

## Influence of papain in biofilm formed by methicillin-resistant *Staphylococcus epidermidis* and methicillin-resistant *Staphylococcus haemolyticus* isolates

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Methicillin-resistant *Staphylococcus epidermidis* (MRSE) and methicillin-resistant *Staphylococcus haemolyticus* (MRSHa) are important coagulase-negative staphylococci. They are often isolated from bacteremia in humans mainly due to their ability to form biofilm on the surfaces of medical devices. Papain is a complex mixture of proteolytic enzymes and peroxidases extracted from the latex of *Carica papaya* and it is recognized by accelerating the healing process of wounds. This study aimed to evaluate the ability of the MRSE and MRSHa isolates to produce biofilms. Besides this, the ability of papain to inhibit the formation of biofilms or to disrupt the ones already formed by those bacteria was analyzed. Thirty MRSHa and 30 MRSE were isolated from bacteremia and used in this study. It was observed that papain has ability to reduce biofilms formed by MRSE ( $p < 0.06$ ) and by MRSHa ( $p = 0.0005$ ). In addition, papain was able to disrupt mature biofilms made by MRSE ( $p = 0.014$ ). No antibacterial activity of papain was observed for any isolates of MRSE and MRSHa tested. Papain has been demonstrated as a potential product for reducing biofilm.

**Uniterms:** *Staphylococcus epidermidis*/methicillin-resistant. *Staphylococcus haemolyticus*/methicillin-resistant. Biofilm/production. Papain/reducing biofilm.

*Staphylococcus epidermidis* resistente à metilina (MRSE) e *Staphylococcus haemolyticus* resistente à metilina (MRSHa) são importantes estafilococos coagulase negativa. São frequentemente isolados em bacteremia humana, principalmente devido à capacidade de formar biofilmes nas superfícies de dispositivos médicos introduzidos no organismo. A papaína é mistura complexa de enzimas proteolíticas e peroxidases extraídas do látex de *Carica papaya*, reconhecida por acelerar os processos de cura de feridas. Este estudo teve como objetivo avaliar a capacidade dos MRSE e MRSHa em produzir biofilmes e analisar a capacidade da papaína em inibir a formação de biofilme ou desintegrar biofilmes já formados por essas bactérias. Observou-se que a papaína tem capacidade de reduzir a formação de biofilme por MRSE ( $p < 0,06$ ) e MRSHa ( $p = 0,0005$ ). Além disso, a papaína foi capaz de desintegrar biofilme maduro formado por MRSE ( $p = 0,014$ ). Nenhuma atividade antibacteriana da papaína foi observada para qualquer das duas espécies de bactérias testadas. A papaína mostrou-se produto potencial para reduzir biofilme.

**Unitermos:** Papaína. *Staphylococcus epidermidis*/resistente à metilina. *Staphylococcus haemolyticus*/resistente à metilina. Biofilme/produção. Papaína/redução de biofilme.

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## INTRODUCTION

Coagulase-negative staphylococci, especially *Staphylococcus epidermidis* followed by *Staphylococcus haemolyticus*, have been frequently reported as significant etiological agents that cause nosocomial infections (Keim *et al.*, 2011, Kristóf *et al.*, 2011; Rosado, Romanelli, Camargos, 2011). The most important property of these staphylococci is their capability to form biofilm on the surfaces of foreign bodies introduced into the organism (Donlan, 2011; Sander *et al.*, 2012). The infections are mainly caused by strains that have already colonized parts of the human body, making the colonized person a reservoir that will spread the organisms (Saising *et al.*, 2012).

In biofilms, these organisms are involved in a matrix composed of polysaccharides, proteins, and extracellular DNA, which provides protection against antimicrobial products and immune system (Von Eiff, Heilmann, Peters, 1999; MacCann *et al.*, 2008). The process of biofilm production has not been totally clarified yet, but in *S. epidermidis*, it seems to occur in two important steps: adherence to the inert surface and biofilm accumulation (Götz, 2002). Many bacterial products are thought to be involved in the initial phase of bacterial adherence, including the Atl and Aap proteins, teichoic acid and staphylococcal adhesins, proteins, which play an important role in the plasma-coated biomaterial. In the second phase, the bacteria attach to the surface of the polymer produce and accumulate an extracellular, amorphous, and mucoid polysaccharide material named biofilm (Von Eiff, Heilmann, Peters, 2002). Studies have indicated that the mature biofilm facilitates colonization and persistence of bacteria in the host (Götz, 2002). In *S. epidermidis*, the *ica* operon (encoding enzymes that are involved in biosynthesis of polysaccharide intercellular adhesin; PIA) seems to be essential for the production of biofilm (Araujo *et al.*, 2006).

