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Safranin-O-Mediated Photodynamic Inactivation of Microorganisms Reduces Milk Contaminating Bacteria Count and Prevented New Cases of Mastitis

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ABSTRACT

The objectives of this study were to evaluate the antimicrobial capacity of the photosensitizer (PS) safranin-O (Sf), through antimicrobial photodynamic therapy (PDTa), against strains of *Pseudomonas fluorescens* (PV1, PVh, and PC3) and *Staphylococcus aureus* (SV1, SV3, SV5, SC5, SC6, and SC8) isolated from milk from cows and goats and the biofilm formation on equipment. To evaluate the effectiveness of the formulation, studies were carried out through in situ applications of the Sf hydrogel as post-dipping in lactating cows and goats. Antibiogram tests showed the resistance of *Staphylococcus aureus* isolates to ampicillin, trimethoprim, and tetracycline. In the *in vitro* assays in bovine milk, the PDTa reduced the *P. fluorescens* (PV1) counts by 17.4% ($p<0.05$). In goat milk, the PDTa reduced the growth of *S. aureus* by up to 24.43% (SC8) ($p<0.05$), and for *P. fluorescens* (PC3), the inactivation was 50.25%. In biofilm formation, there were reduction of 66.66% (SV1) and 38.27% (SC5) in the adhesion of *S. aureus* to stainless steel coupons. For *in situ* applications, photoactivated Sf maintained bacterial counts in bovine milk similar to the control treatment (lactic acid). During the experimental period (28 days), there were reductions of 23.23% and 28.85% in staphylococci and *Pseudomonas* spp. in cow's milk and 76.13% for *Pseudomonas* spp. in goat milk ($p<0.05$). There was no significant difference in goat milk's somatic cell count (CCS). These results indicate that photoactivated Sf can help reduce milk contamination and maintain the health of the mammary gland.

Keywords: milk quality; photodynamic therapy; photosensitizer; safranin-o

INTRODUCTION

Among the pathogens in milk, *Staphylococcus aureus (S. aureus)* stands out, a bacterium that spreads through the milking system and is associated with mastitis, which causes a drop in milk production, increased drug costs and even disposal of animals (Ren *et al.,* 2020). As a deteriorating microorganism, *Pseudomonas fluorescens (P. fluorescens)*, considered the biggest obstacle in dairy products, are the main producers of thermo-resistant proteolytic and lipolytic enzymes that change the organoleptic characteristics of milk and reduce the shelf life of dairy products (Lampugnani *et al.,* 2019).

It is also reported in the literature high biofilm formation capacity by *P. fluorescens* and *S. aureus* in processing environments and resistance to sodium hypochlorite, this resistance being one of the biggest challenges of the dairy industry (Martin *et al.,* 2016; Wang *et al.,* 2018).

In the milking routine, the application of post-dipping (sepsis of teats of lactating animals after milking with products based on iodine, lactic acid, and chlorine) is one of the most common and efficient preventive practices to reduce the contamination of the mammary gland, however, when mastitis is established, curative treatment consists of applying antimicrobials (Das *et al.,* 2017). Given the adaptive capacity of microorganisms, the development of new conventional antimicrobials will not solve this problem, requiring alternative techniques (Tünger *et al.,* 2000).

In this context, Antimicrobial Photodynamic Therapy (PDTa) is an efficient option for treating local infections in animals and also appears as a promising alternative for the removal of biofilms and aid in milk quality control (Sellera *et al.,* 2016). Furthermore, *in situ* studies indicate the effectiveness of PDTa in post-dipping procedures aiming at the prevention of bovine mastitis (Silva Junior *et al.,* 2019). PDT is a therapeutic modality that consists of the excitation of a PS by light at an appropriate wavelength, which interacts with cellular oxygen, resulting in the formation of reactive oxygen species (ROS) whose target site is the wall cells of microorganisms (Silva Junior *et al.,* 2019).

Thus, due to the recurrence of mastitis in lactating animals, the objectives of this study were to evaluate the antimicrobial capacity of the PS Sf, through PDTa,

against strains of *P. fluorescens* (PV1, PVh, and PC3) and *S. aureus* (SV1, SV3, SV5, SC5, SC6, and SC8) isolated from milk from cows and goats and the biofilm formation on equipment. To evaluate the effectiveness of the formulation, studies were carried out through in situ applications of the Sf hydrogel as post-dipping in lactating cows and goats.

MATERIALS AND METHODS

To obtain *S. aureus and P. fluorescens* isolates, milk samples from 10 cows and 10 goats were diluted in sterile peptone water, seeded in Agar Sal Mannitol (Kasvi – São José dos Pinhais, Brazil) and in Pseudomonas Agar Base (Acumedia – Neogen, Lansing, USA) and incubated at 37 °C and 28 °C for 48 h, respectively.

