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Safranine-O Incorporated in F127 Nanocarriers Reduces the Contamination of *Staphylococcus aureus* in Sheep's Milk

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ABSTRACT

Mastitis caused by *Staphylococcus aureus* uses antibiotics as conventional treatment, a practice that has led to bacterial resistance. Therefore, the photodynamic inactivation of microorganisms (PDIM), has the advantage of inactivating pathogens without leading to the selection of resistant microorganisms. The objective of the study was to evaluate the antimicrobial activity of the photosensitizer (PS) Safranine-O (Sf), incorporated in the nanocarrier Pluronic® F127 against strains of *S. aureus* isolated from sheep milk. The reduction of contamination in experimentally contaminated milk and Muller Hilton Broth (MHB), the formation of microbial biofilm, and its effect as *post dipping* in the decrease of total mesophilic aerobic and Staphylococcal counts in milk were evaluated. Three strains of *S. aureus* (SO1, SO3, SO4) and a coagulase-negative *Staphylococcus* (CNS) strain (SO2) were identified through the *nuc* and *coa* genes and all were sensitive to PDIM. The minimum inhibitory concentration (MIC) ranged from 1.18 to 18.75 mg/mL in the different isolates. The SO4 strain was resistant to Ampicillin and Trimetropim. When the microorganisms were cultivated in milk and MHB, there was a reduction in staph counts by 97.33% and 99.63%, respectively. In stainless steel coupons, photoinactivation reduced *S. aureus* adhesion by up to 45.92% (milk) and 99.5% (MHB) ($p<0.05$). The photoactivated Sf was similar to commercial lactic acid when applied as a dipping powder. These results showed that Sf mediated PDIM effectively inactivated pathogens that cause mastitis and reduced milk contamination.

Keywords: mastitis; photoinactivation; Staphylococcus

INTRODUCTION

Mastitis is the cause of great economic losses in dairy herds and sheep. *Staphylococcus aureus* (*S. aureus*) which is responsible for 20% to 60% of clinical mastitis cases, is associated with food poisoning due to the production of *Staphylococcal* toxins (Obaidat *et al.*, 2018) and is described by the production of biofilms on processing surfaces of dairy industries, which can lead to the product recontamination (Sharma *et al.*, 2017).

As a consequence, the importance of maintaining hygiene, application of biosafety measures in milking, and the search for new agents or therapeutic alternatives (Longheu *et al.*, 2020) is highlighted since, currently, the most common treatment for mastitis consists of intramammary application and antibiotics, a practice that has led to the emergence of drug-resistant bacteria (Azzi *et al.*, 2020), with the possibility of residues in milk. On the other hand, in the dairy industry, the concern is the bacterial resistance to sanitizers (Kroning *et al.*, 2020).

In this context, PDIM, a therapeutic modality whose basic principle is to absorb a photon of light by the photosensitizing agent (PS), reacts with molecules in its environment by transferring electrons or energy to molecular oxygen, therefore producing reactive oxygen species (ROS). These ROS damage the cell membrane of microorganisms (Galstyan & Dobrindt, 2019), hence being considered a promising alternative. Sf, a synthetic dye of the phenazine class, has favorable characteristics to be used as PS and has gained prominence in PDIM applications (Silva *et al.*, 2019).

In veterinary medicine, recent studies indicate prime results for treating pathologies such as mastitis and reducing the use of antibiotics and, consequently, residues in milk (Couto *et al.*, 2020). *In vitro* studies by Sellera *et al.* (2016) and dos Anjos *et al.* (2020) verified the efficacy of PDIM in *S. aureus* isolated from bovine

mastitic milk. Moreira *et al.* (2018), Silva *et al.* (2019), and Silva (2020) have shown the efficiency of PDIM in inactivating *in vivo* microorganisms that cause bovine and caprine mastitis.

Therefore, aiming to reduce the microbial contamination of milk and prevent mastitis in lactating animals, the objective of the study was to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Safranine-O in *S. aureus* isolated from sheep's milk, as well as the effectiveness of PDIM in reducing contamination of milk and in the formation of biofilm. The application effect of the stimulus-responsive hydrogel based on Safranine-O photoactivated as *post dipping* in sheep was also evaluated.

MATERIALS AND METHODS

Isolation and Genetic Identification of *Staphylococcus aureus*

To obtain the cultures, milk samples from four sheep were diluted in sterile peptone water, sown on Mannitol Salt Agar (MAN) (Kasvi, São José dos Pinhais, Brazil), and incubated at 37 °C for 48 h. The characteristic colonies were transferred to tubes containing Brain Heart Infusion (BHI) broth (Kasvi, São José dos Pinhais, Brazil) and incubated at 37 °C for 24 h. Coagulase, catalase, and Gram tests were carried out, and positive isolates and cocci were selected (Angelidis *et al.*, 2020).

