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BIOTERAPIA COM *Lucilia cuprina*: ATIVIDADE ANTIMICROBIANA E EFICÁCIA EM FERIDAS INFECTADAS EXPERIMENTALMENTE

Santa Maria, RS, Brasil
2022

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Tese apresentada ao Curso de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Doutor em Medicina Veterinária.**

Orientadora: Profª Drª. Silvia Gonzalez Monteiro

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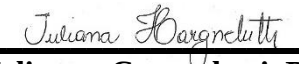

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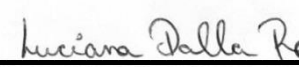

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“Medicus curat,
natura sanat.”

RESUMO

BIOTERAPIA COM *Lucilia cuprina*: ATIVIDADE ANTIMICROBIANA E EFICÁCIA EM FERIDAS INFECTADAS EXPERIMENTALMENTE

AUTORA: Janaína Brand Dillmann

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As feridas cutâneas são frequentes em animais devido a fatores comportamentais e de manejo, tema de estudo constante devido seu interesse clínico, científico e econômico. Embora bactérias façam parte da microbiota da pele e de feridas, sua proliferação exacerbada e potencial formação de biofilme retarda o processo de cicatrização. Além disso, o uso inapropriado de terapia antibiótica é a principal causa da disseminação de microrganismos resistentes. Com isso, a terapia larval surge como uma alternativa no tratamento de feridas, uma vez que larvas necrobiontófagas como da espécie *Lucilia cuprina* possuem potencial de desbridamento, promovem a limpeza das lesões através do consumo do tecido morto local e diminuem o grau de infecção, já que as bactérias são ingeridas e destruídas no trato digestório das larvas. Além desses benefícios, a mudança de pH, estímulo da neovascularização e lavagem tecidual pela exsudação local induzida aceleram o processo cicatricial. Dessa forma, os objetivos desse estudo foram: (i) avaliar o potencial antimicrobiano *in vitro* de larvas de *L. cuprina* estimuladas por bactérias e (ii) avaliar a eficácia e segurança terapêutica de larvas de *L. cuprina* em feridas infectadas induzidas em ratos Wistar. O objetivo (i) foi alcançado através de coleta de extratos do corpo inteiro das larvas incubadas durante 24 horas com uma suspensão bacteriana de *Staphylococcus aureus*, *Pseudomonas aeruginosa* ou *S. aureus* resistente à meticilina (SARM); e análise antimicrobiana através de ensaio de Unidade Formadora de Colônia (UFC) em dois tempos de incubação (0 e 6 horas) com grupos de extratos larvais pré-tratados e não tratados (PBS), em comparação com um grupo de controle do crescimento bacteriano. Os resultados do experimento (i) mostraram significativa atividade contra *P. aeruginosa*, uma ligeira diminuição do crescimento bacteriano para *S. aureus* e SARM. Já os resultados do objetivo (ii) foram alcançados através de análises clínicas, microbiológicas, histopatológicas das feridas, e hematológicas e bioquímicas dos animais. Uma contração significativa da área da ferida (>95%) no grupo tratado com larvas foi alcançado ao 9º dia, em comparação com o 15º dia no grupo tratado com antibiótico. Houve eliminação completa das colônias bacterianas de SARM após o segundo tratamento (6º dia) em comparação com um aumento na proliferação bacteriana do grupo controle. Na histopatologia, observou-se uma ferida limpa no grupo tratado com larvas, formação adequada de colágeno e reepitelização no 15º dia, além de aumento nos níveis de plaquetas na avaliação hematológica. Concluiu-se então, que as larvas de *L. cuprina* são seguras e eficazes na cicatrização da lesão e na eliminação de SARM e que podem representar uma alternativa para a utilização em cicatrização de feridas resistentes aos tratamentos convencionais.

Palavras-chave: Calliphoridae, Feridas, *Staphylococcus aureus* resistente a meticilina, Terapia larval

ABSTRACT

BIOTHERAPY WITH *Lucilia cuprina*: ANTIMICROBIAL ACTIVITY AND EFFECTIVENESS IN EXPERIMENTALLY INFECTED WOUNDS

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Skin wounds are frequent in animals due to behavioral and management factors, and a subject of constant study due to their clinical, scientific, and economic interest. Although bacteria are part of the normal microbiota of the skin and wounds, the onset of an infection by their exacerbated proliferation and potential biofilm formation slows the healing process. In addition, inappropriate use of antibiotic therapy is a major cause of the spread of resistant microorganisms. Larval therapy emerges as an alternative for wound treatment, since necrophagous larvae such as the species *Lucilia cuprina* have present debridement potential, promote lesion cleansing by consuming local dead tissue and decrease the degree of infection, since bacteria are ingested and destroyed in the gut of the larvae. In addition to these benefits, pH change, stimulation of neovascularization, and tissue washing by induced local exudation accelerate the healing process. Thus, the objectives of this study were: (i) to evaluate the *in vitro* antimicrobial potential of *L. cuprina* larvae challenged by bacteria and (ii) to evaluate the efficacy and therapeutic safety of *L. cuprina* larvae on induced infected wounds in Wistar rats. Objective (i) was achieved by collecting whole body extracts from larvae incubated for 24 hours with a bacterial suspension of *Staphylococcus aureus*, *Pseudomonas aeruginosa* or methicillin-resistant *S. aureus* (MRSA). An antimicrobial analysis by Colony Forming Unit (CFU) assay was performed at two incubation times (0 and 6 hours) with pretreated and untreated (PBS) groups of larval extracts compared to a control group of bacterial growth. The results of experiment (i) showed significant activity against *P. aeruginosa*, a slight decrease in bacterial growth for *S. aureus* and MRSA. The results of objective (ii) were achieved through clinical, microbiological, histopathological analyses of the wounds and hematological and biochemical analyses of the animals. A significant shrinkage of the wound area (>95%) in the larvae-treated group was achieved at 9th day compared to 15th day in the antibiotic-treated group. There was complete elimination of MRSA colonies after the second treatment (6th day), compared to an increase in bacterial proliferation in the control group. On histopathology, a clean wound was observed in the larval treated group, as also adequate collagen formation and re-epithelialization on 15th day, and an increase in blood platelet levels in the hematological evaluation. It is then concluded that *L. cuprina* larvae are safe and effective in accelerating wound treatment and eliminating MRSA, and may represent an alternative for use in wound healing resistant to conventional treatments.

Keywords: Calliphoridae, Maggot debridement therapy, Methicillin-resistant *Staphylococcus aureus*, Wound.

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1 INTRODUÇÃO

Uma ferida é representada pela perda da integridade anatômica, fisiológica e funcional dos tecidos (AMALSADVALA & SWAIN, 2006) e pode ser classificada quanto a apresentação clínico-cirúrgica, tempo de exposição e contaminação bacteriana (WALDRON; ZIMMERMAN-POPE, 2007). A função primária da pele normal e intacta é controlar a colonização de tecidos subjacentes por populações microbianas que vivem na sua superfície, além de potenciais agentes patogênicos ambientais (BOWLER et al., 2001). Assim, embora as bactérias façam parte da microbiota normal da pele, a contaminação bacteriana ocorre, pois, a perda da integridade e exposição do tecido subcutâneo proporciona um ambiente úmido, quente e nutritivo para sua proliferação.

Com a finalidade de reconstituir a continuidade tecidual que foi interrompida, o processo de cicatrização envolve uma série de reações físicas, químicas e biológicas que ocorrem logo após a lesão (MODOLIN; BEVILACQUA, 1985). Esse processo é basicamente dividido em inflamação, proliferação e remodelamento (HEDLUND, 2008). Quando a ferida é considerada limpa, a cicatrização pode ocorrer por primeira intenção, pois há perda mínima de tecido, reduzido potencial de infecção e possibilidade de aproximação das bordas da lesão por suturas. Porém, após a instalação de uma infecção, seu fechamento se restringe a segunda intenção, o que acaba por retardar o processo de cicatrização (CARVALHO; BORGES, 2011).

O tratamento de feridas infectadas através de terapia antibiótica indiscriminada na prática clínico-cirúrgica é a principal causa do aparecimento crescente de microrganismos resistentes, como o *Staphylococcus aureus* resistente à meticilina (SARM) (CEFAI et al., 1994; WEESE et al., 2000; 2005; 2007; ALVES et al., 2008). Relatos da transmissão de microrganismos entre humanos e animais aumentam a preocupação frente ao papel do animal em infecções de humanos, e vice-versa, sendo necessária a busca de tratamentos alternativos que evitem a disseminação desses patógenos (CEFAI et al., 1994; MANIAN, 2003; WEESE et al., 2005; MOODLEY et al., 2008; LOEFFLER et al., 2010; JORDAN et al., 2011; EVEILLARD et al., 2015; WORTHING et al., 2018).

Deixada de lado, pelo advento dos antibióticos e do desbridamento cirúrgico nos anos 40 (KERRIDGE et al., 2005), a terapia com larvas de mosca (MDT) não é nenhuma nova descoberta, mas reemerge como uma excelente alternativa no tratamento de feridas

intratáveis tanto em humanos quanto em animais. As larvas de moscas necrófagas da família Calliphoridae, fornecem desbridamento rápido e seletivo, por liquefação e ação mecânica de digestão do tecido desvitalizado (SHERMAN, 2009), bem como aceleração da cicatrização de feridas devido a secreção de enzimas proteolíticas que levam à produção de grandes quantidades de fluído os quais auxiliam na irrigação, migração de fibroblastos e oxigenação tecidual (BLAKE et al., 2007; NIGAM et al., 2010; PATRICIA, 2011). Além disso, possuem marcado efeito antimicrobiano, inclusive contra a SARM, bem como um efeito inibitório na formação do biofilme de bactérias (DU PLESSIUS; PRETORIUS, 2011). A espécie mais estudada mundialmente e de escolha para utilização em MDT é a *Lucilia sericata* (MEIGEN, 1826) (Diptera: Calliphoridae) (WEIL et al., 1933). No entanto, outras moscas já foram relatadas para aplicação da MDT, entre elas, *Lucilia cuprina* (WIEDEMANN, 1830) (Diptera: Calliphoridae) que tem sido utilizada com sucesso no tratamento de feridas humanas (PAUL et al., 2009; TANTAWI et al., 2010; NAIR et al., 2021). Essa espécie de díptero possui distribuição mundial, grande potencial terapêutico, baixo custo e fácil manutenção em laboratório.

O presente estudo teve como objetivo desenvolver dois experimentos: um "*in vitro"* para identificação do potencial antimicrobiano de extratos totais de larvas de *L. cuprina* previamente incubadas com bactérias frente aos microrganismos gram-positivos e gram-negativos mais comumente isolados em feridas: *Staphylococcus aureus*, *Staphylococcus aureus* resistente à meticilina e *Pseudomonas aeruginosa;* e outro "*in vivo*" para a avaliar a eficácia e segurança da utilização de larvas de *L. cuprina* em feridas infectadas induzidas em ratos Wistar.

2 REVISÃO DA LITERATURA

2.1 FERIDAS

Feridas correspondem a uma ruptura da continuidade normal da estrutura corpórea e podem ser classificadas em fechadas ou abertas, sendo que feridas abertas são classificadas com base no tempo de exposição e contaminação bacteriana (WALDRON; ZIMMERMAN-POPE, 2007). Considera-se classe 1, feridas com até seis horas de duração, com contaminação mínima; classe 2, feridas de seis a 12 horas com contaminação significativa; e classe 3, feridas de 12 horas ou mais de evolução, macroscopicamente contaminadas. Com relação ao grau de contaminação, são classificadas em: ferida limpa, criada cirurgicamente, sob condições assépticas; ferida limpa-contaminada, a que apresenta mínima contaminação que pode ser eficazmente removida; ferida contaminada a que apresenta intensa contaminação com presença de corpos estranhos; e ferida suja (infectada) caracterizada por um processo infeccioso em curso. Por definição as feridas infectadas possuem mais de 10^5 microrganismos por grama de tecido (WALDRON; ZIMMERMAN-POPE, 2007). Já a cicatrização de feridas cutâneas pode ser classificada em cicatrização de primeira intenção, quando os bordos da ferida estão próximos e não há contaminação ou de segunda intenção, quando os bordos estão distantes e contaminados (McGAVIN; ZACHARY, 2007).

De acordo com Neto (2003), alguns fatores podem retardar o processo de reparação cutânea, tais como: desnutrição, hipovolemia, hipotensão, hipóxia, hipotermia, infecção e o uso de medicamentos de ação anti-inflamatória. Quanto a infecção das feridas, a integridade do tecido e sua perfusão, os processos cicatriciais, o desafio bacteriano e a resposta do hospedeiro têm fortes influências. A contaminação ambiental, particularmente com matéria fecal e o contato humano com animais podem alterar a flora cutânea normal. Estes contaminantes muitas vezes desempenham um papel importante na infecção de feridas, tanto as de origem traumática quanto infecções de feridas no pós-operatório. Segundo Pope (1996), todas as feridas se contaminam, independentemente das precauções tomadas. Se a infecção irá ocorrer ou não, dependerá do tipo e número de bactérias, da presença de corpos estranhos e tecidos desvitalizados e das condições de defesa imunológica do paciente. Uma infecção bacteriana tende a retardar o processo cicatricial, dificultando o tratamento e, consequentemente, aumentando morbidade, mortalidade e custos (HANSON, 2008; WEEZER et al., 2000).

2.1.1 Processo de cicatrização cutânea

O processo de cicatrização é universal, de modo que após o ferimento, ocorre uma sequência de reações físicas, químicas e biológicas cuja finalidade é reconstituir a continuidade tecidual que foi interrompida (MODOLIN; BEVILACQUA, 1985). A interação entre fatores solúveis, tipos celulares e componentes da matriz extracelular desencadeia a cascata de eventos necessária para que ocorra o reparo tissular. O processo de cicatrização é influenciado por fatores gerais, como idade, estado nutricional, a existência de doenças de base, como diabetes, alterações cardiocirculatórias e de coagulação, disfunção renal, quadros infecciosos sistêmicos e uso de drogas sistêmicas; e de fatores locais, como técnica cirúrgica, formação de hematomas, infecção, reação de corpo estranho, uso de drogas tópicas e ressecamento durante a cicatrização (MANDELBAUM et al., 2003). Alguns autores (ORTONE; CLÉVY, 1994) consideram três estágios no processo de cicatrização: inicialmente um estágio inflamatório, seguido por um de proliferação e finalizando como reparo em um estágio de remodelação. No entanto, outros autores (FAZIO et al., 2000) descrevem uma classificação mais completa, dividindo o processo em cinco fases principais: 1 - coagulação; 2 - inflamação; 3 - proliferação; 4 - contração da ferida; 5 – remodelação.