Papain is a complex mixture of proteolytic enzymes and peroxidases extracted from the papaya latex of *Carica papaya*. It has been used in Brazil since 1983 to treat wounds (Leite *et al.*, 2012). Several studies have recognized that papain accelerates wound healing processes, especially chronic ones. It can be used in different concentrations according to the type of the wounded tissue (Leite *et al.*, 2012). The treatment of wounds and burns occurs due to papain debriding and anti-inflammatory action (Monetta, 1987; Silva *et al.*, 2007, Chukwuemeka, Anthoni, 2010). Because of antitrypsin  $\alpha$ -1, an antiprotease that inhibits the action of enzymes in healthy tissues, papain can be safely used in skin without damages (Flindt, 1979). Further, proteolysis in necrotic tissues and cell fragments from a wound aid the healing process (Sanchez Neto *et al.*, 1993;

Leite *et al.*, 2012). In addition, biofilms can also be found in wounds, which makes the treatment more difficult and increases wound bioburden (Kim, Steinberg, 2012; Percival *et al.*, 2012; Dowd, 2008). Moreover, previous experimental and clinical data have recommended using papain to reduce wound bioburden and to promote wound healing (Payne *et al.*, 2008).

Although bactericidal activity of papain has been reported for gram-positive and gram-negative organisms (Dawkins *et al.*, 2003; Chukwuemeka, Anthoni, 2010; Bhardwaj, Ballal, Velmurugan, 2012), there is no consensus about this function. Ferreira *et al.* (2008) found this activity for *Staphylococcus aureus* and *Pseudomonas aeruginosa* only in 10% of papain. However, Lima *et al.* (2009) verified that papain has no antibacterial activity for *Enterococcus faecalis*, *P. aeruginosa*, *S. aureus*, *Klebsiella pneumoniae*, and *Salmonella typhi* in different concentrations.

Considering the proteolytic characteristic described for papain and the biopolymer matrix composition of bacterial biofilms, this study aimed to evaluate the ability of papain to act as an inhibitor of biofilms in different concentrations. Moreover, given the disagreement of reports analyzed, we also evaluated the antibacterial activity of the papain.

## MATERIAL AND METHODS

### Bacterial strains

To evaluate whether papain had the capacity to interfere in staphylococcal biofilm, 60 hospital isolates (30 methicillin-resistant *Staphylococcus epidermidis*-MRSE and 30 methicillin-resistant *Staphylococcus haemolyticus*-MRShA) were analyzed. After that, 78 more isolates (53 MRSE and 25 MRShA) were included to determine the minimal inhibitory concentration. The isolates were collected from bloodstream infection and were previously identified as oxacillin resistance. All bacteria were preserved at -20 °C at 10% glycerol stock on Tryptic soy broth (TSB).

### Papain solution

Papain (Prozyn, Brazil) was solubilized in water Milli-Q sterilized to appropriate dilutions and kept in refrigerated for 7 days.

### Biofilms assay

The biofilm formation was induced by TSB supplemented with 1% glucose (TSB+G) and was formed on

inert polystyrene surfaces (96-well microtitre plates, Nuclon; NalgeNunc International, Roskilde, Denmark) as described by Araújo *et al.* (2006). *Staphylococcus epidermidis* 70D (strong biofilm producer) and *Streptococcus pyogenes* ATCC 75194 (biofilm non-producer) were used as positive and negative controls, respectively. The microtitre plates were incubated for 20 h at  $36 \pm 1$  °C. The optical density (OD) of the stained biofilm was measured at 560 nm using a microtitre plate reader. The isolates were classified according to Amaral *et al.* (2006) as follows: biofilm non-producers ( $BU \leq 0.182$ ), weak biofilm producers ( $0.182 < BU \leq 0.364$ ), moderate biofilm producers ( $0.364 < BU \leq 0.728$ ), and strong biofilm producers ( $BU > 0.728$ ).