To identify *S. aureus*, through the nuc gene, primers 5'-GCGATTGATGGTGATACGGTT-3' and 5'-AGCC AAGCCTTGACGAACTAAAGC-3' were used (Lúcio *et al.*, 2018). The isolates were also identified through the 3 'terminal region of the Coa gene, using a pair of primers: Coag2, 5'-ACCACAAGGTACTGAATCAACG-3', and Coag 3, 5'-TGCTTTCGATTGTTCGATGC-3' (Cardoso *et al.*, 2013). *S. aureus* was considered an isolate, with a DNA band size 267 bp (*nuc* gene) and 579 bp (*coa* gene).

Analysis of PDIM *In Vitro*

For conducting *in vitro* studies, Safranine-O ([3,7-diamino-2,8-dimethyl-5-phenylphenazine chloride] (Sf) 85% MM= 350.84 g.mol^{-1} - Sigma-Aldrich - São Paulo Brazil) (300 μg/mL) was incorporated into an aqueous copolymeric matrix of Pluronic® F127 nanocarrier (PEO106-PPO70-PEO106, 12 600 g mol^{-1} - Sigma-Aldrich - São Paulo, Brazil) at 4.0% (m/V), pH 7.2. For irradiation, a green LED source (λ= 520 nm) was used with a light dose of 12.9 J/cm^2 (Figure 1A).

Analysis of the Sensitivity of Isolates to Antimicrobials

The isolates were tested for sensitivity to five antimicrobials by the disk diffusion technique being tested Ampicillin 10 μg, Trimethoprim 5 μg, Ciprofloxacin 5 μg, Tetracycline 30 μg, and Ceftiofur 30 μg. For the sensitivity to Sf, the technique of diffusion in wells was used. The plates were illuminated with a green LED source for 30 min and incubated at 37 °C for 24 hours. The diameter of the inhibition halos was measured with a caliper (Silva *et al.*, 2019).

Analysis of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The isolates were grown in BHI Broth (Kasvi, São José dos Pinhais, Brazil) at 37 °C for 24 h, and the cell density was standardized until equivalent turbidity of 0.5 MacFarland solution (Bordignon-Junior *et al.*, 2012). The determination of MIC was performed using the microdilution technique in Mueller Hilton Broth (MHB) (Kasvi - São José dos Pinhais, Brazil), in 96-well plates, with an initial Sf concentration of 150 μg/mL (first well). The dilution serial continued to the last well (1.18 μg/mL) (Souza *et al.*, 2017) as well as the inoculation of 5 μL of standardized crops. The microplates were illuminated for 30 min (clear); other microplates were kept without illumination (in the dark) and then incubated at 37 °C for 24 h (Silva *et al.*, 2019). MBC was determined by the subculture Mueller Hilton Agar (MHA) (Kasvi, São José dos Pinhais, Brazil) by removing 10 μL from each well where there was growth inhibition and a positive control (Campanholi *et al.*, 2020).

Photoinactivation Procedure of Experimentally Contaminated Milk and Stainless Steel

For *in vitro* photoinactivation, suspensions of *S. aureus* were added in two culture media: sterile sheep's milk and MHB, added in 24-well plates and subjected to 4 treatments in a 4x4 factorial design (4 bacterial isolates and 4 treatments), with 4 repetitions by treatment, being: Treatment 1: Control (milk/broth only); Treatment 2: PDIM (milk/broth + photosensitizing agent (Sf) + LED

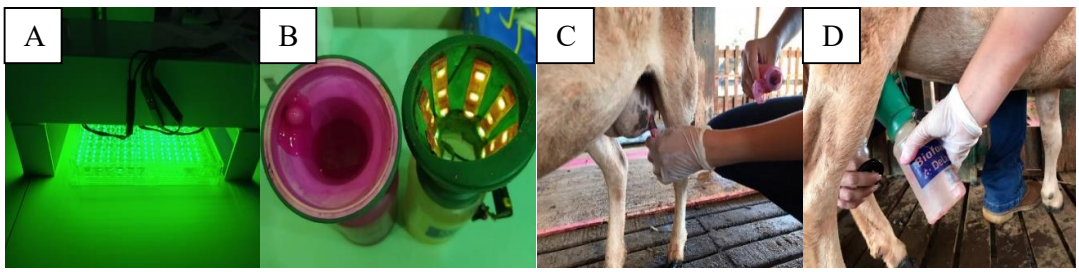

Figure 1. Application of Safranin-O as a photosensitizer in post dipping. A) Green LED light source (λ= 520 nm) 7.2 mW/cm^2; B) Liner for application of the dipping control post (lactic acid Ekomilk post gel film) and liner containing green LED for the irradiation of the Sf; C) Application of hydrogel to sheep; D) LED irradiation.