A primeira fase, de coagulação, tem início 5 a 10 minutos após o surgimento da lesão, quando ocorre a vasoconstrição para minimizar a hemorragia, sendo que a formação de coágulo garante resistência e proteção a ferida (STASHAK, 2004) e também permite o cruzamento da fibronectina, permitindo o ingresso de fibroblastos, células endoteliais e queratinócitos na ferida (CLARK et al., 1982). Essa fase depende da atividade plaquetária e da ativação da cascata de coagulação (TERKELTAUB; GINSBERG, 1998) que inicia a liberação de sustâncias que desencadeiam as próximas fases, tais como vasoativos, proteínas adesivas, citocinas, fatores de crescimento e proteases (CLARK, 1985).

Em sequência inicia a fase dois, inflamatória, caracterizada pela vasodilatação e aumento da permeabilidade capilar estimulada por agentes quimiotáticos e de células inflamatórias, como os leucócitos polimorfonucleares (PMN), macrófagos e linfócitos (MANDELBAUM et al., 2003). OS PMN, principalmente os neutrófilos, são responsáveis pela fagocitose das bactérias nos momentos iniciais da lesão tecidual e são o primeiro subconjunto a entrar na ferida, chegando ao local da lesão no prazo de 24 a 48

horas. São estimulados por prostaglandinas, complemento, interleucina-1 (IL-1), interleucina-6 (IL-6), fator de necrose tumoral alfa (TNF-α), fator de crescimento transformador-beta (TGF-β) e produtos bacterianos (EFRON et al., 2005). O papel secundário de neutrófilos é manter a fase inicial do processo inflamatório através da excreção de citocinas (EFRON et al., 2005).

Os macrófagos, células inflamatórias mais importantes da fase, surgem 48 a 96 horas após o ferimento e permanecem até o fechamento da lesão. Além de fagocitar bactérias e desbridar corpos estranhos, direcionam o desenvolvimento do tecido de granulação (DIEGELMANN, 1981). A migração de macrófagos é influenciada por IL-1, IL-6, IL-8 e o TNF-α. São fonte primária de diversas citocinas e fatores de crescimento e necessários para apoiar o recrutamento e ativação celular, a síntese da matriz extracelular, a angiogênese e a remodelação (MEDEIROS; FILHO, 2016). Os linfócitos vão ter influência sobre os macrófagos e aparecem na ferida em torno de uma semana após a lesão inicial. Além disso, a fibronectina, sintetizada por fibroblastos, queratinócitos e células endoteliais, realiza importante papel durante essa fase, aderindo simultaneamente a fibrina, ao colágeno e a outros tipos de células, formando uma "cola" responsável por consolidar o coágulo (MOSHER; FURCHT, 1981). Suas propriedades quimiotáticas promovem a opsonização e fagocitose de corpos estranhos e bactérias.

A terceira fase, de proliferação é dividida em três subfases: a) reepitelização; b) fibroplasia, c) neovascularização. Durante a reepitelização (a) ocorre a migração de queratinócitos não danificados das bordas de anexos epiteliais ou das margens epidérmicas em caso de feridas de espessura parcial e apenas das margens quando a ferida é total (MANDELBAUM et al., 2003). A IL-6 promove a indução mitótica de queratinócitos e seus movimentos também são determinados pelo conteúdo de água no leito da ferida, sendo que em feridas superficiais abertas e ressecadas a reepitelização é mais lenta do que nas ocluídas (SATO et al., 1999; WINTER, 1962).

A fase b da proliferação, inclui a fibroplasia e a formação da matriz, processo importante na formação do tecido de granulação, sendo a última dependente de fibroblastos. O recrutamento de fibroblastos vem de produtos derivados de plaquetas, tais como: fator de crescimento derivado de plaquetas (PDGF), fator de crescimento insulin-like (IGF-1) e fator de crescimento epitelial (MEDEIROS; FILHO, 2016). O fibroblasto é responsável por produzir elastina, fibronectina, glicosaminoglicana e proteases, responsáveis pelo desbridamento e remodelamento fisiológico (VAN WINKLE, 1967) e que promoverão a substituição da matriz provisória por glicoproteínas, proteoglicanos,

colágeno tipo III e por fim, colágeno tipo I (STASHAK, 2004). Juntos, macrófagos e fibroblastos liberam fatores de crescimento e citocinas que contribuem para a migração de fibroblastos: fator de crescimento fibroblástico (FGF), IGF-1, fator de crescimento endotelial vascular (VEGF), IL-1, IL-2, IL-8, PDGF, fator de crescimento transformador-α e β (TGF-α, TGF-β) e TNF-α (EMING et al., 2007; SHUGART et al., 2008). A última fase da proliferação é a neovascularização/angiogênese (c), essencial para o suprimento de oxigênio e nutrientes para a cicatrização. Os eventos da angiogênese são regulados por meio de fatores de crescimento (TNF-a, TGF-b, VEGF, FGF, PDGF) derivados de plaquetas, macrófagos e células endoteliais danificadas (RAJA et al., 2007).

A neovascularização fornece nutrientes e oxigênio para nutrir o tecido em reparação. À medida que o fluxo sanguíneo e a oxigenação são restabelecidos, o principal fator desencadeador da angiogênese é reduzido e os vasos neoformados começam a diminuir (NETO, 2003). Dá-se início então a penúltima fase, de contração da ferida, onde fibroblastos ativados se diferenciam em miofibroblastos, que por sua vez aproximam as margens da ferida, forçando as fibras de colágeno a se sobreporem e se entrelaçarem (PAGANELA et al., 2009).

A fase final do processo cicatricial consiste na remodelação tecidual. Ao contrário das outras fases de cicatrização, a remodelação dos componentes do colágeno e matriz, como ácido hialurônico e proteoglicanos, persiste por longo tempo após o fechamento da ferida, é o período no qual os elementos reparativos da cicatrização são transformados em tecido maduro com características diferenciadas (NETO, 2003). É responsável pelo aumento da força de tensão e pela diminuição do tamanho da cicatriz e do eritema. A neovasculatura diminui, a cicatriz formada é considerada avascular e ocorrem reformulações dos colágenos, melhoria nos componentes das fibras colágenas e reabsorção de água, eventos que permitem aumento da força da cicatriz e diminuem sua espessura (DOLLOIN et al., 1985).

2.1.2 Uso de antibióticos e resistência

A microbiota normal da pele é constituída por bactérias, tais como *Micrococcus* spp., *Staphylococcus* sp. não hemolítico, *Actinetobacter* spp., entre outras. Porém, as bactérias encontradas variam de acordo com a região geográfica, sendo que há ocorrência de organismos transitórios, isolados com menor frequência ou em menor número, que incluem *Corynebacterium* sp., *Streptomyces* sp., *Streptococcus* sp. não hemolítico,

Bacillus sp., e outros gêneros não entéricos (MILLER et al., 2013). Em sua pesquisa, Westgate et al. (2010), isolaram bactérias presentes em feridas de pele de equinose encontraram prevalentemente *Pseudomonas aeruginosa* e *Enterococcus faecium*. *Staphylococcus,* entretanto, foi a bactéra mais comumente isolada em pele íntegra e lesionada. Em pequenos animais, os organismos infecciosos habituais incluem *Staphylococcus* e espécies de *Streptococcus,* embora *Clostridium* e espécies de *Pasteurella* são frequentemente vistas em feridas de mordedura/punção e *Pseudomonas* é ocasionalmente vista em pacientes com queimaduras (BOWLT; FRIEND, 2011).

A terapia antibiótica de largo espectro em feridas infectadas, como combinações de penicilina G e gentamicina, é uma abordagem comum na clínica de rotina. Em geral, essa abordagem é feita antes que resultados de cultura e susceptibilidade sejam realizados. No entanto, sabe-se que a penicilina G pode não ser a escolha apropriada em algumas circunstâncias, uma vez que diversos patógenos comuns em feridas, como exemplo o *Staphylococcus* sp., produzem enzimas capazes de inativar esse fármaco (GOMEZ & HANSONS, 2005). Esse uso inapropriado de terapia antibiótica é a principal causa da disseminação de microrganismos resistentes, como o *S. aureus* resistente à meticilina (SARM) (CEFAI et al., 1994; WEESE et al., 2000; WEESE et al., 2005; WEESE et al., 2007). O SARM é resistente a todos os antimicrobianos b-lactâmicos e frequentemente a uma ampla gama de classes antimicrobianas devido à presença de uma proteína alterada de ligação à penicilina. Por conseguinte, as infecções podem ser difíceis de tratar e estão associadas a um aumento da morbilidade, mortalidade e custos de tratamento em comparação com infecções causadas por cepas de *S. aureus* sensíveis à meticilina (CEFAI et al., 1994; WEESE et al., 2000).

Estafilococos resistentes à meticilina, incluindo SARM e *S. pseudintermedius* resistente à meticilina (SPRM) representam uma ameaça à saúde animal e humana em todo o mundo. Pela maior proximidade com animais enfermos, médicos veterinários que tratam animais de companhia possuem maior risco de portar SARM do que proprietários ou veterinários que não tratam animais (MOODLEY et al., 2008; LOEFFLER et al., 2010; JORDAN et al., 2011; EVEILLARD et al., 2015; WORTHING et al., 2018). Sua presença em feridas infeccionadas foi isolada em um amplo número de cães e gatos na Austrália (WORTHING et al., 2018) e as linhagens de SARM isoladas desses animais foram semelhantes às encontras em veterinários australianos que tratam essas espécies (GROVES et al., 2016; WORTHING et al., 2018). Embora médicos humanos atribuam aos animais de estimação a função de fonte de infecções de SARM aos seus tutores

(MANIAN, 2003), essas infecções ocorrem de forma bidirecional, sendo os humanos, fontes de infecção para os animais também. A transmissão de *S. pseudintermedius* entre animais de companhia e humanos não está bem caracterizada, porém, sabe-se que o SPRM pode causar infecções em pessoas imunodeprimidas (STARLANDER et al., 2014). Assim, médicos veterinários e seus próprios animais de estimação estão em um grupo de risco como carreadores da bactéria (MORRIS et al., 2010), bem como tutores que trabalham na área da saúde são fatores de risco no transporte de SPRM e SARM para cães (BOOST et al., 2008; NIENHOFF et al., 2011).

Em equinos, o primeiro caso relatado de SARM foi descrito em 1996 (ANZAI et al., 1996) e os relatos só tem aumentado. Apesar de envolver vários sítios em equinos, a ocorrência mais comum é em feridas acidentais e cirúrgicas (MADDOX et al., 2010). Além disso, Maddox et al. (2010), demonstraram que cepas de SARM equinas tem um perfil de resistência maior do que a vista em isolados de humanos e pequenos animais, aumentando a preocupação frente ao papel do animal em infecções de humanos (CEFAI et al., 1994; WEESE et al., 2000; 2005; MANIAN, 2003).

A administração de ceftiofur, aminoglicosídeos e penicilina foram considerados como fatores de risco associados a maior colonização de SARM durante hospitalizações, sendo que cavalos que receberam pelo menos 72 horas de tratamento com penicilina tiveram 5 a 8 vezes mais chance de abrigar SARM comparado aos animais que não receberam o mesmo tratamento (SCHNELLMANN et al., 2006; WEESER et al., 2007). Os mesmos autores propõem que o uso de terapia antibiótica seja evitado em tratamentos de feridas superficiais, uma vez que a limpeza regular e o desbridamento da ferida é eficaz para reduzir a carga bacteriana. Alternativas como a aplicação de mel, açúcar e o desbridamento através da terapia larval vem sendo estudadas (BEXFIELD et al., 2004; SCHNELLMANN et al., 2006; VISAVADA et al., 2008; WRIGHT et al., 1998).

2.2 ORDEM DIPTERA

A ordem Diptera (di= dois, ptera= asa), é assim denominada, por incluir insetos que contém um par de asas membranosas anteriores bem desenvolvidos e um segundo par reduzido denominado halteres ou balancins, que possuem a função de equilíbrio, atribuindo estabilidade durante o voo (MELLO, 2003). Essa ordem possui uma abrangente diversidade, apresentando cerca de 153.000 espécies no mundo

(THOMPSON, 2008) e em torno de 8.700 espécies no Brasil (AMORIM, 2009; CARVALHO et al., 2012). A ordem tem ampla distribuição mundial, abrangendo todos os continentes, porém apresenta maior diversidade nas regiões tropicais, sendo catalogadas mais de 30 mil espécies, distribuídas em 118 famílias (AMORIM, 2009; MELLO, 2003), tornando-a a segunda maior da classe Insecta (WIEGMANN et al., 2011). Estes insetos desempenham papel ecológico como polinizadores, podem ser vetores de doenças e são parte da dieta de diversos animais. Seus hábitos alimentares variados também possuem importante papel decompositor, sendo algumas espécies saprófagas, outras necrófagas e algumas biontófagas, podendo estar presentes em excrementos, material vegetal e animais em decomposição (MAVÁREZ-CARDOZO et al., 2005).

Duas subordens são amplamente reconhecidas: Nematocera e Brachycera (CARVALHO et al., 2012; MAcALPINE, 1981), sendo a subordem Brachycera, em seu conjunto, a mais diversa das famílias de Diptera (GRIMALDI; ENGEL, 2005). A diferenciação das subordens se dá pelo número de segmentos da antena, onde em Brachycera, as mesmas apresentam três a cinco artículos com flagerômeros distais modificados em arista, podendo apresentar sutura pedicelar (=sutura antenal) (CARVALHO et al., 2012). A mesma é dividida em quatro infraordens: Stratiomyomorpha, Xylophagomorpha, Tabanomorpha e Muscomorpha. A infraordem Muscomorpha é separada em duas divisões: “Aschiza” e “Schizophora”, em função da ausência ou presença de uma sutura ptilineal, respectivamente (CARVALHO et al., 2012).

O desenvolvimento é do tipo holometábolo, ou seja, possuem metamorfose completa (ovo, larva, pupa, adulto). Os ovos assumem formas diversas, como globular, esférico, cônico ou elipsoide, sendo a última mais comum em moscas (COSTA et al. 2006). As larvas desta ordem constituem de indivíduos com característica vermiforme, apódes, e as mudanças de um instar ao outro são caracterizadas pela substituição da cutícula e outras estruturas como as sensilas do tórax (COSTA et al., 2006; BORROR et al., 2011). A pupa é formada pelo tegumento do último instar larval, o qual é chamado de pupário. Nessa fase o inseto não se alimenta e não se movimenta até atingir a forma adulta e emergir do pupário (COSTA et al. 2006). Os adultos de Schizophora emergem do pupário utilizando o ptilíneo, que é uma estrutura membranosa localizada entre os olhos, que pressiona o pupário, formando uma fenda de formato circular, pela qual o adulto emerge (CARVALHO et al., 2012).

