UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

INATIVAÇÃO FOTODINÂMICA DE MICRORGANISMOS CONTAMINANTES DO LEITE

Autora: Bruna Moura Rodrigues
Orientadora: Profª Drª Magali Soares dos Santos Pozza
Coorientador: Prof Dr Geraldo Tadeu dos Santos

MARINGÁ
Estado do Paraná
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Tese apresentada, como parte das exigências para obtenção do título de DOUTOR EM ZOOTECNIA, no Programa de Pós-graduação em Zootecnia da Universidade Estadual de Maringá – Área de concentração Produção Animal.

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TITULAÇÃO: Doutor em Zootecnia - Área de concentração Produção Animal

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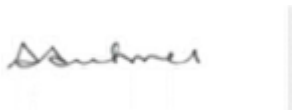

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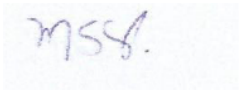

Profª Drª Magali Soares dos Santos Pozza
(Orientadora)

Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente.

Roger Von Oech

A minha família e aos amigos especiais

DEDICO

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BIOGRAFIA

BRUNA MOURA RODRIGUES, filha de Ademir Antônio Rodrigues e Ilza Moura Rodrigues, nasceu em Maringá, Paraná, no dia 16 de agosto de 1989.

Em dezembro de 2013, concluiu o curso de Zootecnia pela Universidade Estadual de Maringá.

Em 2015 trabalhou na empresa Biomax Homeopatia Animal, onde exerceu a função de auxiliar administrativo.

Em 2016 iniciou o mestrado em Zootecnia na área de concentração Produção Animal, e em março de 2018, tornou-se mestre na Universidade Estadual de Maringá, realizando estudos na área de Tecnologia dos Produtos de Origem Animal.

Em março de 2018, iniciou o curso de doutorado no Programa de Pós-graduação em Zootecnia, área de concentração Produção Animal, na Universidade Estadual de Maringá, com estudos na área de Tecnologia dos Produtos de Origem Animal.

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RESUMO

Objetivou-se avaliar a eficácia da Terapia Fotodinâmica antimicrobiana (TFDa) utilizando o fotossensibilizador (FS) Safranina-O (Sf) em leite e cupons de aço inox experimentalmente contaminados com cepas de *Staphylococcus aureus* e *Pseudomonas fluorescens*, isolados de leite bovino, caprino e ovino, assim como a eficiência do uso de um hidrogel termorresponsivo e fotoativo de Sf em aplicações *pós dipping*, visando a redução da contaminação do leite de vacas, cabras e ovelhas. No primeiro estudo, cepas de *S. aureus* (SV1, SV3, SV5, SC5, SC6, SC8) e *P. fluorescens* (PV1, PVh, PC3) foram previamente isoladas de leite bovino e caprino, identificadas geneticamente e submetidas a quatro tratamentos: Tratamento 1: Controle (somente leite/caldo); Tratamento 2: TFDa (leite/caldo + agente fotossensibilizador (Sf) + iluminação com LED); Tratamento 3: LED (leite/caldo + iluminação com LED); Tratamento 4: Fotossensibilizador (Sf) (leite/caldo + agente fotossensibilizador Sf). Para verificar o efeito da fotoinativação na eliminação de biofilmes bacterianos, em cada poço foi adicionado um cupom de aço inox (8 mm x 8 mmx 1 mm). Os ensaios de antibiograma evidenciaram a resistência dos isolados de *S. aureus* frente a Ampicilina, Trimetoprim e Tetraciclina. Nos ensaios *in vitro* em leite bovino, houve redução de 17,4% nas contagens de *P. fluorescens* (PV1) quando comparado ao tratamento controle ($p<0,05$). Em leite caprino, a TFDa reduziu o

crescimento de *Staphylococcus aureus* em até 24,43% (SC8) ($p<0,05$), e para *P. fluorescens* (PC3) a inativação foi de 50,25%. Na formação de biofilmes, houve redução de 66,66% (SV1) e 38,27% (SC5) na adesão de *Staphylococcus aureus* em cupons de aço inox. Para aplicações *in vivo*, a Sf fotoativada manteve as contagens bacterianas no leite bovino semelhantes ao tratamento controle (ácido lático) ($p>0,05$). Ao longo do período experimental (28 dias) houve redução de 23,23% e 28,85% nas contagens de estafilococos e de *Pseudomonas* spp. no leite de vacas, e de 76,13% para *Pseudomonas* spp. no leite de cabras ($p<0,05$). Não houve diferença significativa na contagem de células somáticas (CCS) no leite de vacas e cabras. No segundo estudo, leite e caldo Muller Hinton (CHM) foram experimentalmente contaminados com cepas de *S. aureus* (SO1, SO3, SO4) e uma cepa de *Staphylococcus* coagulase negativo (SO2) previamente isolada e identificada geneticamente através dos genes *nuc* e *coa*. Para verificar o efeito da fotoinativação na eliminação de biofilmes bacterianos, em cada poço foi adicionado um cupom de aço inox (8 mm x 8 mmx 1 mm). Todos os microrganismos se mostraram sensíveis a TFDa. Quando submetidas à análise de resistência a antibióticos, a cepa SO4 foi resistente a Ampicilina e Trimetropim. Quando cultivados em leite e CMH, houve redução nas contagens de estafilococos em até 97,33% e 99,63%, respectivamente. Em cupons de aço inox, a fotoinativação reduziu em até 45,92% (leite) e 99,5% (CMH) a adesão de *S. aureus* ($p<0,05$). A Sf fotoativada foi similar ao uso de ácido lático comercial quando aplicada como *pós dipping*. Estes resultados indicam que a TFDa, mediada por Sf, é eficaz na inativação de microrganismos deteriorantes e patógenos do leite, assim como é eficaz em reduzir a contaminação do leite quando aplicada como pós-*dipping*.

Palavras-chave: biofilme, fotoinativação, mastite, pós-*dipping*

ABSTRACT

This study aimed to evaluate the effectiveness of antimicrobial Photodynamic Therapy (PDTa) using the photosensitizer (PS) Safranin-O (Sf) in milk and stainless-steel coupons experimentally contaminated with *Staphylococcus aureus* and *Pseudomonas fluorescens* isolated from bovine, goat and sheep milk, as well as the efficiency of using a thermo- responsive and photoactive Sf hydrogel in *post dipping* applications, aiming to reduce the contamination of milk from cows, goats and sheep. In the first study, the strains of *S. aureus* (SV1, SV3, SV5, SC5, SC6, SC8) and *P. fluorescens* (PV1, PVh, PC3) were previously isolated from bovine and goat milk, and then genetically identified and subjected to four treatments: Treatment 1: Control (only milk/broth); Treatment 2: PDTa (milk/broth + photosensitizing agent (Sf) + LED lighting); Treatment 3: LED (milk/broth + LED lighting); Treatment 4: Photosensitizer (Sf) (milk/broth + Sf photosensitizing agent). To verify the effect of photoinactivation in eliminating bacterial biofilms, in each well a stainless-steel coupon (8 mm x 8 mm x 1 mm) was added. Antibiogram tests showed the resistance of *S. aureus* isolates to Ampicillin, Trimethoprim and Tetracycline. In *in vitro* assays in bovine milk, there was a

reduction of 17.4% in *P. fluorescens* (PV1) counts when compared to the control treatment ($p<0.05$). In goat milk PDTa reducedthe growth of *Staphylococcus aureus* by up to 24.43% (SC8) ($p<0.05$), and for *P. fluorescens* (PC3) the inactivation was 50.25%. In the formation of biofilms, there was a reduction of 66.66% (SV1) and 38.27% (SC5) in the adhesion of *S. aureus* to stainless steel coupons.For *in vivo* applications, photoactivated Sf maintained bacterial counts in bovine milk similar to the control treatment (lactic acid) ($p>0.05$). During the experimental period (28days) there was a reduction 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 somatic cell count (CCS) in milk from cows and goats. In thesecond study, the Muller Hinton milk and broth (CHM) were experimentally contaminated with strains of *S. aureus* (SO1, SO3, SO4) and a strain of coagulase negative *Staphylococcus* (SO2) was previously isolated and genetically identified through the gene *nuc* and *coa*. To verify the effect of photoinactivation in eliminating bacterial biofilms, in each well a stainless-steel coupon (8 mm x 8 mm x 1 mm) was added. All microorganisms were sensitive to PDTa. When submitted to antibiotic resistance analysis, the SO4 strain was resistant to Ampicillin and Trimethoprim. When cultivated in milk and HCM, there was a reduction in staphylococcal counts of up to 97.33% and 99.63%, respectively. In stainless-steel coupons, photoinactivation reduced up to 45.92% (milk) and 99.5% (CMH) theadhesion of *S. aureus* ($p<0.05$). Photoactivated Sf was similar to the use of commercial lactic acid when applied as a dipping post. These results indicate that Sf- mediated PDTa is effective in inactivating spoilage microorganisms and milk pathogens,as well as being effective in reducing milk contamination when applied as a *post-dipping.*

Key words: biofilm, photoinactivation, mastitis, *post dippin*

I - INTRODUÇÃO GERAL

O leite é considerado um alimento altamente nutritivo, e os parâmetros microbiológicos são fundamentais para garantia de um produto de qualidade. Durante a cadeia de produção, fatores zootécnicos e sanitários, como a saúde da glândula mamária,higiene no processo de ordenha, armazenamento e transporte, influenciam diretamente na qualidade do leite produzido (Vliegher et al., 2012). Dentre as principais causas de perdas da indústria de laticínios destacam-se a produção de enzimas proteolíticas e lipolíticas microbianas (que comprometem as propriedades físico-químicas e sensoriais do produto)(Nornberg et al. 2009), e a mastite (infecção provocada na glândula mamária pela ação de microrganismos patogênicos) (Roberson, 2012).

A Instrução Normativa nº 76 de 2019, ao estabelecer o resfriamento do leite a 4°C no posto de refrigeração e recebimento na indústria com uma temperatura máxima de 7°C, teve como finalidade fornecer um produto de qualidade e seguro ao consumidor (Brasil, 2019). A baixa temperatura inibe a proliferação de microrganismos mesófilos que se multiplicam à temperatura ambiente e são responsáveis pela acidificação do alimento.

Porém, bactérias psicrotróficas estão entre as principais causas da deterioração

físico-química no leite cru. Dentre os principais microrganismos que contaminam o leite destacam-se espécies de *Acinetobacter* spp., *Achromobacter* spp., *Aeromonas* spp., *Alcaligenes* spp., *Bacillus* spp., *Enterobacter* spp., *Flavobacterium* spp., *Pseudomonas* spp., sendo *Pseudomonas fluorescens* a bactéria predominante (Zhang et a. 2019).

As bactérias mesófilas de origem infecciosa, como *Staphylococcus aureus, Staphylococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* e *Escherichia coli*, são consideradas os principais microrganismos causadores de infecçõesintramamárias (Bradley e Green, 2005). Dentre eles, *S. aureus* recebe destaque por ser o microrganismo isolado com maior frequência em casos de mastite e por produzirem enterotoxinas resistentes à pasteurização, e que estão relacionadas a casos de intoxicaçãoalimentar no homem (Cunha e Cunha, 2007).

Além da deterioração dos constituintes do leite e infecção intramamária, *P. fluorescens* e *S. aureus* possuem a capacidade de colonizar a superfície das instalações de processamento de lácteos, formando biofilmes microbianos. A formação de biofilmes pode levar à recontaminação em produtos processados (Millezi et al., 2012), sendo este um dos maiores desafios da indústria em razão da resistência que estes microrganismos desenvolvem quando se aplicam sanitizantes comuns.

Neste contexto, a Terapia Fotodinâmica (TFD) destaca-se como uma modalidade terapêutica promissora no combate a doenças e pode ser aplicada em infecções bacterianas, fúngicas ou virais (Silva-Junior, 2020). Quando aplicada em microrganismos, a TFD é conhecida como Inativação Fotodinâmica de Microrganismos (IFDMO), e a técnica baseia-se na utilização de um composto fotossensível, que quandoativado por irradiação de uma fonte de luz visível em comprimento de onda adequado, gera espécies reativas de oxigênio e induz à morte celular (Dolmans et al., 2003).

