Rev Argent Microbiol. 2017;49(4):305–310

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

ORIGINAL ARTICLE

Streptococcus uberis: *In vitro* biofilm production in response to carbohydrates and skim milk

Silvana A. Dieser[a], **Aluminé S. Fessia**[a], **Miriam P. Ferrari**[b], **Claudia G. Raspanti**[a], **Liliana M. Odierno**[a,*]

[a] *Department of Microbiology and Immunology, Faculty of Exact, Physico-Chemical and Natural Sciences, National University of Río Cuarto, National Route 36 at Km 601, X5804ZAB Río Cuarto, Córdoba, Argentina*
[b] *Department of Chemical Technology, Faculty of Engineering, National University of Río Cuarto, National Route 36 at Km 601, X5804ZAB Río Cuarto, Córdoba, Argentina*

Received 6 November 2016; accepted 27 April 2017
Available online 31 July 2017

KEYWORDS
Streptococcus uberis;
Biofilm;
Glucose;
Sucrose;
Skim milk

Abstract *Streptococcus uberis* has become one of the most important environmental pathogens associated with clinical and subclinical bovine mastitis. Biofilm confers to bacteria more resistance to physical and chemical agents as well as to different mechanisms of the innate immune system. The aim of this work was to evaluate the ability of *in vitro* biofilm production in 32 *S. uberis* isolates from bovine mastitis and identified by biochemical tests and subsequently confirmed by the amplification of the *pauA* gene. The isolates were cultivated in TMP broth and TMP broth with the addition of 0.5% glucose, 1% sucrose, 1% lactose or 0.5% skim milk in microtiter plates stained with crystal violet. We demonstrated that *S. uberis* isolated from bovine mastitis are able to produce biofilms in TMP broth and, also that biofilm formation by *S. uberis* can be significantly enhanced by the addition of 0.5% glucose or 1% sucrose to TMP broth. This may suggest that the carbohydrates in milk or within the ruminant gut might affect the growth mode of *S. uberis*. In addition, our results showed that *in vitro* biofilm production under different conditions of supplementation displays variation among the isolates and that each isolate shows a particular profile of biofilm production. This phenotypic heterogeneity in biofilm production exhibited by *S. uberis* could at least partly explain why this bacterium has the ability to adapt to different niches facilitating survival to diverse and stressful conditions.

* Corresponding author.
E-mail address: lodierno@exa.unrc.edu.ar (L.M. Odierno).

http://dx.doi.org/10.1016/j.ram.2017.04.007

PALABRAS CLAVE
Streptococcus uberis;
Biofilm;
Glucosa;
Sacarosa;
Leche descremada

Streptococcus uberis: producción _in vitro_ de biofilm en respuesta a carbohidratos y leche descremada

Resumen *Streptococcus uberis* es uno de los más importantes patógenos medioambientales asociados a la mastitis bovina clínica y subclínica. El *biofilm* confiere a las bacterias resistencia a agentes físicos y químicos, como así también a diferentes mecanismos del sistema inmune innato. El objetivo del presente estudio fue evaluar la habilidad de producción de *biofilm in vitro* de 32 aislamientos de *S. uberis* recuperados de mastitis bovina, previamente identificados por pruebas bioquímicas y confirmados por la amplificación del gen *pauA*. Los aislamientos fueron cultivados en caldo TMP sin carbohidratos, y además en caldo TMP con la adición de 0,5% de glucosa, 1% de sacarosa, 1% de lactosa o 0,5% de leche descremada, en placas de microtitulación teñidas con cristal violeta. Se demostró que dichos aislamientos son capaces de producir *biofilm* en caldo TMP, y además se observó un incremento significativo en la producción de *biofilm* en caldo TMP suplementado con 0,5% de glucosa o con 1% de sacarosa. Así, los carbohidratos de la leche o los presentes dentro del intestino de los rumiantes podrían afectar el modo de crecimiento de *S. uberis*. Además, nuestros hallazgos mostraron que la producción de *biofilm in vitro* en diferentes condiciones de suplementación presenta variabilidad entre los aislamientos de *S. uberis* y que cada aislamiento muestra un perfil particular de producción de *biofilm*. Esta heterogeneidad fenotípica en la producción de *biofilm* de *S. uberis* podría explicar, al menos en parte, por qué esta bacteria tiene la habilidad de adaptarse a diferentes nichos, lo que le facilita la supervivencia frente a condiciones diversas y estresantes.

