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Original article

Biofilm-formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates from subclinical mastitis in conditions mimicking the udder environment

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Abstract

Staphylococcus is the genus most commonly isolated from bovine mastitis in many countries. It may express several virulence factors including biofilm formation, which may protect the bacterial community from antimicrobials' action, preventing these compounds from reaching its interior, where they reach subinhibitory concentrations (subMIC).

Most biofilm production assays are performed in static conditions, while studies regarding antimicrobial resistance usually do not resemble the udder environment because they are performed at high concentrations. In this study we evaluated the influence of dynamic conditions and media, including Mueller Hinton Broth (MHB) and UHT whole milk (WM), as well as the effect of subMIC concentrations of five different antimicrobial agents on biofilm formation by staphylococci isolated from subclinical mastitis. Results suggest that dynamic conditions and media may influence biofilm formation and revealed that milking simulation may significantly increase biofilm production. SubMIC concentrations decrease biofilm formation in MHB but increase in WM, suggesting a protective role of milk against antimicrobial compounds' action. Therefore, *in vitro* conditions that simulate the udder environment and *in vivo* conditions should be included as one of the parameters in evaluation of biofilm producing strains, in order to provide more reliable results.

Key words: Biofilm, bovine mastitis, dynamic conditions, subMIC, staphylococci, milk

Introduction

Bovine mastitis has a high economic impact on the dairy industry and can be caused by many different microorganisms (Seixas et al. 2014b). It is defined as the inflammation of the udder and is mostly caused by bacterial intra-mammary infections (Barkema et al. 2009). Among the potential pathogens, *Staphylococcus* is the genus most commonly isolated (Seixas et al. 2014b). Staphylococci can develop an infection rapidly, persist in the udder and are able to remain undetected for long periods, frequently in subclinical mastitis (Taponen and Pyorala 2009). According to their epidemiological characteristics, masti-

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tis pathogens are divided into two main categories: contagious pathogens such as *Staphylococcus aureus* with the main reservoir in infected quarters, and environmental pathogens like coagulase-negative *Staphylococcus* (CNS), which can be found frequently in the cow's environment (Barkema et al. 2009). Mastitis caused by *S. aureus* may be subclinical or clinical, while CNS usually cause subclinical mastitis with elevated milk somatic cell count (Taponen and Pyorala 2009). In most countries, CNS (like *Staphylococcus epidermidis*), predominates over *Staphylococcus aureus*, previously described as one of the most important mastitis pathogens in cattle (Taponen and Pyorala 2009).

Both species are capable of producing highly organized multicellular complexes known as biofilms, which are recognized as important virulence factors in staphylococci (Clutterbuck et al. 2007, Oliveira et al. 2007). These complex bacterial structures are attached to a surface and enclosed in an extracellular matrix. The role of this matrix is complex, including nutrient acquisition and protection against environmental stresses (Oliveira et al. 2007). Biofilms can also protect the bacterial community from antimicrobial compounds, which may impair the success of antimicrobial therapy (Melchior et al. 2006). Treatment of cows with chronic mastitis due to *S. aureus* often fails, regardless of *in vitro* antimicrobial susceptibility profile (Melchior et al. 2007). Antimicrobial therapy's success depends on several factors, such as animal age, its immune status, simultaneous occurrence of other disorders, route of antibiotic administration and presence of biofilm-forming bacteria (Mestorino and Errecalde 2012). Due to these factors, an antimicrobial compound may not reach the target site of infection in its required effective concentration, the Minimal Inhibitory Concentration (MIC), being present only at subinhibitory concentrations (subMIC) (Amini et al. 2009).

Since its development, the 96-well microtiter plate test has been the most frequently used assay for quantitative evaluation of bacterial biofilm-forming ability, with modifications over the years, to improve its accuracy (Stepanowić eta al. 2000, Stapanowić et al. 2004, Peeters et al. 2008). Other characteristic of most *in vitro* studies regarding biofilm-producing staphylococci is the fact that they are usually performed under static conditions (Stapanowić et al. 2004, Pettit et al. 2005). These static conditions are rarely found *in vivo* (Stapanowić et al. 2001) and results may not reflect the true ability of biofilm formation by bacteria. Udder bacteria are subjected to intensive dynamic conditions during milking, which may influence adhesion, growth and survival (Tremblay et al. 2014). Besides, most biofilm assays are performed with standard culture media (Amorena et al. 1999, Stapanowić et al. 2004, Tremblay et al. 2013), which compositions do not correlate with milk's constituents.

In this study, the influence of subMIC concentrations of five antimicrobial compounds commonly used on mastitis antimicrobial therapy upon biofilm formation by staphylococci isolates was evaluated. Also, an essay mimicking dynamic conditions was performed and the influence of milk in biofilm-formation was also evaluated. These modifications, which better simulate the udder environment, may provide a better insight into the influence of *in vivo* conditions on mastitis staphylococci biofilm formation.