Os compostos fotossensibilizadores (FS) devem apresentar algumas características principais para serem empregados em TFD. Dentre essas características destacam-se a baixa toxicidade no escuro, absorção de luz na região visível, e deve apresentar formulação simples, ser reprodutível, apresentar alto rendimento de oxigênio singlete ($^1O_2$), além dobaixo custo (Allisson, 2004).

Estudos recentes, como o de Sellera et al. (2016), e Silva Junior et al. (2019), mostraram eficiência na inativação *in vitro* de *S aureus*, pelo sensibilizador azul de metileno e Safranina-O (Sf), respectivamente. Além disso, Silva Junior et al (2019), em estudo *in vivo,* observaram redução no número de células somáticas no leite quando a Sffoi utilizada em aplicações pós-*dipping* para vacas.

O objetivo deste trabalho foi o de isolar cepas de *S. aureus* e *P.fluorescens*, do leite de vacas, cabras e ovelhas, e determinar a concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) da Sf frente a estes microrganismos, assim como determinar os potenciais de formação de biofilmes destas bactérias em cupons de inox e posterior fotoinativação, e avaliar a IFDMO na redução da contaminação do leite e dos tetos dos animais por patógenos deteriorantes e causadores de mastite caprina, bovina e ovina, visto que o emprego de corantes fotossensibilizadores na IFDMO mostram resultados satisfatórios *in vitro* no controle de infecções microbianas, e há escassez de estudos *in vivo*.

1. Revisão bibliográfica

1.1. Qualidade do leite

De acordo com a definição do Ministério da Pecuária e Abastecimento (MAPA), leite é um "produto oriundo da ordenha completa e ininterrupta, em condições de higiene de vacas sadias, bem alimentadas e descansadas", e quando oriundo de outras espécies como cabras e ovelhas, deve receber a sua identificação (Brasil, 2011).

A qualidade do leite está associada a parâmetros físico-químicos, composição inalterada em seus constituintes, livre de sabores e odores não característicos, adequados padrões higiênicos-sanitários, reduzida contagem bacteriana total (CBT), baixa contagem de células somáticas (CCS), além da ausência de água e outros adulterantes (Murphy et al., 2016).

Para garantir as características físico-químicas, a Instrução Normativa n° 76 de 2019 preconiza que a composição do leite de vaca tenha aproximadamente 3,2 % de proteínas, 3,6% de lipídeos, 4,7% de lactose e água (Brasil, 2019). A composição físico-química do leite pode variar de acordo com a espécie de que ele proceda, como exemplo o leite de cabra que é composto por 3,4% de proteínas, 3,8% de lipídeos e 4,1% de lactose (Park et al., 2007). O leite de cabra contém uma fração de caseína composta principalmente por α-S2 caseína e β-caseína, podendo ser consumido por indivíduos que apresentem alergia ao leite de vaca, em que a predominância é de α-S1 caseína, que é a proteína responsável, na maioria das vezes, por gerar resposta alérgica nos consumidores (Tomotake et al., 2006). O leite de ovelha é composto por aproximadamente 7,6% de gordura, 5,6% de proteína e 5,3% de lactose, 4,7% de lactose, sendo que é muito apreciado em vários países para produção de queijos, além de sua qualidade ser fundamental para a produção de cordeiros destinados ao corte (Ribeiro et al. 2007).

Já para os padrões microbiológicos, a CBT por contagem padrão em placas (CPP) e a CCS são fundamentais. A maioria dos contaminantes patogênicos e deteriorantes do leite é considerada microrganismos mesófilos, ou seja, bactérias capazes de se multiplicarem em uma faixa de temperatura compreendida entre 20 e 45°C, com temperatura ótima de 32 °C, e a Contagem Padrão em Placas (CPP) é um procedimento padrão que representa um ótimo indicador de avaliação das condições higiênico sanitárias em que o leite foi obtido. Estes tipos de bactérias crescem predominantemente onde não

há higienização eficiente e ausência da refrigeração adequada do leite (Teixeira et al. 2000).

Já as células somáticas (CS) que são constituídas por leucócitos (neutrófilos, linfócitos e macrófagos) e células epiteliais são indicativos de infecção intramamária, pois em quadros infecciosos, elas migram do sangue para a glândula mamária. Nos quartos mamários sadios os macrófagos são predominantes (66 – 68%) enquanto os neutrófilos representam de 1 a 11%, porém durante quadros infecciosos, os neutrófilos podem compor até 90% das células somáticas no leite (Pyorala, 2003).

Com isto, a Instrução Normativa (IN) n° 76 de 2019 estabeleceu o limite máximo para a contagem de células somáticas (CCS) de 500.000 CS/mL e contagem padrão em placas (CPP) de 300.000 UFC/mL para garantir a inocuidade do leite de vaca (Brasil, 2019). Para cabras, segundo a IN n° 37 de 2000, admite-se CPP de até 500.000 UFC/mL(Brasil, 2000), e não há limite máximo estabelecido para CCS, porém sabe-se que o valoré maior do que para vacas, e contagens a partir de 10^6 células/mL tem sido usada como base para a detecção de mastite (Paes et al. 2003). Já para o leite de ovelha, a legislação brasileira não estipula valores de CCS e CBT, seguindo então os valores descritos para cabras (Paape et al. 2007).

Além das condições higiênico-sanitárias adequadas, outro fator limitante para a conservação da qualidade do leite é o binômio tempo x temperatura de refrigeração do leite pós-ordenha até o recebimento no laticínio. De acordo com a IN n° 76 de 2019, o leite na propriedade rural deve ser armazenado a 4°C por um período máximo de 48 horas, e chegar na plataforma de laticínio com no máximo 7°C (Brasil, 2019). Processo que reduziu perdas econômicas pela acidificação provocada pelas bactérias mesófilas, porém passou a favorecer o desenvolvimento de microrganismos psicrotróficos, que mesmo em temperatura abaixo de 7 °C, encontram ambiente favorável para multiplicação. Segundo Lafarge et al. (2004), a população destes microrganismos pode aumentar em até 50% após 24 horas de armazenamento, e 90% com 48 horas.

Neste contexto, *Pseudomonas fluorescens* e *Staphylococcus aureus* são considerados os principais microrganismos deteriorantes e patogênicos do leite, respectivamente, pois além de reduzir a qualidade do produto, acarreta perdas

econômicas e coloca em risco a saúde dos consumidores; são capazes de produzir biofilmes microbianos que podem contaminar novamente o produto após processamento(Alves, et al. 2018; Abebe et al. 2016, Abdeen et al. 2020).

Então, devem ser evitados fatores que prejudicam a qualidade como doenças no rebanho, falta de higiene durante a ordenha, higienização ineficiente em equipamentos e utensílios de ordenha, armazenamento e transporte em temperatura e inadequada higienização em superfícies de processamento, para garantir a qualidade do produto que sai da fazenda e chega ao consumidor (Santos e Fonseca 2007).

1.2. *Pseudomonas fluorescens*

Pseudomonas fluorescens são bactérias pertencentes à família *Pseudomonadaceae,* são microrganismos aeróbios em forma de bastonetes, Gram negativos, apresentam crescimento em pH neutro, com temperatura ótima de crescimento entre 25 a 30°C, porém capazes de proliferar-se em temperaturas abaixo de 7°C, não resistentes à pasteurização, produtoras de enzimas proteolíticas e lipolíticas que comprometem as propriedades físico-químicas e sensoriais do produto final (Nornberg et al. 2009).

Logo após a ordenha, as bactérias psicrotróficas representam uma pequena fração da população bacteriana de leite cru, cuja composição microbiana está relacionada ao estadode saúde dos animais e às condições de higiene durante a ordenha, e depois, sua prevalênciaé influenciada pelas condições de transporte e armazenamento do leite (Vithanage et al.,2016).

Segundo Jay et al. (2011) e Pinto et al. (2013), a adaptação de *Pseudomonas* ao frio,está associada à quantidade de lipídeos insaturados (entre 59 a 72%) presentes na membrana celular, que provoca redução do ponto de fusão, com consequente manutençãoda membrana, a estrutura química de suas enzimas e a alta atividade específica em baixastemperaturas, assim como a síntese de Proteínas do Choque Frio (CSPs), que sãoproteínas que se ligam aos ácidos nucleicos de fita única, como mRNA, e permitem a transcrição e proteção dos componentes celulares de alterações relacionadas ao frio.

A atividade proteolítica e lipolítica em alimentos está associada à atividade de microrganismos Gram negativos e não fermentadores de glicose, sendo *Pseudomonas fluorescens* a espécie de maior importância (Marchand et al., 2008).

As proteases produzidas por *P. fluorescens* são as metaloenzimas, que dependem deíons divalente, como o Ca^{2+}, para sua atividade e estabilidade e são codificadas pelo gene*aprX*, e hidrolisam todos os tipos de caseína, com preferência pela κ-caseína por sua maiorexposição nas micelas (Matèos et al., 2015; Glück et al., 2016).

A hidrólise da κ-caseína causa desestabilização das micelas de caseína do leite, resultando em coagulação e formação de gel, comprometendo o consumo do mesmo, esta hidrólise promove também a produção de pequenos peptídeos hidrossolúveis, que são liberados no soro ao invés de formarem o coágulo, comprometendo o rendimento na produção de queijos (Nielsen, 2002).

Colantuono et al. (2020), ao avaliarem a proteólise de diferentes cepas de *P. fluorescens*, em diferentes substratos de leite (leite pasteurizado, leite UHT e leite em póreconstituído), observaram que o fator que afeta o crescimento microbiano é a temperatura e que a extensão da proteólise depende não somente da cepa como também da composição dos substratos.

Já as lipases microbianas, codificadas pelo gene *lipA*, cuja principal lipase é a lecitinase (representada pela fosfolipase C), são capazes de hidrolisar triglicerídeos (quecompõem de 96 a 98% da gordura do leite), ésteres sintéticos, monoglicerídeos efosfolipídeos (Ordóñez-Pereda et al., 2005). Assim, a lipólise se dá pela hidrólise enzimática dos lipídeos no leite, liberando moléculas de glicerol, ácido graxos de cadeiacurta (C-4 a C-8), que conferem sabor de ranço aos produtos lácteos, e ácidos graxos de cadeia longa (C-10 a C-12), que produzem sabor e odor de sabão (Chen et al. 2003).

1.3. *Staphylococcus aureus*

Staphylococcus aureus é uma bactéria pertencente à família *Micrococcaceae*,

e ao gênero *Staphylococcus* (do grego “staphyle” = cachos; “cocos” = grão). São microrganismos imóveis, Gram positivos, anaeróbios facultativos, com diâmetro que varia entre 0,5 a 1,5 µm, que se agrupam em massas irregulares ou em forma de cachos de uva, e que estão associados a surtos de doenças transmitidas por alimentos causados pelas enterotoxinas estafilocócicas que podem contaminar carnes, leite e queijos (Lancettie Tanini, 2001; Umeda et al. 2017).

São microrganismos mesófilos com temperatura ótima de crescimento a 37°C, crescem em faixa de atividade de água (Aw) de 0,86 a 0,99, com pH ideal de 6,0 a 7,0, porém podem variar de 4,0 a 9,8; são capazes de fermentar glicose, maltose e manitol,e são tolerantes a concentrações de 10 a 20% de NaCl e nitratos, sendo então os alimentos curados potenciais veículos para esta bactéria (Hennekinne et al., 2012).

São também considerados catalase e coagulase positivos. A catalase é uma enzima que converte o peróxido de hidrogênio (H_2O_2) em água (H_2O) e gás oxigênio (O_2). A coagulase estafilocócica reage com uma substância presente no plasma, denominada de “fator de reação com a coagulase”, formando um complexo que reage com o fibrinogênio, que forma fibrina e promove uma rápida aglutinação das células bacterianas (Koneman et al., 2012).

Estes microrganismos podem ser de origem ambiental, presentes no ar, água, poeirae superfícies ambientais, contaminando o leite através dos manipuladores de alimentos, infecções intramamárias dos animais e do meio ambiente como resultado de condições de higiene inadequadas durante o processo de ordenha (Kümmel et al., 2016; Dittmann el al., 2017); fazem parte da microbiota natural de seres humanos e animais, e são encontrados em pêlos, pele, e trato respiratório. *S. Aureus* não causa infecções no estado imunológico normal, porém pode causar infecções graves ao invadir a corrente sanguínea e tecidos internos (Cong et al., 2020) por possuírem fatores de virulência que contribuem para o estabelecimento e manutenção da infecção (Zecconi e Scali 2013).