lighting); Treatment 3: LED (milk/broth + LED lighting); Treatment 4: Photosensitizer (Sf) (milk/broth + photosensitizing agent Sf). The experiment was repeated 4 times in the proportion of 1 mL milk/broth, 100 µL of the bacterial suspension, and 100 µL of 300 µg/mL Sf solution were used. To verify the effect of photoinactivation on the elimination of bacterial biofilms, a stainless-steel coupon (8 mm x 8 mm x 1 mm) was added to each well. Milk samples were evaluated at time 0 and after 48 h at 7 °C, and the stainless-steel coupons were evaluated after 48 h at 7 °C and 35 °C. The number of cells adhered to the stainless-steel coupons was determined according to Dutra *et al.* (2020).

Post Dipping Procedures: *In Vivo* Tests

For the preparation of the Sf and F127 hydrogel (Pluronic®) for *in vivo* studies, the proportions of 20.0% F127 and 0.20% Carbopol (C934P) (Lubrizol Advanced Materials - São Paulo, Brazil) in Peptone water (Himedia Laboratories, Mumbai, India) were used, as described by Silva *et al.* (2020).

The experiment was conducted in the sheep sector (FEI-UEM). The project was approved by the Animal Ethics Committee of the State University of Maringá - CEUA, under process No. 6171171120. In this experiment, 10 Santa Inês sheep were used, with an average weight of 60.5 kg each and an average production of 1.5 liters of milk/day, in lactation phase 2, maintained in a pasture with the predominance of forage Cynodon spp. and supplemented with 0.4% (PV/MS) of corn concentrate.

The experiment was carried out in a completely randomized design, where T1, control treatment, had the application of lactic acid (Ekomilk post gel film, Alto da Pedra Branca, Brazil) as *post dipping*, as well as T2, Sf photoactivated with green LED light illumination (λmax= 520 nm, 12.7 mW cm^{-2}), which was coupled to a conventional plastic liner for one min per ceiling (Figure 1B).

Milk samples were collected at times 0, 3, 6, 9, and 12 days after application of *post dipping*. The samples were sown on Plate Count Agar (PCA) (Kasvi, São José dos Pinhais, Brazil) for the total mesophilic aerobic count and in MAN (Kasvi, São José dos Pinhais, Brazil) for *Staphylococccus* spp count with incubation at 35 °C for 48 h.

Statistical Analysis

The data obtained *in vitro* and *in vivo* were subjected to analysis of variance (ANOVA), and the significant difference between the means ($p<0.05$) was determined using the Tukey test, with the software SAS 9.3 (Statistical Analysis System Institute, Cary, NC).

RESULTS

Sf-Mediated PDIM: *In Vitro* Assays

Twenty strains of *Staphylococcus* were isolated and selected according to preliminary tests. The microorganisms that were amplified in the primers nuc were named SO1, SO2, SO3, and SO4, for the coa gene. The isolates SO1, SO3, and SO4 were positive, and SO2 was a coagulase-negative (CNS) (Figure 2).

The antimicrobial sensitivity of *S. aureus* to different antimicrobials is defined by the Clinical Laboratory Standard Institute (CLSI). Resistance to Ampicillin and Trimethoprim by SO4 was observed. The photoactivated Sf was shown to be as efficient as antibiotics in inhibiting microbial growth (Table 1).

