysate which decreased the biofilm significantly. In other studies, milk proteins have been shown to play an important role in the initial phase of the internalization and infection of *S. dysgalactiae* (Almeida *et al*., 2003), and the addition of milk and casein proteins has been reported to improve biofilm formation (Varhimo *et al*., 2011). However, it has also been shown that no *S. dysgalactiae* isolate is capable of biofilm production when tested in BME-UVI complete medium, and in BME-UVI complete medium supplemented with casein (Tassi, 2015), and the same has been proven for *Escherichia coli* and *S. aureus* (MTCC 96) which were unaffected by lactose and casein (Atulya, 2014). Biofilm formation is likely to have been unaffected in those bacterial isolates in this study due to the reduced fat content in skimmed milk relative to raw and whole milk. The addition of BSA slightly deceased biofilm production in the present study. These results corroborate those obtained in a study by Abureema (2013) in which BSA addition to the culture medium failed to significantly enhance the formation of biofilm, and the bacterial isolates tested were related (Abureema, 2013). In the current work, the results exhibited 78% of the tested bacterial isolates had the *gfba* gene. This result conforms with the result of a study by Luther *et al*. (2008) who found the *gfba* gene to be present in all isolates of *S. dysgalactiae* obtained from geographically varied locations, which indicates conservation of this gene in many of the isolates (Luther, 2008). Previous studies thus indicate the significance of this gene's protein as a putative virulence agent, and consequently as an antigen for controlling *S. dysgalactiae* mastitis (Almeida, 2006).

The existence of eDNA in the biofilms has been previously reported in many types of bacterial isolates as *Pseudomonas aeruginosa*, *Streptococcus mutans* and *Streptococcus intermedius* (Whitchurch, 2002), and eDNA was recognized in biofilm extracellular matrix as a main key structural component (Whitchurch, 2002). Biofilm formation has also been demonstrated to involve a system capable of functional DNA-binding uptake (Petersen, 2005). The cell supernatants of *S. dysgalactiae* biofilms were found to contain eDNA in this work, and the DNA seems to have been released into the medium spontaneously under the experimental circumstances tested. This release indicates the possible importance of the DNA in biofilm formation, and suggests an autolysis mechanism exists by which the DNA is released. The results of this study are in conformity with results obtained previously in other studies. For instance, the addition of *DNaseI* has been shown to influence stable biofilm formation in *P. aeruginosa* (Whitchurch *et al*., 2002), and the biofilm formed at 60 h solved when treated with *DNaseI*, whereas the biofilm formed at 84 h were shown to be more resistant when treated (Whitchurch, 2002). As the results indicate, substances other than eDNA are capable of reinforcing mature biofilm, and of producing sufficient mature biofilm proteolytic exoenzymes for inactivating *DNaseI*. The *D. Nasel* was therefore demonstrated to not impact on mature biofilm isolates of *S. dysgalactiae* at 48 h. *DNaseI* has been reported to inhibit the production of biofilm whilst existence in the culture medium when seeding the bacterial isolates by Montanaro *et al*. (2011) (28), and in another study by D′Urzo *et al*. (2014), displayed that the addition of *DNasel* (200 mg/mL) was demonstrated to result in partial disarrangement and low inhibition of the biofilm production present in *Streptococcus agalactiae* (D'Urzo *et al*., 2014).

Conclusion

Various agents and additives have been demonstrated in the present work to be capable of affecting the formation of biofilm in isolates of *S. dysgalactiae* that could have been affecting the growth of *S. dysgalactiae* and development of mammary gland infection. This study is considered one of those rare works that made a useful contribution to the field by investigating the impact of several factors such as pH, temperature, time and eDNA existence in the formation of biofilm (*in vitro*) between the isolates of *S. dysgalactiae* obtained from cattle with mastitis. The results of this study provide an improved comprehension of agents that potentially affect biofilm formation in the case of *S. dysgalactiae* isolates present in mastitis, which could assist in the design of new therapeutic methods for dealing with this pathogen.

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