2.2.1 Família Calliphoridae

A família Calliphoridae é cosmopolita e tem reconhecidas mais de 150 gêneros e 1000 espécies (VARGAS; WOOD, 2010). Por conta de sua diversidade ecológica é capaz de ocupar e se adaptar a diversos habites (SKEVINGTON; DANG, 2002). São dípteros de médio a pequeno porte, de coloração, em geral, metálica, em tons de azul, violeta, verde e cuprinos. No Brasil, recebem popularmente o nome de "moscas varejeiras" (BUZZI, 1994; LENKO; PAPAVERO, 1996). É composta por 12 subfamílias: Auchmeromyinae, Bengaliinae, Calliphorinae, Chrycomyinae, Helicoboscinae, Luciliinae, Melanomyinae, Mesembrinellinae, Phumosiinae, Poleniinae, Rhiniinae e Toxotarsinae (ROGNES, 1997), dentre as quais, 29 gêneros e 99 espécies ocorrem na região neotropical agrupados nas seguintes subfamílias: Chrysomyinae, Calliphorinae, Lucilliinae, Mesembrillinae, Polleniinae, Rhiniinae e Toxotarsinae (KOSMANN et al., 2013).

Adultos possuem arista plumosa com cerdas longas até o ápice, calipteras torácicas e meron com cerdas bem desenvolvidas, pós-escutelo ausente ou pouco desenvolvido, segmentos abdominais sem cerdas distais ou, se presentes, pouco desenvolvidas, duas cerdas na notopleura e sutura ptilineal, por se tratar da divisão Schizophora (SERRA-FREIRE; MELLO, 2006). As larvas podem ser biontófagas, necrófagas ou necrobiontófagas, podendo causar miíases obrigatórias (primárias) e facultativas (secundárias), sendo importantes como decompositoras e também para uso na saúde animal e humana (BAUMHOVER, 1966).

Na Região Neotropical são reconhecidos os seguintes gêneros: *Cochliomyia* Tonwsend, 1915, *Compsomyiosps* Townsend, 1918, *Lucilia* Robineau-Desvoidy, 1830 (incluindo *Phaenicia* Robineau-Desvoidy), *Calliphora* Robineau-Desvoidy, 1830, e *Chrysomya* Robineau-Desvoidy, 1830. Espécies pertencentes ao gênero *Chrysomya* Robineau-Desvoidy e ao gênero *Lucilia* Robineau Desvoidy, são consideradas invasoras no território brasileiro, tendo registros de origem Oriental e Australiana e Afrotropical no caso de *Lucilia* (STEVENS et al., 2002; KOSMANN et al., 2013).

2.2.1.1 *Lucilia cuprina*

Atualmente são conhecidas 17 espécies do gênero *Lucilia* Robineau-Desvoidy 1830, existentes na região Neotropical (KOSMANN et al., 2013). A espécie *L. cuprina*

(WIEDEMANN, 1830) (Figura 1), é nativa da região Afrotropical ou Oriental (AUBERTIN, 1933; STEVENS; WALL, 1996). Tem distribuição ampla nas regiões de clima tropical e de clima temperado, ocorrendo do sul dos Estados Unidos até o Uruguai e ao norte da Argentina (LINHARES, 1981). Tem alta preferência por áreas densamente habitadas/urbanas e não apresenta preferência por determinada estação do ano (LINHARES, 1981; VIANNA et al., 1998).

Suas larvas se alimentam de tecido morto, o que a caracteriza como miíase secundária/facultativa, entretanto, quando há restrição de opções alimentares, podem se tornar invasoras de tecido vivo (VISCIARELLI, 2007). Em rebanhos ovinos, *L. cuprina* é identificada como causadora de efeitos deletérios (WALL, 2012). Porém, nesse caso, a miíase não se aprofunda e ocorre devido ao acúmulo de gordura, sujeira e umidade entre a lã e a pele, que torna o cheiro atrativo para a deposição de ovos. Além disso, pode ser encontrada associada a cadáveres humanos, o que a torna importante na entomologia forense, principalmente durante a fase ativa de decomposição (SUKOTASON et al., 2007; PAES et al., 2005).

Figura 1 - *Lucilia cuprina*. Fonte: Janaína Brand Dillmann.

2.3 TERAPIA LARVAL

A utilização de larvas de certas espécies de moscas na limpeza de feridas é conhecida há séculos. A primeira observação de larvas e seus efeitos benéficos em feridas foi feita em 1557 por Ambrose Paré (PARÉ, 1557). Em 1829, o Barão D.J Larrey, cirurgião militar do exército de Napoleão, usou as larvas em feridas para reduzir a

infecção bacteriana e afirmou que "as larvas promoviam a cura sem deixar nenhum dano" (GOLDSTEIN, 1931). Os primeiros a aplicar clinicamente larvas em feridas foram Zacharias e Jones durante a Guerra Civil Americana (CHAN et al., 2007).

Porém, o primeiro relato de utilização de forma intencional em um hospital, ocorreu nos Estados Unidos em 1931, pelo cirurgião ortopédico William Baer (BAER, 1931). Durante a primeira guerra mundial, Baer (1931), observou que soldados recolhidos no campo de batalha com feridas infestadas por larvas de moscas apresentavam uma melhora significativa em relação aos outros e associou isso à ação de desbridamento realizado naturalmente por essas larvas. A partir dessa observação, ele aplicou o mesmo princípio no tratamento de pacientes acometidos por osteomielite, 10 anos mais tarde. Apesar do êxito obtido (sucesso em 90% dos casos), a utilização dessa técnica teve de ser interrompida pois alguns pacientes apresentaram tétano.

Foi então que pesquisas visando a desinfecção das larvas e a esterilização de ovos das moscas começaram a se desenvolver (MARTINI; SHERMAN, 2003). A terapia com larvas medicinais, conhecida internacionalmente como Maggot Debridement Therapy (MDT), foi utilizada rotineiramente até meados da década de 40 em mais de 300 hospitais. Porém, com o advento dos antibióticos e o aperfeiçoamento das técnicas de desbridamento cirúrgico, a terapia larval foi considerada obsoleta até o início de 1980 (ECHEVERRI et al., 2010).

No entanto, a resistência bacteriana a inúmeros antibióticos, o custo nos tratamentos e a dificuldade de cicatrização proporcionaram o ressurgimento do interesse pela terapia larval a partir da década de 80 nos Estados Unidos, Reino Unido e Israel (MUMCUOGLU et al., 1999). Atualmente, a terapia larval é utilizada em mais de 30 países ao redor do mundo e cerca de 24 laboratórios fornecem larvas estéreis para esse tipo de tratamento. No Brasil existem somente biotérios com disposição de larvas estéreis para utilização em tratamentos em laboratórios de pesquisa, tais como o Laboratório de Insetos e Vetores na Universidade Federal do Rio Grande do Norte (UFRN) e o Laboratório de Parasitologia Veterinária (LAPAVET) na Universidade Federal de Santa Maria (UFSM).

Para a bioterapia as larvas mais comumente utilizadas são as pertencentes a família Calliphoridae (SHERMAN, 2000), dentre elas, as larvas de *Lucilia* sp. e *Chrysomya* sp. que são necrófagas e comuns em toda região das Américas. As larvas possuem propriedades biológicas vantajosas, não penetram profundamente nos tecidos e dependem de condições aeróbias, permanecendo assim nas superfícies da ferida, usando

suas partes da boca em forma de gancho para se locomover (PATRICIA, 2011). Durante a movimentação sobre a superfície da ferida secretam uma mistura de enzimas digestivas como carboxipeptidases A e B, leucina aminopeptidase, colagenase, e proteases serinas, que quebram o tecido morto, liquefazendo-o, permitindo a ingestão do material resultante (DHOLARIA et al., 2014). Pela ingestão desse material, mecanicamente, as bactérias são eliminadas no trato digestivo das larvas (VIV, 2011).

Além disso, através da secreção de alantoína, amônia, ureia, ácido fenilacético, fenilacetaldeído e carbonato de cálcio, alcalinizam o leito da ferida, tornando-o impróprio para a habitação bacteriana, incluindo a SARM (BLAKE et al., 2007; DU PLESSIS et al., 2011; DHOLARIA et al., 2014). Concomitantemente, a ação mecânica das larvas, a secreção de enzimas proteolíticas e a resposta do tecido do hospedeiro levam à produção de grandes quantidades de fluído que auxiliam na irrigação da ferida, a migração de fibroblastos e a oxigenação tecidual (BLAKE et al., 2007; NIGAM et al., 2010; PATRICIA, 2011). Portanto, a MDT ajuda a desbridar o tecido necrótico do leito subjacente, elimina e interrompe a formação de biofilme das bactérias e acelera a cicatrização da ferida através da adesão de leucócitos, produção de fatores de crescimento, produção de colágeno, aumento da angiogênese, aumento da responsividade dos macrófagos, aumento da fibrinólise e dos níveis de óxido nítrico (CORNELL et al., 2010).

2.3.1 Terapia larval na medicina veterinária

Poucas pesquisas são vinculadas ao uso da terapia larval por médicos veterinários, sendo que a literatura existente é baseada em relatos com pequenos números de pacientes, sem comparação de tratamentos, portanto, com baixo poder estatístico. No entanto, alguns estudos retrospectivos de uso, principalmente em feridas problemáticas, aumentam o reconhecimento de que as larvas necrófagas da família Calliphoridae possuem um bom potencial de desbridamento e desinfecção não traumática (JUKEMA et al., 2002; SHERMAN, 2000; 2002a). Seu tamanho pequeno (1 mm de diâmetro) no primeiro estágio, combinadas com sua capacidade de digerir e remover o tecido necrótico e eliminar as bactérias, permitem acesso e desbridamento eficientemente em determinadas infecções e tratos sinusais (SHERMAN et al., 2007). As secreções também são reconhecidas por promoverem a cicatrização de feridas, possivelmente pelo estímulo

ao crescimento do tecido (SHERMAN et al., 1995; 2000; PRETE, 1997; SHERMAN, 2002, 2003) e ao aumento do fluxo sanguíneo local (WOLLINA et al., 2002).

O uso de terapia larval foi descrito por Dicke (1953) e Bell (2001), com sucesso em um caso de actinomicose em um touro e paniculite em um burro. Thieman (2003), também relatou o uso de larvas esterilizadas no tratamento de uma infecção profunda em um abscesso de um burro. Em equinos, a MDT foi utilizada no tratamento de bursite supraespinhosa (LEPAGE; DOUMBIA, 2012), bursite séptica navicular, casos de laminite severas (WOLF; HANSSON, 2005; BRASS; MORRISON, 2009), abscesso infectado com osteomielite e outras doenças do casco (JURGA; MORRISON, 2004). Investigações mais abrangentes foram descritas por Morrison (2010), que relatou o uso de bioterapia no desbridamento de infecções do casco de equinos, como a laminite, onde a necrose tecidual é uma sequela comum a infecção. O desbridamento cirúrgico profundo nesses casos é impraticável, pois comprometeria a frágil arquitetura do casco equino. Além disso, Morrison (2010), relatou o uso de terapia larval em 41 casos de osteomielite, laminite crônica, sepse da bursa do navicular, sepse crônica da articulação interfalangeana distal, úlceras e abscessos de casco que não cicatrizavam.

Posteriormente, Sherman et al. (2002; 2007), utilizaram a MDT para tratar vários casos, entre eles, um cavalo de 6 anos de idade com extensa laceração do membro pélvico proximal esquerdo, um potro com vasculite obliterativa envolvendo o casco e uma égua de 9 anos com ferida de punção envolvendo a bursa do navicular e tendão flexor digital. Após a MDT, todas as infecções foram controladas e erradicadas, sendo que apenas um cavalo teve que ser sacrificado. Nenhum efeito colateral foi atribuído à terapia larval nesses casos, apenas desconforto devido a cicatrização. Lepage et al. (2012), trataram 41 equinos com diversas lesões (35 cavalos, 4 burros e 2 pôneis de todas as idades e ambos os sexos) através da MDT. As lesões envolviam atrite séptica da bursa do navicular, fístulas, queratomas, osteíte séptica, feridas cutâneas, lacerações, abscessos e feridas infectadas por SARM. Em 38 casos, um resultado favorável foi alcançado em <1 semana. Em todos os casos, pode-se observar o desbridamento, desinfecção e melhora da cicatrização. Em outros dois cavalos, um carcinoma de células escamosas e um melanoma estavam envolvidos em feridas infectadas crônicas e a cicatrização completa não foi alcançada devido à recorrência de tumores subjacentes (LEPAGE et al., 2012).

Kocisova et al. (2003), utilizaram larvas de *Phormia regina* para tratar coelhos com feridas ulceradas crônicas, numa densidade de 8-12 larvas por cm^2 e descreveram o desbridamento promovido como “rápido e seletivo”, sendo bem tolerado pelos animais.

Em pequenos animais, Sherman et al. (2007), trataram dois cães, quatro gatos e um coelho e obtiveram ótimos resultados de desbridamento e retorno de cicatrização em animais cujas feridas não apresentaram retorno positivo com tratamentos convencionais. Além disso, os dados coletados na pesquisa permitiram afirmar que o uso de terapia larval em cães e gatos é segura e pode ser útil mesmo em infecções graves.

A MDT em pequenos animais e equinos promoveu a cicatrização completa em feridas que já haviam falhado em responder ao tratamento médico e cirúrgico convencional. Como dito anteriormente, a terapia auxilia na cicatrização de feridas por desbridamento, desinfecção e formação de tecido de granulação. Caso o desbridamento completo da ferida não ocorra, uma segunda aplicação de larvas é necessária. Em alguns casos é necessário um prévio desbridamento cirúrgico leve para remoção de debris e tecido necrótico seco (LEPAGE et al., 2012). Através do potencial efeito antibacteriano, principalmente referente a SARM e outras bactérias multirresistentes, a terapia larval é uma forma eficaz de prevenir o estabelecimento de infecções graves e disseminação de patógenos.

3 MANUSCRITO 1

(Manuscrito submetido ao Indian Journal of Microbiology)

In vitro antibacterial activity of whole body extracts from bacteria-pretreated *Lucilia cuprina* maggots

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Statements and Declarations

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Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by JBD, TRRL, ARV, AFIMM. The first draft of the manuscript was written by JBD, and all authors critically revised all versions of the manuscript. Resources and supervision were provided by SGM and JFC.