Segundo Kropec et al. (2005), *S. aureus* pode produzir mais de 30 fatores de virulência, como as α-hemolisinas, que possuem ação necrosante em pequenos vasos, alterando a permeabilidade da membrana celular. A cápsula é uma camada

de polissacarídeo que protege a bactéria da fagocitose. Já as proteases, lipases e proteínas de captação de ferro atuam como mediadores da resistência aos antibióticos. E as MSCRAMMs *(Microbial Surface Components Recognizing Adhesive Matrix Molecules),* são proteínas associadas à parede celular bacteriana, que se ligam às proteínas da matriz extracelular do hospedeiro, como o fibrinogênio, fibronectina e colágeno, passo importante para o início da infecção, entre outros (Acosta et al., 2017).

Este microrganismo pode também secretar enzimas e citotoxinas que possuem a função de converter os tecidos do hospedeiro em nutrientes para o crescimento bacteriano. E são produtores de exotoxinas, como a toxina da síndrome do choque tóxicoe enterotoxinas associadas a casos de intoxicação alimentar (Carfora et al., 2015).

As enterotoxinas estafilocócicas (Es) são proteínas com baixo peso molecular (25 a30 mil daltons), solúveis em água e solução salina, com composição de aminoácidos semelhantes, porém com propriedades imunológicas diferentes, resistentes à atividade deproteases gastrointestinais (Cretenet et al., 2011) e que mantém as atividades biológicas mesmo após submissão a altas temperaturas (Senger e Bizani, 2011).

As Es apresentam período de incubação de 02 a 06 horas, seguido por sintomas como náusea, vômito, dor abdominal e diarreia provocada pelo aumento do peristaltismo intestinal e perda de líquidos pelo organismo (Balaban e Rasooly, 2000), ou também pelo contato da enterotoxina com o epitélio intestinal, que aumenta a liberação de cálcio intracelular, comprometendo a função do tecido (Hu et al., 2005). Então, visando a saúdedo consumidor, a Instrução Normativa n° 60 de 2019, de forma complementar a Resolução da Diretoria Colegiada (RDC) n° 331 de 2019, que estabelecem os padrões microbiológicos para os alimentos, dispõem que as enterotoxinas estafilocócicas devem estar ausentes em leite e seus derivados (Brasil, 2019).

Na produção leiteira, o *S. aureus* é considerado o principal microrganismo infeccioso. Essas bactérias produzem toxinas que destroem as células, danificam os tecidos produtores de leite. Inicialmente *S. aureus* danificam os tecidos que revestem o interior dos tetos e da cisterna da glândula mamária, em seguida migram para os dutos e estabelecem infecções nos alvéolos secretores de leite. Neste processo formam-se abcessos que evitam a propagação da bactéria, porém impedem também a ação dos antibióticos sobre o microrganismo. Outra forma de proteção dos *S. aureus* é através dosneutrófilos, que na tentativa de remover as bactérias, podem mantê-las em estado de latência, e quando este neutrófilo morre, as bactérias voltam ao estado de infecção (Petersson-Wolfe et al. 2010).

Esta bactéria é um desafio global, pois encontra-se em rebanhos leiteiros do mundointeiro. Abebe et al. (2016) verificaram mastite subclínica em 59,2% do rebanho avaliado, e *S.aureus* foi isolado em 51,2% das amostras de leite estudadas. Abdeen et al(2020), identificaram presença de *S. aureus* em 44% das amostras de leite avaliadas e em 20% das amostras de sorvete. Estes autores observaram alta resistência dos microrganismos aos antibióticos Cefoxitina, Trimetoprim, Tetraciclina, Norfloxacina, Penicilina e Cefradina. Angelidis et al. (2020) detectaram *S. aureus* em 29% das amostras de leite cru de cabras com mastite subclínica, além de capacidade de formação de biofilme em 92% destes microrganismos.

Esta alta incidência revela o potencial de perda econômica pela diminuição da produção de leite e aumento nos custos com a saúde dos animais, além de poder levar a problemas de saúde pública em razão de a produção de enterotoxinas que contaminam o alimento e estão associadas a intoxicações alimentares (Abebe et al. 2016, Abdeen et al.2020).

1.4. Mastite

Mastite é a denominação do processo de inflamação na glândula mamária e representa uma das características de saúde mais importantes na produção leiteira, devido aprejuízos pela diminuição da produção e perda da funcionalidade da glândula mamária, sendo a principal preocupação do setor pecuário (Persson e

Olofsson, 2011), além de serconsiderada um risco para a saúde pública em razão da transmissão dos patógenos ou suas toxinas do leite aos humanos (Radostits et al., 2007).

Segundo Sordillo e Streicher (2002), a patogênese da mastite acontece em cinco etapas, em que primeiramente o microrganismo penetra o canal do teto; multiplica-se usando o leite como substrato; alcança o seio lactífero dos ductos coletores e alvéolos, atrai as células de defesa, podendo levar à formação de edemas e abcessos, e muitas vezesna cura, o tecido glandular é substituído por tecido fibroso.

A origem da mastite pode ser ambiental, provocada principalmente por enterobactérias como *Escherichia coli, Klesbsiella pneumoniae, Enterobacter aerogenes, Pseudomonas* spp., *Proteus* spp., fungos e leveduras presentes no ambiente. E de origem infecciosa, causada principalmente por *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus dysgalactiae, Corynebacterium sp.*(Radostits et al. 2002).

Quanto ao processo inflamatório, a mastite pode ser classificada como clínica ou subclínica. A mastite clínica provoca alterações visíveis na glândula mamária como

edema e vermelhidão, além da presença de grumos, sangue e pus no leite. Já a mastite subclínica não provoca alterações visíveis na glândula, porém leva à redução na produçãodo leite (Rodrigues et al., 2018).

De forma geral, bactérias do gênero *Staphylococcus* spp. são isoladas com maior frequência nos casos de mastite bovina, caprina, ovina e bubalina. Este gênero é divididoem dois grupos de acordo com a capacidade de síntese da enzima coagulase, quetransforma o fibrinogênio em fibrina (Pyorala e Taponen, 2009). Bactérias pertencentes ao grupo coagulase negativo são isoladas com maior frequência, porém bactérias do grupo coagulase positivo, representado por *Stapylococcus aureus*, é a espécie de maior importância pela sua alta patogenicidade.

Vários trabalhos têm sido realizados no intuito de identificar a presença de *S. aureus* nos animais e no leite. Fontana et al. (2010) observaram que 55% das vacas avaliadas apresentavam resultado positivo para mastite subclínica, sendo o gênero *Staphylococcus* predominante entre as cepas (40,9%), com destaque para *Staphylococcus aureus.* Soltau et al. (2017) encontraram *Staphylococcus aureus*, *Streptococcus dysgalactiae*, e *Streptococcus uberis* em amostras de leite obtidas diretamente do tanque de resfriamento. Akkou et al. (2018), avaliando a ocorrência de mastite em cabras, observaram que 32,3% dos animais apresentavam infecção, sendo 32,5% dos microrganismos representados por *Staphylococcus*.

Segundo Silva e Silva et al. (2010), na ovinocultura há uma relação inversa entre o número de cordeiros e o desenvolvimento da mastite, sendo que ovelhas com somente um borrego ao pé são mais susceptíveis ao desenvolvimento de mastite clínica e subclínica. Waage e Vatn (2008) observaram que 40% dos casos de mastite clínica acontecem sete dias antes e sete dias depois do parto, e que a incidência de mastite subclínica pode chegar a 24,8% mesmo no sistema extensivo de produção.

O mecanismo de defesa da glândula mamária pode se dar pela imunidade inata e imunidade adquirida. A imunidade inata é predominante nas primeiras fases da infecção, e é formada pela barreira física do esfíncter do teto e a barreira química formada pela queratina, elementos solúveis como as citocinas e elementos celulares como os macrófagos, neutrófilos e células *natural killer* (Oviedo-Boyso et al. 2007). Quando a imunidade inata não é capaz de eliminar o microrganismo patógeno, em um curto espaço de tempo, a resposta imune é ativada e mediada por fatores solúveis como as imunoglobulinas e pela defesa celular conferida pelos linfócitos T e B (Sordillo e Streicher, 2002).

Uma maneira de avaliar a saúde da glândula mamária é através da contagem de células somáticas (CCS), que compreendem macrófagos, células polimorfonucleares, linfócitos e células epiteliais por mL de leite. Em um úbere sadio, com exceção da primeira semana pós-parto, a CCS secretada no leite se mantém constante, porém em um quadro de infecção as células somáticas sinalizam as células sanguíneas, e há uma migração de células polimorfonucleares e neutrófilos para o leite. Estas células matam as bactérias, e quando a infecção é eliminada, a CCS volta ao normal (Malik et al. 2018).

A composição de célula somática sofre variação de acordo com a espécie; nos bovinos os macrófagos representam 80%, seguido por linfócitos (16%), células polimorfonucleares (3%) e 2% de células do epitélio glandular (Sharma et al. 2011). Nos caprinos a predominância é de células polimorfonucleares (72,6%), seguida por 14,9 % de macrófagos, 12,4% de linfócitos e 0,2% de células do epitélio glandular (Boulaaba et al. 2011). Em ovelhas sadias, a CCS é composta de 2 – 3% de células epiteliais, 2 – 28% de células polimorfonucleares, 45 – 85% de macrófagos e 10 – 17% de linfócitos (Bergonier e Berthelot, 2003).

Durante a mastite pode-se observar também mudança na concentração de proteína, gordura, lactose, minerais e vitaminas do leite, pelà redução na secreção de componentes sintetizados na glândula mamária. A lactose pode diminuir de 5 a 20%, enquanto a caseína, principal proteína do leite pode ser reduzida de 6 a 18%, consequentemente há redução de até 13% nos sólidos totais (Rodrigues et al. 2018). Também é possível observar alteração em características físico-químicas como o pH, que se torna mais alcalino pelo aumento da permeabilidade capilar que permite a passagem de constituintes do sangue para o leite (Moura et al. 2017).

Diante de todos os entraves provocados pela mastite, um controle higiênico-sanitário é a melhor forma de evitar a propagação dos patógenos. Os cuidados se iniciam com o ordenhador, que deve estar sempre com a mão limpa e desinfetada na sala de ordenha; higienizar as teteiras com água morna e detergentes ácidos e alcalinos após todas as ordenhas, além de deixar que as vacas que apresentam mastite sejam ordenhadas por último (Amaral et al. 2004).

Na linha de ordenha, um método eficaz para controle da mastite ambiental é a aplicação do pré-*dipping* com soluções desinfetantes (Fonseca e Santos, 2001). Já após a ordenha, a prática mais importante para evitar as infecções intramamárias é o pós-*dipping*, comumente realizado com soluções à base de iodo, clorexidina e cloro, cuja finalidadeé impedir a entrada de microrganismos patogênicos através do esfíncter do teto (Coser et al. 2012), e é importante também que os animais recebam alimento fresco após a ordenha, a fim de estimular que eles permaneçam em pé enquanto o esfíncter do teto não esteja totalmente fechado (Fonseca e Santos, 2001).

Porém, quando os métodos preventivos não são eficientes e a mastite é identificada, o principal tratamento consiste na aplicação de fármacos antimicrobianos que podem levar à resistência bacteriana aos princípios ativos (Moon et al., 2007). Tais medicamentos podem acarretar a presença de resíduos no leite, afetando a fabricação de lácteos, pois uma pequena quantidade de antibiótico no leite já e capaz de inibir o crescimento de culturas lácteas, prejudicando a fabricação de queijos, iogurtes e outros produtos, e a saúde do consumidor por provocarem reações como hipersensibilidade e alteração na microbiota intestinal (Freitas et al., 2005).

1.5. Biofilmes

Nos equipamentos de ordenha e na indústria de alimentos, a contaminação microbiana em superfícies de contato é uma grande preocupação, pois os biofilmes podem funcionar como um reservatório para a contaminação ou recontaminação dos alimentos, levando à deterioração e consequente redução na vida de prateleira dos produzidos, resultando em perdas econômicas (Galié et al. 2018).