### Effect on bacteria attachment assay

To determine whether papain directly inhibited attachment of the bacteria to the polystyrene, MRSE and MRSHa isolates were grown for 20 h in TSB+G medium in the presence or absence of 2.5% or 5% of papain added to well polystyrene microtitre plates (Nuclon; NalgeNunc International, Roskilde, Denmark), followed by staining with crystal violet (Araújo *et al.*, 2006).

### Effect on preformed biofilm assay

We investigated whether papain could affect preformed biofilm produced by 11 MRSE and 11 MRSHa. Thus, after biofilm was established, 2.5% of papain were added to well polystyrene microtitre plates and incubated for 20 hours at  $36 \pm 1$  °C. After the treatment, the wells were gently washed and stained with violet crystal as described (Araújo *et al.*, 2006).

In all biofilm experiments, each isolate was performed at least twice.

### Scanning with inverted microscopy

Biofilms formatted on the polystyrene microtitre plates were analyzed in 10 isolates (5 MRSE and 5 MRSHa) by scanning microscopy (Nikon Eclipse TE 2000-U model), in order to control the results of the spectrophotometric test. At least five different microscopy fields of each isolate were examined.

### Determination of minimal inhibitory concentration (MIC)

The MIC values were determined by the agar dilution method standardized by the Clinical and Laboratory Standards Institute (CLSI, 2011). Papain

was incorporated into the Muller Hinton Agar medium (Difco, Laboratories, USA). Each plate contained different concentrations of papain (1%, 2.5%, 5%, and 10%; Prozyn, São Paulo, Brazil). The bacterial suspensions containing approximately  $10^7$  CFU/mL were inoculated onto agar plates using a Steers replicator. The final inoculum on the agar was  $10^4$  CFU per spot.

*Staphylococcus aureus* ATCC 6538P and *Staphylococcus epidermidis* ATCC 12228 were used as microorganisms control, and drug-free plates were prepared and used as growth controls. After incubation for 24 h at 37 °C, the lowest concentration of the products able to inhibit bacterial growth was considered the MIC.

### Statistical analysis

The null hypothesis using Student's *t* test, for a significance level of 0.1, was performed either for the analyses of the effect of papain on biofilm formation or biofilm disruption.

## RESULTS AND DISCUSSION

### Biofilm study

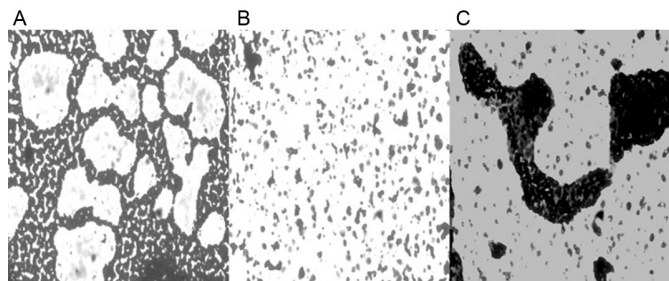
Biofilms is the most important virulent factor for coagulase-negative staphylococci. Our data showed that 16 (53.3%) of MRSE were able to form biofilm and 17% of them displayed high ability to form biofilm (Table I). Previous study realized with isolates in the same hospital showed similar results (Araújo *et al.*, 2006). In relation to MRSHa, 21 (70%) isolates were able to produce biofilm, of which 26.6% of them were classified to be a strong biofilm producer (Table I). Similar to our results, Fredheim *et al.* (2009) found that 74% of the *S. haemolyticus* analyzed were able to produce biofilms. However, the biofilm formed by *S. haemolyticus* has not been subject to more detailed studies so far.