Abstract

Maggot debridement therapy is an alternative method for treating of infected wounds in both human and veterinary medicine. Sterile larvae, such as from *Lucilia cuprina* (Wiedemann 1830) (Diptera: Calliphoridae), stimulate wound healing through debridement of necrotic tissue and disinfection. Hence, we evaluated the antimicrobial activity from bacteria-pretreated *L. cuprina* maggot extracts against the most commonly isolated Gram-positive and Gram-negative bacteria from wounds. In short, whole body extracts were collected after the sterile larvae were incubated for 24 hours with a bacterial suspension of *Staphylococcus aureus* (Rosenbach 1884) (Bacillales: Staphylococcaceae), *Pseudomonas aeruginosa* (Schroeter 1872) (Pseudomonadales: Pseudomonadaceae) or Methicillin-resistant *S. aureus* (MRSA). Larvae were cut into multiple pieces in a microtube containing sterile phosphate-buffered saline (PBS), centrifuged, and the supernatant was filtered and used in antibacterial assays against the same bacteria. A

Colony-Forming Unit (CFU) assay was performed at two incubation times (0 and 6 hours) with pre-treated and non-treated (PBS) larval extracts groups, compared to a bacteria growth control group. The results showed effective activity against *P. aeruginosa*, a slight decrease in bacteria growth for *S. aureus*, and no significant differences for MRSA. With higher antibacterial activity for *P. aeruginosa*, *L. cuprina* may represent a powerful tool to the clinical approach of gram-negative wound healing.

Keywords: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Maggot debridement therapy, MRSA, larvae, antimicrobial.

Introduction

Wound cleansing by fly maggots has been known for centuries. Ambrose Paré [1] made the first observation of the beneficial effects of larvae in wounds in 1557, but only in 1931, orthopedic surgeon William Baer [2] applied it in a hospital environment. Since then, research on disinfection of larvae, sterilization of fly eggs [3] and use of medicinal larvae, internationally known as Maggot Debridement Therapy (MDT), started to be applied routinely. However, it became an obsolete technique until the early 1940s due to the advent of antibiotics and improvements in surgical debridement techniques [4]. Interest resurged in the '80s with the emergence of bacterial resistance to numerous antibiotics, costly treatments, and difficulty in wound healing [5].

Today, MDT is applied in both human and veterinary medicine in more than 30 countries worldwide, and about 24 laboratories provide sterile larvae for this type of treatment. Although considered an alternative therapy, larval therapy alone promotes a set of actions capable of disinfecting, debride the necrotic tissue and stimulate the healing of wounds that would only be achieved with a series of conventional techniques [6].

Larvae from the Calliphoridae family is the most frequently used in therapy due to their several advantageous biological properties, like generally feeding on necrotic tissue, rapid development in the host, and easy rearing in the laboratory [7]. Among them, maggots of *Lucilia sericata* (Meigen 1826) (Diptera: Calliphoridae) and *L. cuprina* are considered as a core of the MDT [8], in all tropical and temperate regions of the world.

Research indicates that the ingestion of bacteria (mechanical act) is the primary method by which the maggots cleaned the wounds of infection [5]. In addition, alkalinization of the wound bed by ammonia by-products of the maggot's digestion makes it unsuitable for bacteria growth [9]. Primarily seeking new alternatives to the use of antibiotics, other studies point out antimicrobial products produced in the larvae gut and secreted/excreted by them [6, 10-11]. Although several studies evaluate those antimicrobial activities of larval excretions/secretions (ES) or whole-body extracts, only a few focused-on *L. cuprina* [12-14], compared to *L. sericata* [11, 15-19]. However, those *in vitro* studies differ between positive and negative antimicrobial activity of ES and larval extracts against Gram-positive and Gram-negative bacteria [12, 20-22].

Considering the practical use in MDT, where the wound bed is always contaminated, maggots activate its immune mechanism and produce an antagonistic response to stress to protect themselves from the environment. The question raised is whether this response would have a direct influence on reducing or eliminating bacteria. Thus, this study aimed to evaluate an extract of *L. cuprina* maggots pre-treated or not with bacteria, to identify antimicrobial activity against the most commonly isolated Gram-positive and Gram-negative aerobes from wounds, Methicillin-resistant *S. aureus*, *S. aureus*, and *P. aeruginosa* [11].

Material and methods

Rearing of Lucilia cuprina in the laboratory and larvae obtainment

The colony of *L. cuprina* originated from adults collected in traps in Santa Maria, Rio Grande do Sul, Brazil. Flies were kept in entomological cages (30cm x 30cm x 30cm) at 25 ± 1°C under a light/dark cycle (12:12 H) and relative air humidity of 70% ± 5%. Adult flies were fed with honey made available on a Petri dish (80 x 15mm) with rice, and water *ad libitum*. For oviposition, a bovine liver was available in a Petri dish (80 x 15mm). Eggs were carefully removed through a moistened brush and placed in a Petri dish (80 x 15mm) with the larval substrate, consisting of commercial pasty lamb feed for dogs (Pedigree®). After hatching, larvae were fed daily with the same pasty feed and kept under the same environmental conditions as the adult flies. Third instar larvae migrated to the sand below the larval substrate for pupation, where they remained until the emergence of adults.

For the tests, third instar larvae were collected from the substrate and starved for 12 hours before washing with distilled water. Sterilization was carried out by washing with 0.5% sodium hypochlorite for 5 min and then rinsed with sterile distilled water three times, to ensure its removal. The sterility of the larvae was confirmed by incubating the larvae in Blood Agar [Tryptone soy agar (TSA) with Sheep Blood].

Bacteria

The following bacterial strains were tested: two American Type Culture Collection (ATCC) [*S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853)] and one clinical isolate [Methicillin-resistant *S. aureus* (MRSA)]. Bacteria in the logarithmic growth phase on TSA were suspended in sterile phosphate-buffered saline (PBS) at OD_{550} =0.5 before using the larval pre-treatment assay.

Larval pre-treatment with bacteria

An adapted Kawabata et al. [11] pre-treatment of the larvae with each of the bacteria strains was performed to activate their immune system. Approximately 50–60 sterilized larvae in the treated group were mixed in a 15 mL test tube containing 4 mL of bacterial suspension, OD_{550} =0.5 (Methicillin-resistant *S. aureus, S. aureus,* or *P. aeruginosa*) and 2 mL of tryptone soy broth (TSB). In the control group (non-treated), we replaced bacterial suspension with PBS. Tubes were closed with sterile gauze and kept for 30 min at room temperature (25 ± 1ºC). The mixture was then transferred to new sterile liver pure agar in a Petri dish (100 x 15 mm), sealed with Parafilm M®, and incubated at 35 ºC for 24 h before collecting of larval extracts.

Collection of larval extracts

After 24 hours of incubation, both pre-treated and non-treated larvae were anaesthetized at -3ºC for approximately 60 seconds, removed from the liver puree agar, and transferred separately to microtubes of 2 mL containing 500 µL of PBS. The larvae were then cut into multiple pieces with sterile scissors. The obtained mixture was vortex-mixed and centrifuged at 20.000 g for 15 min at 4 ºC. The collected supernatant was filtered with a 0.22 µm sterile syringe filter (Kasvi Syringe Filters-K18-230) and stored in 1.5 mL sterile microtubes, sealed with Parafilm M®, at −80 ºC until use.

Antibacterial assay – Colony-forming unit (CFU) assay

A colony-forming unit (CFU) assay was applied to evaluate the antibacterial activity against *S. aureus*, *P. aeruginosa,* and Methicillin-resistant *S. aureus*. Bacteria were allowed to grown on TSA 24 hours before the test, then suspended in sterile PBS at

OD_{550} = 0.5, and 50µL of each bacterial suspension were added to 6 mL of TSB in Falcon tubes.

Five microtubes, in duplicate, were prepared: larval extract pre-treated with bacteria (LE T); larval extract non-treated (LE N/T); bacteria control (C BAC); larval extract pre-treated control (C LE T); and larval extract non-treated control (C the LE N/T). In microtube 1 (LE T), 100 µL of the TSB bacterial suspension and 100 µL of larval extract pre-treated were added; in microtube 2 (LE N/T), 100 µL of the TSB bacterial suspension and 100 µL of the larval extract with PBS were added; in microtube 3 (C BAC), 100µL of the TSB bacterial suspension and 100 µL of PBS were added; in microtube 4 (C LE T), 100 µL aliquot of the larval extract pre-treated and 100 µL of PBS were added; and, in microtube 5 (C LE N/T), 100 µL of the larval extract with PBS and 100 µL of PBS were added. All larval extracts were kept on ice during the assay.

Microtubes 1, 2, and 3 were diluted in PBS (1:10) until reaching a dilution of 10^{-7}, and 50 µL of dilutions 10^{-3}, 10^{-5}, and 10^{-7} were plated in triplicates in TSA (time 0). Microtubes 4 and 5 were larval extract sterility controls, and 50µl of each were plated in TSA. The duplicate of each microtube was incubated at 37 ° C for 6 hours (time 1) then the same procedure described above was performed. All plates were incubated overnight at 37 ° C, and the number of colonies was counted and compared with controls.

Statistical analysis

The variables were previously analyzed for normality and homogeneity with the Shapiro-Wilk and Bartlett tests, respectively. The statistical analyses were performed by analysis of variance with a degree of significance of 5% ($p < 0.05$) and the differences between the groups were evaluated with the t student test by software GraphPad Prism (Version 6.05). Data were expressed as mean ± standard deviation of the mean (SD).

Results

Collection of larval extracts

The average of larval extract obtained was 3.5 mL for each treatment, and their color differed between pre-treatments, being light brown for both *S. aureus* and dark brown for *P. aeruginosa* (which was expected since *P. aeruginosa* produces its pigment). Moreover, the untreated group (PBS) was yellowish. Larval extracts were kept on ice during the tests and did not show bacterial growth in the control plates.

Effect of larval extracts on Methicillin-resistant S. aureus growth

The number of CFUs of Methicillin-resistant *S. aureus* at time 0, for both bacteria-pre-treated (LE T) and non-treated (LE N/T) larval extracts, were not significantly different between the control group (C BAC) (Fig. 1), although it tended to be smaller. The growth of bacteria remained stable and after 6 hours of incubation were no significant differences between the groups (Fig. 1).

Effect of larval extracts on S. aureus growth

At time 0, CFU number of all groups differed significantly among them (Fig. 2). There were differences between both groups of larval extracts (LE T and LE N/T) and the control group (C BAC), as well as between the pre-treated (LE T) and untreated groups (LE N/T) (Fig. 2). The non-treated group (LE N/T) showed a significant decrease of CFU compared to the pre-treated group (LE T) by *S. aureus*. Both groups of larval extracts showed significantly lower CFU compared to the control group (C BAC).

After 6 hours of incubation (time 1), there were no significant differences between the pre-treated and non-treated groups. Nevertheless, there was a statistical difference

between the control group (C BAC) and the larval extract groups (LE T and LE N/T), although with higher growth of bacteria in the extract groups (Fig. 2).

Effect of larval extracts on P. aeruginosa growth

In both times, the numbers of CFUs of *P. aeruginosa* for both larval extracts were significantly lower concerning the positive control (Fig. 3). At hour 0, the difference between the pre-treated group (LE T) by *P. aeruginosa* and the non-treated group (LE N/T) were not statistically significant (Fig. 3), although bacteria-pretreated larval extract (LE T) tended to have a lower number of CFUs. However, after six hours of incubation, the treated group (LE T) showed a significant decrease in bacterial growth compared to the control group (C BAC) (Fig. 3), as also showed a statistical difference between the larval extracts, with the pre-treated group presenting the lowest numbers of CFUs.

Discussion

In the present study, we evaluated the antibacterial activity of *L. cuprina* larval extract, pre-treated with *S. aureus*, *P. aeruginosa* and MRSA. The primary hypothesis was that the incubation of larvae in a bacterial suspension for 24 hours could activate its immune mechanism and produce an antagonistic response to stress [23], thereby benefiting antibacterial activity.

The fly species and incubation temperature were modified in the current study, in addition to testing with a clinical isolate MRSA. All modifications were intended to make the methodology close to a natural infection. For example, we incubated larvae at 35°C, close to the body temperature of animals/humans, which allowed the assessment of larvae survival against bacteria as in a clinical wound. This proved to be an applicable modification for collecting larval extracts without interfering in the amounts obtained.

Furthermore, two incubation times (time 0 and 1, of 6 hours) allow evaluate if the larval extracts have only momentary action or if they remain in the long term.

Whereas our results demonstrate a decrease in CFU numbers of the bacteria-pretreated (LE T) group compared to the non-treated group (LE N/T) against *P. aeruginosa* and MRSA, *S. aureus* demonstrated the opposite, with LE N/T showing the lower results. Although antibacterial activity may be dose-dependent, preliminary study from Kawabata et al. [11] with *L. sericata* showed a more potent antibacterial activity with larvae grown in an infected environment against *S. aureus* and none for *P. aeruginosa*, and bacterial concentration was the same used in our work. Therefore, the probability is that the species itself directly influence our results, and *L. cuprina* larvae have a different immune response to bacteria species since *L. sericata* have already a marked antibacterial capacity against *S. aureus* supported by previous studies *in vitro* [20, 24].

We obtained a significant decrease in the CFU count of *S. aureus* at hour 0, and although there was a decrease in the CFU count of Methicillin-resistant *S. aureus*, no significant difference was found. Nevertheless, after the 6 hours incubation period, there was a higher growth of bacteria in the *S. aureus* groups containing larval extract, rather than a decrease. Cazender et al. [22] also showed an increase in bacterial growth, when using both live maggots and ES of *L. sericata,* and no antibacterial activity for several bacteria strains, including *S. aureus* and *P. aeruginosa*. They explain that the enhanced growth of bacteria might be due to the nutritious values of the maggot's excretions, which possibly corroborates with the results found in our study.

Regarding the species employed in our study, *L. cuprina*, there is also a divergence of results among described methodologies. Whereas the ES was effective against *S. aureus*, Arora et al. [12] found no antibacterial activity using live maggots of *L. cuprina*.

Conversely, The et al. [14], demonstrated significant action from the ES against all seven bacteria tested (including *S. aureus*, MRSA, and *P. aeruginosa*) through a turbidimetric assay, but in the CFU test, they found activity only against *P. aeruginosa*. The last results support our findings since the highest antimicrobial activity of our *L. cuprina* larval extract was against *P. aeruginosa*. These results show a direct influence by collection methodology and antimicrobial activity and at the same time reinforces that species diverge from each other. Furthermore, the contradictory results between studies of larval antibacterial activity demonstrate the need to standardize the preparation of larval products and employed bioassays.