Os biofilmes são estruturas de células microbianas ligadas a uma superfície, e durante o desenvolvimento e crescimento destes microrganismos, há a excreção de produtos de origem polimérica, que envolvem as células em uma matriz que as protegem de agressões exteriores (Ciccio et al., 2015). Estes biofilmes podem se formar em poucas horas, por múltiplas espécies ou espécie única, e são resistentes a medicamentos e desinfetantes, tornando difícil sua remoção (D'acunto et al., 2015).

Esta matriz é composta por água e substâncias poliméricas extracelulares que atuam como barreira de proteção e permite que os nutrientes, que são partículas pequenas, cheguem às células através de canais e poros, e impedem a passagem de partículas maiores, como os produtos tóxicos (Nan, Yang e Ren, 2015).

O desenvolvimento do biofilme é caracterizado por cinco estágios, incluindo a fixação inicial, fixação irreversível, formação de microcolônia, maturação e dispersão do biofilme (Coughlan et al. 2016).

A adesão inicial de um microrganismo é um processo reversível que envolve a aproximação das células bacterianas a uma superfície. Esta adesão é consolidada pela produção de polissacarídeos extracelulares que, além de permitir a adesão celular, agem como trocadores de íons para captura de nutrientes e água, e atuam como substrato para a aderência de microrganismos secundários, formando então as microcolônias, tornando esta adesão irreversível. Durante a maturação, conforme as células microbianas se dividem, a densidade e a complexidade do biofilme aumentam atingindo alta atividade metabólica; os componentes extracelulares produzidos pelas bactérias interagem com moléculas orgânicas e inorgânicas do ambiente onde estão inseridas para formar o glicocálix (Medonline, 2008). Os biofilmes quando atingem uma massa crítica começama liberar as células em estado planctônico que podem colonizar novas superfícies e formar novos biofilmes (Nam, Yang e Ren, 2015) (Figura 1).

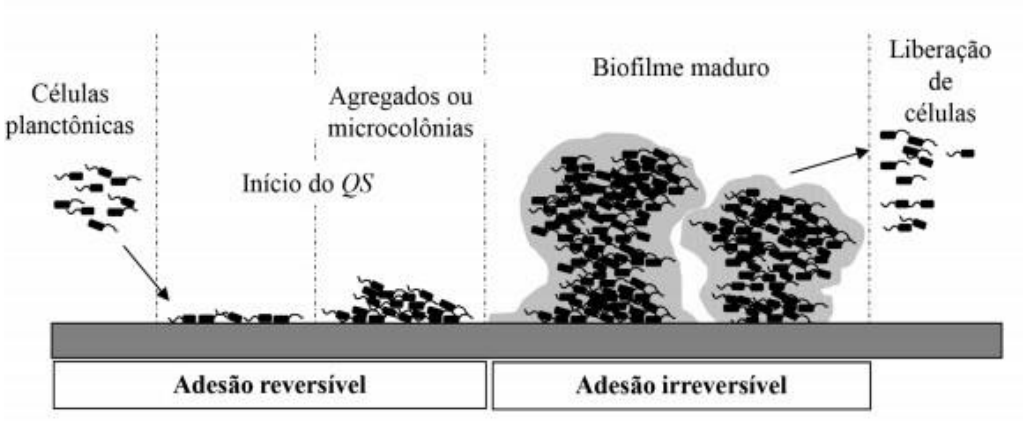

Figura 1. Estágio de desenvolvimento dos biofilmes: adesão reversível com a produção de microcolônias, adesão irreversível com a produção de exopolissacarídeo, ruptura do biofilme (Trentin et al. 2013).

Dentro das espécies de microrganismos que podem participar do processo de adesão, gerando problemas econômicos, estão: *Pseudomonas aeruginosa, Pseudomonas fragi, P. fluorescens, Micrococcus sp. e Enterococcus faecium.* Já dentre os microrganismos patogênicos que causam problemas de saúde pública, podem-se citar: *Listeria monocytogenes, Yersinia enterocolitica, Escherichia coli, Staphylococcus aureus e Bacillus cereus* (Parizzi et al., 2004).

O *S. aureus* é o patógeno mais comum relacionado a surtos de intoxicação alimentar (Lira et al., 2016). Meira et al. (2012), avaliando a adesão de *S. aureus* em superfície de aço inoxidável e polipropileno, a 7°C e 28°C, revelaram alta capacidade de adesão e formação de biofilme em ambas as superfícies e temperaturas, e também a ineficiência de hipoclorito de sódio e ácido peracético na remoção destes biofilmes.

Rodrigues et al. (2017) relataram alta capacidade de formação de biofilme de cepas de *S. aureus* isoladas de superfícies de contato com alimentos, e a eficácia de óleo essencial de orégano e carvacrol na inibição de células sésseis e planctônicas.

O gênero *Pseudomonas* está altamente associado à deterioração dos alimentos, sendo que *P. fluorescens* é considerada a principal deteriorante de leite e derivados (Ge et al. 2017). Rossi et al. (2016) verificaram que a formação de biofilme foi favorecida por temperaturas mais baixas, ao estudarem a capacidade de formação de biofilme de cepas de *P. fluorescens* em superfície de poliestireno, a 10°C e 30°C por 24 e 48 horas.

Wang et al. (2018) observaram início de adesão de *P. fluorescens* em superfície de aço inox logo após 10 minutos de exposição, colonização de 4,5 log UFC/cm^2 após 5 horas, e cinco dias; depois o biofilme foi caracterizado com mais de 9,5 log UFC/cm^2 e 120 µm de espessura. Neste referido estudo, os microrganismos se mostraram resistentes aos desinfetantes de hipoclorito de sódio, dióxido de cloro, água eletrolisada levemente ácida e água eletrolisada ácida, tornando-se a maior preocupação e desafio da indústria laticinista.

1.6. Inativação fotodinâmica de microrganismos

A luz tem sido utilizada como agente terapêutico desde a antiguidade, em que o sol erautilizado como fonte de irradiação, porém é recente o uso da luz como terapia, com embasamentocientífico (Hamblin e Huang, 2014). Atualmente, a Terapia Fotodinâmica (TFD)visa a necrose ou apoptose de tecidos vivos com crescimento anormal, podendo ser aplicada em infecções bacterianas, virais e fúngicas, já que estas são causadas por alta taxa de multiplicação das células (Dai et al. 2009; Dovigo et al. 2011).

A IFDMO consiste na combinação de um corante fotossensível, que na presença de oxigênio celular é ativado por uma luz visível em comprimento de onda adequado, e esta reação então produz uma resposta tóxica, capaz de danificar as estruturas celulares, levando a morte celular (Maisch, 2009).

Segundo de Melo et al (2013), a luz é responsável por excitar o fotossensibilizador que interage com moléculas vizinhas por dois mecanismos representados pelo diagramade Jablonski (Figura 2).

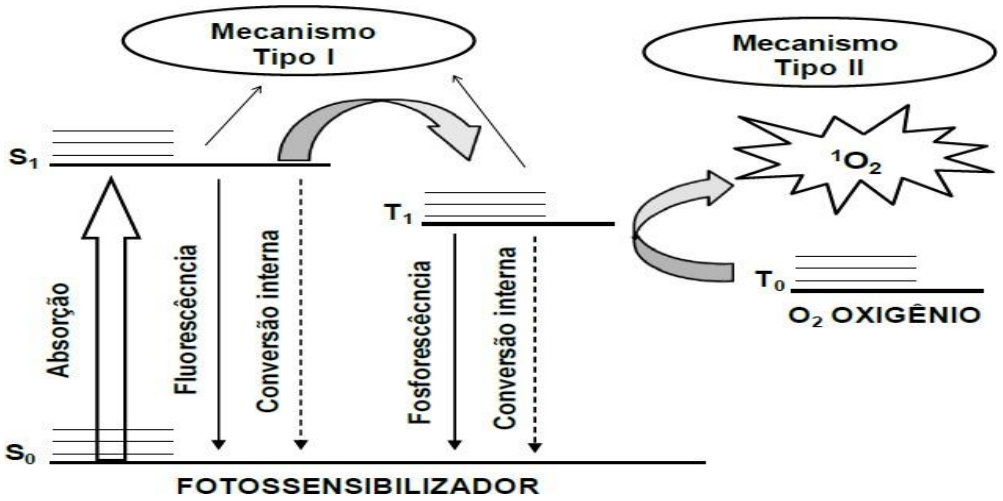

Figura 2: Representação do diagrama de Jablonski (Henderson e Dougherty, 1992).

Inicialmente, no mecanismo Tipo I, que ocorre principalmente em moléculas insaturadas como os fosfolipídeos da membrana celular, o fotossensibilizador (FS), após absorver um fóton de luz, pode reagir ao remover um átomo de hidrogênio de uma molécula do substrato biológico, passando do seu estado fundamental (S_0) para seu estado excitado singlete (S_1), podendo retornar ao seu estado fundamental ao emitir energia, ou então alterar seu spin, indo do estado singlete (S_1) para o estado triplete (T_1). Duranteestas reações, peróxido de hidrogênio (H_2O_2), superóxido (O_2^-) e radicais hidroxila (OH)são produzidos e provocam a peroxidação dos lipídeos da membrana celular, afetando sua estrutura e funcionamento (Henderson e Dougherty, 1992; Plaetzer et al. 2009).

No mecanismo Tipo II, o FS em seu estado triplete (T_1), ao reagir com o oxigênio molecular, produz espécies reativas de oxigênio (EROS), como $^1O_2$, que reage com proteínas, ácido nucleico e lipídeos, sendo considerado então o principal responsável pelo

dano oxidativo e morte das células microbianas (Handerson e Dougherty, 1992; Vatansever et al. 2013; Allison e Moghissi, 2013).

Quando comparada aos tratamentos antimicrobianos tradicionais, a TFD apresenta vantagens como largo espectro de ação, pois o FS pode agir sobre bactérias, fungos, vírus e protozoários, eficiência mesmo em cepas microbianas resistentes a antibióticos e também falta de seleção de microrganismos fotorresistentes por não haver efeito tóxico cumulativo (Hamblin e Hasan, 2004; Jori et al. 2006).

Estudos recentes vêm mostrando a eficácia da IFDMO *in vitro* e *in vivo*. Dos Anjos et al. (2020), ao avaliarem o efeito da luz azul sobre atividade *in vitro* de *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella entérica* e *Mycobacterium fortuitum,* verificaram redução de mais de 5 $\log_{10}$ em todos os microrganismos em um período de 2 horas, sem alteração significativa nos constituintes do leite. Já Silva Junior et al (2019) verificaram *in vivo* a viabilidade do uso da Safranina fotoativada como terapia alternativa na prevenção de mastite bovina, quando utilizada em aplicações pós- *dipping*, especialmente com relação à redução nas contagens de *S. aureus.*

Segundo Jori et al. (2006), um dos principais problemas enfrentados pela inativação fotodinâmica de microrganismos é a ampla variação na morfologia da parede celular microbiana. As bactérias Gram positivas, como o *Staphylococcus aureus*, apresentam a porção mais externa da parede celular, composta por peptideoglicanos e ácido lipoteicoico, mais porosa, permitindo que o fotossensibilizador atinja mais facilmente a membrana citoplasmática, tornando estas bactérias mais sensíveis à terapia (Melo et al. 2013).

No entanto, as bactérias Gram negativas, como *Pseudomonas fluorescens*, possuem além da camada de peptideoglicano uma camada composta por lipopolissacarídeos, lipoproteínas e proteínas, que atuam como uma barreira física, dificultando a incorporação do fotossensibilizador (Dai et al. 2009). Porém, em estudo realizado por Galstyan e Dobrindt (2019), foi verificado que a associação do fotossensibilizador e EDTA (Ácido etilenodiamino tetra acético) melhorou a atividade inibitória da terapia diante de *Escherichia coli,* pois a membrana deixou de representar uma barreira significativa.

Outro fator limitante à IFDMO é a formação de biofilmes por microrganismos, pois além da diferença morfológica, a presença de outros componentes, como os polissacarídeos extracelulares, dificulta a interação do FS com os microrganismos (Kostakioti et al. 2013).

Malacrida et al. (2020), avaliando a fotoinativação de biofilme formado por *S. aureus* com o FS hipericina, obtiveram redução de 0,9 log/UFC/cm^2, e concluíram que a irradiação por períodos mais longos (30 minutos) foi eficaz contra células planctônicas, podendo ser aplicada no controle deste microrganismo.