To identify *P. fluorescens* by Polymerase chain reaction (PCR), the amplification of the specific region of the 16S gene was performed using the 16SPSEfluF and 16SPSER primer set (16SPSEfluF 5′-TGCATTCAAAACTGACTG-3′; 16SPSER 5′-AAT CACACCGTGGTAACCG-3′), and considered as *P. fluorescens* the isolates that presented an ADN band with 850 base pairs (bp) (Scarpellini *et al.*, 2004). To identify *S. aureus*, through the nuc gene, the primers 5′-GCGATTGATGGTGATACGGTT-3′ and 5′-AGCCAAGCCTTGACGAACTAAAGC-3′ were used (Brakstad *et al.*, 1992). The isolates were also identified through the 3′ terminal region of the coa gene, using a pair of primers: Coag2, 5′-ACCACAAGGTACTGAATCAACG-3′, Coag3, 5′-TGCTTTCGATTGTTCGATGC-3′. Isolates that presented an ADN band with a size of 267 bp (nuc gene) and 579 bp (coa gene) were considered as *S. aureus* (da Silva & da Silva, 2005).

As PS compound, Sf was used ([3,7-diamino-2,8-dimethyl-5-phenylphenazine chloride] 85% MM = 350.84 g.mol-1 - Sigma-Aldrich – São Paulo, Brazil). To obtain the concentration of 300 μg/mL of Sf, PS was incorporated into an aqueous copolymer matrix of Pluronic® (F127) (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 was used (λ=520 nm) with a light dose of 12.9 J/cm^2 (Figure 1A).

The isolated strains of *S. aureus* were subjected to antibiogram tests for sensitivity to five antimicrobials commonly used for mastitis treatment (Bauer *et al.*, 1996). The well diffusion technique (Silva Junior *et al.*, 2019) was used for sensitivity to an aqueous Sf solution, with irradiation for 30 minutes.

The minimal inhibitory concentration (MIC) was determined using the microdilution technique in Mueller Hilton Broth (CMH) (Kasvi - São José dos Pinhais, Brazil), in 96-well plates, with an initial concentration of 150 μg/mL aqueous Sf solution (first well), continuing the serial dilution until the last well (1.18 μg/mL) and inoculation of 5 μL of the activated *S. aureus* and *P. fluorescens* cultures and standardized at 1 x 10^8 cfu/mL. The microplates were illuminated for 30 minutes (light), and other microplates were kept unlit (dark) and then incubated at 37 °C and 28 °C for 24 h. Minimum bactericidal concentration (MBC) was determined by subculture in Agar Mueller Hilton (AMH) (Kasvi – São José dos Pinhais, Brazil), removing 10 μL from each well where there was growth inhibition and positive control (Silva Junior *et al.*, 2019).

For *in vitro* photoinactivation, suspensions of *S. aureus* and *P. fluorescens* were added to two culture media: sterile milk (from cow or goat) and CMH, added in 24-well plates and subjected to four treatments in a factorial scheme, with four repetitions per treatment: Treatment 1: Control (milk or broth); Treatment 2: PDTa (milk or broth + PS (Sf) + LED lighting); Treatment 3: LED (milk or broth + LED lighting); Treatment 4: PS (Sf) (milk or broth + PS Sf).

The proportion of 1 mL milk or broth, 100 μL of bacterial suspension and 100 μL of 300 μg/mL Sf solution and irradiation for 30 minutes was used (Figure 1A). To verify the effect of photoinactivation on bacterial biofilm removal, in each well, a stainless-steel coupon (AISI 304, simulating the surface of equipment used in the dairy industry) (8 mm x 8 mm x 1 mm) was added (Dutra *et al.*, 2020).

For the preparation of hydrogel of Sf and F127 (Pluronic®) for *in situ* studies, the proportions of 20.0% F127 and 0.20% Carbopol (C934P) and Sf concentration of 1 mmol L^{-1} (Lubrizol Advanced) – São Paulo, Brazil, Peptone water (Himedia Laboratories, Mumbai, India)

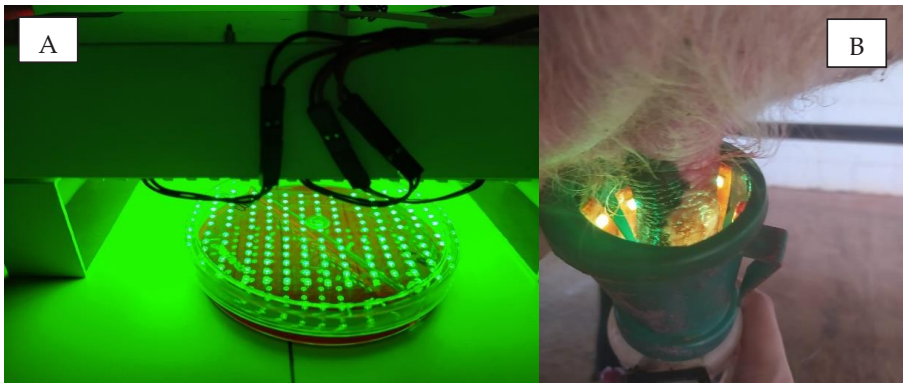

Figure 1. A) Green LED source (λ=520 nm) with a light dose of 12.9 J/cm^2 for *in vitro* antimicrobial photodynamic therapy. B) Green LED light source (λmax = 520 nm, 12.7 mW/cm^2) coupled to a conventional plastic teat for application of antimicrobial photodynamic therapy *in vivo*.

F127 and C934P were dispersed in 25 mL of distilled water and stored under cooling for 24 hours. Sf was added to the dispersion and solubilized in an ice bath. The hydrogel obtained was transferred to an amber bottle with a lid having the pH adjusted to 7.0 with triethanolamine (TEA) and stored under refrigeration (4 °C) (Silva Junior *et al*., 2020).