It is important to point out that none of the larval extracts and ES has shown a full inhibitory effect, but a decrease in some bacteria growth. This result indicates that the larvae' mechanical action and physiological response act together to eliminate bacteria from the wound bed. Nevertheless, the higher antibacterial activity against *P. aeruginosa* obtained in the present work raises questions of how to use these findings in favor of the clinical application of MDT. Clinical reports indicate that MDT may be more effective against Gram-positive than Gram-negative infections, requiring higher concentrations of larvae and more extended periods of use [25]. However, *L. sericata* is the species of choice for the application and has no significant antibacterial activity against Gram-negative bacteria *in vitro* tests [11, 22]. With this in mind, it may be appropriate to indicate different fly species for each clinical case, depending on the predominant bacterial species in the wound bed.

In conclusion, *L. cuprina* larvae exposed to an infected environment showed higher antibacterial activities against *P. aeruginosa* and may represent a powerful tool to the clinical approach of this bacteria in wound healing. The natural infection method by Kawabata et al. [11] with our modifications proved to be an effective way to evaluate

the antibacterial activities of larvae used for MDT and should be considered for further studies with different fly species and bacteria, seeking its clinical use.

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Figures

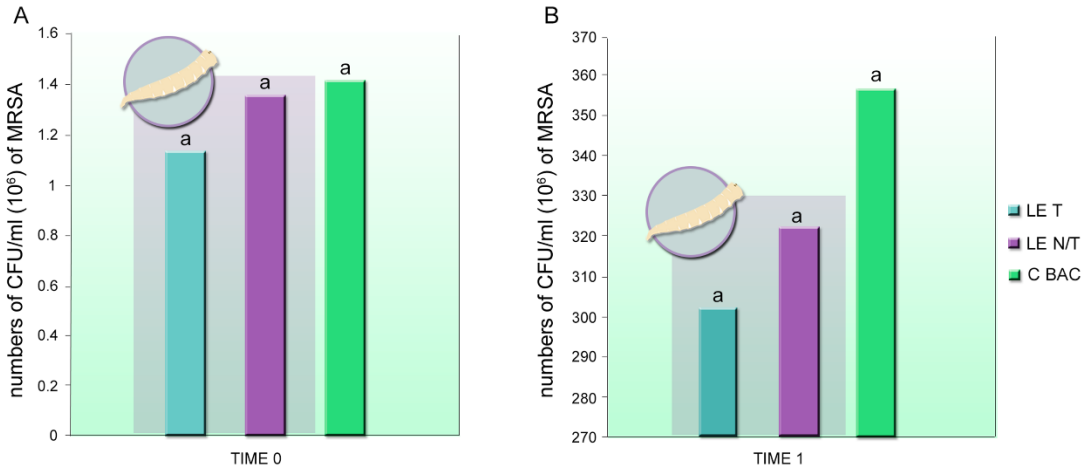

Fig. 1 Number of colony-forming units of MRSA incubated for time 0 (Fig 1.A) and 1 (6 hours) (Fig 1.B) with extract collected from non-treated larvae (LE N/T) and larvae treated (LE T) to 4 mL of MRSA suspension for 24 h. C BAC: bacteria growth control. *Post hoc analysis: significant differences ($p < 0.05$) between treatments are indicated by different letters.

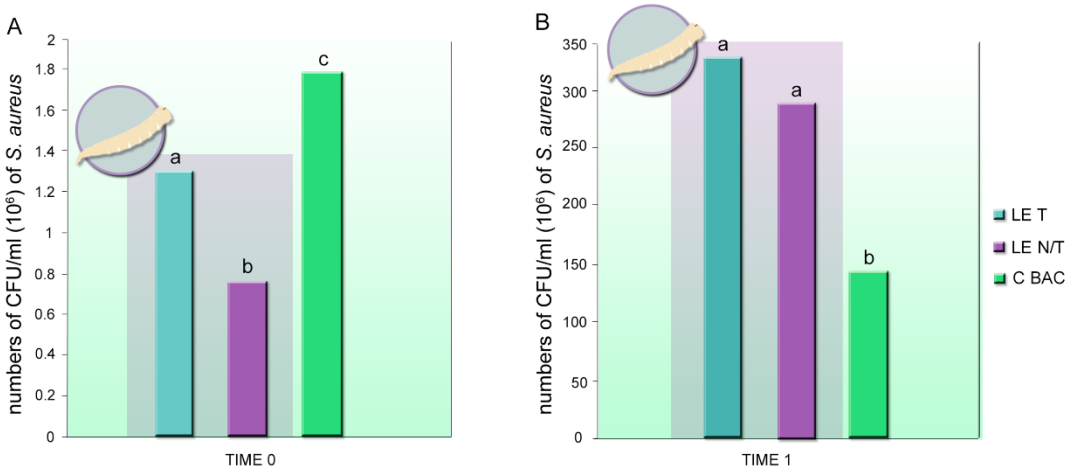

Fig. 2 Number of colony-forming units of *Staphylococcus aureus* incubated for time 0 (Fig. 2A) and 1 (6 hours) (Fig. 2B) with extract collected from non-treated larvae (LE N/T) and larvae treated to 4 mL of *S. aureus* suspension (LE T) for 24 h. C BAC: bacteria growth control. *Post hoc analysis: significant differences ($p < 0.05$) between treatments are indicated by different letters.

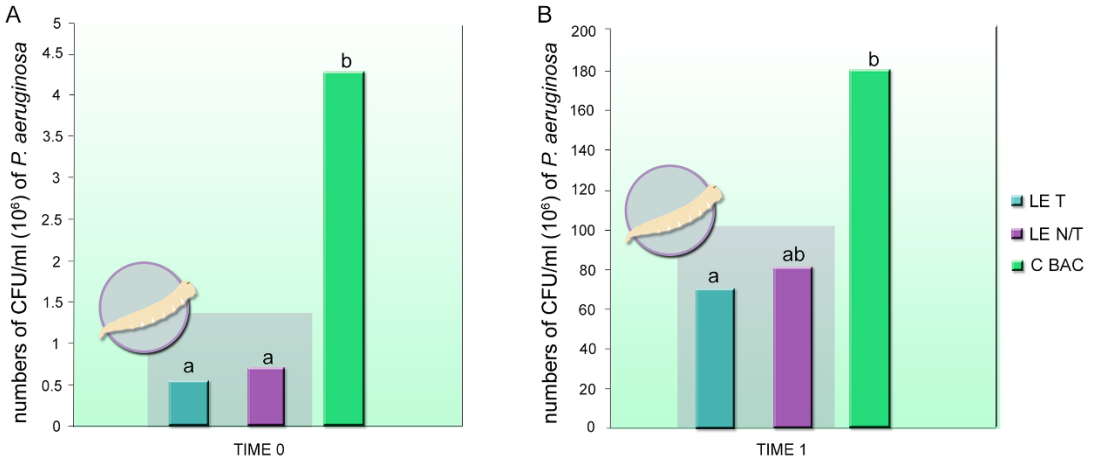

Fig. 3 Number of colony-forming units of *Pseudomonas aeruginosa* incubated for time 0 (Fig. 3A) and 1 (6 hours) (Fig 3B) with extract collected from non-treated larvae (LE N/T) and larvae treated to 4 mL of *P. aeruginosa* suspension (LE T) for 24 h. C BAC: bacteria growth control. *Post hoc analysis: significant differences ($p < 0.05$) between treatments are indicated by different letters.

4 MANUSCRITO 2

(Manuscrito submetido – Acta Tropica)

Safety and efficacy of *Lucilia cuprina* maggots on treating an induced infected wound in Wistar rats

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Abstract

Infection is one of the main complications that hinder wound healing. Currently, antibiotic-resistant bacteria, such as Methicilin-resistant *Staphylococcus aureus* (MRSA), are a concern worldwide for both humans and animals. Maggot debridement therapy is re-emerging as an alternative to intractable wounds and maybe an option to the traditional antibiotic treatment. Thus, we evaluated the efficacy and safety of using *Lucilia cuprina* larvae on induced infected wounds in Wistar rats. In short, 32 male Wistar rats were divided into 4 groups: Group I - saline solution treated; Group II - antibiotic-treated; Group III - treated with larval debridement, and Group IV - without wound and treatment. Skin wounds were induced in groups I, II and III. All treatments were performed once and held for 48 hours. Clinical, microbiological, histopathological, hematological, and biochemical analyses were done. Significant wound area contraction was found (>95%) in group III on the 9th day compared to the 15th day in group II. Complete elimination (0.0 ± 0.0 CFU/mL) of bioburden was achieved after the second treatment (6^{th} day) in both II and III groups, compared to an increase in Group I (6.51 ± 0.37 CFU/mL). A cleaner wound was also observed in the histopathological evaluation of group III, with adequate collagen formation and re-epithelialization on the 15th day. Furthermore, larvae increased blood platelet levels after the first treatment. *L. cuprina* larvae have proven safe and effective in accelerating wound treatment and eliminating MRSA.

Keywords: Wound, infection, MRSA, larvae, blowflies, Maggot debridement therapy

Introduction

Wound healing in humans and animals is a topic of constant study due to its clinical, scientific, and economic interest, aiming to accelerate the healing process and reduce potential complications. One of the main complications that hinder wound healing is infection. Although bacteria are part of the skin's microbiota, the onset of infection occurs due to the loss of integrity and exposure of the subcutaneous tissue providing a favorable environment for proliferation allowing secondary contamination by more pathogenic bacteria. One of the predominant opportunist bacteria in animal and human skin is *Staphylococcus aureus.* The main issue is that inappropriate treatment through indiscriminate antibiotic therapy by tutors and practitioners increases the emergence of resistant microorganisms, such as methicillin-resistant *S. aureus* (MRSA) (Cefai et al. 1994; Weese et al. 2000, 2005, 2007).

Reports of the transmission of microorganisms between humans and animals increase the concern worldwide, calling for alternative treatments to traditional antibiotics to prevent the spread of these pathogens (Cefai et al. 1994; Manian 2003; Weese et al. 2005; Moodley et al. 2008; Loeffler et al. 2010; Jordan et al. 2011; Eveillard et al. 2015; Worthing et al. 2018). Sidelined by the advent of antibiotics and surgical debridement in the 1940s (Kerridge et al. 2005), fly larvae therapy is certainly not a new discovery. Still, it is re-emerging as an excellent alternative in treating intractable wounds. With a set of actions that includes disinfecting, debriding the necrotic tissue and stimulating wound healing, maggot debridement therapy (MDT) today is applied in both human and veterinary medicine in more than 30 countries worldwide (Sherman 2009).

For centuries, the species of choice for MDT has been *Lucilia sericata* Meigen, 1826 (Diptera: Calliphoridae) (Weil et al. 1933). However, other calliphorid flies have a few MDT application reports. Among them, *Lucilia cuprina* Wiedemann, 1830 (Diptera:

Calliphoridae*)* has successfully been used in treating human wounds (Paul et al. 2009; Tantawi et al. 2010; Nair et al. 2021).

With great therapeutic potential, low cost and easy management at the laboratory, and approaching its use in animal wounds, the present study aimed to evaluate the efficacy and safety of using *L. cuprina* larvae in induced infected wounds in Wistar rats. For that, we assessed the wounds clinical, pathological and microbiological aspects in the animals.

Material and methods

Rearing of Lucilia cuprina in the laboratory

The colony of *L. cuprina* were kept in entomological cages (30cm x 30cm x 30cm) at 25 ± 1°C under a light/dark cycle (12:12 H) and relative air humidity of 70% ± 5%. Adult flies were fed with honey made available on a Petri dish (80 x 15mm) with rice and water *ad libitum*. For oviposition, a bovine liver was available in a Petri dish (80 x 15mm). Eggs were carefully removed through a moistened brush and placed in a Petri dish (80 x 15mm) with the larval substrate, comprising commercial pasty lamb feed for dogs (Pedigree®). After hatching, larvae were fed daily with the same pasty feed and kept under the same environmental conditions as the adult flies. Third instar larvae migrated to the sand below the larval substrate for pupation, where they remained until the emergence of adults.

Egg sterilization for MDT

Fly eggs were sterilized by magnetic stirring in a flask containing 0.5% sodium hypochlorite for 5 minutes. After being placed on a paper filter, they were washed with distilled water to remove excess hypochlorite and placed in a moistened sterile gauze inside sterile Petri dishes for hatching. After 24 hours, the larvae were checked for

sterility, packaged and transported in 50 ml collection bottles sealed to the application site.

Experimental animals

Thirty-two healthy male Wistar rats (*Rattus norvegicusalvinus*, Rodentia, Mammalia) weighing 250-300 ± 25 grams, with 12 ± 2 weeks old, were obtained from the Central Biotherapy of the Federal University of Santa Maria (UFSM). The animals were housed in the Experimental Animal Facility in separate polypropylene cages (41x34x18 cm), kept in a controlled environment with an average temperature of 25ºC ± 2, with a 12-hour light/dark cycle, relative humidity of 60% ± 10, abundant water and balanced commercial feed. To ensure the wellbeing of the animals, an adaptation period to the environment for 7 days before the procedures were performed and daily evaluations to check for stress and clinical changes. The protocols used in this study were reviewed and approved by the Ethics Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) (CEUA Nº: 5414110220). Animals were divided randomly into four groups of eight animals each, categorized as Group I (CN) for control negative of mechanical debridement with 0.9% NaCl saline solution; Group II (ATB) for antibiotic control treated by the topical ointment of Bacitracin (250 UI/g) + Neomycin (5mg/g) 0.5 ml per day; Group III (MDT) for maggot debridement treatment and Group IV (WW) without wound or treatment for comparison of intact skin.

Bacteria

A Methicillin-resistant *S. aureus* (MRSA) clinical isolated bacterial strain was grown on Brain Heart Infusion (BHI) for 24 hours and then suspended in sterile

trypticase soy broth (TSB) to OD_{550} =0.5 before using to activate infection on the wound.

Excisional wound model

Induced infected wounds were based on Fallon et al. (1999) methodology with modifications. Animals underwent dissociative injectable anesthesia, with the association of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (90 mg/kg) intraperitoneally. Analgesia was performed with tramadol hydrochloride (8mg/kg) subcutaneously. After, the dorsal skin was trichotomized (6 x 6 cm) 2 cm below the scapula, and antisepsis was performed with povidone-iodine and 70% alcohol. A 2 x 2 cm incision (4 cm^2), 1 cm deep, previously demarcated with a template, was made using a 15-scalpel blade and surgical scissors. Sterile sand (100 mg) was introduced into the wounds as a potentially infectious foreign body, and the lesions were inoculated with 200 µl of a solution containing MRSA at 5.0 x 10^7 UFC to obtain an infection. Within three days, the wounds became infected, presenting purulent discharge, crusts, and areas of necrosis. The 3rd day after the procedure was considered day 0, and the treatments were started.