Para que a IFDMO seja efetiva, a escolha da fonte de luz e do FS é fundamental para que haja interação entre eles. Na literatura, o FS mais estudado é o azul de metileno irradiado com luz vermelha emitida por lasers de baixa potência ou então por diodos emissores de luz (LEDs). Fontes de laser ou LED podem ser utilizadas, porém cada uma apresenta uma característica específica: o laser possui monocromaticidade (fótons com o mesmo comprimento de onda), colimação (fótons emitidos na mesma direção) e coerência (fótons emitidos em sincronismo no tempo e espaço), sendo indicado para irradiação que necessita de maior profundidade, já os LEDs apresentam somente a monocromaticidade, indicados para tratamentos superficiais (Eduardo et al. 2015).

1.7. Fotossensibilizadores

Os fotossensibilizadores (FS) são definidos como moléculas capazes de interagir com a luz e gerar espécies reativas de oxigênio, que são tóxicas a tecidos vivos e podem induzir à morte celular através de necrose ou apoptose (Machado, 2000), e são utilizados na TFD para a remoção de contaminantes ambientais, inativação de microrganismos, descontaminação de alimentos e tratamento de infecções bacterianas, fúngicas e virais localizadas (Navntoft, et al., 2007; Perussi, 2007; Luksiene, 2005).

O efeito de um corante como fotossensibilizador foi observado primeiramente por Raab, em 1900, que notou inativação do protozoário causador de malária (*Paramecium caudatum*), após exposição do mesmo ao corante laranja de acridina e luz solar, atribuindo o efeito à transferência de energia da luz para a substância química que gerava um efeito citotóxico, visto que de forma isolada, luz e corante não apresentavam qualquer efeito separadamente (Brovko, 2010).

Desde então, inúmeros compostos com atividade fotodinâmica vêm sendo testados, porém, características como ausência de toxicidade no escuro (sem irradiação), inativação de células bacterianas sem causar danos às células do hospedeiro, acúmulo em células patogênicas e rápida eliminação de células não alvo, além de alto rendimento e baixo custo, são fundamentais para a viabilidade de aplicação (Allison, et al. 2004).

Segundo Garcez et al (2003), os Fs mais utilizados são os derivados da hematoporfirina, fenotiazinas (azul de metileno e azul de toluidina), cianinas (indicianina verde), fitoterápicos (azuleno) e ftalocianinas.

Sellera et al (2016), avaliando a ação do Fs azul de metileno na inativação *in vitro* de microrganismos causadores de mastite bovina, observaram maior sensibilidade de *Corynebacterium bovis* após 10 segundos de irradiação, e inativação total de *Staphylococcus aureus* após de 30 segundos. Gándara et al. (2016), ao aplicar azul de toluidina em *Staphylococcus aureus*, observaram inativação celular com baixas e altas doses de irradiação (4,2 J/cm^2 e 10 J/cm^2 respectivamente).

Porém, outras substâncias que apresentam características compatíveis como um agente fotossensibilizador, como a Safranina (Sf), vêm sendo estudadas. A Sf é um corante utilizado nas indústrias têxtil, farmacêutica, de papel e cosmética, que apresenta caráter anfifílico que confere maior interação com os substratos biológicos, não apresenta toxicidade e gera espécies altamente reativas de oxigênio (EROS), que inativam microrganismos (Silva Junior et al. 2019).

Dadras et al. (2006), ao fotoinativar *P. aeruginosa* com o azul de toluidina e Safranina-O, observaram diminuição da população bacteriana na presença de ambos os fotossensibilizadores. Voos et al. (2014) observaram atividade antimicrobiana de Safranina-O em bactérias periodontopatogênicas (*Streptococcus gordonii*; *Streptococcus mutans*; *Fusobacterium nucleatum; Aggregatibacter actinomycetemcomitans* e Porphyromonas gingivalis), com morte bacteriana superior ao tratamento com antibiótico clorexidina.

Em estudos recentes com aplicação *in vitro* e *in vivo,* Silva Junior et al. (2019) mostraram eficiência na inativação *S. aureus,* e redução no número de células somáticas no leite quando a Safranina foi utilizada como pós- *dipping* para vacas.

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Visando a aplicação dos fotossensibilizadores e sua liberação controlada nos tecidos-alvo, os géis e hidrogeis, vem sendo investigados (Yu e Ding, 2008). Uma das formas de se conseguir um sistema adesivo termo-sensível é através da utilização do Carbopol® 934P (C934P), que são polímeros sintéticos do ácido acrílico, utilizados como géis, suspensões e emulsões (Allen Jr. 2007).

Já os hidrogeis são polímeros que absorvem grande quantidade de água e que apresentam a capacidade de liberar o fármaco de forma constante e por um período prolongado, se assemelham aos tecidos naturais pelo teor de água, resultando em biocompatibilidade e bioadesão (Amin et al. 2009).

Os hidrogeis com gelificação *in situ*, como o Pluronic® F127, são os mais vantajosos, visto que são aquosos em temperatura de resfriamento e assumem textura de gel quando administrados em temperatura corporal, resultando em aumento na aderência (Yu e Ding, 2008). O F127 é um agente surfactante composto por monômeros de poli(óxido de etileno co-óxido de propileno-co-óxido de etileno (PEO-PPO-PEO)), que se gelifica pelo processo de formação de micelas, em que com o aumento da temperatura, a concentração micelar crítica (CMC) destes polímeros diminui, resultando em desidratação dos domínios hidrofóbicos (Dumortier et al. 2006) (Figura 3)

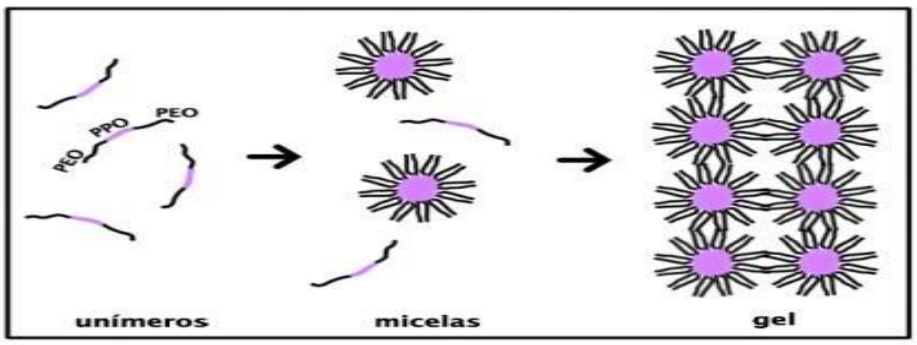

Figura 3: Esquema do mecanismo de gelificação do PF127 em água

O F127 vem sendo estudado a bastante tempo em formulações de uso injetável e tópico (REF). Veyries et al (1999), utilizando um gel injetável de F127 com vancomicina em ratos, observaram aumento de 9 horas no tempo de residência do fármaco no local.

Liu et al. (2007), com aplicação subcutânea, observaram que o hidrogel de F127 dobrou o tempo de biodisponibilidade de um polipeptídeo antitrombótico em ratos.

Assim, os fotossensibilizadores, quando associados a géis e hidrogeis, podem formar um produto fototóxico que possui alta taxa de adesão na superfície a ser tratada e alto grau de penetração nas bactérias (Escobar-Chavez et al., 2005).

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Prevention of bovine and caprine mastitis via Safranin-O-mediated Photodynamic Inactivation of microorganisms: *in vitro* and *in situ* studies

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Abstract

The objectives of this work were to evaluate the antimicrobial capacity of the photosensitizer (PS) Safranin-O (Sf), through Antimicrobial Photodynamic Therapy (PDTa), in milk and stainless steel coupons experimentally contaminated with Staphylococcus aureus and Pseudomonas fluorescens strains. The efficiency of using a thermo-responsive and photoactive Sf hydrogel in post-dipping applications was also evaluated in order to reduce the contamination of milk from cows and goats. Strains of *S. aureus* (SV1, SV3, SV5, SC5, SC6, SC8) and *P. fluorescens* (PV1, PVh, PC3) were isolated from bovine and goat milk. Antibiogram tests showed the resistance of *S. 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 the formation of biofilms, there was a 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) ($p>0.05$). During 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 76.13% for *Pseudomonas* spp. in goat milk ($p<0.05$). There was no significant difference in somatic cell count (CCS) in goat milk. These results indicate that photoactivated Sf can help reduce milk contamination and maintain the health of the mammary gland.

Key words: Photosensitizer, Safranin-O, Mastitis, Photodynamic Therapy, Milk quality.

Milk is a nutrient rich food, but its constituents provide an excellent growing medium for pathogenic and spoilage microorganisms. Among the pathogens, *Staphylococcus aureus* stands out, a bacterium that spreads through the milking system and is associated with mastitis (inflammation of the mammary gland), 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*, 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). The biggest problem related to *S. aureus* in dairy production is the application of these antimicrobials, as the practice has caused bacterial resistance to drugs and disposal of raw material due to the high levels of leukocytes and antibiotics in milk (Ma et al., 2018).

In this context, Antimicrobial Photodynamic Therapy (PDTa) has been shown to be an efficient option in the treatment of 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 the application 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 in the excitation of a photosensitizer (FS) 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).

Several FS compounds have been used in PDTa, especially Safranin (Sf), a synthetic dye of the phenazine class, which when used in the form of a hydrogel with adhesive and thermo-responsive characteristics, showed great potential for inactivating microorganisms during the treatment of cases of caprine mastitis (Silva Junior et al., 2020).

Thus, due to the recurrence of mastitis in lactating animals, the aim of this work was to explore the in vitro antimicrobial activity of photoactivated Sf against strains of *P. fluorescens* and *S. aureus* isolated from the milk of cows and goats. Sf was also used to reduce milk contamination and reduce microbial biofilm formation. To evaluate the effectiveness of the formulation in small and large ruminant animals, studies were conducted via in situ applications of the Sf hydrogel as post-dipping in lactating cows and goats.

Materials and methods

Isolation and genotypic identification of S. aureus and P. fluorescens

To obtain the 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) for cultivation of *Staphylococcus* spp. and *Pseudomonas* spp., and incubated at 37 °C and 28 °C for 48 h, respectively.

Characteristic staphylococcal 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, calatase and Gram stain tests were then performed. For Pseudomonas, characteristic colonies on Pseudomonas Agar Base were isolated for colony characteristics. Selected microorganisms were frozen at -20 °C in BHI broth and glycerol for further genetic identification.

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'-AATCACACCGTGGTAACCG-3'), and considered as *P. fluorescens* the isolates that presented a 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 a ADN band with a size of 267 bp (nuc gene) and 579 bp (coa gene) were considered as *S. aureus* (da Silva and da Silva, 2005).

In vitro antimicrobial photodynamic therapy

For in vitro analysis, strains of *S. aureus* and *P. fluorescens* were cultivated in Mueller Hinton Broth (CMH) (Kasvi – São José dos Pinhais, Brazil) at 37 ºC and 28 °C respectively, for 24 h, for three successive activations, and cell density was standardized with decimal dilution in tubes of sterile 0.9% saline solution, until turbidity equivalent to 0.5 MacFarland solution, (Bordignon Junior et al., 2012). 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, FS 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/cm2 (Supplementary material Figure 1 A).

Sensitivity of *S. aureus* isolates to different antimicrobials and photoactivated Sf

The isolated strains of *S. aureus* were tested for sensitivity to five antimicrobials commonly used for the treatment of mastitis. Using the disk diffusion technique, Ampicillin 10 µg/mL, Trimethoprim 5 µg/mL, Ciprofloxacin 5 µg/mL, Tetracycline 30 µg/mL and Ceftiofur 30 µg/mL (Bauer et al., 1996) were evaluated. For sensitivity to aqueous Sf solution, the well diffusion technique (Silva Junior et al., 2019) was used, with irradiation for 30 minutes. The diameter of the inhibition halos was measured by a caliper after 24 hours of incubation at 37 °C (Bradn New – TopStar).

Minimum inhibitory concentration and minimum bactericidal concentration

The 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), other microplates were kept unlit (dark) and then incubated at 37 ºC and 28 °C for 24 h. CBM 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 a positive control (Silva Junior et al., 2019).