Ten Holstein (7 heads) and Jersey (3 heads) cows were used, with an average weight of 500 kg and average production of 20 L/day, in different stages of lactation (beginning, peak, and end), kept in pasture and supplemented with corn silage and concentrate corn grain. Ten goats of the Saanen (7 heads) and Boer (3 heads) breeds, with an average weight of 55 kg and an average production of 3.0 L/day at the peak of lactation (average of 70 days), kept in a confinement system and fed with silage of corn as roughage, and corn grain concentrate, soy bran, and vitamin-mineral supplement. The experiment was carried out in a completely randomized design, T1: control treatment – application of lactic acid (Ekomilk after gel film, Alto da Pedra Branca, Brazil) and T2: application of photoactive Sf hydrogel. The animals' teats were irradiated with green LED lighting (λmax = 520 nm, 12.7 mW cm – 2) coupled to a conventional plastic teat cup for one minute per teat) (Figure 1B).

The studies were approved by the Committee on Ethics in the Use of Animals (CEUA) for cows (nº 1287230920) and goats (nº 6266030920). For each animal, the sample consisted of four-quarters of the udder. Milk samples (100 mL) were collected at 0, 7, 14, 21, and 28 days of product application for the microbiological count, somatic cell count (CCS) (Bentley Somacount FCM), and physicochemical composition (Ekomilk). The samples were sowed in Plate Count Agar (PCA) (Kasvi – São José dos Pinhais, Brazil), Agar Sal Mannitol (Kasvi – São José dos Pinhais, Brazil) and *Pseudomonas* Agar Base (Acumedia – Neogen, Lansing, USA).

Data obtained *in vitro* (factorial scheme) and *in situ* (cross over) were submitted to analysis of variance (ANOVA), and the significant difference between means ($p<0.05$) was determined by Tukey test using SAS 9.3 software (Statistical Analysis System Institute, Cary, NC).

RESULTS

After genetic identification, the isolates of bovine *S. aureus* that amplified the nuc and coa genes were named SV1, SV3, and SV5, and the isolates that amplified the specific region for the 16S gene of *P. fluorescens* (16SPSEfluF and 16SPSER) were named PV1 and PVh (Figure 2A). For the strains from goat milk, the isolates SC5, SC6, and SC8 were identified as *S. aureus*, and for *P. fluorescens* the PC3 (Figure 2B).

For isolates from bovine milk, resistance to Ampicillin (SV3 and SV5), Trimethoprim (SV1 and SV5), and Tetracycline (SV3 and SV5), and resistance to these three active principles by all goat isolates (SC5, SC6, and SC8) was observed (CLSI, 2005). The photoactivated Sf showed inhibition halos ranging from 14 mm to 19 mm for bovine *S. aureus* and from 17 mm to 22 mm for *S.*

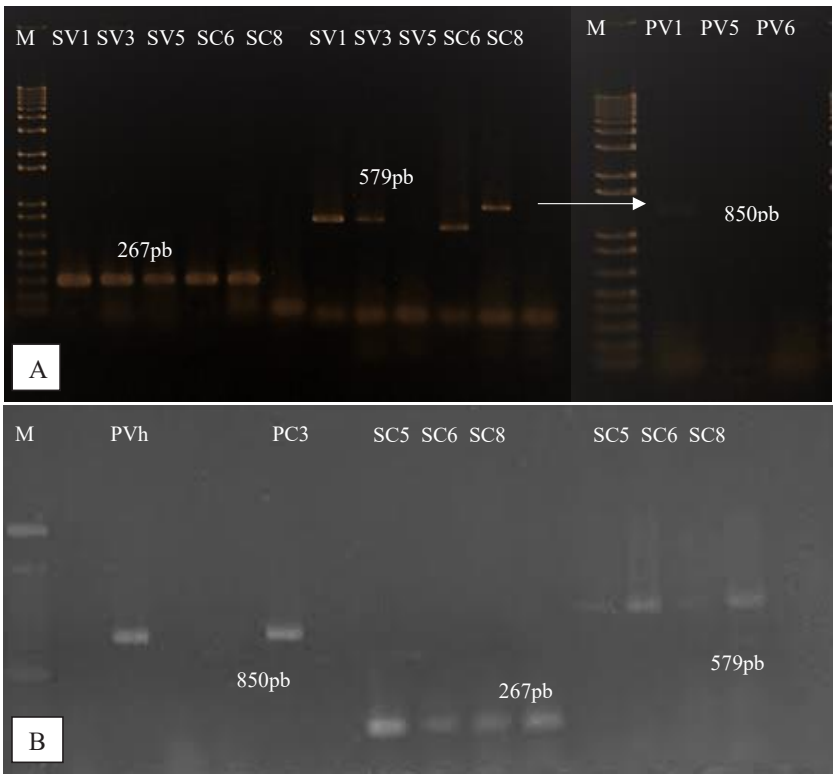

Figure 2. A) Agarose gel electrophoresis image of the nuc gene (267 bp), coa gene (579 bp), and 16S gene of different isolates of *Staphylococcus aureus* and *Pseudomonas fluorescens* from cow's milk. B) Agarose gel electrophoresis image of the nuc gene (267 bp), coa gene (579 bp), and 16S gene of different isolates of *S. aureus* and *P. fluorescens* from goat milk. M= 1Kb plus DNA ladder (Invitrogen).

aureus of goat origin, results superior to inactivation by lactic acid (10 mm to 13 mm), a commonly used product as post-dipping in milking (Table 1).

