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Bibiana Martins Barasuol

Novos bacteriófagos de *Staphylococcus aureus* **oriundos de leite bovino: isolamento, caracterização e atividade antibiofilme bacteriano**

Santa Maria, RS
2021

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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 do título em **Doutor em Ciência Animal.**

Orientadora: Profª. Dra Sônia de Avila Botton

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RESUMO

AUTORA: Bibiana Martins Barasuol

Orientadora: Sônia de Avila Botton

Os bacteriófagos ou fagos são vírus que possuem a capacidade de infectar e destruir as bactérias, por meio da lise celular. Esses vírus podem representar uma alternativa promissora para o tratamento das infecções bacterianas, especialmente àquelas causadas por agentes mutirresistentes às terapias antimicrobianas, como as mastites bovinas por *Staphylococcus aureus*, e no controle de biofilmes bacterianos. Sendo assim, os objetivos deste estudo foram: isolar e caracterizar fagos de *S. aureus* provenientes de leite bovino; testar a eficiência dos fagos frente aos isolados bacterianos de mastites bovinas; avaliar a capacidade dos fagos isolados de inibir e remover biofilmes bacterianos de isolados oriundos de mastites bovinas e cepas bacterianas. Na seção correspondente ao Capítulo 1 desta tese, apresenta-se um artigo científico de meta-análise, realizado com objetivo de investigar a eficiência de vários fagos de *S. aureus* frente a diferentes bactérias oriundas de mastites bovinas, confirmando a alta eficiência (80%) e especificidade da maioria dos fagos dos estudos incluídos na meta-análise. A seguir, no Capítulo 2, no manuscrito 1 objetivou-se realizar o isolamento de fagos oriundos de leite bovino. Estes fagos foram denominados B_UFSM1, B_UFSM3, B_UFSM4, e B_UFSM5 e, a partir da caracterização genômica e fenotípica de dois fagos temperados de *S. aureus* (B_UFSM4 e B_UFSM5), verificou-se que os mesmos pertencem à ordem *Caudovirales*, família *Siphoviridae* e gênero *Biseptimavirus*. Adicionalmente, com análise da eficiência de plaqueamento, verificou-se que os fagos B_UFSM4 e B_UFSM5 são capazes de infectar isolados de sua espécie hospedeira alvo e, também, possuem a habilidade de infectar outra espécie bacteriana, *Rothiae terrae*. Em seguida, no Capítulo 3, referente ao manuscrito 2, foi investigado se os fagos e duas combinações A (B_UFSM4 e B_UFSM5) e B (B_UFSM1, B_UFSM3, B_UFSM4 e B_UFSM5), apresentavam a capacidade de inibir e remover biofilmes bacterianos de *Staphylococcus* spp. e de *Pseudomonas aureginosa* oriundos de mastite bovina, bem como, de cepas-padrão bacterianas. Neste sentido, foi possível observar que os fagos e as combinações tiveram atividade antibiofilme e somente o fago B_UFSM4 não foi capaz de inibir a formação e/ou remover os biofilmes dos isolados e cepas-padrão bacterianas testadas. Dessa forma, conclui-se, que os fagos isolados, caracterizados e analisados deverão ser mais estudados para verificar o potencial uso como alternativa terapêutica clínica e no controle de biofilmes de *S. aureus* oriundos de mastite bovina. Adicionalmente, devido a pandemia COVID-19, ocorreram atrasos nos prazos estabelecidos para a finalização da avaliação e caracterização morfológica e molecular, dos fagos B_UFSM1 e B_UFSM3; sendo assim, estes resultados não puderam ser incluídos nesta tese.

Palavras-chave: bacteriófagos, fagos, *Staphylococcus aureus*, mastites bovinas.