Treatment application

Treatments were applied according to each group, and dressings were the same, except for the control group without a wound. All animals received sterile gauze dressing and elastic strap bandage, closed by three-point anchored adhesive tape. We lightly dampened all gauze with 0.9% saline solution, and in the case of the group treated with larval therapy (Group III - MDT), a small opening in the gauze was made to allow the larvae to receive oxygen.

In Group III (MDT), stage 1 larvae were applied directly to the infected wound bed (day-0) at a rate of 5 to 10 larvae/cm² and left for 48 hours as recommended by Sherman et al. (2007). After that, larvae recovered alive were discarded as infectious dressing waste, and a new application and dressing were made. Group II (ATB), through bacterial strain sensitivity testing, received treatment with Bacitracin (250 UI/g) + Neomycin (5mg/g). At each dressing change, 0.5 ml of ointment was applied. Group I (C) was kept as a control and the wound was only cleaned with 0.9% saline solution.

Clinical evaluation of the wound and measurement of wound area and contraction

Clinical evaluation was performed every three days, using the following parameters: the presence of granulation (low, moderate, excessive), colour (pale, pink and hyperemic) and sensitivity (absent or present). Wound size was measured using a digital pachymeter, and wounds were photographed for measurement using ImageJ software. The wound area was measured every three days from day 0 to day 15, and we performed euthanasia in each group on days 3rd (n=4) and 15th (n=4). Wound contraction (%), wound healing, was determined using Okoli et al. (2009) formula, which is defined by the wound diameter in day 0 (WD0) and the wound diameter on the day of assessment (WDt), according to the following formula:

Wound contraction (%):

$$\frac{WD_0 - WD_t}{WD_0} \text{ x } 100$$

Microbiological analysis

Sterile swabs were collected from the wounds on days 0 (before application of treatments), 3 (after the first treatment with MDT) and 9 (after the second treatment with MDT) for the evaluation of the bacterial load and identification of the bacteria

present in the wound. Swabs were gently scraped over the center of the lesion, away from the skin and hair, and placed into a tube containing PBS. Samples were diluted (until 10^{-3}) in PBS and plated on nutrient agar. After 24 hours of incubation, colony identification and colony-forming unit (CFU) counting were performed and then corrected to ml (number of colonies x correction factor x dilution).

Histopathological analysis

Two euthanasia at different times were performed, with half of the animals in each group euthanized after the first treatment (3 days) and the remaining after wound healing (15 days). Euthanasia was done by associative anesthesia with ketamine hydrochloride (90 mg/kg IP) and xylazine (10 mg/kg IP) to abbreviate pain or suffering and exsanguination by cardiac puncture for blood collection. Animals were fixed on the operating table, and by cutting with a scalpel, the complete wound area with a safety margin was removed for histopathological analysis. The samples were immediately fixed in 10% formalin and routinely processed for histopathology, cut to 5 µm thick and stained with hematoxylin and eosin (HE). Two pathologists, under a light microscope, blindly performed the histopathological analyses. Variables analyzed were: inflammation intensity, granulation tissue, collagen maturation, neovascularization, and reepithelialization, using a subjective scale of mild (+), moderate (++), and accentuated (+++).

Hematological and biochemical analysis

Blood samples from the euthanized animals were collected by cardiac puncture in EDTA and blood serum tubes. Counting of leukocyte, platelet, hemoglobin concentration and RDW value were determined using an automated hematology counter model BC-

VET 2800 (Mindray-São Paulo, SP, Brazil). The hematocrit (Ht) determination was obtained in a micro-hematocrit centrifuge at a rotation of 14,000 r.p.m. for 15 minutes. The mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) were determined by indirect calculation: MCV = (Ht x 10)/RBCs; MCHC = (hemoglobin x 100) / Ht. The erythrocyte morphological evaluation and the leukocyte differential were performed on a blood smear stained with routine Romanowsky-type stains (Fast Panotic- Laborclin-Pinhais, PR, Brazil), using light microscopy with a 100x objective.

For biochemical analysis, the blood samples used were sent in tubes without anticoagulant and centrifuged at 900xg speed for 4 minutes (Combate-CELM-São Paulo, SP, Brazil) to obtain serum. According to the manufacturer's instructions, biochemical analyses were performed using commercial kits (Bioclin-Belo Horizonte, MG, Brazil) with serum samples from the animals. These analyses were processed by an automatic biochemical analyzer (BS 120-Mindray-São Paulo, SP, Brazil).

Statistical analysis

The numerical data obtained from the experiment were analyzed statistically on GraphPad Prism 5.0 software. All values were analyzed, and their results were expressed as media (M) ± standard error of media (SEM). Differences between the treated and control groups were compared using a one-way analysis of variance (ANOVA), followed by Dunnett’s t-test to determine their significance level. Differences at $p<0.05$ were considered statistically significant.

Results

Clinical evaluation of the wound and measurement of wound area and contraction

The macroscopic evaluation of the wounds is presented in Figure 1. All groups initially had seropurulent secretion due to the onset of infection, a blackened crust (fibrin-hemorrhagic) and punctual areas of necrosis. After the first treatment, on the 3rd day, it was identified significant differences between the groups. The formation of scabs in Group I (CN) and Group II (ATB) remained. After removal, Group I had a seropurulent secretion, with some spots of a smooth white film over the wound, and Group II had a serosanguineous secretion. Moreover, the edges were irregular, while Group III (MDT) had edges that retracted regularly. In addition, Group III (MDT) already had a drier, pinker wound, showing granulation tissue formation.

On day 6, after the second treatment, Group I (CN) still had a smooth film over the wound, with some whitish spots and areas with crusting and irregular edges. Group II (ATB) remained essentially after the first treatment but was already showing signs of wound retraction and less formation of scabs. Group III (MDT) already had a moist, reddish-pink wound with regular borders. Between days 9 and 12, Group I (CN) lost the whitish film above the lesion and had a redder wound, but still with crust formation. On the other hand, Group II (ATB) had a clean wound, without scabs, with more regular edges.

Group III (MDT) was already entering the final stage of healing, with the return of fur around the lesion. After 15 days, removing the crusts, Group I (CN) had a small wound region of red color, apparently exposing the dermal region. In contrast, the wounds in Group II (ATB) and III (MDT) were pink and homogeneous with no signs of exposure of the dermal region, and Group III (MDT) presented new hair coverage.

Table 1 shows the results of the treatments in a total area of the wound (mm^2) and contraction measurement of excisional infected wounds at different days of observation. Group III (MDT) reduced the wound size faster and more completely than the other

treatments among all the groups. A significant difference ($p < 0.05$) among the other groups and Group III (MDT) was found after the second treatment, on the 6th day and 9th day. Larval therapy decreased the wound means from 3rd day (247.3 ± 47.18 mm²) to (40.18 ± 4.01 mm²) on the 6th day and (21.35 ± 7.48 mm²) on the 9th day, achieving a 90.25 ± 0.85% wound contraction already on the 6th day. On the 9th-day maggot, the treatment achieved a 94.50 ± 1.84% wound contraction, turning to complete healing (>95%) at day 12th with a 98.0 ± 0.81% contraction.

Group II (ATB) also achieved complete healing of the wound on the 15th day (99.5 ± 1.0%); however, the treatment with antibiotic ointment only matched the larval therapy from the 12th day on. The wound area of Group I (CN) remained close ($p > 0.05$) to the Group II (ATB) from the 6th day (160.8 ± 48.17 mm² and 122.1 ± 18.39 mm² respectively), to the 9th day (89.94 ± 14.88 mm² and 68.0 ± 9.33 mm² respectively), differing significantly ($p < 0.05$) on the 12th day (51.03 ± 7.68 mm² and 26.23 ± 6.3 mm² respectively). Group I (CN) differed significantly when compared to Group III (MDT) from day 6 to day 15 and failed to achieve complete wound closure (<95%).

Microbiological analysis

The average total bacterial colony counts are presented in Table 2, expressed in mean logarithm-transformed values of colony-forming units per millilitre of initial suspension (log/CFU/mL). The bacteria concentration at 0-day of initiation of treatment did not differ significantly, although they were not very similar amongst the groups. Infection of wound decreased significantly ($p < 0.05$) at 3rd day for maggot debridement therapy Group III (MDT) and antibiotic control Group II (ATB) when compared to Group I (CN), remaining at 7th day.

However, the reduction in bacterial load from day-0 (4.82 ± 0.66) to 3rd (2.52 ± 2.90) within Group II (ATB) only showed a significant difference ($p < 0.05$) for the 7th day (0.0 ± 0.0). Within Group III (MDT), larval treatment reduced the bacterial count from 6.20 ± 0.29 on day-0 to 2.34 ± 1.35 on day 3rd, and 0.00 ± 0.00 on day 7th. In contrast, Group I (CN) of control negative treatment with 0.9% saline solution showed a significantly ($p < 0.05$) increase in the total bacterial counts from day-0 (5.57 ± 1.22) at 3rd day (7.18 ± 0.17), remaining in 7th day (6.51 ± 0.37).

Histopathological analysis

To evaluate the histological changes in the groups with wounds, group IV (WW) was used as a reference and showed a normal epidermis with well-organized dermal collagen (eosinophilic fibers and with normal amounts of fibroblasts) (Fig 2a) and adnexa in both times. The first evaluation (3 days) showed in all wound-induced groups (CN, ATB, MDT) an abundant polymorphonuclear (PMN) cellular infiltration inflammatory process (Fig 2d), with apparent neovascularization, granulation tissue formation, but with mild signs or absence of re-epithelialization and collagen organization. However, some differences between groups could be observed. The negative control group (Group I - CN) (Fig 2b) contained a large amount of superficial cellular debris (serocellular crusts), exudate, neutrophils and clustering of bacteria (Fig 2c) and was considered a dirty wound. The collagen was disorganized, immature and very cellular, just below the crust, loaded with fibroblasts and neutrophils. In comparison, the maggot treated Group III (MDT) was found to have a clean wound with no crusting (Fig 2e). Despite this, both Groups II (ATB) and III (MDT) still showed immature loose collagen, signs of PMN inflammatory cells and neovascularization.

Although none showed complete collagen maturation, all wounds were considered totally or almost re-epithelized on day 15th (second euthanasia). However, Group I (CN) (Fig 2f) had a thin epithelium followed by a serocellular crust and bacterial clusters in a few animals. Both Groups II (ATB) (Fig 2g) and III (MDT) (Fig 2h-i) showed organized collagenous tissue, more eosinophilic and less cellular.

Hematological and biochemical analysis

The treatments with MDT and antibiotic ointment did not significantly affect hematological or biochemical parameters compared to the animals without wound or control negative group. Only a few of the parameters showed to be significant ($p<0.05$) and, in general, are associated with acute infection. The effects on hematological and biochemical values were considered after the first treatment (3rd day) and after complete wound closure, (15th day) and are illustrated in Tables 3, 4, 5 and 6. After the first treatment, all wound-induced groups presented significant change ($p < 0.05$) in total plasma protein (PPT g/dL) compared to group IV (WW) (Table 3.1). Group III (MDT) also increased in the platelet count (PLT x 103/µL) significantly to 1247.00 ± 04.36 when compared to Group IV (WW) 999.00 ± 10.06. However, Group I (CN) and Group II (ATB) also increased PLT, but values were not significant. Neutrophils (NEUT) increased in all three treatment groups compared to their control group without wound, but this parameter was insignificant. Biochemical results after the first treatment also show a significant ($p < 0.05$) decrease in alkaline phosphatase (ALP U/L) for Group I (CN), II (ATB) and III (MDT), and in CK, but no significance.

After complete closure, parameters remained stable compared to the control group without wound (WW), except for a significant increase in NEUT for all three groups

treated. In addition, biochemical results showed an increase in CK for all treatments groups, unlikely the first treatment, but we found no significance.

Discussion

L. cuprina larvae proved promising and safe for therapeutic application, preserving healthy tissues unharmed, removing only necrotic/devitalized areas, and promoting antimicrobial action. The acceleration in tissue regeneration was evident in the group of animals treated with larvae. In the initial macroscopic evaluations, maggot application, already after the first treatment (3rd day), showed little or no exudate in the wound bed (Fig 1) since larvae liquefy the medium to ingest necrotic tissue and bacteria. The presence of exudate generally interferes in the epithelialization process (Borges and Chianca 2000) since it favours bacterial growth, which competes with healing cells for nutrients and oxygen. With their secretions/excretions, maggots tend the oxygen perfusion, cell proliferation and fibroblast migration, allowing for matrix remodeling and granulation tissue propagation (Figueroa et al. 2006; Sherman 2014). Both control (Group I) and antibiotic ointment (Group II) treatment presented a lot of exudates. It seemed to start the healing process only after subsided, about the 9th day of treatment.

Maggot treatment winded another macroscopically visible feature reduction, one of the basic principles in evaluating the healing process (Borkataki et al. 2021). With an evaluation at each dressing change, i.e. every three days, the area and contraction of the wound made it clear that the larvae treatment decreased the size of the wounds more rapidly and more thoroughly than the antibiotic-treated and control group. By the 6th day, Group III (MDT) had already achieved a shrinkage percentage of 90.25%, compared to 70.25% in Group II (ATB) and 60.5% in Group I (CN). Even though there was no absolute difference between the antibiotic and maggot treated groups, both wounds were

considered completely closed (>95%) at the end of the 15th day. In this context it is much more beneficial to use maggots than medication, as larvae are not at risk of causing resistance, damaging the environment, and providing debridement.

Researchers who applied *L. sericata* larvae to infected wounds in diabetic Wistar rats (Borkataki et al. 2018, 2021) and *Cochliomyia macellaria* (Masiero and Thyssen 2016) found similar results, reducing the healing time to about 20 days. Borkataki et al. (2021) raise an interesting question about free larvae in the wound compared to biological bags (bio bags) where the maggot body has no direct contact with the wound (Dumville et al. 2009). They associate the use of free larvae with faster healing, assuming that the small spines present on the larvae's body and their movement across the wound bed cause debridement and scraping of necrotic tissues, aiding deep access to the enzymes secreted, facilitating liquefaction and ingestion of debris and bacteria, reducing healing time (Sherman 2009, 2014). In addition, the maggot movement stimulates angiogenesis, a complex series of events that promotes endothelial cell proliferation in the wounded area (Bexfield et al. 2010).