Introduction

Streptococcus uberis is commensal at many body sites and has been isolated from the skin, gut, tonsils and genital tract of asymptomatic ruminants such as bovines[21]. In recent decades, *S. uberis* has become one of the most important environmental pathogens associated with clinical and subclinical bovine mastitis[24], given the constant challenge of the environment with multiple strains of *S. uberis* which have previously colonized the ruminant gut and later gain access to the mammary gland[16,21]. *S. uberis* infections may persist in the same cow for up to 5 months[11], and they have been observed during both non-lactating and lactating periods as well as during the antimicrobial treatment[17,23]. One of the major reasons for recurrence of infection is bacterial biofilm formation inside the udder tissue[15]. Biofilm formation makes bacteria more resistant to physical and chemical agents as well as to innate immune mechanisms[5]. Biofilm is a structured community of bacterial cells added and embedded in an extracellular polymeric matrix composed of polysaccharides, proteins and/or extracellular DNA[7,9]. Biofilm formation is caused in response to fluctuating environmental conditions[19]. Previous studies evidenced that the biofilm-forming capacity of *S. uberis* strains varies from nonproducing strains to strong biofilm-producing strains in supplemented media with milk or carbohydrates[1,4,12,20]. Varhimo et al.[20], demonstrated that extracellular proteins play a crucial role in biofilm formation by *S. uberis* strains. In addition, sugar metabolism has been suggested to be an important process in the early growth of biofilm in *S. uberis* 0140J[4]. As yet, nothing has been reported about *in vitro* biofilm formation among *S. uberis* isolates from cattle with mastitis in Argentina. The purpose of this study was to investigate the ability of *in vitro* biofilm production in *S. uberis* isolated from bovine mastitis in TMP broth without carbohydrates, and also in response to supplements such as glucose, lactose or sucrose, and skim milk.

Materials and methods

Bacterial isolates

A total of 32 *S. uberis* isolates collected from 19 herds located in the central dairy region of Argentina were used in this study. One to three isolates were isolated from each herd.

The isolates were obtained from individual mammary quarters of cows with clinical and subclinical mastitis with a somatic cell count of (SCC) > 250 000 cells/ml between December 2014 and March 2015. These isolates were presumably identified as *S. uberis* by biochemical tests of hippurate hydrolysis, esculin hydrolysis, growth on 6.5% sodium chloride and growth on bile[13,14], and were confirmed by the amplification of the *pauA* gene by PCR[18].

Biofilm assay

The ability of *S. uberis* to form *in vitro* biofilm was determined using 96-well polystyrene microtiter plates, according to what was described by Christensen[3], with minor changes. A preculture of each isolate was carried out in 6 ml of TMP broth (1.5% tryptone, 0.3% meat peptone, 0.5% sodium chloride, 0.25% dibasic potassium phosphate) (Britania, BA,

Table 1 Most frequent profiles of *in vitro* biofilm production in *Streptococcus uberis* isolates

Biofilm production profiles	TMP[a]	0.5% Glucose	1% Sucrose	1% Lactose	0.5% Skim milk	No. of isolates
A	W[c]	W	W	W	W	6
B	W	M[d]	M	W	W	6
C	W	M	W	N	W	4
D	W	W	W	W	N	2
E	W	W	M	W	W	2
F	W	M	W	W	W	2
G	N[b]	W[c]	W	W	W	2

[a] **TMP** medium was made on the basis of individual components Britania (**T**ryptone, **M**eat peptone, Sodium chloride, dibasic potassium **P**hosphate).
[b] N, negative (ODs $\leq$ ODnc).
[c] W, weak (ODnc < ODs $\leq$ 2 ODnc).
[d] M, moderate (2 ODnc < ODs $\leq$ 4 ODnc).