ABSTRACT

AUTHOR: Bibiana Martins Barasuol

ADVISOR: Sônia de Avila Botton

Bacteriophages or phages are viruses that have the ability to infect and lyse bacteria through cell lysis. These viruses may represent a promising alternative for the treatment of bacterial infections, especially those caused by strains multiresistant to antimicrobial therapies, such as bovine mastitis by *Staphylococcus aureus* and in control of bacterial biofilms. Therefore, the objectives of this study were: isolating and characterizing *S. aureus* phages from bovine milk; test the efficiency of phages against bovine mastitis bacterial isolates; evaluating the ability of isolated phages to inhibit and remove bacterial biofilms from bovine mastitis isolates and standard bacterial strains. In the section corresponding to Chapter 1 of this thesis, a scientific article of meta-analysis is presented, carried out with the aim of investigating the efficiency of several *S. aureus* phages against different bacteria from bovine mastitis, confirming the high efficiency (80%) and specificity of most phages from the studies included in the meta-analysis. Subsequently, in Chapter 2, in manuscript 1, aimed to isolate phages from bovine milk. These phages were named B_UFSM1, B_UFSM3, B_UFSM4, and B_UFSM5 and, with the genomic and phenotypic characterization of two temperate *S. aureus* phages (B_UFSM4 and B_UFSM5) it was found that they belong to the order *Caudovirales*, family *Siphoviridae* and *Biseptimavirus* genus. In addition, with the analysis of plating efficiency, it was found that the phages B_UFSM4 and B_UFSM5 are able to infect their target host species e also have the ability to infect another bacterial species, as *Rothiae terrae*. Then, in Chapter 3, referring to manuscript 2, it was investigated whether phages and two combinations A (B_UFSM4 and B_UFSM5) and B (B_UFSM1, B_UFSM3, B_UFSM4 and B_UFSM5) had the ability to inhibit and remove bacterial biofilms of *Staphylococcus* spp. and *Pseudomonas aeruginosa* from bovine mastitis, as well as standard bacterial strains. It was possible to observe that the phages and combinations had antibiofilm activity and only the B_UFSM4 phage was not able to inhibit the formation and/or remove the biofilms of the tested bacterial isolates and standard strains. Thus, it is concluded that the isolated, characterized and analyzed phages should be further studied to verify their potential use as clinical therapeutic alternatives and in the control of *S. aureus* biofilms from bovine mastitis. Additionally, due to the COVID-19 pandemic, there were delays in the deadlines established for the completion of the morphological and molecular evaluation and characterization of the phages B_UFSM1 and B_UFSM3; therefore, these results could not be included in this thesis.

Keywords: bacteriophages, phages, *Staphylococcus aureus*, bovine mastitis.

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APRESENTAÇÃO

Esta tese de doutorado está composta pelos seguintes itens: 1. INTRODUÇÃO, 2. REVISÃO BIBLIOGRÁFICA, 3. CAPÍTULO 1, 4. CAPÍTULO 2, 5. CAPÍTULO 3, 6. DISCUSSÃO e 7. CONCLUSÕES, REFERÊNCIAS e ANEXOS. O artigo 1, manuscrito 1 e manuscrito 2 compõem a íntegra deste estudo. Separadamente, no artigo 1, manuscrito 1 e manuscrito 2, estão descritos os objetivos de cada estudo, bem como as respectivas seções de Materiais e Métodos, Resultados, Discussão e Referências. Adicionalmente, no item DISCUSSÃO estão apresentadas as considerações finais relativas aos pontos em destaque da tese. No item REFERÊNCIAS estão contempladas as citações contidas nos itens INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA e DISCUSSÃO desta tese.

1. INTRODUÇÃO

Os bacteriófagos ou fagos são vírus capazes de infectar e destruir bactérias; os bacteriófagos são reconhecidos a mais de século como agentes antimicrobianos conhecidos (D'HERELLE, 1931) e podem ser ferramentas importantes no controle da resistência antimicrobiana e, de bactérias multirresistentes (TORRES-BARCELÓ, 2018).

Nesse sentido, os fagos apresentam certas características como especificidade da célula hospedeira, ausência de danos em humanos e animais, e mecanismo de ação diferente dos antimicrobianos convencionais. Além disso, esses vírus têm sido eficazes contra bactérias multirresistentes e, ainda, podem ser encontrados com certa facilidade em alguns ambientes naturais, por exemplo, o marinho e solo (BATINOVIC et al., 2019; SULAKVELIDZE; ZEMPHIRA; JR, 2001; VEIGA-CRESPO; BARROS-VELÀZQUEZ; VILLA, 2010). As características previamente descritas tornam os fagos uma possível solução para a crise de resistência antimicrobiana encontrada em diversas bactérias de origem humana e animal (ALMEIDA; SUNDBERG, 2020).

Na medicina veterinária destaca-se a resistência aos antimicrobianos devido ao uso indiscriminado para tratar diferentes infecções na rotina clínica e no tratamento e prevenção das mastites bovinas (ECONOMOU; GOUSIA, 2015). As mastites bovinas são um grande problema nas fazendas leiteiras em todo mundo, sendo uma das doenças mais comuns, resulta em grandes perdas econômicas através da redução da qualidade e da produção de leite e dos altos custos no tratamento (HALASA et al., 2007; JONG et al., 2018; SEEGERS; FOURICHON; BEAUDEAU, 2003; VIGUIER et al., 2009). Dentre os micro-organismos envolvidos na etiologia das mastites bovinas, a bactéria *Staphylococcus aureus* é reconhecida como sendo o agente de maior ocorrência mundial (BARKEMA; SCHUKKEN; ZADOKS, 2006; MESQUITA et al., 2019; SEYOUM et al., 2018; SOUZA et al., 2016; SULEIMAN; KARIMURIBO; MDEGELA, 2018).