Materials and Methods

Forty three biofilm-producing isolates obtained from bovine subclinical mastitis cases including *S. aureus* (n=21) and *S. epidermidis* (n=22) were used in this study, belonging to a collection of mastitis isolates from dairy cows of 12 Portuguese commercial dairy farms (Oliveira et al. 2006). All staphylococci isolates belonged to different clonal types detected by PFGE (data not shown). *Staphylococcus epidermidis* ATCC35984 and ATCC12228 were used as positive and negative controls of biofilm formation, respectively.

For each isolate, quantification of biofilm formation by bacterial suspensions in Mueller-Hinton broth (MHB, Liofilchem) and UHT whole milk (WM) was performed, using a 96-well microplate Alamar Blue (AB, Invitrogen) assay with a few modifications (Pettit et al. 2005). Briefly, microplates (Orange Scientific) were inoculated with 100 µl of each bacterial suspension ($5x10^5$ CFU/ml) in MHB and WM and incubated under four different conditions, as follows: 1 – incubation at 37°C, 24h, in static conditions; 2 – incubation at 37°C, 24h, with agitation (minishaker apparatus (VWR) at 50 rpm); 3 – incubation at 37°C, 24h, with a medium change at 12h, to mimic milking dynamic conditions; 4 – incubation at 37°C, 24h, in the presence of subMIC concentrations of gentamicin (GN), enrofloxacin (ENR), oxytetracycline (OXT), penicillin G (P) and sulfamethoxazole/trimethoprim (SXT). The subMIC concentrations applied corresponded to half of the MIC values established by CLSI (Clinical and Laboratory Standards Institute 2007). All antimicrobial compounds were purchased from Oxoid.

After incubation, microplates were processed and 5 µl of AB was added to each well, and incubated at 37°C for 1h. Absorbance at 570 nm was determined using a Spectra MAX 340PC microplate reader (Molecular Devices, Sintra, Portugal) (Pettit et al. 2005). Assays were performed in triplicate and repeated on three different occasions.

Influence of the different conditions on biofilm formation (Table 1) and also of media (MHB and WM), with and without several antimicrobial compounds (Table 2), were evaluated by paired sample t-test using the SPSS 20.0 software (IBM Corporation, NY, USA). Results are presented by mean value ± standard deviation. A P value ≤ 0.05 was considered statistically significant.

Table 1. Biofilm production by all isolates under different conditions. MHB – Mueller Hinton Broth; WM – Whole Milk.

Conditions	OD mean values	
	MHB	WM
Static	0.660 ± 0.165	2.170 ± 0.213
Agitation	0.629 ± 0.169	2.067 ± 0.167
Milking simulation	0.745 ± 0.153	2.228 ± 0.150

Results

Higher OD values of staphylococci biofilm production were observed in milking simulation (3rd condition), followed by static (1st condition) and agitation conditions (2nd condition) for both media studied (Table 1). Higher OD values of biofilm in WM in comparison with MHB were observed in all conditions evaluated (Table 1). These differences were statistically significant (p<0.001), with an exception for the comparison between static and agitation conditions in MHB (p=0.142).

Regarding the influence of subMIC concentrations on biofilm production, it was observed that almost all antimicrobials led to a decrease in biofilm formation for both bacterial species in MHB (Table 2). For *S. aureus*, it was observed that a subMIC concentration of SXT increased biofilm formation in MHB; on the contrary, in WM a decrease in biofilm production was observed. In WM, an increase of biofilm production in presence of subMIC concentrations was detected in both species, with the exception of SXT in *S. aureus*, as pointed previously (Table 2). Significant statistical differences for *S. aureus* were observed for ENR, GN, P, OXT in MHB but not in WH, and in SXT in WH but not in MHB. For *S. epidermidis* all the differences observed between the antimicrobials and the media, were statistically significant (Table 2).

Discussion

In this study we aimed to mimic the conditions found in the udder environment to characterise biofilm production by staphylococci from subclinical mastitis isolates, including the presence of subMIC concentrations of five antimicrobial compounds in two media and in the presence of dynamic conditions.

The modified microtiter biofilm assay staining with Alamar Blue, revealed that staphylococci isolates possess a high ability for biofilm formation on plastic surfaces, which is in accordance with previous studies (Oliveira et al. 2006).

Results also showed that dynamic conditions influence *in vitro* results, considering that biofilm formation by field isolates differed when dynamic conditions were applied. Milking simulation influenced biofilm formation by staphylococci isolates by increasing its production. Milking simulation by replacing the growth media every 12h during the course of the experiment enabled biofilms to continue growing in both media most likely due fresh nutrients being provided and toxic compounds being removed (Kwasny and Opperman 2010). Higher OD mean values were obtained in WM in comparison with MHB. These results were expected, as components of WM, like lactose, may contribute to capsule polysaccharide and biofilm formation in *S. aureus* (Poutrel et al. 1995, Melchior et al. 2009). A previous study showed that tryptic soy broth medium supplemented with low concentrations of milk or lactose, upregulated *ica* operon genes in two strains of *S. aureus* associated with bovine mastitis; in one strain milk also promoted an increase in the transcription of surface proteins such as Bap, the biofilm-associated protein (Xue et al. 2014). These studies suggest that bovine staphylococci isolates can adapt to the environment found in the udder, with milk influencing biofilm production and therefore, promoting bacterial survival.