The bactericidal and bacteriostatic action of Sf was investigated via MIC and MBC. Sf irradiated with a green LED light source (λ= 520 nm) with a light dose of 12.9 J/cm^2, at concentrations ranging from 9.38 to 18.75 mg/mL led to growth inhibition of all isolated. Concentrations of 18.75 mg/mL to 37.50 mg/mL caused cell death. Without irradiation, Sf was not efficient in inhibiting bacterial growth (Table 2).

When the inactivation of microorganisms cultivated in cow's milk was evaluated, for *S. aureus* isolates, there was no significant difference between the control treatment and the photoactivated Sf, with maximum growth inhibition of 18.86% (0.33 log cfu/mL) (SV1). Still, when the substrate was CMH, the inhibition rate of *S. aureus* was up to 90.26% (1.39 log cfu/mL) (SV1) ($p<0.05$). In the formation of biofilms, there was a significant difference for the PDTa when compared to the control treatment ($p<0.05$), with a reduction of up to 66.66% (2.00 log cfu/mL) (SV1) in the adhesion of *S. aureus* cultivated and bovine milk (Table 3). The PDTa reduced the growth of *P. fluorescens* cultivated in cow's milk by up to 17.4% (0.87 log cfu/mL) (PV1) ($p<0.05$), whereas in CMH the inactivation was 23.5% (PV1) (Table 4).

In goat milk, PDTa reduced the growth of *S. aureus* by up to 24.43% (0.54 log cfu/mL) (SC8) when compared to the control treatment ($p<0.05$), in CMH the inhibition of *S. aureus* isolated from goat milk was 84.88% (1.74 log cfu/mL) (SC6). The reduction in *S. aureus* biofilm formation in stainless steel coupons cultivated in goat milk was up to 38.27% (1.24 log cfu/mL) (SC5) ($p<0.05$) (Table 5). For PC3 in milk the inactivation was 50.25% (3.03 log cfu/mL), and in CMH 11.11% (0.25 log cfu/mL) ($p<0.05$) (Table 6). No adhesion of bovine and goat *P. fluorescens* isolates to stainless steel coupons was observed.

Table 1. Diameter of inhibition halos (mm) and antimicrobial sensitivity profile of *Staphylococcus aureus* strains isolated from bovine and goat milk

Active principle	*Staphylococcus aureus* isolated from cow milk		
	SV1	SV3	SV5
Ampicillin	30.00±0.00 (S)	22.00±0.00 (R)	21.67±0.58 (R)
Trimetoprim	20.67±0.58 (R)	26.00±0.00 (S)	21.00±0.00 (R)
Ciprofloxacim	33.00±0.00 (S)	29.33±0.58 (S)	31.67±0.58 (S)
Tetraciclim	30.00±0.00 (S)	24.33±0.58 (R)	19.67±1.15 (R)
Ceftiofur	30.00±0.00 (S)	28.33±0.58 (S)	26.67±2.08 (S)
	Staphylococcus aureus isolated from goat milk		
	SC5	SC6	SC8
Ampicillin	20.00±0.00 (R)	22.00±0.00 (R)	22.00±0.00 (R)
Trimetoprim	21.67±0.58 (R)	22.00±0.00 (R)	22.00±0.00 (R)
Ciprofloxacim	34.00±1.00 (S)	34.67±0.58 (S)	40.67±0.58 (S)
Tetraciclim	20.00±0.00 (R)	20.67±0.58 (R)	20.00±0.00 (R)
Ceftiofur	30.67±0.58 (S)	29.00±0.00 (S)	34.33±0.58 (S)

Note: SV1, SV3, SV5: *Staphylococcus aureus* grant from bovine milk. SC5, SC6, SC8: *Staphylococcus aureus* isolated from goat milk. S: Sensitive, R: Resistant.

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration (µg/mL) of Safranin-O against *Staphylococcus aureus* and *Pseudomonas fluorescens* strains isolated from cow and goat milk

	Treatments			
	PDTa		Sf no irradiation	
	MIC	MBC	MIC	MBC
Bacteria isolated from bovine milk				
SV1	18.75±00.00	37.50±00.00	> 150	> 150
SV3	18.75±00.00	18.75±00.00	> 150	> 150
SV5	18.75±00.00	37.50±00.00	> 150	> 150
PV1	18.75±00.00	37.50±00.00	> 150	> 150
PVh	18.75±00.00	37.50±00.00	> 150	> 150
Bacteria isolated from goat milk				
SC5	18.75±00.00	37.50±00.00	> 150	> 150
SC6	18.75±00.00	37.50±00.00	> 150	> 150
SC8	18.75±00.00	37.50±00.00	> 150	> 150
PC3	18.75±00.00	18.75±00.00	> 150	> 150

Note: SV1, SV3, SV5: *Staphylococcus aureus* isolated from bovine milk. SC5, SC6, SC8: *Staphylococcus aureus* isolated from goat milk. PV1, PVh: *Pseudomonas fluorescens* isolated from control bovine milk (milk/broth only). PC3: *Pseudomonas fluorescens* isolated from goat milk MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; PDTa: Antimicrobial photodynamic therapy.