A principal característica de *S. aureus* é persistir na glândula mamária causando infecção subclínica, através de importantes mecanismos intrísecos e de evasão do sistema de defesa do hospedeiro. Sendo assim, destaca-se a capacidade de formação de biofilmes, a presença de cápsulas polissacarídicas constituintes do micro-orgsnismo e a sua capacidade de invadir células epiteliais, consequentemente evadindo da resposta imune inata e adaptativa da fêmea (ZAATOUT; AYACHI; KECHA, 2020).

Além disso, a bactéria *S. aureus* demonstra potencial para desenvolver resistência às diferentes classes de antimicrobianos disponíveis (YANG et al., 2015). Logo, estudos vêm

demonstrando isolados de *S. aureus* de mastites bovinas resistentes à meticilina e, também, a penicilina (AQIB et al., 2017; SILVA et al., 2020).

Diante disso, os antimicrobianos habitualmente empregados na clínica veterinária não estão sendo eficazes no tratamento e controle das mastites bovinas, ocasionadas por este agente bacteriano. Portanto, torna-se necessário a busca por alternativas de tratamento eficazes no controle desta importante doença. Nesta perspectiva, os fagos vêm assumindo um importante papel alternativo para o tratamento das infecções intramamárias bovinas (ALLUWAIMI, 2004; MONK et al., 2010; SILLANKORVA; AZEREDO, 2014; SULAKVELIDZE, 2004).

Fagos têm sidos isolados e caracterizados e, estes apresentam-se eficientes com grande potencial lítico contra as bactérias causadoras de mastite em vacas, assim como, as suas enzimas líticas. Porém, há necessidade da inclusão de estudos clínicos (testes *in vivo*) sobre a eficácia destes na terápia fágica para mastite bovina (ZDUŃCZYK; JANOWSKI, 2020).

Dessa forma, estudos demonstram o isolamento e a caracterização de fagos de *S. aureus* e análises *in vitro* da eficiência e/ou infectividade dos fagos contra isolados bacterianos provenientes de mastites bovinas; contudo, os resultados são bastante variáveis (BASDEW; LAING, 2011). Sendo assim, no artigo 1 desta tese, objetivou-se apresentar um estudo de meta-análise avaliando a eficiência *in vitro* de fagos isolados e caracterizados em isolados bacterianos oriundos de mastites bovinas.

Além disso, a constituição de bancos de fagos contendo diversificados isolados virais é necessária para a aplicação dos fagos como alternativa aos agentes antimicrobianos. Os bancos de fagos reunindo diferentes tipos de fagos são importantes, pois fornecem o material biológico para pesquisas, incluindo desde o isolamento e a caracterização de fagos, bem como, a infectividade validando o possível melhor fago e/ou combinação de fagos a serem empregados no tratamento de doença bacteriana (ABEDON; KUHL; KUTTER, 2011; CHAN; ABEDON; LOC-CARRILLO, 2013). Portanto, no manuscrito 1 desta tese, objetivou-se realizar o isolamento de fagos oriundos e leite bovino com mastite. Posteriormente, procedeu-se a seleção de fagos de *S. aureus* para estudos de caracterização fenotípica e genotípica dos fagos e investigou-se a eficiência lítica *in vitro* dos vírus em bactérias isoladas de mastite bovina.

No manuscrito 2, pesquisou-se a capacidade dos fagos de *S. aureus* (B_UFSM1, B_UFSM3, B_UFSM4, B_UFSM5) isolados neste estudo, isoladamente ou em combinação, de atuarem como agentes de controle de biofilmes de bactérias provenientes de mastites bovinas, tanto na inibição e/ou formação desses biofilmes bacterianos.

2. REVISÃO BIBLIOGRÁFICA

2.1 BACTERIÓFAGOS OU FAGOS

Os bacteriófagos, também denominados fagos, são vírus capazes de infectar e replicar em bactérias, arqueas ou arqueobactérias (D'HERELLE, 1917). Os fagos foram co-descobertos por Frederick W. Twort e Felix D'Herelle (D'HERELLE, 1917; TWORT, 1915). Inicialmente, Twort, em 1915, observou zonas claras (que seriam bactérias mortas) em suas culturas de vacina para varíola. Dois anos mais tarde, D'Herelle publicou observações semelhantes à efetuadas por Twort e denominou os micro-organismos como sendo bacteriófagos ou fagos (DUCKWORTH, 1976). Os fagos foram descritos como potenciais antimicrobianos, primeiramente, no ano de 1921 (BRUYNOGHE; MAISIN, 1921).

Os fagos são o maior grupo de vírus existentes e a maioria absoluta de todos os organismos da biosfera (ACKERMANN; DUBOW, 1987). Estima-se que existem 4-6x10^{30} células bacterianas em todos os ecossistemas e aproximadamente 10 vezes mais partículas de fagos de cauda do que bactérias, ou seja, pressupõe -se que há 10^{31} fagos na biosfera (WHITMAN; COLEMAN; WIEBE, 1998; BERGH et al., 1989; HATFULL et al., 2008). Além disso, os pesquisadores calculam que existem cerca de 10^{23} infecções fágicas por segundo em uma escala global, indicando que a população não é apenas grande, mas também altamente dinâmica (SUTTLE, 2007).