The effectiveness of photoinactivation is related to the photodynamic effects caused by the interaction of light with the PS agent. For the three strains of *S. aureus* (SO1, SO3, and SO4) and SCN (SO2), the Sf without irradiation could not inhibit the growth of microorganisms at the evaluated concentration. A cytotoxic effect was

Table 1. Diameter of halos inhibition (mm) and antimicrobial sensitivity profile of *Staphylococcus aureus* strains and coagulase negative *Staphylococcus* isolated from sheep's milk

Active principle	*Staphylococcus* spp.			
	SO1	SO2	SO3	SO4
Ampicillin	30 (S)	30 (S)	31 (S)	15 (R)
Trimethoprim	30 (S)	30 (S)	31 (S)	15 (R)
Ciprofloxacin	30 (S)	30 (S)	31 (S)	32 (S)
Tetracycline	30 (S)	30 (S)	31 (S)	20 (S)
Ceftiofur	30 (S)	30 (S)	31 (S)	31 (S)
Sf (PDIM)	30 (S)	30 (S)	31 (S)	26 (S)

Note: S= Sensitive; R= Resistant; I= Intermediate; SO2= *Staphylococcus* coagulase negative; SO1, SO3, SO4= three strains of *Staphylococcus aureus.*

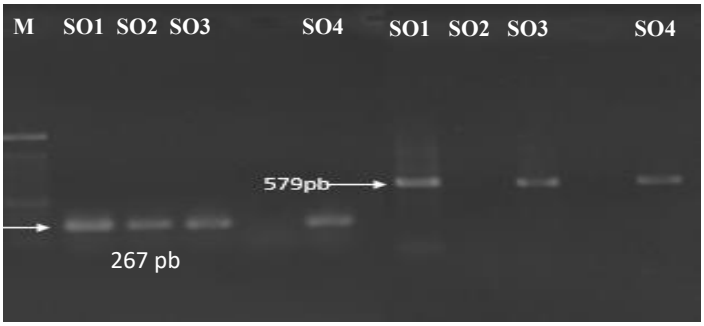

Figure 2. Image of agarose gel electrophoresis of the nuc (267 bp) and coa gene (579 bp) of the different isolates of *Staphylococcus aureus* from sheep's milk. M= 1 Kb Plus DNA Ladder (Invitrogen).

observed when Sf was associated with irradiation with a green LED light source (λ= 520 nm) (Table 2).

Photoinactivation in Milk and Stainless-Steel Coupons

The photoactivated Sf was able to reduce the growth of all strains (SO1; SO2; SO3; SO4) compared to the control treatment (p<0.05). In milk, coagulase-negative Staphylococcus (SO2) was more sensitive to PDIM (treatment 2), with an inhibition rate of 97.33% (2.92 log cfu/mL) (SO2). As for *S. aureus*, the maximum inhibition rate was 50.00% (1.50 log cfu/mL) (SO4) after 30 min of irradiation. *Staphylococcal* counts remained low even after simulating the maximum cooling time and temperature of the milk (48 h at 7 °C) before processing (IN 58 of 2019), which evidences cell death (Table 3). When subjected to LED irradiation (Treatment 3), there was a significant reduction of 57.33% (1.72 log cfu/ mL) only for coagulase-negative *Staphylococcus* (SO2). The FS Safranine-O (Treatment 4) could not inactivate bacterial growth.

When MHB broth was used as the substrate, the SO2 isolate was more sensitive to PDIM, with 99.66% (2.99 log cfu/mL) inhibition; in *S. aureus* the maximum inhibition was 1.42 log cfu/mL (50.53%) (SO1) (Table 4). These results indicate that SCNs are more sensitive to PDIM than coagulase-positive strains of *S. aureus*, which may be associated with the lack of production of the enzyme coagulase, which is one of the virulence factors that protect the microorganism from the action of antimicrobials.

The formation of biofilms by *S. aureus* represents a concern in the food industry for their resistance to sanitizers. For milk, PDIM reduced the adhesion of *S. aureus* by up to 45.92% (SO3) after 48 h of incubation at 7 °C. In contrast, in MHB, PDIM showed greater efficiency in reducing the adhesion of microorganisms, with inhibition of *S. aureus* up to 1.99 log UFC/mL (99.5%) (Table 5).

When the coupons were incubated at 35 °C to simulate the optimal temperature conditions for *Staphylococcus*, there was a reduction in the adhesion of the different strains of *S. aureus* submitted to PDIM (Table 6).

Application of Photo-Activated Safranine-O as *Post Dipping* in Sheep

There was no significant difference between treatments regarding microbiological counts in milk when using photoactivated Sf or the commercial product based on lactic acid. During the period of application of the *post dipping* (12 days), a more noticeable reduction was observed in the microbiological counts of milk of animals treated with lactic acid (p<0.05) (Figure 3).