The microbiological evaluation in the present study also yielded excellent results. Since the goal was to evaluate the MRSA load post-treatments, evaluations were done on day 0, before treatment, and on days 3rd and 6th right after the larval treatments. Initial values (day-0) were statistically similar, and all were above 10^{-3} CFU/collected swab, which is the minimum to cause infection and interfere with wound healing (Borkataki et al. 2018). After the first treatment, there was a decrease in MRSA CFU in both groups II (ATB) and III (MDT), but in control negative group, we observed bacterial proliferation (7.18 ± 0.17 log/CFU/mL). As followed macroscopically, the group I (CN) started to show occasional areas with a smooth, creamy film over the wound, possibly being biofilm formation. At the second analysis, the negative control began to show a decrease in

bacterial proliferation. However, the values were still high, unlike what groups II (ATB) and III (MDT) showed since we did not detect bacteria in swab culture after the second treatment. Although equivalent, the antibiotic treatment started with lower loads than the larval treatment. It is important to emphasize that the antibiotic of choice was tested to assess the sensibility of the MRSA strain used in the present study. As such, the larvae successfully decreased MRSA load as much as the antibiotic and yet provided faster healing due to their other properties.

Some researchers have already reported the antibacterial activity of excretion/secretion of *L. cuprina* for some bacteria (Arora et al. 2011; The et al. 2017), but none has shown a full inhibitory effect alone, only a decrease in bacteria growth. In addition, larval secretion contains deoxyribonuclease (DNAse), which degrade microbial DNA and inhibit microbial growth and biofilm formation (Brown et al. 2012). However, we consider larvae's actions responsible for eliminating bacteria and interrupting biofilm formation. As described above, crawling and wandering of the maggots, ingestion of liquefied necrotic tissues and bacteria, alkalinization of the wound bed by ammonia by-products of the maggot's digestion makes it unsuitable for bacteria growth (Blake et al. 2007).

The histopathological findings reinforce the value of the set of actions provided by the use of *L. cuprina* larvae. Although after the first treatment, all groups had a PMN cellular infiltration inflammatory process, mostly neutrophils due to the onset of the infection and apparent neovascularization, the maggot treated group (MDT) was found to have a clean wound, with no crusting, and mild signs of re-epithelialization and collagen organization. Evidence suggests that maggots clean the wound and stimulate the growth of granulation tissue by its movements (Rayner 1999) and secretions that enhance macrophage activity (Thomas 1988), visible in the macroscopic evaluation as well. As in

macroscopic observation, Group I (CN) was the only group considered a dirty wound, with clustering of bacteria and lots of exudates and superficial debris. It was considered almost re-epithelized on the 15th day, still maintained bacterial clusters in some animals. Despite remaining crusted, the antibiotic treatment had no significant histological differences compared to the larval treatment on day 15th, with both having more organized collagen and re-epithelization.

Few significant differences ($p<0.05$) were found in haematological and biochemical parameters between the treated and control groups without a wound. As the first defenders of the innate immune response and a rapid and robust response against infection and harmful agents, neutrophils remained increased from the beginning to the end of healing (Wang 2018). This maintenance of elevated levels is associated with physiological tissue regeneration, as the neutrophil response depends on the context of the injury, such as inflammatory response, tissue environment, and other cells involved (Wang 2018). The slight decrease of PPT is associated with wound infection (Powanda and Moyer 1981) and normalized after wound healing. One of the most significant results was the increase in PLT after the first larval treatment. It is well known that upon tissue injury, platelets are responsible for providing a provisional scaffold for inflammatory cells, besides harbouring a reservoir of cytokines, chemokines, and growth factors that lead the repair, among which the recruitment of neutrophils (Einsinger et al. 2018). This is the first time that MDT has been associated with high PLT at blood levels during wound healing, a finding that only reinforces the benefit of larvae. After wound debridement, CK levels usually decrease in the first 24 to 48 hours (Nordmann et al. 2009); this justifies the findings in the present study.

In conclusion, the benefits of treatment with *L. cuprina* larvae outweigh conventional treatments to infected wounds, providing a continuous and selective form

of debridement, which removes the necrotic tissue, in addition, to interrupting bacteria proliferation and biofilm formation, beyond their complete elimination. In addition, MDT facilitated the healing process, making it faster.

Statements and Declarations

Conflict of interest: The authors declare that they have no conflict of interest.

Consent for Publication: All authors consented for publication and approved the final manuscript.

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Ethical approval: The protocols used in this study were reviewed and approved by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) (CEUA Nº: 5414110220).

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Tables

Table 1. Wound contraction in area and percent in Wistar rat over 15 days, starting at day 3 of wound infection (0 DAY). CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

Group	0 day	3th day		6th day		9th day		12th day		15th day	
	Area (mm^2)	Area (mm^2)	% Contr	Area (mm^2)	% Contr	Area (mm^2)	% Contr	Area (mm^2)	% Contr	Area (mm^2)	% Contr
Gr I (CN)	421.5 Ad ± 17.76	291.4 Ac ± 22.01	29.9 Ba ± 8.20	160.8 Bb ± 48.17	60.5 Ab ± 13.57	89.94 Bb ± 14.88	78.25 Ab ± 4.46	51.03 Ba ± 7.68	88.0 Ab ± 1.58	28.13 Ba ± 7.69	93.25 Ab ± 1.79
Gr II (ATB)	414.5 Ad ± 20.07	374.2 Bd ± 17.65	9.45 Aa ± 1.68	122.1 Bc ± 18.39	70.25 Ab ± 4.53	68.0 Bb ± 9.33	83.75 Ac ± 1.6	26.23 Aa ± 6.3	93.75 Bc ± 1.31	2.2 Aa ± 2.2	99.5 Bc ± 1.0
Gr III (MDT)	419.5 Ac ± 23.11	247.3 Ab ± 47.18	39.48 Ba ± 13.19	40.18 Aa ± 4.01	90.25 Bb ± 0.85	21.35 Aa ± 7.48	94.50 Bb ± 1.84	7.87 Aa ± 3.17	98.0 Bb ± 0.81	1.0 Aa ± 1.0	99.75 Bb ± 0.25

Similar superscript does not differ significantly ($P < 0.05$). Smaller superscript denotes within the groups between the days and capital superscript denotes between the groups within different days.

Table 2. Total bacterial colony counts in log (CFU)/ml of initial suspension in different days of observation in Wistar rat. CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

Group	log (CFU)/ml of initial suspension		
	Before treatment (day 0)	1st treatment (3th day)	2nd treatment (7th day)
Gr I (CN)	$5.57^{Aa} \pm 1.22$	$7.18^{Bb} \pm 0.17$	$6.51^{Bb} \pm 0.37$
Gr II (ATB)	$4.82^{Ab} \pm 0.66$	$2.52^{Aab} \pm 2.90$	$0.0^{Aa} \pm 0.0$
Gr III (MDT)	$6.20^{Ab} \pm 0.58$	$2.34^{Aa} \pm 2.70$	$0.0^{Aa} \pm 0.0$

Similar superscript does not differ significantly ($p < 0.05$). Smaller superscript denotes within the groups between the days and capital superscript denotes between the groups within different days.

Table 3. Hematological data of Wistar rats excisional infected wounds after the first treatment (3^{th} day) with maggot debridement therapy (MDT). WW: Without wound; CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

Hematological Parameters	WW	CN	ATB	MDT
WBC (× 10^3/µL)	6.47 ± 1.21	6.57 ± 0.96	7.05 ± 1.39	6.97 ± 1.46
RBC (× 10^6/µL)	6.37 ± 0.11	6.45 ± 0.31	6.61 ± 0.38	6.20 ± 0.28
HGB (g/dL)	12.33 ± 0.33	12.38 ± 0.59	12.15 ± 0.98	11.75 ± 0.71
HCT (%)	40.53 ± 0.49	40.60 ± 2.31	42.30 ± 2.92	39.35 ± 2.54
MCV (fL)	63.75 ± 1.50	62.98 ± 1.07	64.00 ± 1.47	63.43 ± 1.57
MCHC (g/dL)	30.38 ± 0.68	30.45 ± 0.34	28.93 ± 0.80*	29.83 ± 0.66
PLT (x 10^3/µL)	999.00 ± 10.06	1177.00 ± 19.10	1110.00 ± 13.63	1247.00 ± 04.36*
PPT (g/dL)	6.5 ± 0.11	6.05 ± 0.10*	6.0 ± 0.36*	6.0 ± 0.19*
RDW-CV (%)	11.90 ± 0.29	11.93 ± 0.28	11.60 ± 0.66	12.08 ± 0.54
NEUT (%)	6.75 ± 1.5	13.0 ± 4.08	13.0 ± 4.24	12.25 ± 3.3
LYMPH (%)	91.5 ± 1.29	84.0 ± 5.47	85.5 ± 5.26	87.5 ± 3.3
MONO (%)	1.5 ± 1.0	2.5 ± 1.91	0.75 ± 0.95	0.25 ± 0.5
EO (%)	0.25 ± 0.5	0.5 ± 1.0	0.75 ± 0.5	0.0 ± 0.0
BASO (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are given as Mean ± S.E.M. for each group. The mean difference is considered significant at $p<0.05$.*=significant ($p<0.05$).

Table 4. Biochemical data of Wistar rats excisional infected wounds after the first treatment (3^{th} day) with maggot debridement therapy (MDT). WW: Without wound; CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

Biochemical Parameters	WW	CN	ATB	MDT
Albumin (g/dl)	3.47 ± 0.1	3.22 ± 0.22	3.0 ± 0.46	3.22 ± 0.1
ALT (U/L)	42.50 ± 2.38	48.75 ± 9.17	39.25 ± 11.12	57.25 ± 6.18
ALP (U/L)	241.5 ± 28.02	176.5 ± 32.06*	181.8 ± 23.49*	182.5 ± 26.80*
GGT (U/L)	0.75 ± 0.9	0.0 ± 0.0	0.75 ± 0.95	0.5 ± 1.0
CK (U/L)	840.3 ± 191.9	608.8 ± 246.2	602.5 ± 448.2	550.5 ± 112.6
Cr (mg/dL)	0.52 ± 0.05	0.47 ± 0.05	0.45 ± 0.1	0.50 ± 0.05
PT (g/dL)	5.6 ± 0.16	5.02 ± 0.49	4.72 ± 1.11	5.07 ± 0.2
Urea (mg/dL)	36.75 ± 7.5	37.0 ± 1.6	37.5 ± 6.6	32.25 ± 8.3

Values are given as Mean ± S.E.M. for each group. The mean difference is considered significant at $p<0.05$.*=significant ($p<0.05$).

Table 5. Hematological data of Wistar rats excisional infected wounds after the complete closure (15th day) with maggot debridement therapy (MDT). WW: Without wound; CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

Hematological Parameters	WW	CN	ATB	MDT
WBC (× 10^3/µL)	6.75 ± 2.36	6.77 ± 0.66	7.10 ± 1.40	7.25 ± 2.12
RBC (× 10^6/µL)	6.29 ± 0.32	6.28 ± 0.52	6.32 ± 0.14	6.40 ± 0.25
HGB (g/dL)	11.88 ± 0.83	11.70 ± 1.08	11.85 ± 0.56	12.18 ± 0.65
HCT (%)	39.38 ± 1.84	38.88 ± 3.32	40.40 ± 1.08	40.35 ± 1.38
MCV (fL)	62.73 ± 1.97	61.98 ± 0.90	63.98 ± 0.61	63.10 ± 0.66
MCHC (g/dL)	30.10 ± 1.59	30.03 ± 0.43	29.25 ± 0.81	30.13 ± 1.02
PLT (x 10^3/µL)	791.00 ± 182.8	852.00 ± 80.71	1016.00 ± 136.1	864.00 ± 155.6
PPT (g/dL)	6.1 ± 0.34	5.55 ± 0.34	6.0 ± 0.28	5.8 ± 0.3
RDW-CV (%)	11.65 ± 0.66	12.03 ± 0.56	12.05 ± 0.30	12.03 ± 0.45
NEUT (%)	7.5 ± 2.6	15.0 ± 8.7*	14.25 ± 1.25*	15.75 ± 3.40*
LYMPH (%)	88.75 ± 3.30	80.75 ± 9.10	82.00 ± 2.82	80.50 ± 2.51
MONO (%)	3.0 ± 0.81	3.75 ± 0.95	3.75 ± 2.62	2.25 ± 0.50
EO (%)	0.5 ± 0.57	0.5 ± 0.57	.0 ± .0	0.5 ± 0.57
BASO (%)	0.25 ± 0.5	.0 ± .0	.0 ± .0	.0 ± .0

Values are given as Mean ± S.E.M. for each group. The mean difference is considered significant at $p<0.05$.*=significant ($p<0.05$).

Table 6. Biochemical data of Wistar rats excisional infected wounds after the complete closure (15^{th} day) with maggot debridement therapy (MDT). WW: Without wound; CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

Biochemical Parameters	WW	CN	ATB	MDT
Albumin (g/dl)	3.45 ± 0.37	3.45 ± 0.23	3.52 ± 0.09	3.15 ± 0.43
ALT (U/L)	37.25 ± 10.72	54.50 ± 8.66	55.0 ± 12.68	40.0 ± 10.8
ALP (U/L)	179.8 ± 40.94	180.0 ± 17.36	215.0 ± 30.24	145.3 ± 28.15
GGT (U/L)	0.5 ± 0.57	2.5 ± 2.51	0.5 ± 0.57	1.5 ± 2.38
CK (U/L)	676.3 ± 326.3	1032.0 ± 409.9	1024.0 ± 79.55	700.3 ± 371.0
Cr (mg/dL)	0.5 ± 0.08	0.52 ± 0.05	0.5 ± 0.1	0.42 ± 0.09
PT (g/dL)	5.77 ± 1.19	5.60 ± 0.82	5.90 ± 0.33	5.17 ± 1.26
Urea (mg/dL)	39.0 ± 2.58	35.25 ± 5.31	46.25 ± 10.63	37.25 ± 6.44

Values are given as Mean ± S.E.M. for each group. The mean difference is considered significant at $p<0.05$.*=significant ($p<0.05$). WW: Without wound; CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

Figures

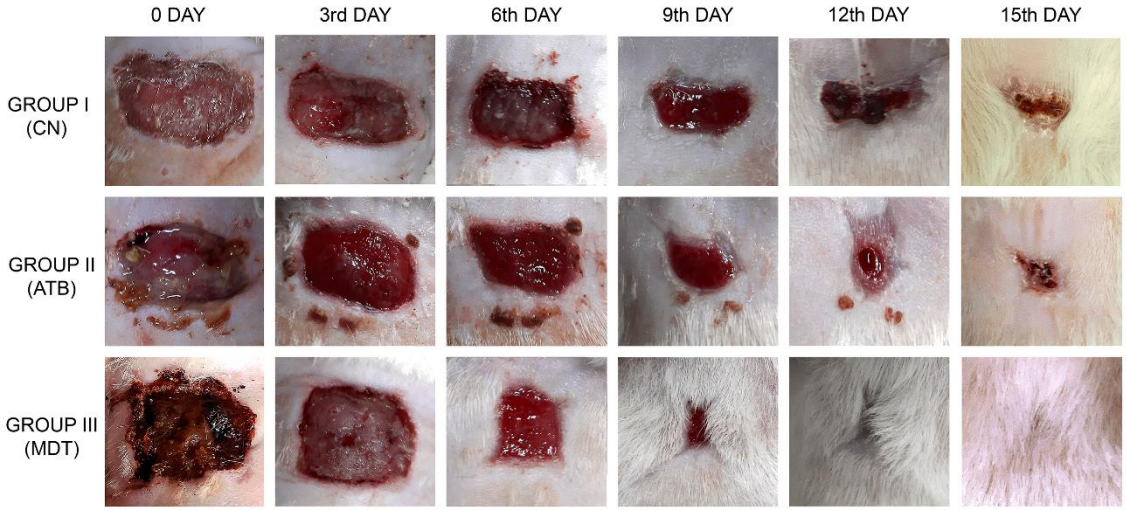

Figure 1. Macroscopic evaluation of induced wound in Wistar rats over 15 days, starting at day 3 of wound infection (0 DAY). CN: negative control group (without treatment); ATB: group submitted to antibiotic treatment; MDT: group treated with larval therapy.