Adicionalmente, os fagos são normalmente encontrados em ambientes onde seu hospedeiro está presente (WEINBAUER, 2004). Eles são tipicamente altamente específicos, muitas vezes sendo restritos à determinada espécie bacteriana. No entanto, alguns fagos têm uma gama de hospedeiros relativamente ampla, infectando várias espécies dentro de um gênero e podem até infectar membros de outros gêneros intimamente relacionados aos seus hospedeiros. Em alguns casos, isso reflete a natureza incerta da taxonomia e, em outras, a presença de receptores comuns (MANILOFF; DYBVIG, 2006).

Estudos genômicos comparativos de fagos, especialmente com os fagos com cauda, e os estudos ambientais, fornecem um quadro novo e dramático do tamanho, estrutura genética e dinâmica da população deste micro-organismo. As comparações de sequências genômicas revelam alguns mecanismos detalhados pelos quais os fagos evoluem e influenciam na evolução de seus hospedeiros bacterianos e arqueológicos. Revelando, troca horizontal desenfreada de

sequências entre os genomas, mediada por recombinação homóloga e não-homóloga (HENDRIX, 2003).

Com a revolução de novas tecnologias de sequenciamento, muitos sequenciamentos de genomas procarióticos foram realizados. Todavia, o sequenciamento de genoma completo de fagos não acompanhou essa revolução, apesar de seus genomas serem pequenos em comparação com as bactérias e outros organismos, o sequenciamento de genomas de fago apresenta vários desafios como, a obtenção de material genômico de fago puro, vieses de amplificação de PCR e a natureza complexa de seu material genético, devido apresentarem bases metiladas e repetições que são inerentemente difíceis de sequenciar e realizar a montagem de genomas (KLUMPP; FOUTS; SOZHAMANNAN, 2012).

Atualmente, são 11.489 sequências de genoma disponíveis no NCBI (National Center for Biotechnology Information) Viral RefSeq database (version 94). Sendo que, a maioria dos fagos caracterizados genotipicamente possuem genoma de DNA de fita dupla (dsDNA) e são fagos de cauda (NORMAN et al., 2015; MANRIQUE et al., 2016; BARYLSKI et al., 2020). Sendo assim, os fagos podem conter material genético de DNA ou RNA, de cadeia dupla ou simples, além disso, a maioria dos fagos possuem uma cabeça, contendo material genético envolto por um capsídeo lipoproteíco e uma cauda constituída de proteínas (ICTV, 2017).

2.1.1 Classificação dos fagos

Ainda não está definido um método universal para a classificação dos fagos, até momento, são agrupados de acordo com sua morfologia e com a composição de seu material genético (ICTV, 2017). Entretanto, devido a razões históricas, toda essa diversidade está confinada a uma única ordem de vírus, a *Caudovirales*, inicialmente composta por quatro famílias: *Myoviridae*, *Siphoviridae*, *Podoviridae* e *Ackermannviridae*. Nos últimos anos, esse esquema de classificação com base na morfologia tornou-se obsoleta devido às inúmeras sequências de genomas de fagos analisadas, revelando que os fagos com cauda são amplamente diversos geneticamente. Portanto, o Comitê Internacional de Taxonomia de Vírus (ICTV), iniciou a reorganização geral da taxonomia de fagos, uma vez que a morfologia e o tipo de genoma das famílias de fagos são altamente variáveis (BARYLSKI et al., 2020).

Além dos fagos previamente descritos, um grupo notável com genomas de DNA de fita simples estão agrupados na família *Inoviridae* e possuem a forma de filamentos. Além disso, a morfologia e o tipo de genoma do restante das famílias de fagos são altamente variáveis (MANILOFF; DYBVIG, 2006).

2.1.2 Mecanismos de infecção dos fagos

A infecção por fagos ocorre quando há a ligação entre a partícula viral e a célula bacteriana hospedeira. Inicialmente, ocorre a adsorção da partícula viral aos receptores de superfície da bactéria. Esse processo é específico e pode ser reversível ou irreversível. A adsorção reversível é influenciada por ações eletrostáticas e na adsorção irreversível há ligações mais estáveis e ação de enzimas hidrolíticas (RAKHUBA et al., 2010).

Os principais receptores bacterianos são os componentes da superfície celular da bactéria. A natureza e localização dos receptores da célula hospedeira reconhecidos por fagos variam muito dependendo do fago e da célula hospedeira. Portanto, incluem desde sequências de peptídeos às porções de polissacarídeos celulares. Sendo assim, foi demonstrado que os fagos se ligam aos receptores localizados nas paredes de bactérias Gram-positivas e Gram-negativas (MARTI et al., 2013; XIA et al., 2011), podendo reconhecer moléculas nas cápsulas bacterianas (FEHMEL et al., 1975) e nos, apêndices, como pili (GUERRERO-FERREIRA et al., 2011) e flagelos (SHIN et al., 2012).