Argentina), and following overnight incubation at 37 °C, a 1/100 dilution in TMP broth was performed. The optical density ($OD_{660\,nm}$) was adjusted to 0.009, and a volume of 10 μl of the culture was transferred to the wells of a microplate (Nunc, Roskilde, Denmark) containing 190 μl of TMP broth or TMP broth supplemented with 0.5% glucose, 1% sucrose, 1% lactose or 0.5% skim milk (Sigma–Aldrich, MO, USA). Following incubation at 37 °C for 24 h, the medium and the planktonic cells were removed, and the wells of each plate were gently washed three times with sterile PBS. Each microplate was set at 60 °C for 1 h and stained using 100 μl of Hucker's crystal violet solution (2%, p/v) (Britania, BA, Argentina). Finally, 100 μl of 100% ethanol was added, and an OD_{560nm} reading was done using an ELISA reader (Labsystems Multiskan MS). Each isolate was tested in triplicate, and the assay was repeated two times. *Staphylococcus aureus* strain V329 *Staphylococcus epidermidis* ATCC 12228 were used as positive and negative controls, respectively. The optical density (ODs) of each isolate was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (ODnc). The following classification was used for the determination of *in vitro* biofilm formation: no biofilm production (ODs $\leq$ ODnc), weak biofilm production (ODnc < ODs $\leq$ 2 ODnc), moderate biofilm production (2 ODnc < ODs $\leq$ 4 ODnc) and strong biofilm production (4 ODnc < ODs).

Statistical analysis

Data analyses were performed using the InfoStat statistical software[6]. Descriptive statistics (the arithmetic mean, standard deviation and standard error) were used to assess *in vitro* biofilm production in different media (TMP broth and TMP broth supplemented with 0.5% glucose, 1% sucrose, 1% lactose or 0.5% skim milk). The statistical analysis was performed using one-way factorial ANOVA with the least significant difference (LSD) test for comparison between multiple groups. *p*-values of < 0.05 were considered to be statistically significant.

Results

In this study, we evaluated for the first time *in vitro* biofilm production of *S. uberis* in TMP broth, without a carbohydrate source. We also determined the response of the isolates to different carbohydrates or skim milk added to this medium on *in vitro* biofilm formation by using the crystal violet staining method in 96-well polystyrene plates. All *S. uberis* isolates showed different ability to produce *in vitro* biofilm in TMP broth or TMP broth with different supplements. Further analysis showed that 15 different biofilm profiles, named with a capital letter from A to O, were found in all 32 *S. uberis* isolates, and 16 (50%) strains belonged to

Table 2 Proportion of *in vitro* biofilm production by 32 *Streptococcus uberis* isolates in TMP broth and in response to carbohydrates and skim milk according to the qualitative analysis

Culture media	Ability to produce biofilm			
	Non-producers	Weak	Moderate	Strong
	Proportion of isolates (%)			
TMP[a]	6.25%	87.5%	6.25%	0%
TMP 0.5% glucose	3.12%	46.87%	43.75%	6.25%
TMP 1% sucrose	3.12%	59.3%	31.25%	6.25%
TMP 1% lactose	18.75%	75%	6.25%	0%
TMP 0.5% skim milk	9.37%	87.5%	3.12%	0%

[a] TMP, broth without carbohydrates.

Table 3 *Streptococcus uberis* isolates with statistically significant *in vitro* biofilm production

Isolates	Arithmetic means[a] (values $OD_{560\ nm}$)	Condition	*p* values[b,c]
RC13	1.690	1% sucrose	0.000
RC19	0.220	0.5% glucose	0.000
RC20	0.223	1% sucrose	0.043
RC21	0.180	0.5% glucose	0.000
RC22	0.299	0.5% glucose	0.000
RC23	0.290	1% sucrose	0.019
RC25	0.323	1% sucrose	0.023
RC27	0.189	0.5% glucose	0.030
RC29	0.360	0.5% glucose	0.025
RC30	0.661	1% sucrose	0.000
RC31	0.311	1% sucrose	0.000
RC32	0.171	1% sucrose	0.038
RC37	0.217	1% sucrose	0.001
RC38	1.330	0.5% glucose	0.000
RC39	0.1365	1% sucrose	0.000

[a] Two different determinations were carried out in triplicate for each strain.
[b] ANOVA with the least significant difference (LSD).
[c] $p < 0.05$.

the three most frequent profiles. Data regarding these three most frequent biofilm profiles are summarized in Table 1. In general, it was observed that a high percentage of isolates was classified as weak biofilm producers in a wide range of values (46.7–87.5%), considering all culture media tested. The percentage of isolates, which were non-biofilm producers, was equal or lower than 18.75% in all culture media used, while strong and moderate biofilm producers were observed in 50% or less of the isolates (Table 2). The quantitative analysis was carried out in 32 *S. uberis* isolates growing in all the above-mentioned conditions. The addition of 0.5% glucose or 1% sucrose to cultures in TMP broth significantly improved *in vitro* biofilm formation, exhibiting arithmetic means of 0.179 and 0.180, respectively. According to our results, biofilm formation by *S. uberis* isolates in the presence of 1% lactose (OD_{560} 0.050) was observed. Importantly, there was no significant difference in the arithmetic mean values of the biofilm production as compared with those obtained in TMP broth (OD_{560} 0.063) or with skim milk (OD_{560} 0.042). Fifteen of the 32 isolates (46.8%) showed $OD_{560\ nm}$ values with statistical significance ($p < 0.05$) in at least one of the media tested (Table 3). Nine of the 15 isolates exhibited a significant *in vitro* biofilm production in TMP broth supplemented with 1% sucrose, while the remaining isolates showed this behavior in TMP broth supplemented with 0.5% glucose. In contrast, none of the isolates showed $OD_{560\ nm}$ values with statistically significant differences in TMP broth and in TMP broth supplemented with 1% lactose or 0.5% skim milk. In order to assess whether there are differences in the effect caused by glucose or sucrose on *in vitro* biofilm production, all isolates that demonstrated a positive effect to glucose and sucrose were pooled, and a simple way ANOVA test with two levels (0.5% glucose and 1% sucrose) was performed. This analysis led to the conclusion that there was not a significant difference ($p = 0.868$) between the mean values of the isolates in TMP broth supplemented with sucrose or glucose. A multiple contrast test revealed a greater ability to produce *in vitro* biofilm in 3 isolates selected among 15 isolates that showed a significant increase in their ability to form *in vitro* biofilm in TMP broth supplemented with 0.5% glucose or 1% sucrose.

Discussion

Biofilms are sessile and attached forms of bacterial growth that enable better survival in hostile environments such as antimicrobial treatments or the immune response of the host, and to colonize new niches through dispersal mechanisms[9,25]. Cell signaling allows bacteria to sense and phenotypically respond to their environment, for example, environmental chemical cues[7]. The nutrient content of the growth medium regulates the development *in vitro* of biofilms in several organisms[2,8,10]. Bovine milk is the natural growth medium of *S. uberis*, and lactose is the major carbohydrate constituent in bovine milk. A diversity of metabolic pathways involved in the utilization of different carbohydrates available within the bovine gut and also in mammary gland secretions have been identified in *S. uberis*[7,21]. Thus, the effect of carbohydrates present in milk or in different environmental niches on *S. uberis* biofilm formation is of great importance. The purpose of this study was to investigate the ability of *in vitro* biofilm production in *S. uberis* isolated from bovine mastitis in TMP broth, and also in response to supplements such as glucose, lactose or sucrose, and skim milk. Thirty-two *S. uberis* isolates were tested for their ability to form *in vitro* biofilms on 96-well polystyrene microtiter plates using crystal violet staining. It should be also pointed out that the results of the present study were not strictly comparable to those obtained in previous studies. As yet, nothing has been reported about *in vitro* biofilm formation in *S. uberis* in broth medium without carbohydrate such as TMP broth. Previous studies determined biofilm formation in conventional Tryptic Soy Broth (TSB) or Todd–Hewitt broth (THB) media[1,4,12] or Todd–Hewitt broth with 1% yeast extract (THY)[20]. Our

findings demonstrate that *S. uberis* isolates are able to produce biofilms in TMP broth. Furthermore, *in vitro* biofilm formation by *S. uberis* was enhanced when TMP broth was supplemented with 0.5% glucose. Here, a low glucose concentration as found in bovine milk, was sufficient to improve *in vitro* biofilm formation by *S. uberis*. Previous results obtained with *S. uberis* support this idea by indicating that the glucose (1–2%) added to THB medium can have a significant increase in biofilm formation[1]. A significant increase in biofilm formation in the presence of 1% sucrose was observed in this study. This result agrees with the work of Abureema[1], who reported a significant increase *in vitro* biofilm formation in the presence of sucrose at a similar concentration. In contrast to our results, Moore[12] did not find an effect on biofilm production by *S. uberis* when sucrose was added to TSB medium. A possible explanation for this inconsistency could be due to the different concentrations of sucrose that could lead to different results in the amount of biofilm production. The latter study was performed using 5% sucrose, while the current study used 1% sucrose. In the present study, the biofilm generated in the presence of 0.5% glucose was not significantly different from that obtained with 1% sucrose. Less biofilm formation by *S. uberis* in the presence of 1% lactose as compared with the above-mentioned carbohydrates was observed in this study. This result is consistent with previous studies that suggested that 1% or 2% lactose in THB markedly reduced biofilm formation[1], or did not have an effect on biofilm production by *S. uberis* when 0.5% lactose was added to TSB medium[12]. A possible explanation of why lactose did not show an inductive effect on biofilm formation in several investigations including ours could be due to a low concentration of this sugar added to the medium, as compared with approximately 5% lactose naturally found in bovine milk. Since adhesion is a prerequisite for biofilm formation, a host factor such as bovine milk could be useful in establishing biofilms. The inductive effect of sterile skim milk at high concentrations on *S. uberis* biofilm formation has been described previously by Aburema[1] and Crowley[4]. However, these milk concentrations led to the coagulation of milk proteins in the 96-well microtiter plates in the present study (data not shown). Thus, the effect of 0.5% sterile skim milk on *in vitro* biofilm formation was investigated in this study. The results showed less biofilm formation by *S. uberis* in the presence of 0.5% skim milk as compared with glucose or sucrose. In the present study, the results of *in vitro* biofilm formation in the presence of skim milk are inconsistent with those described by other authors[1,4]. Crowley et al.[4] demonstrated *in vitro* biofilm formation in all *S. uberis* isolates when they were grown with 20% sterile skim milk in TSB medium without glucose, whereas Abureema[1] showed a statistical significant production of biofilm by *S. uberis* in THB medium at higher milk concentrations (12.5%, 25% and 50%) than those used in our study. A possible explanation as to why skim milk showed a lower inductive effect on biofilm formation in our research could be due to a markedly lower concentration of milk added in the medium compared to other studies mentioned before[1,4]. In the present study, the biofilm generated in the presence of 0.5% skim milk was not significantly different from those obtained in TMP or TMP with 1% lactose. Moreover, a previous study suggested that the extracellular proteolytic activity of *S. uberis* contributes to an increased biofilm formation[20]. In this work, we showed that 100% of *S. uberis* harbored the gene *pauA* (data not shown). This gene codes for plasminogen activator which is able to activate bovine plasminogen to the serine protease plasmin[22]. Varhimo et al.[20] demonstrated that serine-type proteolytic activity is necessary for biofilm formation in *S. uberis* due to the inhibition of milk-stimulated biofilm formation by 1-mM serine-(dichloroisocoumarin) protease inhibitor. In this study, at least to our knowledge, it is the first time that *in vitro* biofilm formation by *S. uberis* growing in TMP broth without carbohydrates has been reported. Furthermore, the response to supplements such as glucose, lactose or sucrose, and skim milk in biofilm formation has been investigated. In conclusion, we demonstrated that *S. uberis* isolated from bovine mastitis are able to produce biofilms in TMP broth and, also that biofilm formation by *S. uberis* can be enhanced by the addition of glucose or sucrose to TMP broth. This may suggest that the carbohydrates in milk or present within the ruminant gut might affect the growth mode of *S. uberis*. In addition, our results showed that *in vitro* biofilm production under different conditions of supplementation displays variation among the isolates and each isolate shows a particular profile in biofilm production. This phenotypic heterogeneity in biofilm production exhibited by *S. uberis* could at least partly explain why this bacterium has the ability to adapt to different niches facilitating survival to diverse and stressful conditions.

Ethical disclosures

Protection of human and animal subjects. The authors state that no human or animal experiments have been performed for this research.

Confidentiality of data. The authors state that no patient data appears in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Acknowledgements

We thank M.V. Liliana Tirante (LactoDiagnóstico Sur, Olivos, Buenos Aires) for providing the isolates. This work was supported by grants from SECyT (Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto) and FONCyT (Agencia Nacional de Promoción Científica y Tecnológica). S. Dieser holds a postdoctoral fellowship with the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), A. Fessia holds a doctoral fellowship with the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

References

1. Abureema S [Doctor of Philosophy thesis] Characterization of *Streptococcus uberis* from bovine milk. Victoria, Australia: School of Applied Sciences, RMIT University Melbourne; 2013.
2. Assaf D, Steinberg D, Shemesh M. Lactose triggers biofilm formation by *Streptococcus mutans*. Int Dairy J. 2015;42:51–7.
3. Christensen G, Simpson W, Janara J, Baddour L. Adherence of Coagulase-Negative *Staphylococci* to plastic tissue a quantitative model for the adherence of *Staphylococci* to medical devices. J Clin Microbiol. 1985;22:996–1006.
4. Crowley R, Leigh J, Ward P, Lappin-Scott H, Browler L. Differential protein expression in *Streptococcus uberis* under planktonic and biofilm growth conditions. Appl Environ Microbiol. 2011;77:382–4.
5. Dantas G, Sommer MO, Oluwasegun RD, Church GM. Bacteria subsisting on antibiotics. Science. 2008;320:100–3.
6. Di Rienzo JA, Casanoves FB, Balzarini MG, González L, Tablada M, Robledo CW, Grupo Infostat, FCA. InfoStat versión. Argentina: Universidad Nacional de Córdoba; 2014.
7. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15:167–93.
8. Fabres-Klein MH, Santos MJC, Klein RC, de Souza GN, Ribon ADOB. An association between milk and slime increases biofilm production by bovine *Staphylococcus aureus*. BMC Vet Res. 2015;11:1.
9. Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. Cel Microbiol. 2009;11:1034–43.
10. Kurola P, Tapiainen T, Sevander J. Effect of xylitol and other carbon sources on *Streptococcus pneumoniae* biofilm formation and gene expression in vitro. APMIS. 2011;119:135–42.
11. Milne MH, Biggs AM, Barrett DC, Young FJ, Doherty S, Innocent GT, Fitzpatrick JL. Treatment of persistent intramammary infections with *Streptococcus uberis* in dairy cows. Vet Rec. 2005;157:245.
12. Moore GE [University of Tennessee Honors Thesis Projects] Biofilm production by *Streptococcus uberis* associated with intramammary infections. Knoxville, TN: Animal Science: Science/Technology Pre Veterinary Medicine Department of Animal Science at the University of Tennessee; 2009.
13. National Mastitis Council Current concepts of bovine mastitis. In: Proceedings of the 43rd annual meeting of NMC. 2004.
14. Odierno L, Calvinho L, Traversa P, Lasagno M, Bogni C, Reinoso E. Conventional identification of *Streptococcus uberis* isolated from bovine mastitis in Argentinean dairy herds. J Dairy Sci. 2006;80:3886–90.
15. Olson ME, Ceri H, Morck DW, Buret AG, Read RR. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res. 2002;66:86–92.
16. Pryor SM [Doctor of Philosophy thesis] Bovine mastitis and ecology of *Streptococcus uberis*. New Zealand: University of Waikato; 2008.
17. Pullinger GD, Coffey TJ, Maiden MC, Leigh JA. Multilocus-sequence typing analysis reveals similar populations of *Streptococcus uberis* are responsible for bovine intramammary infections of short and long duration. Vet Microbiol. 2007;119:194–204.
18. Raemy A, Meylan M. Phenotypic and genotypic identification of streptococci and related bacteria isolated from bovine intramammary infections. Acta Vet Scand. 2013;55:53.
19. Ramírez-Mata A, Fernández-Domínguez IJ, Nuñez-Reza KJ, Xiqui-Vázquez ML, Baca BE. Redes de señalización en la producción de biopelículas en bacterias: quorum sensing, di-GMPc y óxido nítrico. Rev Arg Microbiol. 2014;46:242–55.
20. Varhimo E, Varmanen P, Fallarero A, Skogman M, Pyorola S. Alpha-and-beta casein components of host milk induce biofilm formation in the mastitis bacterium *Streptococcus uberis*. Vet Microbiol. 2011;149:381–9.
21. Ward PN, Holden MT, Leigh JA, Lennard N, Bignell A, Barron A, Egan SA. Evidence for niche adaptation in the genome of the bovine pathogen *Streptococcus uberis*. BMC Genom. 2009;10:1.
22. Ward PN, Leigh JA. Genetic analysis of *Streptococcus uberis* plasminogen activators. Indian J Med Res. 2004;119:136.
23. Zadoks RN, Gillespie BE, Barkema HW, Sampimon OC, Oliver SP, Schukken YH. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. Epidemiol Infect. 2003;130:335–49.
24. Zadoks RN, Middleton JR, McDougall S, Katholm J, Schukken YH. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. J Mammary Gland Biol Neoplasia. 2011;16:357–72.
25. Zhang L, Mah TF. Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. J Bacteriol. 2008;190:4447–52.