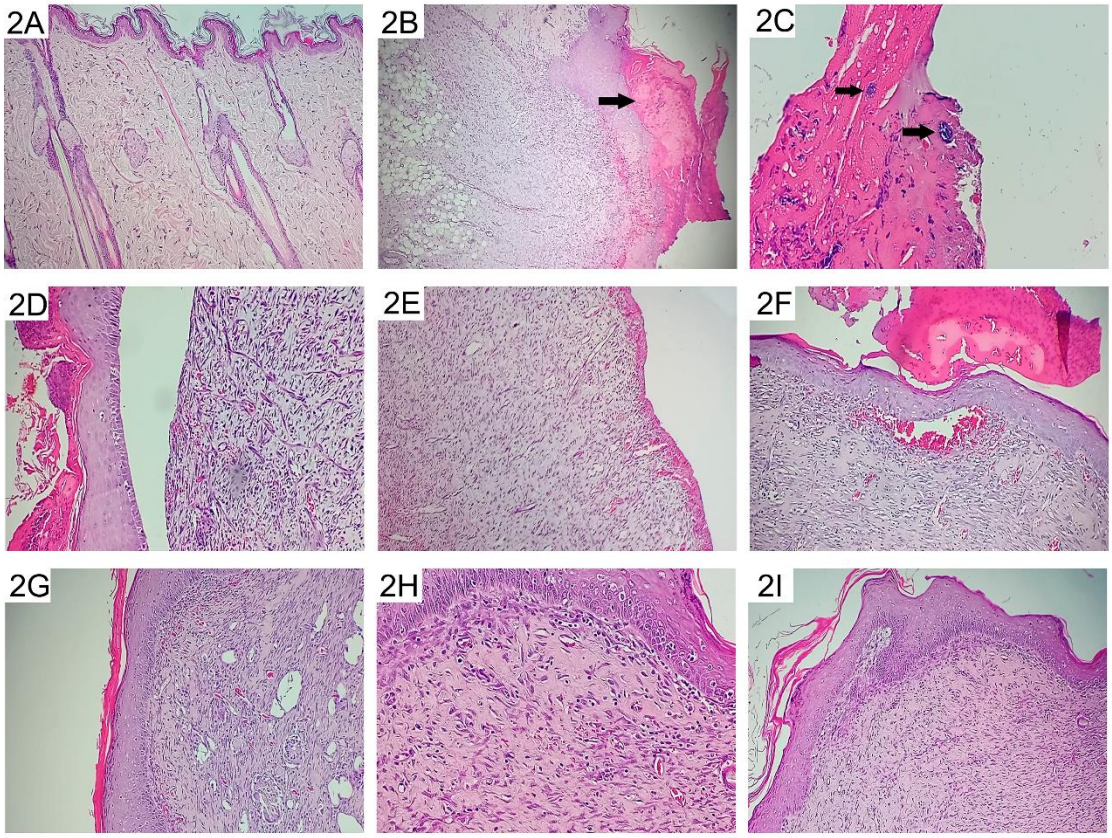

Figure 2. 2a. Normal epidermis with well-organized dermal collagen (eosinophilic fibers and with normal amounts of fibroblasts). H&E 10X; **2b**. Group I (CN) – 7th day: large amount of superficial cellular debris (serocellular crusts), exudate and neutrophils. H&E 4X; **2c**. Group I (CN) – 7th day: arrows points clustering of bacteria. H&E 40X; **2d**. Group II (ATB) – 7th day: serocellular crust, abundant polymorphonuclear (PMN) cellular infiltration inflammatory process, with apparent neovascularization, granulation tissue formation. H&E 10X; **2e**. Group III (MDT) – 7th day: No serocelluar crust, abundant PMN, collagen disorganized. H&E 10X; **2f**. Group I (CN) – 15th day: Serocellular crust, signs of re-epithelization. H&E 10X; **2g**. Group II (ATB) – 15th day: Small crust, re-epithelization, more organized collagenous tissue, more eosinophilic and less cellular. H&E 10X; **2h**. Group III (MDT) – 15th day: organized collagenous tissue, more eosinophilic and less cellular. H&E 20X; **2i**. Group III (MDT) – 15th day: Wound completely epithelialized, organized collagen fiber. H&E 10X.

5 DISCUSSÃO

O objetivo dessa tese foi avaliar a atividade *in vitro* dos extratos de larvas (EL) frente a microrganismos, desafiadas/incubadas previamente ou não com bactérias, e elucidar a ação das larvas de *L. cuprina* sobre feridas infectadas induzidas em ratos Wistar. O primeiro estudo foi baseado na hipótese primária de Wang et al. (2014) de que a incubação de larvas com uma suspensão bacteriana durante 24 horas, poderia ativar o seu mecanismo imunitário a produzir uma resposta antagônica ao stress da incubação. A questão levantada foi se essa resposta teria uma influência direta na redução ou eliminação de bactérias. A literatura abrange a utilização de extratos larvais e secreções larvais frente a bactérias, inclusive com a espécie *L. cuprina* (ARORA et al. 2010; TEH et al. 2013), no entanto há divergências de resultados e metodologias, o que implica na necessidade de padronizar a preparação dos produtos larvais e dos bioensaios utilizados. Assim, no primeiro artigo foram avaliados os extratos larvais de *L. cuprina* pré-incubadas ou não com *S. aureus*, *P. aeruginosa* e *S. aureus resistente à meticilina*, contra as mesmas bactérias, as quais são frequentemente isoladas de feridas (KAWABATA et al., 2010).

Nos ensaios de atividade antibacteriana do EL, foi observada uma diminuição no número de unidades formadoras de colônia (UFC) do grupo das larvas pré-incubadas (EL T) em comparação com o grupo não tratado (EL N/T) contra *P. aeruginosa* e SARM, porém, frente a *S. aureus*, EL N/T demonstrou resultados pouco expressivos. Ambos grupos de extratos larvais de *L. cuprina* tiveram uma diferença estatística e melhores resultados de atividade antimicrobiana contra a *P. aeruginosa*. Estudos preliminares de Kawabata et al. (2010) utilizando larvas de *Lucilia sericata* mostraram atividade antibacteriana mais potente com larvas cultivadas num ambiente infectado contra *S. aureus,* mas nenhuma para *P. aeruginosa*, e a concentração bacteriana foi a mesma utilizada no nosso trabalho. Portanto, é possível que a espécie influencie diretamente os resultados, e que larvas de *L. cuprina* tenham uma resposta imunitária melhor frente a *P. aeruginosa*. Tais resultados devem ser considerados durante a aplicação clínica da terapia larval, podendo ser apropriado indicar diferentes espécies de moscas para cada caso clínico, dependendo da espécie bacteriana predominante no leito da ferida. Alguns autores relatam que a terapia larval utilizando *L. sericata* é mais eficaz contra infecções Gram-positivas do que Gram-negativas (SHERMAN et al., 2014), porém a mesma não tem atividade antibacteriana significativa contra bactérias Gram-negativas em testes *in vitro* (CAZENDER et al., 2009; KAWABATA et al., 2010). Assim, larvas de *L. cuprina*

podem representar uma poderosa ferramenta para a abordagem clínica da cicatrização de feridas infectadas com *P. aeruginosa*.

É importante salientar que nenhum dos extratos larvais teve efeito inibitório total, apenas diminuiu o crescimento de algumas bactérias, indicando que a ação mecânica e a resposta fisiológica das larvas atuam em conjunto para eliminar as bactérias do leito da ferida. Devido a isso, buscamos no segundo artigo estudar a eficácia e segurança da utilização de larvas de *L. cuprina* em feridas induzidas infectadas com SARM em ratos Wistar. Os animais foram avaliados quanto à forma clínica, histológica e microbiológica da ferida, além de realizada a coleta de sangue para a análise dos parâmetros hematológicos e bioquímicos.

As larvas de *L. cuprina* provaram ser promissoras e seguras para aplicação terapêutica, preservando tecidos saudáveis ilesos, removendo apenas áreas necróticas/desvitalizadas e promovendo a ação antimicrobiana. Comparado ao tratamento de desbridamento mecânico com solução fisiológica (NaCl 0,9%) (Grupo I) e de pomada antibiótica Bacitracina (250 Ui/g) + Neomicina (5mg/g) (Grupo II), foi evidente a aceleração no processo de regeneração dos tecidos no grupo de animais tratados com larvas (Grupo III). Após o primeiro tratamento (3º dia) havia pouco ou nenhum exsudato no leito das feridas tratadas com larvas. Isso se deve ao fato de que as mesmas liquefazem o meio para ingerir o tecido necrótico e bactérias, além de secretarem/excretarem propriedades que favorecem a perfusão de oxigênio e a proliferação celular e migração de fibroblastos, permitindo a remodelação da matriz e a propagação do tecido de granulação (FIGUEROA et al., 2006; SHERMAN, 2014). O movimento das larvas através do leito da ferida também se provou benéfico para a cicatrização mais rápida e a presença de pequenos espinhos presentes no corpo da mesma, ajuda no desbridamento e raspagem dos tecidos necróticos, o que auxilia o acesso profundo de enzimas excretadas pelas larvas para a liquefação e ingestão do material (BORKATAKI et al., 2021; SHERMAN 2014). A contração da ferida no 6º dia chegou a 90,25%, comparados a 70,25% no Grupo II (ATB) e 60,5% no Grupo I (CN). Embora ao final do 15º dia não houvesse diferença entre os grupos tratados com pomada antibiótica e com larvas, pode ser considerado melhor benefício o uso de larvas no lugar de medicamentos, uma vez que não há risco de provocar resistência bacteriana ou danos ao ambiente, além de concomitantemente, proporcionar desbridamento seletivo.

De posse dos resultados obtidos, foi possível concordar que a ação mecânica das larvas tem grande influência sobre a redução da carga bacteriana, uma vez que as larvas

foram capazes de inibir completamente o crescimento de SARM no leito das feridas. Porém, como descrito acima, é importante reforçar que a movimentação, a alcalinização do leito por subprodutos de amônia da digestão das larvas (BLAKE et al., 2007), os produtos antimicrobianos das secreções/excreções, como as deoxirribonucleases (BROWN et al., 2012) e a ação mecânica, em conjunto são os responsáveis pela eliminação das bactérias. Reforço também garantido pelas descobertas histopatológicas, que mostraram feridas limpas e sem crostas logo após o primeiro tratamento, com sinais de reepitelização e organização de colágeno, que sugerem o estímulo de crescimento de tecido de granulação por seus movimentos (RAYNER, 1999).

Nessa pesquisa foram evidenciadas pela primeira vez, alterações em parâmetros hematológicos e bioquímicos dos animais tratados com terapia larval. Foram encontradas poucas diferenças significativas ($p<0.05$) entre os grupos tratados e o grupo controle sem ferida, ou seja, houve segurança na utilização da terapia larval, sem danos à saúde do animal. Além disso, o grupo tratado com larvas foi o único a demonstrar aumento significativo do número de plaquetas totais logo após o primeiro tratamento. As plaquetas são responsáveis por fornecer um "andaime" provisório para células inflamatórias, além de abrigar um reservatório de citocinas, como as quimiocinas e fatores de crescimento que conduzem a reparação tecidual e estimulam o recrutamento de neutrófilos (EINSINGER et al., 2018).

6 CONCLUSÃO

Extratos larvais de *L. cuprina* incubadas previamente ou não com bactérias mostraram significativa atividade antibacteriana frente a *Pseudomonas aeruginosa*.

Larvas de *L. cuprina* realizaram um desbridamento contínuo e seletivo da lesão e remoção do tecido necrótico e desvitalizado, facilitando o processo de cicatrização.

As larvas foram capazes de interromper a proliferação bacteriana e eliminar por completo a SARM nas feridas dos animais tratados.

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ANEXO A – Autorização CEUA

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Atividade antimicrobiana de LUCILIA CUPRINA desafiadas por bactérias e seu uso em bioterapia", protocolada sob o CEUA nº 5414110220 (ID 003202), sob a responsabilidade de **Silvia Gonzalez Monteiro** *e equipe; Janaína Brand Dillmann; Letícia dos Santos Petry; Talissa Silva dos Santos* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 06/10/2020.

We certify that the proposal "Antimicrobial activity of LUCILIA CUPRINA challenged by bacteria and its use in biotherapy ", utilizing 32 Heterogenics rats (32 males), protocol number CEUA 5414110220 (ID 003202), under the responsibility of **Silvia Gonzalez Monteiro** *and team; Janaína Brand Dillmann; Letícia dos Santos Petry; Talissa Silva dos Santos* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 10/06/2020.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 10/2020 a 10/2021 Área: Departamento de Microbiologia E Parasitologia

Origem:	Biotério Central UFSM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	6 a 8 semanas	N:	32
Linhagem:	Wistar			Peso:	150 a 250 g		

Local do experimento: O experimento será realizado no Laboratório de Parasitologia Veterinária e os animais serão mantidos no Setor de experimentação animal do Departamento de Microbiologia e Parasitologia do Centro de Ciências da Saúde- UFSM

Santa Maria, 20 de janeiro de 2022

Dra. Patrícia Bräunig
Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Profa. Dra. Vania Lucia Loro
Vice-Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria