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Evaluation of environmental and nutritional factors and *sua* gene on *in vitro* biofilm formation of *Streptococcus uberis* isolates

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ABSTRACT

The pathogenesis of *Streptococcus uberis* is attributed to a combination of extracellular factors and properties such as adherence and biofilm formation. The aim of this work was to evaluate the influence of different factors, additives and bovine milk compounds on *S. uberis* biofilm formation, as the presence of the *sua* gene by PCR. Additionally, extracellular DNA and the effect of *DNaseI* were evaluated in the biofilms yielded. Optimal biofilm development was observed when the pH was adjusted to 7.0 and 37 °C. Additives as glucose and lactose reduced biofilm formation as bovine milk compounds tested. PCR assay showed that not all the isolates yielded *sua* gene. Extrachromosomal ADN was found in cell-free supernatants, suggesting that DNA released spontaneously to the medium. The results contribute to a better understanding of the factors involved in biofilm production of this important pathogen associated with mastitis in order to promote the design of new therapeutic approaches.

1. Introduction

Bacteria are able to form biofilm as a natural kind of growth [1]. Approximately 60% of bacterial infections consist of biofilms, and these communities show an inherent resistance to antibiotics [2].

Different species of *Streptococcus* constitute human and animal microbiota. However, members of this genus are significant pathogens, and they are able to reach a high density may be as a result of forming biofilm-like populations. *Streptococcus uberis* is a ubiquitous bacterium found in the environment of dairy farms capable of infecting the mammary gland [3]. *S. uberis* infections are an emerging problem for pasture-based herds. Mastitis infections caused by this pathogen result in major economic losses attributed to an important diminution in milk yield. Problems in therapies of recurrent mastitis infections are associated to the ability of pathogens to form biofilms [4]. Treatment of the disease is currently based on antimicrobial susceptibility tests according to Clinical and Laboratory Standards Institute (CLSI) [5]. Standard therapy designed for bacteria isolates at planktonic state continues to be applied. However, the pathogenic profile and the antimicrobial resistance are totally different from biofilms, causing reduced cure rates. Furthermore, the ability to form biofilm is important both from the pathogenicity to the animal as for the manufacturing milk industries, where the pathogen can adhere to abiotic food processing structures and persist in adverse conditions through biofilms.

The development and establishment of the biofilm depend on the ability of pathogen to adhere to bovine mammary epithelial cells. *S. uberis* have many virulence factors including the capacity to bind to the host's cells surface by a protein called SUAM, which is involved in adherence and internalization, by its linking to lactoferrin [6].

Previously, we demonstrated that the *S. uberis* isolated from mastitis cows of the central dairy region of Argentina were able to produce biofilm [7]. Economic negative effects in the milk production around the world and the possible involvement of biofilms in *S. uberis* mastitis infections, address the research to the study of factors that may contribute for the biofilm production in order to establish more effective treatment strategies. Different studies report that environmental conditions influence the capacity to form biofilm in many bacterial species [8], [9] and [10]. To our knowledge, there are reports concerning the impact of additives in biofilm of *S. uberis* [11] and [12] but the influence of different factors has not been studied in this pathogen.

The aim of this work was to evaluate the influence of different factors, such as time, temperature, pH of the medium and additives as glucose, lactose, bovine milk compounds as skim milk, casein hydrolysate, α-casein and bovine serum albumin on *S. uberis* biofilm formation, as the presence of *sua* gene by PCR. Additionally, extracellular DNA and the effect of *DNaseI* were evaluated in the *S. uberis* biofilms produced.

2. Materials and methods

2.1. Bacterial isolates

In the present study, 32 *S. uberis* isolated from milk samples were used. The bacterial isolates were collected from bovine mastitis cases from 12 dairy farms located in the central dairy region of Argentina. Bacterial isolates were phenotypically identified [13] and genotypi-

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cally confirmed using restriction fragment length polymorphism analysis of 16S rDNA (16S rDNA RFLP) as described by Jayarao and collaborators (1992) [14] and Khan and collaborators (2003) [15]. All isolates investigated were biofilm producers in a previous study [7].

A previously characterized biofilm producing isolate, *Staphylococcus epidermidis*, was used as a positive control. The isolate was facilitated by the Microbiology laboratory of the Department of Microbiology and Immunology of the National University of Rio Cuarto.