PDTa in milk and experimentally contaminated stainless steel coupons

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. 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. In milk samples, planktonic cells were evaluated right after irradiation, and in stainless steel coupons, sessile cells were evaluated after 48 h at 4°C. The determination of the number of adhered cells took place according to Dutra et al. (2020), with modifications, where after treatment, each coupon was transferred to a microtube containing 1.0 mL of 0.85% saline solution, remaining at rest for 1 minute to remove planktonic cells. Then, each coupon was added to new microtubes containing 1.0 mL of 0.85% saline solution and subjected to constant agitation for 1 minute. The percentage of inhibition was calculated according to the following the next formula:

(1) Diff (log CFU/mL) = Trat 1 (control) - Trat 2 (TFDa)

(2) % inhibition = Dif (log CFU/mL) x 100 / Trat 1 (control),

where Dif is the bacterial count difference.

PDTa as post-dipping in cows and goats

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) 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).

The experiment was conducted in the cattle and goat sector of the Experimental Farm of Iguatemi – State University of Maringá (EFI – SUM) under approval by the Animal Ethics Committee of the State University of Maringá - CEUA, processes nº 1287230920 and 6266030920 respectively.

Ten Holstein and Jersey cows were used, with an average weight of 500 kg and average production of 20 liters/day, in different stages of lactation (beginning, peak and end), kept in pasture and supplemented with corn silage and concentrate. corn grain, and 10 goats of the Saanen and Boer breeds, with an average weight of 55 kg and an average production of 3.0 liters/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 being 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 teatcup for one minute per teat) (Supplementary material Figure 1 B).

Milk samples were collected at 0, 7, 14, 21 and 28 days of product application for microbiological count, somatic cell count (CCS) and physicochemical composition. The samples were sowed in Plate Count Agar (PCA) (Kasvi – São José dos Pinhais, Brazil) to count total mesophilic aerobics, Agar Sal Mannitol (Kasvi – São José dos Pinhais, Brazil) to count *Staphylococccus* spp and *Pseudomonas* Agar Base (Acumedia – Neogen, Lansing, USA), for enumeration of *Pseudomonas* spp., and incubation at 37 °C and 28 °C for 48 h, respectively. The somatic cell count and physicochemical composition of cow's milk were performed in the milk analysis laboratory of the Dutch Association of

Cattle Breeders (APCBRH) using the Bentley Somacount FCM and Bactocount IBC equipment. The physicochemical composition and somatic cell count of the goats' milk were determined by the ultrasomic milk analyzer Ekomilk Total and Ekomilk Scan, respectively.

Statistical analysis

Data obtained in vitro and in situ 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

Isolation and genetic identification of S. aureus and P. fluorescens

Initially, 10 strains of staphylococci and 10 strains of *Pseudomonas* from the milk of cows and goats were selected. After genetic identification, the isolates of bovine *S. aureus* that amplified the nuc and coa gene 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 (Supplementary material 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 (Supplementary material Figure 2 B).

Sensitivity of S. aureus isolates to different antimicrobials and photoactivated Sf

Five antimicrobials commonly used in the treatment of mastitis were evaluated for their effectiveness against isolated *S. aureus*. For isolates from bovine milk, resistanceto 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, SC8) was observed) (CLSI, 2005). The photoactivated Sf showed inhibition halos ranging from 14 to 19 mm for bovine *S. aureus* and from 17 to 22 mm for *S. aureus* of goat origin, results superior to inactivation by lactic acid (10 to 13 mm), a commonly used product as post-dipping in milking (Supplementary material Table S1).

Minimum inhibitory concentration and minimum bactericidal concentration

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 to 37.50 mg/mL caused cell death. Without irradiation, Sf was not efficient in inhibiting bacterial growth (Supplementary material Table 2).

PDTa in milk and experimentally contaminated stainless steel coupons

When the inactivation of microorganisms cultivated in cow's milk was evaluated, for *S. aureus* isolates there was no significant difference ($p>0.05$) between the control treatment and the photoactivated Sf, with a maximum growth inhibition of 18.86% (0.33 log CFU/mL) (SV1), but 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 1). 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) (Supplementary material Table 3).

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 2). For PC3 in milk the inactivation was 50.25% (3.03 log CFU/mL), and in CMH 11.11% (0.25 log CFU/mL) ($p<0.05$) (Supplementary material Table 4).

No adhesion of bovine and goat *Pseudomonas fluorescens* isolates to stainless steel coupons was observed.

PDTa as post-dipping in cows and goats

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%)

(0.90 log CFU/mL) during the application period (28 days) ($p<0.05$) (Figure 1). 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/cm3) and 2.74 log/CCS (549,500 CCS/cm3) for the control and photoactivated Sf treatments respectively at the beginning of the experiment (day 0) and 2.46 log/CCS (288.400 CCS/cm3) for both treatments at the end of the experiment (day 28) (Figure 1).

In goats there was a reduction in *Pseudomonas* counts throughout the experimental period (76.14%) (0.67 log CFU/mL) ($p<0.05$) (Figure 2). 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/cm3) and 5.30 log/CCS (367,900 CCS) /cm3) and final count (day 28) of 5.61 log/CCS (754,300 CCS/cm3) and 5.56 log/CCS (543,000 CCS/cm3) for the control and photoactivated Sf treatments respectively (Figure 2).

Discussion

P. fluorescens are Gram-negative bacteria that produce proteases that destabilize the casein micelle, causing milk to coagulate, and lipases that hydrolyze cell constituents leading to the appearance of undesirable odors in milk, representing a challenge in dairy industries (Scarpellini et al., 2004). Meng et al. (2017) and Lampugnani et al. (2019), identified, through specific genes, the presence of *P. fluorescens* in 42.7% and 86.8% of raw milk samples, respectively, concluding that among the psychrotrophic microorganisms present in milk, *P. fluorescens* is the one prevalent.

S. aureus, on the other hand, are Gram positive bacteria that spread through the milking system and are associated with mastitis, which causes a drop in milk production, increased drug costs and even the disposal of animals (Ren et al., 2020). As in the present study, several studies have reported the presence of *S. aureus* in dairy herds. Hamid et al. (2017) and Rana et al. (2020), found the presence of this microorganism in 22.5% and 34.69% of the cow and goat milk samples evaluated. Obaidat et al. (2018), observed the presence of *S. aureus* in 65.9% and 68.1% of the cattle and dairy goat farms evaluated. Among the isolates, the authors found resistance to penicillin, ampicillin, tetracycline, gentamicin, doxycycline, trimethoprim. This high incidence may be due to the presence of virulence factors such as the ability to synthesize enzymes, such as β-lactamases, which

inactivate and hydrolyze the β-lactam ring of antibiotics of the penicillin, cephalosporin, carbapenem and monobactan class (Kesharwani and Mishra 2019).

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 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 in the cytoplasm, leading to cell death (Silva Junior et al. 2019), (Supplementary material Table S1).

The use of PDTa has been shown to be efficient in inactivating several microorganisms isolated from milk. dos Anjos et al. (2020), observed that the exposure of bacteria to blue light emitting diode (LED) (λ = 413 nm) inhibited the growth of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Mycobacterium fortuitum,* a fact attributed to photosensitizing compounds present in bacterial cells which absorb light with a wavelength in the range of 400 to 480 nm, producing ROS that damage the cell structure (Srimagal et al., 2016).

Greater inactivation efficiency has been reported in Gram positive bacteria. Galstyan and 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 ducts, reducing the shelf life of products and changing the organoleptic characteristics of milk, Sf has proven effective in reduce 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 (1O2), 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, not 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 and 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).

PDTa has also been showing promising results in the veterinary clinic, where it aims to reduce the use of antibiotics and inhibit microorganisms that affect the quality of milk. Moreira et al. (2018), observed a reduction in the count of *Streptococcus dysgalactiae* and coagulase negative *Staphylococcus* after 12 hours of application of toluidine blue PS irradiated with red LED (λ = 635 nm) applied intramammary in cows.

In general, the PDTa has been shown to be an effective alternative for reducing contamination and improving the quality of food, and in view of the concern with 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.

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, the use of carrier copolymers improves the development of Sf by keeping it in its monomeric state and allowing greater interaction with multidrug resistance, in addition to 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 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 and 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, but in cases of mastitis, 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.

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

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Table 1. *Staphylococcus aureus* count (log CFU/ml) in experimentally contaminated cow's milk, CMH and stainless steel coupons, submitted to different treatments after photoinactivation.

Staphylococcus aureus in cow milk							
Treatament	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}	0,000	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							
Teratament	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}	0,000	0,000	0,000	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}				
Stell *Staphylococcus aureus* in cow milk							
Treatament	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}	0,000	0,000	0,000	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}				
Stell *Staphylococcus aureus* Mueller Hinton Broth							
Treatament	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}	0,000	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}				

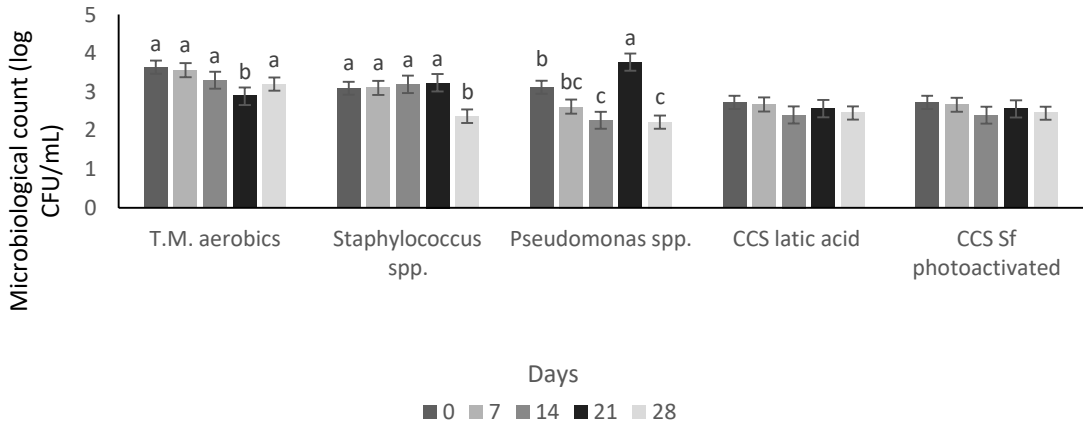
SV1, SV3, SV5: *S. aureus* isolated from bovine milk. Control (only milk/broth); PDTa (milk/broth + photosensitizing agent) Safranin (Sf) + LED lighting); LED (milk/broth + LED lighting); Sf (Safranina Photosensitizer) (milk/broth + Safranina photosensitizing agent), SEM: standard error of the mean,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

Table 2. *Staphylococcus aureus* count (log CFU/ml) in experimentally contaminated goat milk, CMH and stainless steel coupons, submitted to different treatments after photoinactivation.

Staphylococcus aureus in goat milk							
Treatament	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}	0,000	0,000	0,000	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							
Treatament	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}	0,000	0,000	0,000	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}				
Stell *Staphylococcus aureus* in goat milk							
Treatament	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}	0,000	0,000	0,000	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}				
Stell *Staphylococcus aureus* Mueller Hinton Broth							
Treatament	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}	0,000	0,000	0,000	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}				

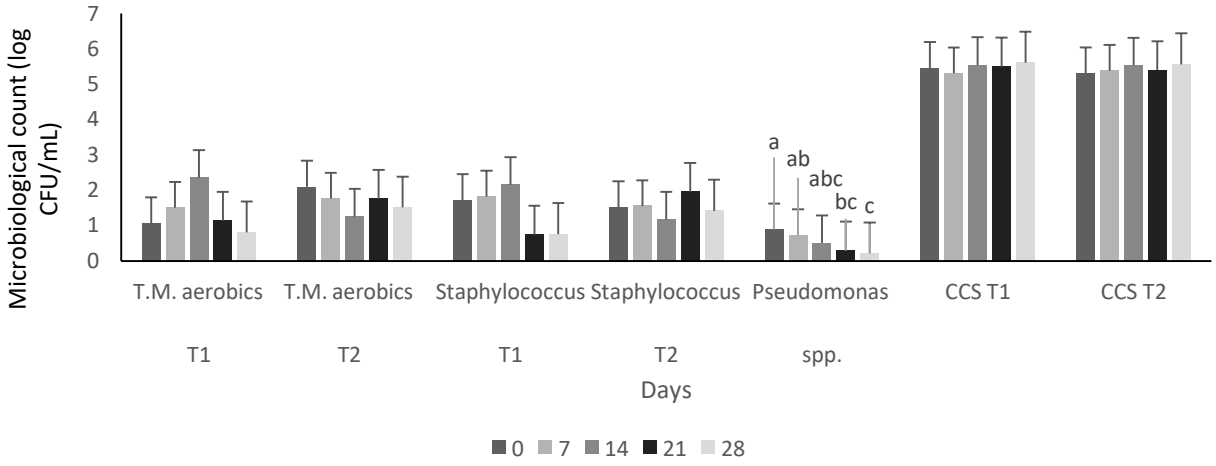
SC5, SC6, SC8: *S. aureus* isolated from goat milk. Control (only milk/broth); PDTa (milk/broth + photosensitizing agent) Safranin (Sf) + LED lighting); LED (milk/broth + LED lighting); Sf (Safranina Photosensitizer) (milk/broth + Safranina photosensitizing agent), EPM: standard error of the mean, ($p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

Figure 1: 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: 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. Total A.M: y=3.793 -0.155x; Stafilo: y=3.399 -0.131x, Pseus: y=2.995 -0.065x.

Figure 2: Count of mesophilic aerobics, S. aureus and Pseudomonas fluorescens (log CFU/ml) in goat milk during 28 days of post-dipping application Lactic acid and photoactivated Safranin.



Total A.M. T1: total mesophilic aerobic count in animals treated with lactic acid, total mesophilic aerobic count T2: total mesophilic aerobic count in animals treated with photoactivated Sf hydrogel, Staphylococcus T11: 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. Pseus: $y = 1.1 - 0.2x$.

Prevention of bovine and caprine mastitis via Safranin-O-mediated Photodynamic Inactivation of microorganisms: *in vitro* and *in situ* studies

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Supplementary file

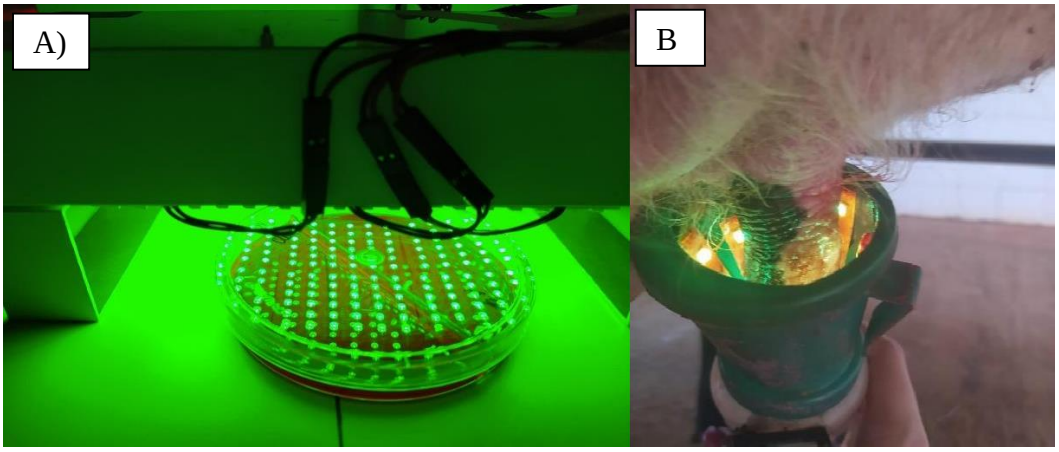

Figure S1. A) Green LED source (λ=520 nm) with a light dose of 12.9 J/cm2 for in vitro antimicrobial photodynamic therapy. B) Green LED light source (λmax = 520 nm, 12.7 mW cm – 2) coupled to a conventional plastic teat for application of antimicrobial photodynamic therapy in vivo.

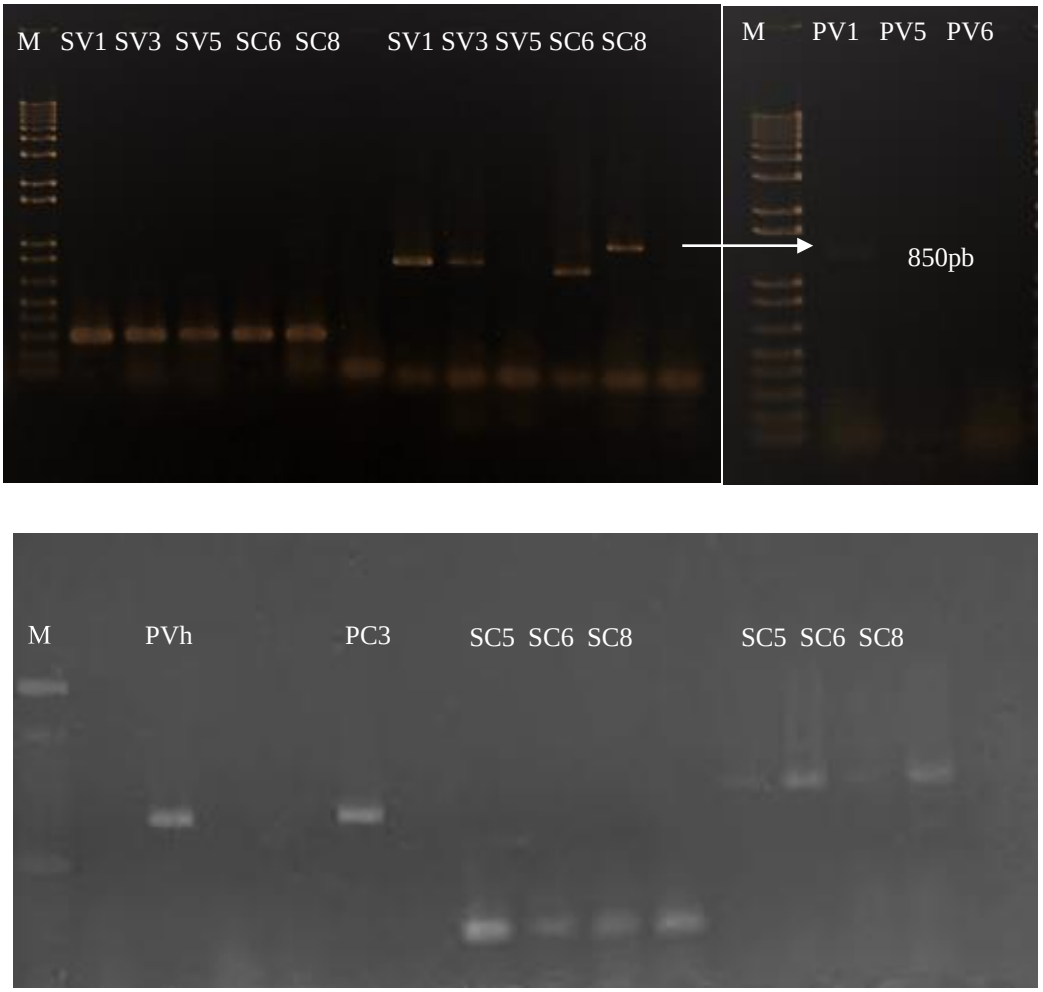

Figure S2. A) Agarose gel electrophoresis image of the nuc gene (267 bp), coa gene (579 bp) and 16S gene of different isolates of S. aureus and P. fluorescens from cow's milk. B) Agarose gel electrophoresis image of the nuc gene (267 bp), coa gene (579 bp) and 16S gene of different isolates of S. aureus and P. fluorescens from goat milk.

Table S1. 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
Ampicilina	30 (S)	22 (R)	22 (R)
Trimetoprim	21 (R)	26 (S)	21 (R)
Ciprofloxacina	33 (S)	29 (S)	32 (S)
Tetraciclina	30 (S)	24 (R)	19 (R)
Ceftiofur	30 (S)	28 (S)	27 (S)

Active principle	*Staphylococcus aureus* isolated from goat milk		
	SC5	SC6	SC8
Ampicilina	20 (R)	22 (R)	22 (R)
Trimetoprim	22 (R)	22 (R)	22 (R)
Ciprofloxacina	34 (S)	35 (S)	41 (S)
Tetraciclina	20 (R)	21 (R)	20 (R)
Ceftiofur	31 (S)	29 (S)	34 (S)

SV1, SV3, SV5: *Staphylococcus aureus* grant from bovine milk. S: Sensitive, A: Resistant.

Table S2. Minimum inhibitory concentration and minimum bactericidal concentration of Safranin-O against *Staphylococcus aureus* and *Pseudomonas fluorescens* strains isolated from cow and goat milk.

Cows										
Treatament	SV1		SV3		SV5		PV1		PVh	
	CIM	CBM	CIM	CBM	CIM	CBM	CIM	CBM	CIM	CBM
PDTa	18,75	37,50	18,75	18,75	18,75	37,50	18,75	37,50	18,75	37,50
Sf no irradiation	> 150	> 150	> 150	> 150	> 150	> 150	> 150	> 150	> 150	> 150

Goat								
Treatament	SC5		SC6		SC8		PC3	
	CIM	CBM	CIM	CBM	CIM	CBM	CIM	CBM
PDTa	18,75	37,50	18,75	37,50	18,75	37,50	9,38	18,75
Sf no irradiation	> 150	> 150	> 150	> 150	> 150	> 150	> 150	> 150

SV1, SV3, SV5: *Staphylococcus aureus* isolated from bovine milk. SC5, SC6, SC8: *Staphylococcus aureus* isolated from goat milk. MIC: Minimum inhibitory concentration; CBM: Minimum bactericidal concentration. TFDa: antimicrobial photodynamic therapy

Table S3. *Pseudomonas fluorescens* count (log CFU/mL) in experimentally contaminated cow's milk and HCM, submitted to different treatments after photoinactivation.

Pseudomonas fluorescens in cow milk						
Treatament	Time 0		P value			SEM
	PV1	PVh	Bac	Trat	Bac*Trat	
Control	5,00±0,01^{Aa}	4,00±0,01^{Ab}	0,000	0,000	0,000	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						
Treatament	Time 0		P value			SEM
	PV1	PVh	Bac	Trat	Bac*Trat	
Control	5,27±0,32^{Aa}	3,74±0,14^{Ab}	0,000	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}				

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 line are different from each other.

Table S4. *Pseudomonas fluorescens* count (log CFU/ml) in experimentally contaminated cow's milk and HCM, subjected to different treatments after photoinactivation

Pseudomonas fluorescens in goat milk			
Treatament	Time 0	P value	SEM
	PC3	Trat	
Control	6,03^{A}	0,00000	0,00083
PDTa	3,00^{D}		
LED	5,84^{C}		
Sf	5,86^{B}		
Pseudomonas fluorescens in Mueller Hinton Broth			
Treatament	Time 0	P value	SEM
	PC3	Trat	
Control	2,25^{A}	0,00617	0,017
PDTa	2,00^{B}		
LED	2,20^{B}		
Sf	2,04^{B}		

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 capital letters in the same column treatments differ from each other.

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

Artigo redigido conforme as normas de publicação do *Tropical Animal Science Journal*

ABSTRACT

Mastitis caused by *Staphylococcus aureus* has the use of antibiotics as a conventional treatment, a practice that has led to bacterial resistance. Thus, the photodynamic inactivation of microorganisms (PDIM), has the advantage of inactivating pathogens without leading to the selection of resistant microorganisms. The objective of these study was to evaluate the antimicrobial activity of the photosensitizer (PS) Safranine-O (Sf), incorporated in the nanocarrier Pluronic® F127 against strains of *Staphylococcus 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 reduction of total mesophilic aerobic and Staphylococcal counts in milk were evaluated. Three strains of *Staphylococcus 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 staphylococcus counts by up to 97.33% and 99.63% respectively. In stainless steel coupons, photoinactivation reduced *Staphylococcus aureus* adhesion by up to 45.92% (milk) and 99.5% (MHB) ($p<0.05$). The photoactivated Sf was similar to the use of commercial lactic acid when applied as a dipping powder. These results show that Sf mediated PDIM is effective in inactivating pathogens that cause mastitis and in reducing milk contamination.

Keywords: Photoinactivation, Mastitis, Staphylococcus

INTRODUCTION

Mastitis is the cause of great economic losses in dairy herds, and in sheep, *Staphylococcus aureus* (*S. aureus*) are responsible for 20% to 60% of cases of clinical mastitis, being associated with food poisoning due to the production of Staphylococcal toxins (Obaidat et al. 2018) and are described by the production of biofilms on processing surfaces of dairy industries, which can lead to a recontamination of the product (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) are 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. In the dairy industry, on the other hand, the concern is with bacterial resistance to sanitizers (Kroning et al. 2020).

In this context, PDIM, a therapeutic modality whose basic principle is the absorption of 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 and Dobrindt, 2019), hence being considered as 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 Junior et al. 2019).

In veterinary medicine, recent studies indicate prime results for the treatment of pathologies such as mastitis and also for reducing the use of antibiotics and consequently residues in milk (Couto et al. 2020). *In vitro* studies of 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 Junior et al. (2019) and Silva Junior (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, calatase and Gram tests were carried out, and positive isolates and coconuts were selected (Angelidis et al., 2020).

For the identification of *S. aureus*, through the nuc gene, primers 5'-GCGATTGATGGTGATACGGTT-3' and 5'-AGCCAAGCCTTGACGAACTAAAGC-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 as isolate, with DNA band in the size of 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 / cm2 (Figure 1. A).

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 Junior et al. 2019).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The isolates were grown in BHI Broth (Kasvi, São José dos Pinhais, Brazil) at 37ºC 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 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 37ºC for 24 h (Silva Junior 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 of milk and coupons from experimentally contaminated 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 four treatments in a 4x4 factorial design (4 bacterial isolates and 4 treatments), with four repetitions by treatment, being: Treatment 1: Control (milk / broth only); Treatment 2: PDIM (milk / broth + photosensitizing agent (Sf) + LED lighting); Treatment 3: LED (milk / broth + LED lighting); Treatment 4: Photosensitizer (Sf) (milk / broth + photosensitizing agent Sf). The experiment was repeated four 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 Peptone water (Himedia Laboratories, Mumbai, India) were used, as described by Silva Junior 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 pasture with 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 1. B).

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* spp. were isolated and selected according to preliminary tests. The microorganisms that were amplified in the primers nuc were named as 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 was not able to inhibit the growth of microorganisms at the evaluated concentration. When Sf was associated with irradiation with a green LED light source (λ = 520 nm), a cytotoxic effect was observed (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) was not able to inactivate bacterial growth.

When MHB broth was used as 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*, a fact that 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, whereas 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, and among them are the thermostable nucleases (nuc 1 and nuc 2) that hydrolyze the DNA or RNA of the host cells, which lead to tissue destruction and dispersion of the host cells. Bacterial cells and coagulase, which leads to the deposition of fibrin around the microorganism, hindering 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 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 to those verified by da Silva and 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 Junior et al. 2019). Thus, small concentrations of this PS (1.18 to 18.75 mg / mL) were able to 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 influence the susceptibility of *S. aureus* to PDIM induced oxidative mechanisms.

Studies with the use of 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 was efficient in inactivating *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 the efficiency of photoinactivation, 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 and 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 and 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 Junior 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 and 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

The use of 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 and 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 and Van Winkle, 2010).

Silva Junior 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 Junior 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, 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 the veterinary clinic are scarce, but 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 are necessary.

CONCLUSIONS

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 it reduced microbial adhesion in stainless steel coupons. When applied *in vivo*, PDIM has shown to be similar to the use of 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 organization related to the material discussed in the manuscript.

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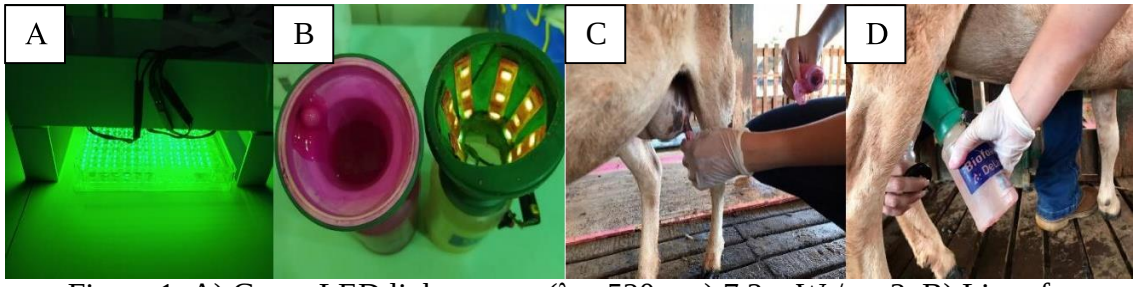

Figure 1. A) Green LED light source (λ = 520 nm) 7.2 mW / cm2. B) Liner for application of the dipping control post (lactic acid Ekomilk post gel film) and liner containing green LED for the irradiation of the Sf. C) Application of hydrogel to sheep. D) LED irradiation.

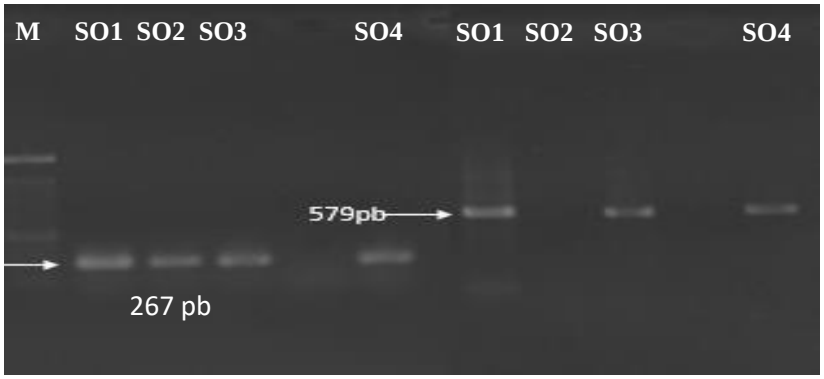

Figure 2. Image of agarose gel electrophoresis of the nuc (267 bp) and coa gene (579 bp) of the different isolates of *S. aureus* from sheep's milk.

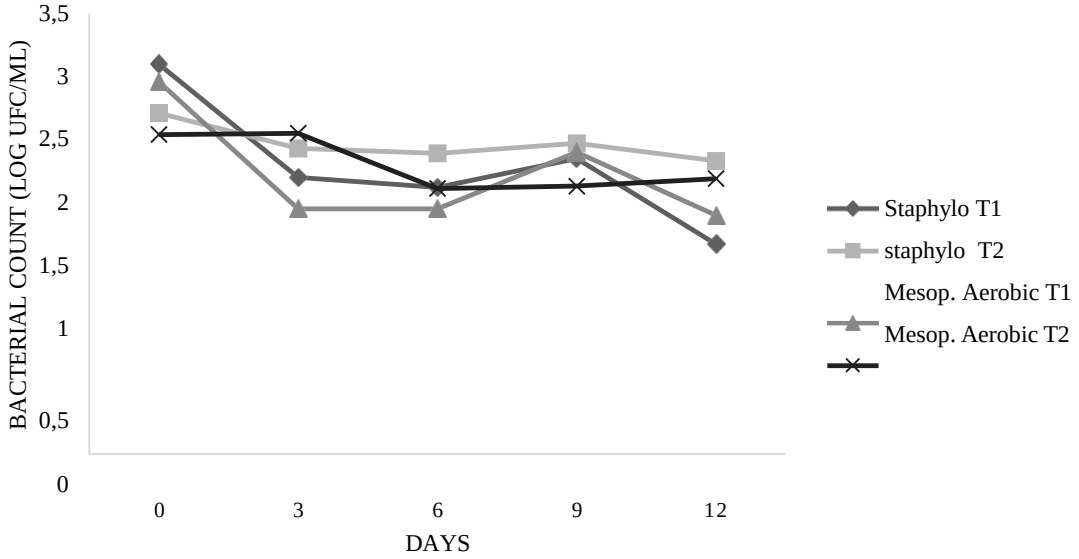

Figure 3: Total bacterial count and *S. aureus* count during 12 days of application of the control: T1: Lactic acid (*S. aureus*: y = 3.102-0.2719x; R2: 68.16% / month total: y = 2, 7505-0.1771x; R2: 36.22%;) and T2: Safranine as *post dipping*. (*S. aureus*: y = 2.6826-0.0722x; R2 = 62.33%/ total mesophilic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

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

Active principle	*Staphylococcus*			
	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)

S: Sensitive, R: Resistant; I: Intermediate

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

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.

Treatment	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
IFDMO	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}				

Treatment	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}				
IFDMO	1,39±0,12^{Cb}	0,00±0,01^{Cc}	2,14±0,13^{Ba}	2,57±0,01^{Aa} LED	0,000	0,000	0,000	7,93
	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}				

Control (milk only); PDIM (milk + Sf photosensitizing agent + LED lighting), LED (milk + LED lighting); PS (Photosensitizer) (milk + photosensitizing agent Sf). CV: Coefficient of variation. P (<0.05). Different capital letters in the same column: the treatments differ between each other. Different lowercase letters on the same line of isolates differ from each other.

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.

Treatment	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}				
IFDMO	1,39±0,12^{Ba}	0,00±0,00^{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}	0,000	0,000	0,003	10,17
PS	2,26±0,17^{Ab}	1,74±0,19^{Bb}	2,35±0,07^{Aab}	2,93±0,02^{Aab}				

Treatment	Time 48 hours							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}				
IFDMO	1,45±0,21^{Ca}	0,00±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}	0,005	0,000	0,001	11,25
PS	2,15±0,01^{BCab}	2,64±0,10^{Aab}	1,87±0,12^{Bb}	2,83±0,06^{Aab}				

Control (broth only); PDIM (broth + Safranine photosensitizing agent) Sf + LED lighting); LED (broth + LED lighting); PS (Photosensitizer) (broth + Safranin photosensitizing agent). CV: Coefficient of variation. P (<0.05). Different capital letters in the same column: the treatments differ between each other. Different lowercase letters on the same line: isolates differ from each other.

Table 5. Count of *Staphylococcus aureus* and coagulase negative *Staphylococcus* sessile cells (log UFC / cm2) present in stainless steel coupons grown in milk and Muller Hilton broth incubated at 7° C for 48 h.

Treatment	Milk				P value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Controle	<3,00±0,01^{Aa}	3,00±0,01^{Aa}	2,57±0,01^{Ab}	<3,00±0,01^{Aa}				
IFDMO	1,71±0,15^{Cbc}	1,99±0,12^{Ba}	1,39±0,12^{Cc}	1,6±0,213^{Cbc}	0,000	0,000	0,000	3,94
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
Controle	<2,00±0,01^{Aa}	<2,00±0,01^{Aa}	<2,00±0,01^{Aa}	<2,00±0,01^{Aa}				
IFDMO	0,00±0,01^{Ba}	0,00±0,01^{Ba}	0,00±0,01^{Ca}	0,35±0,49^{Ba}	0,017	0,000	0,016	16,97
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}				

Control (milk / broth); PDIM (milk / broth + photosensitizing agent) Safranine (Sf) + LED lighting); LED (milk / broth + LED lighting); PS (Photosensitizer) (milk / broth + Safranine photosensitizing agent). CV: Coefficient of variation. P (<0.05). Different capital letters in the same column: the treatments differ between each other. Different lowercase letters on the same line: isolates differ from each other.

Table 6. Count of *Staphylococcus aureus* and coagulase negative *Staphylococcus* sessile cells (log UFC / cm2) present in stainless steel coupons grown in milk and Muller Hilton broth incubated at 35° C for 48 h.

Treatment	Milk				P value			CV
	SO1	SO2	SO3	SO4	Bac	Trat	Bac*Trat	
Controle	5,43±0,09^{Aa}	5,00±0,01^{ABb}	4,00±0,01^{Ac}	4,00±0,01^{Ac}				
IFDMO	4,64±0,06^{Ba}	4,45±0,31^{Ba}	0,00±0,00^{Cb}	0,00±0,00^{Cb}	0.000	0.000	0.000	3,52
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}	NS	0.000	NS	28,45

Control (milk / broth); PDIM (milk / broth + Safranine photosensitizing agent (Sf) + LED lighting); LED (milk / broth + LED lighting); PS (Photosensitizer) (milk / broth + Safranine photosensitizing agent). NS> not significantCV: Coefficient of variation. P (<0.05). Different capital letters in the same column: the treatments differ between each other. Same lowercase letters on the same line: there is no significant difference.