**TABLE I-** Biofilm formation of methicillin-resistant *Staphylococcus epidermidis* (MRSE) and methicillin-resistant *Staphylococcus haemolyticus* (MRSHa)

Biofilm	Microorganism (%)	
	MRSE	MRSHa
Strong	5 (16.6%)	8 (26.7%)
Moderate	6 (20.1%)	5 (16.7%)
Weak	5 (16.6%)	8 (26.7%)
Total producer	16 (53.3%)	21 (70%)
Non-producer	14 (46.7%)	9 (30%)

Because staphylococci are among the most likely, microorganisms to contaminate and adhere to any medical device that penetrates host surfaces (Vuong, Otto, 2002), several compounds have been tested as biofilm inhibitors, and some are already used to coat catheters (Huigens *et al.*, 2009; Juda *et al.*, 2008, Maki *et al.*, 1997). Randomized trials have shown the benefits of using antibiotic-impregnated catheters in hospitalized patients to reduce bacterial colonization on the surface of these devices (Maki *et al.*, 1997). In this paper, we explore the effect of different concentrations of papain (2.5% or 5%) in biofilm production by several MRSE and MRSHa isolates. When papain (2.5% or 5%) was incorporated into the culture, the biofilm formation by MRSE was inhibited ( $p = 0.06$ ). However, the papain action showed to be more effective for MRSHa isolates ( $p = 0.0005$ ). The experiment suggested that papain is able to influence the ability of cells to form biofilm, thus affecting the bacterial attachment. Scanning with inverted microscopy is presented in Figure 1.

It is well known that staphylococcal biofilm formation is multi-factorial and two major enzymes, accumulation-associated protein (Aap) and autolysin

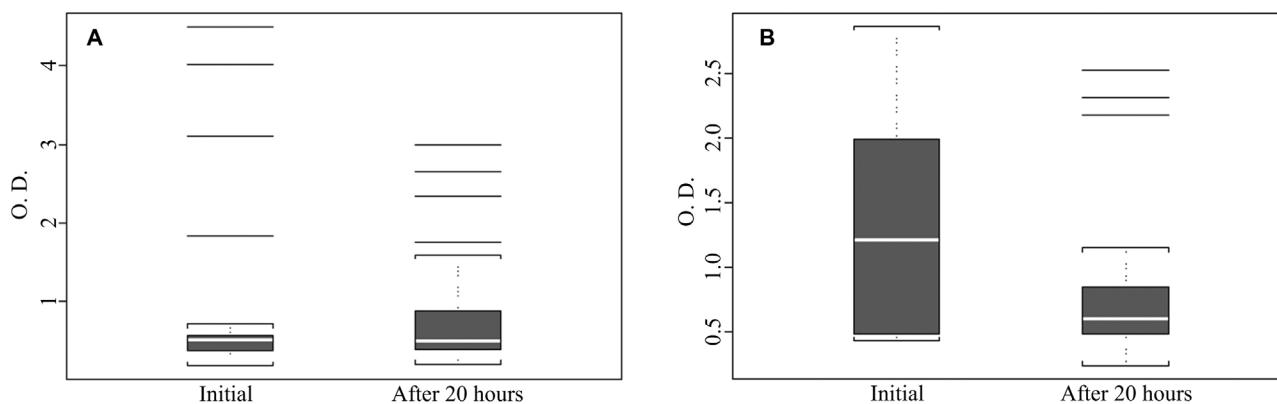


**FIGURE 1** - Inverted microscope of biofilm produced by a MRSHa representative isolate on polystyrene surface. (A) Untreated, (B) 5% papain, and (C) 2.5% papain. Increased 40x.

(Atl), seem to be associated with the first step of biofilm formation (Biswas *et al.*, 2006). In *S. epidermidis*, PIA production and protein factors (i.e., Aap and AtlE) likely contribute strongly to the development of mature biofilms *in vivo* in some isolates (Stevens *et al.* 2009). In their article, it was verified that sodium metaperiodate caused a significant decrease in biofilm formed by PIA and proteinase K treatment was sufficient to significantly disperse the biofilm matrix, suggesting a role for protein in biofilm production. Autolysins such as AtlE play an important role in cell-wall processing and recycling, and it is interesting to speculate that the major role they have in the process of biofilm formation may be to orientate and expose cell-surface adhesins correctly in order to maximize and ensure an effective interaction with either host factors or device surfaces (Stevens *et al.*, 2009).

Karamanos *et al.* (1995) reported that the extracellular slime layer of *Staphylococcus epidermidis* ATCC 35983 contains two non-anionic carbohydrate-containing proteins degradable by papain, and a macromolecule also degradable by papain-bearing acidic carbohydrates covalently bound to protein. Although we did not test the hypothesis in which papain can damage AtlE, it is reasonable to suppose that papain can degrade these biofilm-associated proteins and/or another protein(s) involved in biofilm formation.