Sendo assim, os fagos podem se ligar nos peptioglicanos ou ácidos teicoícos nas bactérias Gram-positivas (WENDLINGER; LOESSNER; SCHERER, 1996) e no lipolissacarídeo (LPS) na maioria das bactérias Gram-negativas (DATTA; ARDEN; HENNING, 1977; LINDBERG, 1973). A exemplo dos fagos da família *Siphoviridae*, tais vírus reconhecem as proteínas da membrana externa, açúcar ou ambos, os fagos da família *Myoviridade* são capazes de ligarem-se nos açúcares da parede bacteriana, assim como, os fagos pertencentes à família *Podorividae* (BERTOZZI; STORMS; SAUVAGEAU, 2016).

A adsorção é uma etapa crucial no processo de infecção e também representa o ponto inicial de contato entre o vírus e o hospedeiro, bem como, indica a especificidade de hospedeiros. Sendo assim, a adsorção dos fagos geralmente consiste em três etapas: contato inicial, ligação reversível e fixação irreversível (DUCKWORTH, 1976). Após, as etapas, o fago libera o seu material genético dentro da célula hospedeira para iniciar a sua multiplicação.

Por outro lado, as bactérias possuem alguns mecanismos para escapar da infecção pelos fagos, como o sistema de repetição palíndrômica curto regularmente intercalado, o sistema CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). Portanto, este é o sistema de defesa mais difundido usado pelas bactérias contra elementos genéticos móveis (MGEs), incluindo os fagos (CHAUDHARY; CHATTOPADHYAY; PRATAP, 2018). Sendo assim, esse sistema CRISPR confere uma resistência específica contra as sequências de DNA

ou RNA dos fagos, consequentemente, constitui um sistema antiviral e pode ter efeito sobre a interação vírus-hospedeiro (RATH et al., 2015).

2.1.3 Ciclo replicativo dos fagos

Após a adsorção, os fagos liberam o seu material genético no interior da célula hospedeira, propagando-se através de dois ciclos replicativos (ou biológicos): lítico e lisogênico. Quando os fagos se multiplicam e matam a célula hospedeira, o ciclo é conhecido como lítico. Por outro lado, alguns fagos conhecidos como temperados podem integrar seu genoma na replicação do cromossomo da bactéria. Neste caso, o ciclo replicativo é o lisogênico (INAL, 2003). Sendo assim, os fagos temperados evoluem juntamente com seus hospedeiros, otimizando seus mecanismos de disseminação e liberação da célula bacteriana para o meio ambiente (CATALÃO et al., 2013).

Adicionalmente, os fagos para serem lisogênicos devem possuir o gene que codifica a enzima integrase para integrar seu material genético no da bactéria, uma vez que, após o DNA viral ser integrado ao DNA da célula hospedeira, é denominado de prófago (FORTIER; SEKULOVIC, 2013; TAO; WU; SUN, 2010). Podendo, em particular, replicar-se lisogenicamente, integrando-se ao cromossomo da célula bacteriana hospedeira (CASJENS; HENDRIX, 2015), ou mantendo-se extracromossomicamente como genoma circular (LOBOCKA et al., 2004) ou genoma linear (RAVIN, 2015). No entanto, os fagos utilizam um sistema de comunicação baseado em peptídeos para decidir se entram no ciclo lítico ou lisogênico. A suposta molécula de comunicação foi denominada arbitrium (EREZ et al., 2017).

Sendo assim, as infecções pelos bacteriófagos variam e dependem da genética tanto do fago, como do hospedeiro, da concentração do fago, da fisiologia do hospedeiro, e das condições ambientais (HOWARD-VARONA et al., 2017). No qual, os estressores ambientais podem ser mudanças de nutrientes, pH ou temperatura e exposição aos antibióticos e/ou peróxido de hidrogênio (BANKS; LEI; MUSSER, 2003; CASJENS; HENDRIX, 2015; MELL; REDFIELD, 2014).

Além disso, no término do ciclo de replicação lítico (onde há lise celular), uma vez que as partículas virais maduras são montadas, a enzima denominada holin é sintetizada em altas concentrações e inserida na membrana bacteriana, formando os poros para a interação das enzimas líticas com a parede bacteriana. Portanto, as enzimas líticas anteriormente acumulada no citoplasma celular, podem atingir a estrutura peptidoglicana, em Gram-positivas, e provocar

a lise celular bacteriana (BARRERA-RIVAS et al., 2017; INAL, 2003) e através da lise celular hospedeira os fagos efetuam a liberação dos seus vírions descendentes (RAJAURE et al., 2015).