2.2. Microtiter plate assay

Microtiter plate assay was carried out to determine the influence of different factors and additives. The effect of time at 2, 5, 24, 48 and 72 h, temperature at 30, 35 and 37 °C, the pH of the medium (5 and 9); the addition of sugars as glucose (5% p/v), lactose (0.5%, 5% p/v) and bovine milk compounds as skim milk (0,1% ó 0,5% p/v), casein hydrolysate (3 mg/ml), α-casein (3 mg/ml) and bovine serum albumin (BSA) (5 mg/ml) (Sigma, St. Louis, MO, USA) were determined according to Christensen and collaborators (1985) [16]. The bacterial isolates were previously tested for bacterial growth at different conditions in planktonic stage. Each isolate was tested for biofilm production in quadruples and the experiment was repeated on 2 different occasions. Four wells in each plate containing uninoculated Tripticase Soy broth (TSB) (Britania) media were used as blanks. Likewise, each plate contained media inoculated with *S. epidermidis* as biofilm positive control. The bacterial isolates were categorized using a scale based on the average optical density of the blank wells plus 3 times the standard deviation of the mean. An isolate was considered negative if the optical density was below the cutoff value.

2.3. PCR amplification

Genomic DNA was isolated as described by Jayarao and collaborators (1992) [14]. A PCR assay was carried out with 25 ng of DNA per reaction. The specific oligonucleotide primers 5′ ACGCAAGGTGCTCAAGAGTT 3′ and 5′ TGAACAAGCGATTCGTCAAG 3′ designed with PRIMER3 software were used for the detection of the *sua* gene of *S. uberis* [17]. Amplifications were conducted in 25 µl 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).

2.4. Analysis of extrachromosomal DNA

Cell-free supernatants were analyzed for the presence of extrachromosomal DNA (eDNA). Briefly, cell-free supernatants were resuspended in 300 µl of isopropanol and centrifuged at 12,000 rpm for 5 min. DNA was precipitated with 300 µl of 100% ethanol. Finally, the resulting DNA was resuspended in TE buffer (10 mM Tris - 1 mM EDTA; pH 7.5). eADNs were stored at −20 °C until use. PCR assays were performed using specific oligonucleotide primers for the detection of *hasA* (5′ GAAAGGTCTGATGCTGAT 3′ and 5′ TCATCCCCTATGCTTACAG 3′), *hasB* (5′ TCTAGACGCCGATCAAGC 3′ and 5′ TGAATTCCTATGCGTCGATC 3′) [18] and *gapC* (5′ GCTCCTGGTGGAGATGATGT 3′ and 5′ GTCACCAGTGTAAGCGTGGA 3′) [19] genes of *S. uberis*.

To evaluate the impact of the eDNA on biofilm formation, *DNaseI* (2U Promega, Madison, WI, USA) was included in the biofilm culture medium for the entire 24 and 48 h of the incubation period. The concentration of *DNAsaI* used was assayed and did not affect planktonic bacterial growth.

2.5. Statistical analysis

Statistical analyses were performed with Infostat software. The data obtained were evaluated by one-way analysis of variance (ANOVA) and the Tukey's multiple comparison tests. P values < 0.05 were considered significant.

3. Results

3.1. Effect of time, temperature and pH on biofilm growth

The ability of the bacterial isolates to form biofilms at different times was tested. Microtiter plate assay revealed that all of bacterial isolates tested produced biofilm at different times. Values of absorbance for biofilm formed after 24, 48 and 72 h were compared. Statistical differences were shown among the production of 2, 5, 24 h and 48 h ($p < 0.05$) and 48 h and 72 h ($p < 0.05$). Not statistical difference was found among biofilm production of 24 and 72 h (Fig. 1A).