On the other hand, to verify whether papain also disrupted the biofilm that accumulated on the polystyrene surface, 22 isolates (11 MRSE and 11 MRSHa) were used. Thus, preformed biofilms were treated with 2.5% papain and incubated for 20 hours at  $36 \pm 1^\circ\text{C}$ . The data obtained for the MRSHa isolates showed no significant influence of the enzyme on biological films ( $p = 0.573$ ) (Figure 2A). However, the results showed a huge reduction of the optical densities obtained for MRSE isolates treated with papain compared to the untreated ones (Figure 2B),



**FIGURE 2** – Boxplot of optical densities of biofilms formed by staphylococcal isolates, both untreated and those treated with 2.5% of papain for a period of 20 hours. (A) *Staphylococcus haemolyticus* – MRSHa, (B) *Staphylococcus epidermidis* – MRSE.

with a significance level  $p = 0.014$ . For Orgaz *et al.* (2011), diverse results against different bacteria may be the consequence of specific features of their biofilm matrix, cell wall or membrane, and/or some intracellular events.

Previous studies have suggested that extracellular proteins seem to be one of the main components of the biofilm matrix formed by *S. haemolyticus* (Fredheim *et al.*, 2009; Silva *et al.*, 2013). Because of that, we expected papain to disrupt biofilm accumulation produced by MRSHa. In the present study, papain disintegrated better biofilm formed by MRSE, the principal component of which is poly- $\beta$ -1,6-*N*-acetyl-D-glucosamine (PNAG), also known as polysaccharide intercellular adhesin (Götz, 2002). According to Jabbouri and Sadovskaya (2010), the sensitivity of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. The authors report that protein components in biofilms play an important role in stabilizing intercellular structure. Our results demonstrate that more studies are still needed to define the roles and the other components of biofilms formed by staphylococci bacteria.

### Determination of minimal inhibitory concentration (MIC)

Papain is a safe agent to use in wounds with high-level bacteria because it is able to reduce bioburden (Payne *et al.*, 2008). Thus, we determined the anti-staphylococcal activity of this agent. Six isolates tested showed no susceptibility for 1%, 2.5%, 5%, or 10% papain. To confirm this result, we decided to add 78 more isolates; hence, all 138 bacteria analyzed demonstrated no activity to papain at different concentrations.

Several studies reported papain antibacterial activity in gram-positive and gram-negative bacteria (Chukwuemeka, Anthoni, 2010; Bhardwaj, Ballal, Velmurugan, 2012). Nonetheless, Ferreira *et al.* (2008) verified that only papain gel 10% was able to inhibit *S. aureus*. Notably, Lima *et al.* (2009) did not find antibacterial activity of papain for various microorganisms. As shown in our study. Seenivasan *et al.* (2010) reported that papain has interacted with the bacterial cell membrane, destabilizing it and resulting in osmotic shock and release of cytoplasmic contents, where the antibacterial activity was established. However, *in vivo*, the association between papain and the reduction of infections observed in the wound seems to be due its anti-inflammatory action, which promotes cellular reproduction, stimulates cytokines production, and recruits phagocytic cells, so that the environment becomes unfavorable for the growth of pathogenic microorganisms (Falanga, 2002; Lima *et al.*, 2009).

## CONCLUSION

Papain seems to be a promising breakthrough in the prevention and disruption of biofilms, a subject of interest of the pharmaceutical industry. Although our experiments showed that papain has no bactericidal activity against *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, it presented a potential product that may reduce biofilm formed by these microorganisms.

## ACKNOWLEDGMENTS

This work was supported, in part, by grants of the Fundação Carlos Chagas de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), Pró-Reitoria de Pesquisa, Pós-graduação e Inovação of the Universidade Federal Fluminense (PROPPi-UFF, Brazil). We thank Professor Dr. Ana Lúcia Marques Ventura from the Neurobiology Department of Biology Institute (UFF) for microscopy experiments and the laboratory technician Wilmar Jorge da Silva for helping us in all experiments.

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Received for publication on 23<sup>th</sup> March 2013

Accepted for publication on 07<sup>th</sup> August 2013