2.1.4 Enzimas líticas dos fagos

As enzimas líticas são moléculas produzidas pelos fagos e utilizadas no processo de infecção bacteriana. Atuam promovendo a degradação do peptidoglicano ou como proteínas solúveis para lisar a célula bacteriana de forma massiva no final do ciclo lítico (FISCHETTI, 2005; BRIERS, 2019).

As enzimas líticas são denominadas de lisinas ou hidrolases de peptidoglicanos, e visam romper a integridade da parede celular, sendo projetadas para degradar as principais ligações do peptidoglicano. Portanto, a endo-b-N-acetilglucosaminidase e N-acetilmuramidase (ou lisozimas) agem sobre a porção de açúcar da parede bacteriana e a endopeptidase, denominada N-acetilmuramoil-L-alanina amidase (ou amidase), atua na fração do peptídeo do peptidoglicano (YOUNG, 1992). Sendo assim, a característica das enzimas líticas de fagos infectantes de bactérias Gram-positivas é a sua estrutura de dois domínios, um domínio catalítico N-terminal e um domínio de ligação C-terminal. Com poucas exceções, o domínio N-terminal contém a atividade catalítica da enzima, que cliva especificamente as principais ligações encontradas no peptidoglicano (NAVARRE et al., 1999).

As lisinas têm importância na infecção das bactérias Gram-positivas, pois agem em contato direto com o peptidoglicano da parede celular; enquanto, nas bactérias Gram-negativas os componentes presentes na constituição celular externa impedem essa interação (FISCHETTI, 2005). Portanto, as lisinas apresentam potencial como agentes antimicrobianos frente às infecções por bactérias Gram-positivas, incluindo as mastites bovinas por *S. aureus* (FISCHETTI, 2010; SCHMELCHER; KOROBOVA et al., 2012; SCHMELCHER; POWELL et al., 2012).

Posteriormente, constatou-se que nos hospedeiros Gram-negativos, a lise pelo fago também requer a ruptura da constituição externa da bactéria. Isso é feito pelas *spanins*, que são proteínas codificadas pelos fagos; contudo o mecanismo pelo qual as *spanins* destrõem os constituintes celulares externos ainda é desconhecido (RAJAURE et al., 2015).

Adicionalmente, foram identificadas pequenas proteínas secretadas pelos fagos, necessárias para que as lisinas tenham acesso ao peptidoglicano. Essas proteínas foram denominadas *holins* (formadoras de orifícios) e as lesões na membrana bacteriana formada pelas *holins* são estáveis e inespecíficas (YOUNG, 1992).

Portanto, as lisinas têm recebido atenção crescente como agentes antimicrobianos nos últimos anos, particularmente devido à alta especificidade conferida pelo fago em relação à célula-alvo (em Gram-positivas) e o desenvolvimento de resistência contra essas enzimas líticas são consideradas improváveis, pois essas enzimas clivam ligações altamente conservadas dentro do peptidoglicano bacteriano (BORYSOWSKI; WEBER-DĄBROWSKA; GÓRSKI, 2006; DONOVAN, 2008; FISCHETTI, 2010; LOESSNER, 2005).

2.1.5 Terapia fágica ou fagoterapia

Com a descoberta dos fagos no início do século XX, muitos pesquisadores acreditavam que os fagos poderiam ser possíveis agentes terapêuticos. Mas, depois da Segunda Guerra Mundial, quando os antibióticos foram descobertos, este potencial agente terapêutico recebeu pouca atenção e foi apenas considerado uma ferramenta de pesquisa por muitos anos (CLARK; MARCH, 2006).

Todavia, apesar da terapia fágica ter sido extensamente substituída pelo uso de fármacos antimicrobianos nos países ocidentais, depois da Segunda Guerra Mundial, os fagos permaneceram como tratamento popular, na Polônia e Rússia, por todo o século XX, resultando em dados sobre os tratamentos com fagos em várias infecções bacterianas em humanos (SULAKVELIDZE; ZEMPHIRA; JR, 2001). Atualmente, com a resistência global das bactérias aos antimicrobianos, somando-se ao aumento da conscientização sobre a importância da microbiota, ressurge o interesse no uso potencial dos fagos para fins terapêuticos, conhecido como terapia fágica ou fagoterapia (MALIK et al., 2017).

De forma mais ampla, os fagos são estudados como agentes biológicos de controle, reduzindo as cargas bacterianas em alimentos (BAI et al., 2016), nas bactérias de animais de produção usados como fonte alimentar (ATTERBURY, 2009), e de bactérias patogênicas tanto de plantas (BUTTIMER et al., 2017) quanto de seres humanos (ABEDON; KUHL; KUTTER, 2011). Sendo assim, os fagos vêm sendoestudados para tratar infecções bacterianas de animais, como as mastites bovinas (BASDEW; LAING, 2011).