Additionally, we tested the ability of the bacterial isolates to form biofilms at three different temperatures. Results revealed that all of isolates were able to produce biofilm at different temperatures, although at 30 °C and 35 °C they remarkably decrease biofilm production. Statistical differences were found among biofilm production at

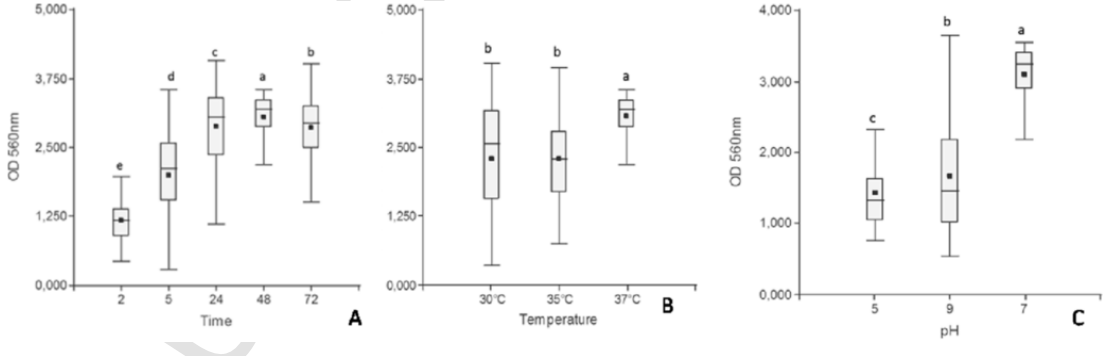

Fig. 1. Effect of different factors on *S. uberis* biofilm formation. A: Time; B: Temperature; C: pH. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$).

30 °C or 35 °C and 37 °C ($p < 0.05$), (Fig. 1B). The best results were obtained at 37 °C.

The influence of pH was assayed also. Optimal biofilm development was observed when the starting pH of the medium was adjusted to 7.0. Statistical difference was found among biofilm production at pH 5 or pH 9 and pH7 ($p < 0.05$) (Fig. 1C).

3.2. Effect of glucose and lactose on biofilm growth

Additives as glucose (5% p/v) and lactose (0.5% and 5% p/v) were evaluated subsequently. Supplementation of the media with glucose reduced biofilm formation, as well as supplementation with lactose. Statistical difference was found among biofilm production with the addition of glucose or lactose and TSB medium ($p < 0.005$). No difference was observed in *S. uberis* bacterial isolates growth in the presence of 0.5% and 5% of lactose in growth medium. Fig. 2 shows the behavior with each carbohydrate source.

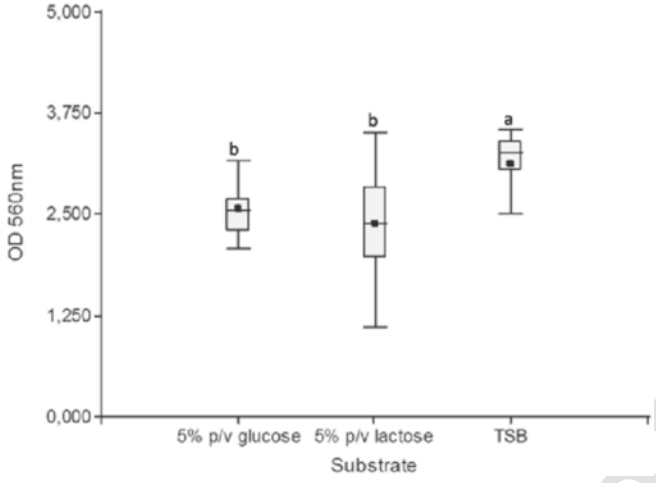

Fig. 2. Effect of glucose and lactose (5% p/v) on *S. uberis* biofilm formation. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$).

3.3. Effect of bovine milk compounds on biofilm growth

The addition of skim milk, α-casein and BSA to the medium had no significant effect on biofilm formation (Fig. 3). The addition of casein hydrolysate significantly reduced biofilm formation ($p < 0.005$).

3.4. PCR amplification

Results revealed that not all the isolates yielded the *sua* gene. PCR assay showed that eighty one percent of the bacterial isolates yielded *sua* gene.

3.5. Extrachromosomal DNA

Presence of eADN was found in the cell-free supernatants. To confirm the nature of eDNA, PCR assays using primers corresponding to *hasAB* and *gapC* genes were performed. All eDNA tested yielded amplification products.

Furthermore, the ability to produce biofilm in the presence of *DNaseI* was assayed. Our results showed that addition of *DNaseI* to the culture medium affected the biofilm at 24 h. However, biofilms were not affected at 48 h. Statistical differences were shown among the biofilm production at 24 h with the addition of *DNAsaI* and the TSB control ($p < 0.05$), and 24 and 48 h ($p < 0.05$) (Fig. 4).

4. Discussion

The first stage in biofilm formation is the adherence of bacteria to a superficial with nutrients. Different environmental factors such as temperature, osmolarity and pH can influence biofilm formation [20].