Table 3. *Staphylococcus aureus* count (log cfu/mL) in experimentally contaminated cow's milk, Mueller Hinton Broth and stainless steel coupons, submitted to different treatments after photoinactivation

Staphylococcus aureus in cow milk							
Treatments	Time			P value			SEM
	SV1	SV3	SV5	Bac	Trat	Bac*Trat	
Control	1.75±0.21^{ABb}	<2.00±0.01^{Aa}	2.13±0.04^{Aa}	0	0.008	0.041	0.023
PDTa	1.42±0.03^{Ba}	<2.00±0.01^{Aa}	1.91±0.03^{Aa}				
LED	1.39±0.12^{Bb}	<2.00±0.01^{Aa}	2.08±0.07^{Aa}				
Sf	1.56±0.02^{ABb}	<2.00±0.01^{Aa}	1.97±0.03^{Aa}				
Staphylococcus aureus Mueller Hinton Broth							
Treatments	Time 0			P value			SEM
	SV1	SV3	SV5	Bac	Trat	Bac*Trat	
Control	1.54±0.11^{Ac}	3.00±0.01^{Aa}	2.00±0.01^{Ab}	0	0	0	0.026
PDTa	0.15±0.21^{Bc}	2.58±0.06^{Ba}	1.00±0.06^{Cb}				
LED	1.60±0.01^{Ac}	3.00±0.01^{Aa}	2.00±0.01^{Ab}				
Sf	1.43±0.05^{Ac}	3.00±0.01^{Aa}	1.69±0.14^{Bb}				
Coupons *Staphylococcus aureus* in cow milk							
Treatments	48 h at 4 °C			P value			SEM
	SV1	SV3	SV5	Bac	Trat	Bac*Trat	
Control	3.00±0.01^{Aa}	2.00±0.01^{Ab}	2.74±0.06^{ABa}	0	0	0	0.034
PDTa	1.00±0.01^{Cb}	2.00±0.01^{Aa}	1.85±0.21^{Ca}				
LED	2.29±0.12^{Bab}	2.00±0.01^{Ab}	2.47±0.26^{ABCab}				
Sf	3.00±0.01^{Aa}	2.00±0.01^{Ab}	2.23±0.26^{BCb}				
Coupons *Staphylococcus aureus* Mueller Hinton Broth							
Treatments	48 h at 4 °C			P value			SEM
	SV1	SV3	SV5	Bac	Trat	Bac*Trat	
Control	1.00±0.01^{A}	1.98±0.18^{A}	1.00±0.01^{A}	0	0.038	0.022	0.082
PDTa	1.00±0.01^{A}	1.00±0.01^{B}	1.00±0.01^{A}				
LED	1.00±0.01^{A}	1.58±0.39^{A}	1.00±0.01^{A}				
Sf	1.00±0.01^{A}	1.58±0.39^{A}	1.00±0.01^{A}				

Note: SV1, SV3, and SV5: *Staphylococcus aureus* isolated from bovine milk. Control (only milk/broth); PDTa (milk/broth + photosensitizing agent) Safranin (Sf) + LED lighting); LED (milk/broth + LED lighting); Sf (Safranina Photosensitizer) (milk/broth + Safranina photosensitizing agent), SEM: Standard error of the mean, ($p<0.05$). Different capital letters in the same column treatments differ from each other. Different lowercase letters on the same row are different from each other.

Table 4. *Pseudomonas fluorescens* count (log cfu/mL) in experimentally contaminated cow's milk and Mueller Hinton Broth, submitted to different treatments after photoinactivation

Pseudomonas fluorescens in cow milk						
Treatments	Time 0		P value			SEM
	PV1	PVh	Bac	Trat	Bac*Trat	
Control	5.00±0.01^{Aa}	4.00±0.01^{Ab}	0	0	0	0.011
PDTa	4.13±0.02^{BCa}	3.58±0.13^{Bb}				
LED	3.94±0.01^{Ca}	3.91±0.12^{Aa}				
Sf	4.30±0.01^{BCa}	4.00±0.01^{Ab}				
Pseudomonas fluorescens in Mueller Hinton Broth						
Treatments	Time 0		P value			SEM
	PV1	PVh	Bac	Trat	Bac*Trat	
Control	5.27±0.32^{Aa}	3.74±0.14^{Ab}	0	0.008	0.015	0.032
PDTa	4.05±0.21^{Ba}	3.71±0.10^{Aa}				
LED	4.71±0.13^{Aa}	3.86±0.31^{Ab}				
Sf	4.80±0.07^{Aa}	4.02±0.04^{Ab}				

Note: PV1, PVh: *Pseudomonas fluorescens* isolated from Control bovine milk (milk/broth only); TFDa (milk/broth + photosensitizing agent) Safranin (Sf) + LED lighting); LED (milk/broth + LED lighting); Sf (Safranine Photosensitizer) (milk/broth + Safranine photosensitizing agent), EPM: Standard error of the mean, ($p<0.05$). Different capital letters in the same column treatments differ from each other. Different lowercase letters on the same row are different from each other.