DISCUSSION

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration and Sensitivity Profile to Antimicrobials

S. aureus can produce important enzymes for the resistance of these microorganisms. Among them are the

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration (mg/mL) for Safranine-O against the strains of *Staphylococcus aureus* and coagulase negative *Staphylococcus*

Sf (mg/mL)	SO1		SO2		SO3		SO4	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
With irradiation	18.75	37.50	4.69	9.38	9.38	9.38	1.18	4.69
Without irradiation	> 150	> 150	> 150	> 150	> 150	> 150	> 150	> 150

Note: Sf= Safranin-O; MIC= minimum inhibitory concentration; MBC= minimum bactericidal concentration; SO2= *Staphylococcus* coagulase negative; SO1, SO3, SO4= three strains of *Staphylococcus aureus.*

Table 3. Count of *Staphylococcus aureus* and coagulase negative *Staphylococcus* (log cfu/mL) in experimentally contaminated milk, subjected to different treatments, at times 0 and 48 hours at 7 °C

Treatments	7 °C – time 0				p value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Control	3.00 ± 0.01^{Aa}	3.00 ± 0.01^{Aa}	3.00 ± 0.01^{ABa}	3.00 ± 0.01^{Aa}	0.000	0.000	0.000	13.39
PDIM	1.50 ± 0.01^{Cb}	0.08 ± 0.021^{Cc}	2.20 ± 0.03^{Ba}	2.45 ± 0.07^{Aa}				
LED	2.35 ± 0.07^{Bb}	1.29 ± 0.01^{Bc}	2.61 ± 0.01^{ABab}	3.00 ± 0.01^{Aab}				
PS	3.00 ± 0.01^{Aa}	2.50 ± 0.01^{Aa}	3.00 ± 0.01^{ABa}	3.00 ± 0.01^{Aa}				
Treatments	7 °C – time 48 hours				p value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Control	3.00 ± 0.01^{Aa}	3.00 ± 0.01^{Aa}	3.00 ± 0.01^{Aa}	3.00 ± 0.01^{Aa}	0.000	0.000	0.000	7.93
PDIM	1.39 ± 0.12^{Cb}	0.01 ± 0.01^{Cc}	2.14 ± 0.13^{Ba}	2.57 ± 0.01^{Aa}				
LED	2.07 ± 0.10^{Bb}	0.50 ± 0.10^{Cc}	2.23 ± 0.04^{Bb}	3.00 ± 0.01^{Aa}				
PS	3.00 ± 0.01^{Aa}	2.00 ± 0.01^{Bb}	3.00 ± 0.01^{Aa}	3.00 ± 0.01^{Aa}				

Note: PDIM= Photodynamic inactivation of microorganisms; LED= light emitting diode; PS= Photosensitizer; SO2= *Staphylococcus* coagulase negative; SO1, SO3, SO4= three strains of *Staphylococcus aureus.* Control (milk only); PDIM (milk + Sf photosensitizing agent + LED lighting), LED (milk + LED lighting); PS (milk + photosensitizing agent Sf). CV= Coefficient of variation. Different capital letters in the same column show different treatments between each other (p<0.05). Different lowercase letters on the same line show different isolates from each other (p<0.05).

Table 4. Count of *Staphylococcus aureus* and coagulase negative *Staphylococcus* (log UFC/mL) in experimentally contaminated broth, submitted to different treatments, at time 0 and after 48 h at 7 °C

Treatments	7 °C - time 0				p value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Control	2.81±0.02^{Aa}	2.70±0.02^{Aa}	2.72±±0.13^{Aa}	2.99±0.06^{Aa}	0.000	0.000	0.003	10.17
PDIM	1.39±0.12^{Ba}	0.01±0.01^{Cb}	1.69±0.12^{Ba}	1.80±0.71^{Ba}				
LED	2.71±0.02^{Aa}	1.51±0.05^{Bb}	2.36±0.40^{Aa}	2.88±0.01^{Aa}				
PS	2.26±0.17^{Ab}	1.74±0.19^{Bb}	2.35±0.07^{Aab}	2.93±0.02^{Aab}				
Treatment	7 °C – time 48 hours				p value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Control	3.01±0.01^{ABa}	2.94±0.02^{Aa}	3.10±0.05^{Aa}	2.96±0.08^{Aa}	0.005	0.000	0.001	11.25
PDIM	1.45±0.21^{Ca}	0.01±0.01^{Cb}	0.70±0.02^{Cb}	0.66±0.91^{Bb}				
LED	2.77±0.16^{ABCa}	1.87±0.04^{Bb}	2.970.08^{Aa}	2.82±0.07^{Aa}				
PS	2.15±0.01^{BCab}	2.64±0.10^{Aab}	1.87±0.12^{Bb}	2.83±0.06^{Aab}				

Note: PDIM= Photodynamic inactivation of microorganisms; LED= light emitting diode; PS= Photosensitizer; SO2= *Staphylococcus* coagulase negative; SO1, SO3, SO4= three strains of *Staphylococcus aureus.* Control (milk only); PDIM (milk + Sf photosensitizing agent + LED lighting), LED (milk + LED lighting); PS (milk + photosensitizing agent Sf). CV= Coefficient of variation. Different capital letters in the same column show different treatments between each other ($p<0.05$). Different lowercase letters on the same line show different isolates from each other ($p<0.05$).