To determine the optimal conditions for biofilm formation of *S. uberis*, biofilm assays were carried out under various conditions representative of the mammary gland. First, the ability of the bacterial isolates to form biofilms at different times was tested. Microtiter plate assay revealed that all of the isolates tested produced biofilm at different times. The results indicated that the greater biofilm production occurred at 48 h and that it would begin to form between 2 and 5 h of incubation. The data suggest that biofilms occur early after the start of culture. On the other hand, mature biofilms can detach after 48 h.

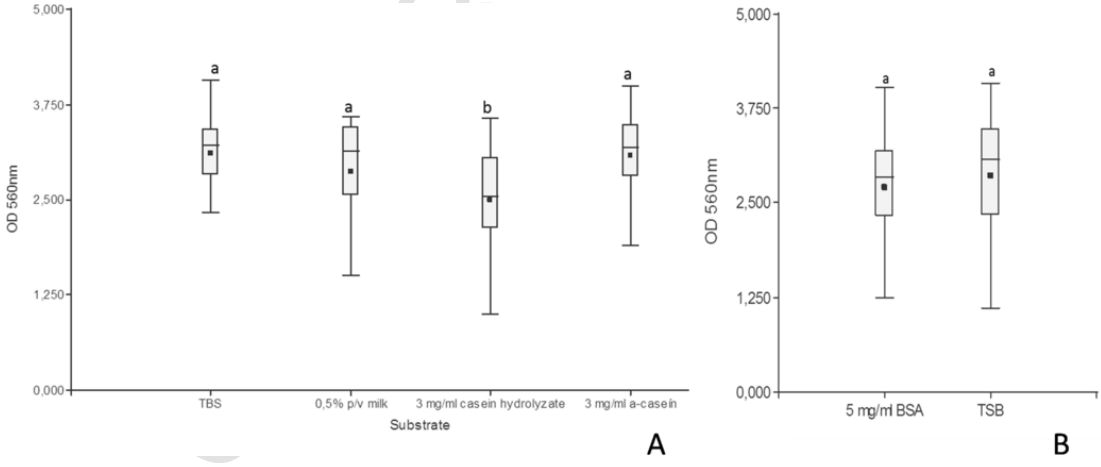

Fig. 3. Effect of bovine milk compounds on *S. uberis* biofilm formation. A: Skim milk (0.5%); Casein hydrolizate (3 mg/ml); α-casein. B: BSA. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$).

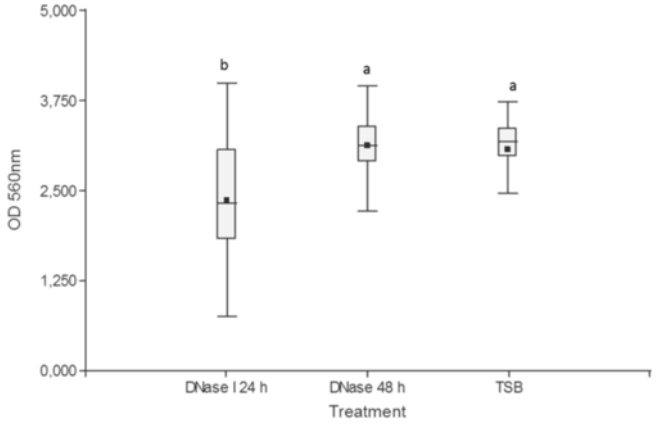

Fig. 4. Effect of *DNAsaI* on *S. uberis* biofilm formation. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$).

When the ability of the isolates to form biofilms at three different temperatures was tested, differences were found among biofilm production at 30 °C or 35 °C and 37 °C, suggesting that *S. uberis* biofilm is formed under the host conditions of the cow.

The result of this work suggests that pH of the milk may be optimal for biofilm production. The findings may indicate that at similar conditions of the mammary gland, there was a tendency for high levels of biofilm formation in *S. uberis*. On the other hand, as suggest Atulya and collaborators (2014) by the fact that high pH has a negative correlation with biofilm formation, pH enhancing agents could be considered to use with the current mastitis therapy to develop effective strategies [21].