When applied as post-dipping in cows, photoactivated Sf did not differ from the lactic acid-based control treatment in relation to bacterial counts in milk ($p>0.05$), however, there was a reduction in total mesophilic aerobic counts (12.05% - (0.44 log cfu/mL)), staphylococci (23.23% - (0.72 log cfu/mL)) and *Pseudomonas* (28.85%)

Table 5. *Staphylococcus aureus* count (log cfu/mL) in experimentally contaminated goat milk, Mueller Hinton Broth and stainless steel coupons, submitted to different treatments after photoinactivation

Staphylococcus aureus in goat milk							
Treatments	Time 0			P value			SEM
	SC5	SC6	SC8	Bac	Trat	Bac*Trat	
Control	0.01±0.01^{Ac}	2.07±0.02^{Ab}	2.21±0.01^{Aa}	0	0	0	0.012
PDTa	0.01±0.01^{Ac}	1.88±0.02^{Ba}	1.67±0.06^{Cb}				
LED	0.01±0.01^{Ac}	2.06±0.02^{Aa}	1.97±0.07^{Bb}				
Sf	0.01±0.01^{Abc}	1.99±0.01^{Ab}	2.18±0.01^{Aa}				
Staphylococcus aureus Mueller Hinton Broth							
Treatments	Time 0			P value			SEM
	SC5	SC6	SC8	Bac	Trat	Bac*Trat	
Control	0.01±0.01^{Ab}	2.05±0.01^{Aa}	2.20±0.07^{Aa}	0	0	0	0.068
PDTa	0.01±0.01^{Ab}	0.31±0.42^{Bb}	0.77±0.10^{Ba}				
LED	0.01±0.01^{Ab}	1.93±0.01^{Aa}	2.060.01^{Aa}				
Sf	0.01±0.01^{Ab}	1.77±0.12^{Aa}	2.09±0.03^{Aa}				
Coupons *Staphylococcus aureus* in goat milk							
Treatments	48 h at 4 °C			P value			SEM
	SC5	SC6	SC8	Bac	Trat	Bac*Trat	
Control	3.24±0.06^{Aa}	3.24±0.06^{Aa}	3.24±0.06^{Aa}	0	0	0	0.006
PDTa	2.00±0.01^{Cb}	3.00±0.01^{Ba}	3.00±0.01^{Ba}				
LED	3.00±0.01^{Ba}	3.00±0.01^{Ba}	3.00±0.01^{Ba}				
Sf	3.00±0.01^{Ba}	3.00±0.01^{Ba}	3.00±0.01^{Ba}				
Coupons *Staphylococcus aureus* Mueller Hinton Broth							
Treatments	48 h at 4 °C			P value			SEM
	SC5	SC6	SC8	Bac	Trat	Bac*Trat	
Control	3.86±0.04^{Aa}	2.02±0.23^{Ac}	3.35±0.04^{Ab}	0	0	0	0.026
PDTa	2.00±0.01^{Ca}	1.00±0.01^{Bc}	1.30±0.01^{Cb}				
LED	2.68±0.06^{Bb}	2.02±0.23^{Ac}	3.15±0.06^{Aa}				
Sf	3.00±0.01^{Ba}	2.00±0.01^{Ac}	2.36±0.13^{Bb}				

Note: SC5, SC6, SC8: *Staphylococcus aureus* isolated from goat milk. Control (only milk/broth); PDTa (milk/broth + photosensitizing agent) Safranin (Sf) + LED lighting); LED (milk/broth + LED lighting); Sf (Safranina Photosensitizer) (milk/broth + Safranina photosensitizing agent), EPM: Standard error of the mean, ($p<0.05$). Different capital letters in the same column treatments differ from each other. Different lowercase letters on the same row are different from each other.

Table 6. *Pseudomonas fluorescens* count (log cfu/mL) in experimentally contaminated cow's milk and Mueller Hinton Broth, subjected to different treatments after photoinactivation

Pseudomonas fluorescens in goat milk			
Treatments	Time 0	P value	SEM
	PC3	Trat	
Control	6.03^{a}	0	0.001
PDTa	3.00^{d}		
LED	5.84^{c}		
Sf	5.86^{b}		
Pseudomonas fluorescens in Mueller Hinton Broth			
Treatments	Time 0	P value	SEM
	PC3	Trat	
Control	2.25^{a}	0.006	0.017
PDTa	2.00^{b}		
LED	2.20^{b}		
Sf	2.04^{b}		

Note: PC3: *Pseudomonas fluorescens* isolated from goat milk. Control (only milk/broth); TFDa (milk/broth + photosensitizing agent) Safranin (Sf) + LED lighting); LED (milk/broth + LED lighting); Sf (Safranine Photosensitizer) (milk/broth + Safranine photosensitizing agent), EPM: Standard error of the mean, ($p<0.05$). Different letters in the same column treatments differ from each other.

(0.90 log cfu/mL) during the application period (28 days) ($p<0.05$) (Figure 3). As for CCS, no significant difference was observed between treatments throughout the experimental period ($p>0.05$), with counts of 2.73 log/CCS (537,310 CCS/cm^3) and 2.74 log/CCS (549,500 CCS/cm^3) for the control and photoactivated Sf treatments respectively at the beginning of the experiment (day 0) and 2.46 log/CCS (288,400 CCS/cm^3) for both treatments at the end of the experiment (day 28) (Figure 3).