Table 5. Count of *Staphylococcus aureus* and coagulase negative *Staphylococcus* sessile cells (log UFC/cm^2) present in stainless steel coupons grown in milk and Muller Hilton broth incubated at 7 °C for 48 h

Treatments	Milk				p value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Control	3.00±0.01^{Aa}	3.00±0.01^{Aa}	2.57±0.01^{Ab}	3.00±0.01^{Aa}	0.000	0.000	0.000	3.94
PDIM	1.71±0.15^{Cbc}	1.99±0.12^{Ba}	1.39±0.12^{Cc}	1.6±0.213^{Cbc}				
LED	2.70±0.07^{Ba}	2.19±0±0.02^{Bb}	2.00±0.01^{Bb}	2.04±0.19^{Bb}				
PS	3.00±0.00^{Aa}	3.00±0.01^{Aa}	2.00±0.01^{Bb}	3.00±0.01^{Aa}				
	Broth				p value			CV
Control	2.00±0.01^{Aa}	2.00±0.01^{Aa}	2.00±0.01^{Aa}	2.00±0.01^{Aa}	0.017	0.000	0.016	16.97
PDIM	0.01±0.01^{Ba}	0.01±0.01^{Ba}	0.01±0.01^{Ca}	0.35±0.49^{Ba}				
LED	0.50±0.70^{Bb}	1.68±0.19^{Aa}	0.70±0.01^{Bb}	1.47±0.10^{Aa}				
PS	2.00±0.02^{Aa}	2.00±0.02^{Aa}	2.00±0.02^{Aa}	2.00±0.02^{Aa}				

Note: PDIM= Photodynamic inactivation of microorganisms; LED= light emitting diode; PS= Photosensitizer; SO2= *Staphylococcus* coagulase negative; SO1, SO3, SO4= three strains of *Staphylococcus aureus.* Control (milk only); PDIM (milk + Sf photosensitizing agent + LED lighting), LED (milk + LED lighting); PS (milk + photosensitizing agent Sf). CV= Coefficient of variation. Different capital letters in the same column show different treatments between each other ($p<0.05$). Different lowercase letters on the same line show different isolates from each other ($p<0.05$).

Table 6. Count of *Staphylococcus aureus* and coagulase negative *Staphylococcus* sessile cells (log UFC/cm^2) present in stainless steel coupons grown in milk and Muller Hilton broth incubated at 35 °C for 48 h

Treatments	Milk – 35 °C				p value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Control	5.43±0.09^{Aa}	5.00±0.01^{ABb}	4.00±0.01^{Ac}	4.00±0.01^{Ac}	0.000	0.000	0.000	3.52
PDIM	4.64±0.06^{Ba}	4.45±0.31^{Ba}	0.01±0.01^{Cb}	0.01±0.01^{Cb}				
LED	4.62±0.22^{Ba}	4.74±0.07^{ABa}	3.00±0.01^{Bb}	3.24±0.34^{Bb}				
PS	4.60±0.02^{Ba}	4.00±0.01^{Cb}	4.00±0.01^{Ab}	4.00±0.01^{Ab}				
Variable	Broth				p value			CV
	Control	PDIM	LED	PS	Bac	Trat	Bac*Trat	
S. aureus	5.55±0.35^{a}	1.21±1.66^{a}	2.17±1.81^{b}	4.74±0.06^{b}	-	0.000	-	28.45

Note: PDIM= Photodynamic inactivation of microorganisms; LED= light emitting diode; PS= Photosensitizer; SO2= *Staphylococcus* coagulase negative; SO1, SO3, SO4= three strains of *Staphylococcus aureus.* Control (milk only); PDIM (milk + Sf photosensitizing agent + LED lighting), LED (milk + LED lighting); PS (milk + photosensitizing agent Sf). CV= Coefficient of variation. Different capital letters in the same column show different treatments between each other ($p<0.05$). Different lowercase letters on the same line show different isolates from each other ($p<0.05$).

thermostable nucleases (nuc 1 and nuc 2) that hydrolyze the DNA or RNA of the host cells, leading to tissue destruction and dispersion of the host cells. Bacterial cells and coagulase, which leads to fibrin deposition around the microorganism, hinder the microorganism's phagocytosis (Lúcio *et al.*, 2018; Cardoso *et al.*, 2013). In the present study, of the 20 strains evaluated, four (20%) were positive for the nuc gene (SO1, SO2, SO3, and SO4), and three (15%) had the coa gene (SO1, SO3, and SO4).