Diante disso, o papel dos fagos não deve ser subestimado e devem ser considerados ao projetar estratégias para enfrentar a crise global de resistência aos antimicrobianos (TETZ; TETZ, 2017). Entretanto, os fagos necessitam de regulamentações farmacêuticas para sua aplicabilidade no tratamento das infecções bacterianas, pois são entidades vivas, dinâmicas, em evolução e específicas, e não se encaixam as categorias, normas e modelos de desenvolvimento atuais dos fármacos convencionais (BRIVES; POURRAZ, 2020). Todavia, os fagos estão

sendo aprovados por agências reguladoras em vários países, fornecendo uma intervenção segura, natural e eficaz contra os agentes patogênicos, principalmente, como biocontrole de itens de produtos frescos ou processados (SHARMA, 2013).

Adicionalmente, os biofilmes bacterianos são um problema devido sua tolerância às defesas do hospedeiro e aos antimicrobianos; sendo necessário desenvolver formas alternativas para prevenir e controlar infecções bacterianas associadas aos biofilmes (AZEREDO; SUTHERLAND, 2008). Estudos, empregando os fagos em biofilmes bacterianos vêm demonstrando eficácia no controle destes, pois o alto número de bactérias presentes nos biofilmes facilita a ação dos fagos. Além disso, os bacteriófagos apresentam a capacidade de produzir a enzima, denominada despolimerase, que degradam a matriz extracelular do biofilme, assim como, apresentam a capacidade de permanecerem dormentes no interior da célula bacteriana e reativando o ciclo replicativo quando necessário (HARPER et al., 2014).

2.2 *Staphylococcus aureus* E A RESISTÊNCIA BACTERIANA EM MASTITES BOVINAS

A busca por novas alternativas de tratamento das bactérias resistentes aos antimicrobianos disponíveis tanto para uso na medicina veterinária quanto na medicina humana, tem impulsionado muitas pesquisas no mundo todo. Dentre as alternativas para a eliminação e/ou minimização dos micro-organismos resistentes aos fármacos antimicrobianos, os bacteriófagos ou fagos são considerados como agentes potenciais para uso terapêutico (MOTLAGH; BHATTACHARJEE; GOEL, 2016), destacando-se as doenças de difícil tratamento, como as mastites bovinas (GOMES; HENRIQUES, 2016).

As mastites bovinas constituem um grande problema mundial na produção de leite, sendo uma das enfermidades mais encontradas nas fazendas leiteiras, resultando em importantes impactos econômicos devido aos custos no tratamento, a baixa produção de leite, bem como o descarte do animal (HALASA et al., 2007; JONG et al., 2018).

A mastite bovina é caracterizada pela inflamação aguda ou crônica da glândula mamária, que cursam com alterações patológicas, fisiológicas e microbiológicas no úbere (SHARMA; SINGH; BHADWAL, 2011). Podendo ocorrer de forma branda, sem alterações macroscópicas, porém, com alterações químicas e microbiológicas do leite, caracterizando a mastite subclínica (BARKEMA et al., 1999). No entanto, a mastite bovina pode acontecer de uma forma mais severa, causando mudanças no aspecto do leite e no tecido mamário, além de alguns efeitos locais (grumos e pus no leite, e edema, dor e hiperemia da glândula mamária) e sistêmicos (hipertermia e prostração) que caracterizam a mastite clínica (HUNT et al., 2013).

Estudos prévios demonstraram uma prevalência maior da mastite na forma subclínica (ACOSTA et al., 2016; BRITO et al., 2014; CUNHA et al., 2015; GETANEH; GEBREMEDHIN, 2017; MARTINS et al., 2010; MOTA et al., 2012; ZERYEHUN; ABERA, 2017). Além disso, a mastite subclínica possui uma grande importância, uma vez que não é clinicamente evidenciada, sendo necessários testes complementares para sua detecção. Além disso, os seus agentes etiológicos podem persistir no tecido mamário durante a lactação, resultando na diminuição da qualidade do leite produzido (PILLA et al., 2013).

Os agentes etiológicos da mastite podem transpor a defesa inata do animal, ultrapassa a barreira anatômica da glândula mamária, composta pelo esfíncter do teto, bem como, a barreira química, formada pela queratina e citocinas (OVIEDO-BOYSO et al., 2007). Consequentemente, os patógenos também podem evadir dos mecanismos da imunidade adaptativa, celular e humoral da glândula mamária, estabelecendo a doença (SORDILLO; STREICHER, 2002).

Dentre os diversos agentes infecciosos e não infecciosos que podem estar associados à etiologia da mastite bovina, destaca-se a ocorrência dos agentes bacterianos (MOTAUNG et al., 2017). A existência de traumas de origem mecânica, térmica e/ou química também pode predispor à infecção intramamária pelos micro-organismos (PETROVSKI et al., 2009; WATTS, 1988). Destaca-se, dentre outros fatores, as condições de higiene do local que podem influenciar no desenvolvimento da infecção (GETANEH; GEBREMEDHIN, 2017). Portanto, sua ocorrência vai depender da interação do agente, do ambiente e do hospedeiro (ZHAO; LACASSE, 2008).