In goats, there was a reduction in *Pseudomonas* counts throughout the experimental period (76.14%) (0.67 log cfu/mL) ($p<0.05$) (Figure 4). There was no significant difference between treatments ($p>0.05$) for somatic cell count, with initial count (day 0) of 5.45 log/CCS (495,500 CCS/cm^3) and 5.30 log/CCS (367,900 CCS/cm^3) and final count (day 28) of 5.61 log/CCS (754,300 CCS/cm^3) and 5.56 log/CCS (543,000 CCS/cm^3) for the control and photoactivated Sf treatments respectively (Figure 4).

DISCUSSION

When subjected to PDTa, Sf at the concentration studied (300 µg/mL) showed greater efficacy in inhibiting the *in vitro* growth of *S. aureus* when compared to

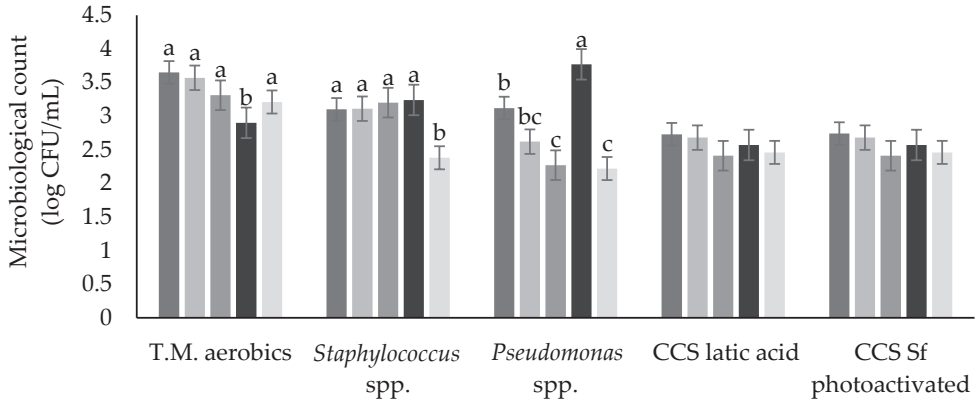

Figure 3. Count of mesophilic aerobics, *Staphylococcus* spp., *Pseudomonas* spp. (log cfu/mL) and CCS (log/cm³) of cow's milk during 28 days of post-dipping application lactic acid and photoactivated Safranin. Total mesophilic aerobics (T.M. aerobics): total mesophilic aerobics count; *Staphylococcus* spp.: *Staphylococcus* spp. count; *Pseudomonas* spp.: *Pseudomonas* spp.; CCS latic acid: somatic cell count treatment 1 (commercial lactic acid); CCS Sf photoactivated: somatic cell count treatment 2 (Sf photoactivated). Different lowercase letters, treatments differ from each other throughout the experimental period. ■ 0 ■ 7 ■ 14 ■ 21 ■ 28.

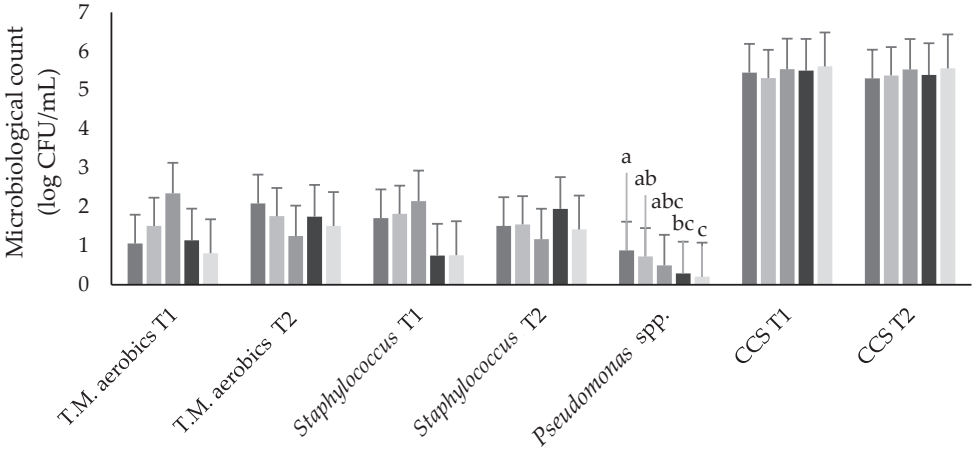

Figure 4. Count of mesophilic aerobics, *Staphylococcus aureus* and *Pseudomonas fluorescens* (log cfu/mL) in goat milk during 28 days of post-dipping application lactic acid and photoactivated Safranin. T.M. Aerobics T1: Total mesophilic aerobic count in animals treated with lactic acid; total mesophilic aerobic count T.M. Aerobics T2: Total mesophilic aerobic count in animals treated with photoactivated Sf hydrogel; *Staphylococcus* T1: *Staphylococcus* spp. in lactic acid treatment; *Staphylococcus* T2: *Staphylococcus* spp. in the hydrogel treatment of photoactivated Sf.; *Pseudomonas* spp.: *Pseudomonas* spp.; CCS T1: somatic cell count treatment 1 (commercial lactic acid); CCS T2: Somatic cell count treatment 2 (photoactivated Sf). Different lowercase letters the treatments differ from each other over the experimental period. ■ 0 ■ 7 ■ 14 ■ 21 ■ 28.

lactic acid. The efficiency in microbial inactivation is due to the amphiphilic character of Sf, which acts in the lipid layer of the cell membrane and the cytoplasm, leading to cell death (Silva Junior *et al.*, 2019).