The spread of resistant *S. aureus* has been reported in several studies, such as Giacinti *et al.* (2017) and Azzi

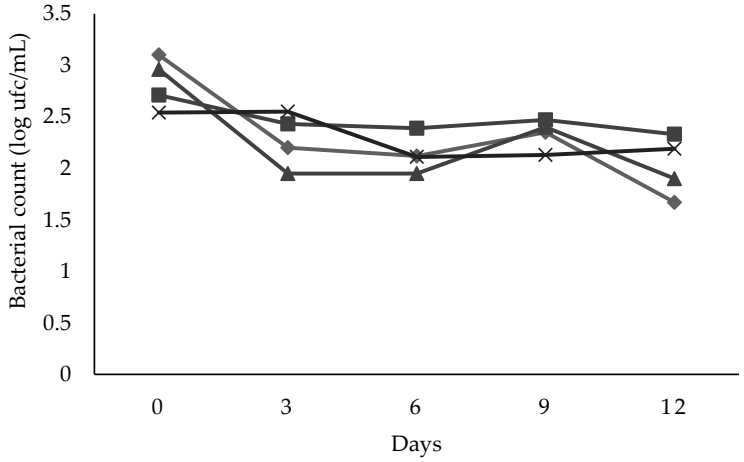

Figure 3. Total bacterial count and *Staphylococcus aureus* count during 12 days of application of the post-dipping: T1= Lactic acid (*S. aureus*: y= 3.102-0.2719x; R2= 68.16%/month total: y= 2, 7505-0.1771x; R2= 36.22%;) and T2= Safranine as post dipping. (*S. aureus*: y= 2.6826-0.0722x; R2= 62.33%/total mesophilic aerobes: y= 2.6358-0.1108x; R2= 63.37%). Staphylo= *S. aureus*. Mesop. Aerobic= total mesophilic aerobes. Staphylo T1= -♦-; Staphylo T2= -■-; Mesop. Aerobic T1= -▲-; Mesop. Aerobic T2= -×-

et al. (2020), who found a high frequency of resistance to Tetracycline, Trimethoprim, Sulfonamides, and Sulfamethoxazole.

The lower resistance of the isolates tested in the present study may be related to the extensive system of young lambs at foot, which reduces the accumulation of milk on the ceilings and, consequently, the risk of mastitis and antibiotic treatment (Table 1). These data corroborate those verified by da Silva *et al.* (2010), in which 100% of the strains of *S. aureus* isolated from sheep's milk were sensitive to the tested antibiotics due to their lesser use.

Sf was shown to be efficient in inhibiting microbial growth (Table 2). This effect can be attributed to the amphiphilic character of Sf, which acts on the lipid layer of the cell membrane or by its dissolution in the cytoplasm (Silva *et al.*, 2019). Thus, small concentrations of this PS (1.18 to 18.75 mg/mL) could inhibit the growth of different strains of *S. aureus*, and concentrations of 4.69 to 37.50 mg/mL led to cell death (Table 2).

Photoinactivation of Milk and Stainless-Steel Coupons

Staphylococcus aureus is an important pathogen associated with diseases in humans and animals that respond to PDIM in a multifactorial and strain-dependent manner. These microorganisms have a variety of virulence factors, such as hemolysin exoproteins, nucleases, and proteases, which facilitate host cell lysis and binding proteins necessary for the colonization of host tissues. According to Grinholc *et al.* (2013), the expression of virulence factors is expressed and regulated by the *quorum-sensing* mechanism by the accessory gene regulator (*agr*), and although the a*gr* locus is conserved among staphylococcal species, it consists of a polymorphic fragment that influences the susceptibility of *S. aureus* to PDIM induced oxidative mechanisms.

Studies using LEDs for microbial inactivation to replace heat treatment have gained prominence. Srimagal *et al.* (2016) and Anjos *et al.* (2020) found that the blue LED efficiently inactivates *Escherichia coli, S. aureus, Pseudomonas aeruginosa, Salmonella Typhimurium,* and *Mycobacterium fortuitum* isolated from bovine milk. The authors attributed this antimicrobial effect to the photosensitizing metabolites present in bacterial cells, such as porphyrins, flavins, cytochromes, and NADH, which absorb light at wavelengths ranging from 400 to 480 nm, resulting in the release of reactive oxygen species that degrade the cellular components.

Photosensitizer are critical elements for photoinactivation efficiency, and desirable characteristics such as low toxicity in the dark, high selectivity, and high yield in the generation of reactive oxygen species are essential (Yin & Hamblin, 2015).

In this context, photosensitizing compounds that are active in contaminating microorganisms from milk started to be studied. Sellera *et al.* (2016) and Galstyan & Dobrindt (2019) evaluated the effect of methylene blue PS irradiated with red LED on *Streptococcus dysgalactiae, Corynebacterium bovis, Staphylococcus aureus, Streptococcus agalactiae, Staphylococcus hominis, Staphylococcus warneri,* and *Escherichia coli,* and verified a significant decrease in viability.

Silva *et al.* (2019), when studying the potential of Sf as a photoactive compound, found that 24% of excited Sf molecules can generate singlet oxygen ($^1O_2$), which reacts with almost all cellular components, leading to cell unfeasibility.

However, even with low production of $^1O_2$ when compared to other photosensitizers, the photoactivated Sf was efficient in inhibiting the growth of *S. aureus* and SCN in experimentally contaminated milk (Table 3), with inhibitions that varied from 0.55 log (18.33 % - SO4) to 2.92 log (93.33% - SO2). In stainless steel coupons grown in milk, the maximum growth inhibition was 1.18 log (45.91% - SO3).

For Galstyan & Dobrindt (2019), the dispersion of light, mainly by fat globules, milk casein micelles and

the interaction of milk components with the PS, reduce the absorption of light by the PS or decrease its availability, diminishing its effectiveness, which explains why in MHB experimentally contaminated with staph strains, as well as in stainless steel coupons grown in MHB, PDIM were more efficient in reducing the formation of biofilms, with inactivation ranging from 82.5% (SO4) to 99.5% (SO1, SO2, SO3), if compared with coupons grown in milk.

In general, PDIM has been shown to be an efficient alternative to replace or complement heat treatments in the food industry to reduce microbial contamination and health problems caused by food poisoning and the resistance of microorganisms to antimicrobials when applied *in vitro*.

Application of Photo-Activated Safranine-O as *Post Dipping* in Sheep

Using antibacterial agents as *post dipping* is essential to form a protective barrier, which prevents the entry and colonization of pathogenic microorganisms in the mammary gland. In recent years, nanotechnology has been highly explored in the pharmaceutical industry, with nanotransporters capable of encapsulating and transporting drugs to target tissues without affecting healthy tissues (Thakor & Gambhir, 2013). Among these nanotransporters, Pluronic® F127 stands out due to its property of forming gel *in situ* depending on its concentration and temperature, since concentrations of 20%-40% of F127 can alternate from a liquid phase to a solid gel (Moore *et al*., 2000; You & Van, 2010).

Silva *et al.* (2019), when evaluating the incorporation of Sf in Pluronic® F127 obtained a hydrogel, which when applied as a dipping powder in goats, assumed the texture of a viscous gel with high adherence capacity and which was effective in the treatment of mastitis, thus reducing the microorganism count (Silva *et al*., 2020). In the present study, there was no significant difference in the count of microorganisms in sheep's milk after the *post dipping* application of the Sf hydrogel followed by PDIM in comparison with lactic acid (Figure 3), therefore indicating its efficiency in maintaining the health of the mammary gland and avoiding the appearance of new mastitis.

When Moreira *et al.* (2018) evaluated the effect of the blue PS of toluidine irradiated with red LED (λ= 635 nm) applied intramammary in cows with subclinical mastitis, they observed a reduction in the count of *Streptococcus dysgalactiae* and coagulase-negative *Staphylococcus* after 12 h of application.

Studies on the application of PDIM in veterinary clinics are scarce. Still, the results are promising, so the search for therapeutic protocols that aim to reduce the use of antibiotics and improve the quality of milk and animal health is necessary.

CONCLUSION

PDIM mediated by Sf and irradiation with green LED light (λ= 520 nm) promoted the reduction of *Staphylococcus aureus* contamination in experimentally contaminated milk and Mueller Hilton broth, as well as reduced microbial adhesion in stainless steel coupons. When applied *in vivo*, PDIM has shown to be similar to commercial lactic acid in bacterial counts of milk.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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