The results showed that carbohydrates as glucose and lactose affect negatively the biofilm formation. Abureema (2003) reported that addition of fructose, glucose, or sucrose to Tood-Hewitt broth as carbohydrate source, promoted biofilm formation by *S. uberis*, but lactose markedly reduced biofilm formation [11]. The latter result is in accordance with our results. However, a study carried out by Xue and collaborators (2014) reported that lactose increased biofilm formation predominantly by inducing polysaccharide intercellular adhesin production in *Staphylococcus aureus* isolates [22]. The results are in contrast with Xue and collaborators (2014) and may indicate a different behavior among bacterial isolates from different regions [22].

As caseins are the major constituents in bovine milk, we evaluated the addition of skim milk, casein hydrolyzed, α-casein and BSA. None of the compounds affected the formation of biofilm, except casein hydrolysate that significantly reduced biofilm.

Almeida and collaborators (2003) have shown that milk proteins are important in the early stage of infection and internalization of the *S. uberis* [23]. Similarly, Varhimo and collaborators (2010) reported that the addition of milk and casein proteins enhanced biofilm production [12]. Neverthless, Tassi and collaborators (2015) reported that none of the *S. uberis* isolates tested in BME-UV1 complete medium and BME-UV1 complete medium supplemented with casein were able to form biofilm [24]. Similarly, a study carried out by Atulya and collaborators (2014) demonstrated that biofilm formation by *Escherichia coli* and *S. aureus* (MTCC 96) was not influenced by any of the milk components tested, including lactose and casein [21]. As skim milk has a reduced fat content compared with whole milk and raw milk, the biofilm formation may not be affected in the bacterial isolates tested in this study.

In this work, the addition of BSA reduced slightly the biofilm formation. The results are in agreement with Abureema (2013), who reported that the addition of BSA to the culture medium did not greatly improve biofilm production and would be related to the bacterial isolates assayed [11].

Findings of the present study show that the 81.3% of the bacterial isolates harbored the *sua* gene. The results are in agreement with Luther and collaborators (2008) who detected *sua* gene in all *S. uberis* isolated from geographically diverse locations suggesting that this gene is conserved in many isolates [25]. Research studies propose that the protein of this gene is an important putative virulence factor and potentially a promising antigen that could be used to better control *S. uberis* mastitis [6].

The presence of eDNA in biofilms has been documented in several types of bacterial isolates as *Pseudomonas aeruginosa*, *Streptococcus intermedius* and *Streptococcus mutans* [26]. The eDNA has been identified as a key structural component of the biofilm extracellular matrix [26], and biofilm formation was shown to involve a functional DNA-binding uptake system [27]. In the present study, we found that the cell-free supernatants of the *S. uberis* biofilms contained eDNA. Under the experimental conditions assayed here, DNA appears to have been released spontaneously into the medium, suggesting that it is an important component of the biofilm and the existence of an autolysis mechanism by which DNA is released.

Our results are according with others reported. Whitchurch and collaborators (2002) have shown that the formation of a stable biofilm in *Pseudomona aeruginosa* was affected by the addition of *DNaseI* [26]. Moreover, they observed that biofim established, at 60 h, were dissolved by treatment with *DNaseI*, while those of 84 h were more resistant to the treatment. The results suggested that mature biofilm matrix can be reinforced by substances other than eDNA, or can produce enough mature biofilm proteolytic exo-enzymes to inactivate *DNaseI*. This finding showed that the *DNaseI* has no effect on mature 48 h biofilm isolates of *S. uberis*. Montanaro and collaborators (2011) reported that *DNaseI* is able of inhibiting biofilm formation in when it is present in the culture medium at the time of seeding the bacterial isolates [28]. Similarly, D′Urzo and collaborators (2014) showed that the addition of 200 μg/ml of DNase resulted in low inhibition and partial disruption of the biofilm in *Streptococcus agalactiae* [29].

5. Conclusion

This study showed how different factors and additives affect the biofilm formation in *S. uberis* isolates, which may be influencing the growth course of *S. uberis* and the progress of the intramammary infection. Although several studies have investigated *in vitro* biofilm formation in Group B Streptococcus, this is the first study that investigates the influence of different factors, as time, temperature, pH and the presence of eDNA among *S. uberis* isolates from cattle with mastitis. The results contribute to a better understanding of the factors involved in biofilm production of this important pathogen associated with mastitis in order to promote the design of new therapeutic approaches.

Declaration of interest

The authors declare that they have no conflicts of interest.

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