The use of PDTa is efficient in inactivating several microorganisms isolated from milk. Greater inactivation efficiency has been reported in Gram-positive bacteria. Galstyan & Dobrindt (2019) evaluated the effect of methylene blue PS associated with red LED irradiation (λ>610 nm) and obtained better results in reducing *Staphylococcus* counts when compared to *Escherichia coli*, according to the authors, Gram negative bacteria because they have a complex outer phospholipid membrane, they make Sf absorption difficult. However, in the present study, when compared to the control treatment, Sf was shown to be an effective photoactive compound by inhibiting the growth of Gram-negative bacteria *P. fluorecens* cultivated in milk from cows and goats by up to 17.4% (PV1), and 50.25% (PC).

When Sf-mediated PDTa was applied to *S. aureus* cultivated in bovine and goat milk, there was a reduction of 18.86% (SV1) and 24.24% (SC8), respectively. On the other hand, in reducing the formation of biofilms, considered microbial ecosystems that in dairy industries form on the stainless steel internal surfaces of storage tanks and pipe, reducing the shelf life of products and changing the organoleptic characteristics of milk, Sf has proven effective in reducing by 66.66% (SV1) and 38.27% (SC5) the adhesion of bovine and goat *S. aureus* to stainless steel coupons, respectively. This phototoxic effect is likely because 24% of excited Sf molecules can generate singlet oxygen, which reacts with cellular components leading to cell death (Silva Junior *et al.*, 2019).

Zarei *et al.* (2020) reported that *P. fluorescens* strains are responsible for recontamination after pasteurization due to their ability to form biofilm on polystyrene and stainless-steel surfaces, but in the present study, for the studied *P. fluorescens* isolates, no microbial adhesion was observed on stainless steel coupons.

Light scattering and interaction of milk components with PS can reduce light absorption by PS or decrease its availability, reducing its effectiveness (Galstyan & Dobrindt, 2019), a fact that explains why when the culture medium was the CMH, the inhibition rates reached 90.26% (SV1) and 84.88% (SC8).

In general, the PDTa is an effective alternative for reducing contamination and improving food quality. Because of the concern for the health and welfare of animals and consumers of milk and its derivatives in relation to residues of antibiotics in dairy products, the present study proposed an alternative to the control of mastitis in cows and goats based on reducing the microbial load of the mammary gland. It was observed that there was no significant difference in the total bacterial count of milk between the control treatment (lactic acid) and the photoactivated Sf hydrogel ($p>0.05$). However, over the experimental period (28 days), there was a reduction of 23.23% and 28.85% in staphylococci and *Pseudomonas* spp. in cow's milk and a 76.14% reduction in *Pseudomonas* counts in goat's milk ($p<0.05$), which indicates that photoactivated Sf can help maintain the health of the mammary gland.

The application of PDTa is beneficial, even though it is equivalent to the use of lactic acid and other conventional methods, because to control antibiotic resistance and the microbiological quality of milk, the mechanism of PDIM action acts on multiple targets and guarantees rapid and rapid cell death. Irreversible, preventing the development of resistance (Kashef & Hamblin, 2017).

These data corroborate the studies by Silva Junior *et al.* (2019) and Silva Junior *et al.* (2020), who obtained a reduction in the count of microorganisms in the milk of cows and goats that received Sf incorporated in Pluronic® F127 as post-dipping. According to the authors, using carrier copolymers improves the development of Sf by keeping it in its monomeric state and allowing greater interaction with multidrug resistance, and improving its solubilization, biodistribution, and protection against degradation.

Pluronic® F127 is a nanocarrier capable of transporting drugs to target tissues without affecting healthy tissues; in addition to having the ability to form a gel *in situ*, where at concentrations of 20% to 40%, it can acquire a liquid texture at low temperatures, and form a solid gel at room temperature (You & Van Winkle, 2010). This feature allows the Sf hydrogel to present itself as a viscous gel capable of forming a protective barrier in the teat, preventing the entry of microorganisms through the sphincter.

Another way to assess the health of the mammary gland is through the Somatic Cell Count (CCS), which comprises macrophages, polymorphonuclear cells, lymphocytes, and epithelial cells per mL of milk, which remains constant in a healthy gland. Still, in mastitis cases, there is an increase in the number of these cells due to the migration of defense cells from the blood to the milk (Malik *et al.*, 2018). Silva Junior *et al.* (2019) found that the Sf hydrogel proved to be as efficient as iodine, commonly used as post-dipping, in maintaining the CCS count in bovine milk, a result that corroborates the present study where there was no significant difference between the CCS of cows and goats treated with Sf hydrogel and commercial lactic acid.

CONCLUSION

PDTa can be a promising alternative to reduce milk contamination by *S. aureus* and *P. fluorescens*, helping maintain the quality and health of the mammary gland, and when associated with adequate hygienic-sanitary milking management, brings results as satisfactory as conventional treatments.

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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