In dynamic conditions a low degree of biofilm formation by staphylococci was observed. This can be explained by the fact that dynamic conditions may impair planktonic bacterial adhesion, the first step of biofilm formation (Stapanowić et al. 2001). Several studies revealed that dynamic conditions affect Gram-negative and positive biofilm-producing isolates, emphasizing the importance of experimental conditions in results' outcome (Stapanowić et al. 2001, Seixas et al. 2014a).

It was observed that subMIC concentrations influence biofilm formation, especially regarding *S. epidermidis* isolates, resulting in a decrease of biofilm formation in MHB but an increase in WM, with the exception for SXT subMIC concentrations in *S.aureus* isolates. In these mastitis isolates, the presence of subMIC concentrations of ENR, GN and P influenced biofilm production in both media. Oxytetracycline subMIC concentrations only influenced biofilm formation in MHB and SXT in WH and previous reports suggested that the administration of subMIC concen-

Table 2. Biofilm production by *S. aureus* and *S. epidermidis* under different subMIC concentrations in MHB and WM.

Conditions		GN			ENR		
Strains	Growth Media	With	Without	*P* value	With	Without	*P* value
S. aureus	MHB	0.291 ± 0.127	0.600 ± 0.132	*P*<0.001*	0.218 ± 0.096	0.552 ± 0.128	*P*<0.001*
	WM	2.243 ± 0.192	2.167 ± 0.159	*P*=0.018*	2.324 ± 0.201	2.100 ± 0.145	*P*<0.001*
S. epidermidis	MHB	0.297 ± 0.166	0.740 ± 0.124	*P*<0.001*	0.253 ± 0.153	0.775 ± 0.076	*P*<0.001*
	WM	2.633 ± 0.179	2.317 ± 0.134	*P*<0.001*	2.452 ± 0.156	2.213 ± 0.162	*P*<0.001*

cont. table 2

Conditions		OXT			P			SXT		
Strains	Growth Media	With	Without	*P* value	With	Without	*P* value	With	Without	*P* value
S. aureus	MHB	0.368 ± 0.168	0.512 ± 0.136	*P*=0.01*	0.549 ± 0.225	0.695 ± 0.178	*P*=0.006*	0.538 ± 0.255	0.405 ± 0.143	*P*=0.105
	WM	2.195 ± 0.265	2.124 ± 0.151	*P*=0.095	2.220 ± 0.219	2.158 ± 0.217	*P*=0.025*	2.139 ± 0.173	2.228 ± 0.136	*P*=0.012*
S. epidermidis	MHB	0.562 ± 0.151	0.787 ± 0.058	*P*<0.001*	0.324 ± 0.215	0.647± 0.253	*P*<0.001*	0.470 ± 0.293	0.791 ± 0.090	*P*<0.001*
	WM	2.479 ± 0.171	2.285 ± 0.135	*P*<0.001*	2.484 ± 0.209	2.303 ± 0.208	*P*<0.001*	2.558 ± 0.224	2.293 ± 0.140	*P*<0.001*

* *P* values were considered statistical significant. GN – gentamicin; ENR- enrofloxacin; OXT – oxytetracycline; P – penicillin; SXT – sulfamethoxazole/trimethoprim.

trations of antimicrobials could increase biofilm formation (Rachid et al. 2000, Kaplan et al. 2012). On the contrary, other studies demonstrated that subMIC concentrations of some antimicrobials are effective in reducing the amount of biofilm formed by staphylococci (Pérez-Giraldo et al. 2003, Cerca et al. 2005a), which was observed for *S. epidermidis* and for the majority of antimicrobials used upon *S. aureus.* SubMIC concentrations may not only contribute to biofilm production but also may influence the composition of the biofilm matrix (Cerca et al. 2005b).

This study also demonstrated a protective role of milk against antimicrobial action at subMIC concentrations. Interestingly, not only milk, *per se*, may promote biofilm formation but also change the effect of subMIC concentrations of antimicrobials. Instead of decreasing biofilm production, as seen for MHB, growth in milk increased the production of this virulence factor, which may come closer to what naturally happens in the udder. SubMIC concentrations are also associated with an increase of mutation rates and horizontal gene transfer which may be responsible for the emergence of multidrug resistant bacteria (Laureti et al. 2013).

In conclusion, exposure of bacterial populations to low concentrations of antimicrobials has ecological effects that may have an important impact on animal and dairy production. SubMIC concentrations of antimicrobials may alter the expression of virulence factors, such as biofilm formation (Laureti et al. 2013), as observed in this study.

In vitro conditions that simulate the udder environment and *in vivo* dynamic conditions to which bacteria are subjected should be included as one of the parameters in evaluation of biofilm producing strains, in order to provide reliable results. The adequate detection and characterization of biofilm-producing strains may provide more relevant information for an adequate control of bacterial infections and strategy for antimicrobial use in animals.

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