Dentre as bactérias causadoras da mastite bovina, *Staphylococcus aureus* é a mais prevalente em todo o mundo (CHAGAS et al., 2012; SEYOUM et al., 2017; SULEIMAN; KARIMURIBO; MDEGELA, 2018). A patogênese de *S. aureus* é conferida pela combinação de fatores extracelulares e toxinas, juntamente com propriedades invasivas, como aderência, formação de biofilme e resistência à fagocitose, através do encapsulamento e formação de microabscessos (SOMPOLINSKY et al., 1985).

Adicionalmente, os biofilmes são a base para infecções bacterianas persistentes e crônicas, como nas mastites subclínicas ou clínicas em vacas leiteiras (COSTERTON; STEWART; GREENBERG, 1999). Sendo assim, o insucesso no tratamento das infecções recorrentes ou crônicas causadas por *S. aureus* é devido aos mecanismos de virulência, como a produção de biofilmes, que permitem que o agente infeccioso seja protegido contra os antimicrobianos, bem como, da resposta imune do hospedeiro (MELCHIOR; FINK-GREMMELS; GAASTRA, 2006).

Antimicrobianos são produtos naturais ou sintéticos elaborados para inibir parcial ou totalmente a multiplicação e crescimento de micro-organismos. Estas substâncias são classificadas como antibióticos (substâncias químicas produzidas durante o metabolismo de micro-organismos, como por exemplo, os fungos e bactérias) e quimioterápicos (substâncias sintetizadas ou produtos microbianos modificados estruturalmente em laboratório). Entretanto, o uso intensivo e indiscriminado destas substâncias, rapidamente dificultou seu uso em infecções por bactérias, devido à progressiva resistência bacteriana a esses fármacos (MONTELLI; SADATSUNE, 2001).

A resistência bacteriana vem se destacando com um dos importantes entraves na clínica veterinária, bem como, nos sistemas de produção, devido às bactérias desenvolverem continuamente mecanismos de resistência para as diversas classes de antimicrobianos, usados na rotina veterinária (ECONOMOU; GOUSIA, 2015).

As bactérias podem ser intrinsecamente resistentes a determinados antimicrobianos, mas também podem adquirir resistência através de mutações em genes cromossômicos ou por meio da transferência gênica horizontal, por elementos móveis como, os transposons, plasmídeos ou fagos, levando à recombinação gênica (BLAIR et al., 2015; HAWKEY, 1998; JOO; FU; OTTO, 2016). Sendo assim, a resistência bacteriana pode ser desenvolvida por vários mecanismos, podendo acontecer entre organismos da mesma ou de diferentes populações microbianas, por exemplo, da microbiota animal para humana ou vice-versa (HAWKEY, 1998).

Estudos relatam resistência aos antimicrobianos em *Staphylococcus* spp. provenientes de mastites bovina, bem como, detecção de seus genes de resistência em todo o mundo (KLIMIENE et al., 2016; MELLO et al., 2017; RUEGG et al., 2015; YANG et al., 2016). A resistência antimicrobiana mais frequentemente relatada é contra a penicilina, onde dois mecanismos de resistência diferentes são responsáveis pela resistência de *Staphylococcus* spp., sendo a hiperprodução da enzima β -lactamase, que hidrolida o antimicrobiano ou, a codificação, pelo gene *mecA*, da proteína alterada denominada PBP 2a da parede bacteriana, com baixa afinidade pelos antimicrobianos β-lactâmico. Portanto, *Staphylococcus* spp. que expressam o gene *mecA* podem ser considerados como resistentes a todos os antimicrobianos β-lactâmicos (ZHANG et al., 2001;WIELDERS et al., 2002; MELLO et al., 2017). Adicionalmente, segue-se maior ocorrência de resistência à tetraciclina (SEYOUM et al., 2018; SREDNIK et al., 2017; YANG et al., 2016) e à meticilina em leite bovino (HARAN et al., 2012; KHAIRULLAH et al., 2020; MAHANTI et al., 2020).

Outro fato importante é o surgimento de linhagens bacterianas de múltipla resistência, isto é, bactérias resistentes aos diferentes antimicrobianos. Portanto, para estas bactérias de mútipla resistência poucos tratamentos são eficazes e dados demonstram alta porcentagem de resistência aos antimicrobianos comumente utilizados para tratar infecções por *S. aureus* (WORLD HEALTH ORGANIZATION, 2015). Fato observado em isolados de *S. aureus* de mastites bovina que demonstraram múltipla resistência aos diferentes fármacos antimicrobainos (YANG et al., 2016).

2.3 FAGOS E ENZIMAS LÍTICAS PARA O TRATAMENTO DAS MASTITES BOVINAS

Os fagos vêm sendo avaliados como uma alternativa aos antimicrobianos para o controle de mastite bovina. Entretanto, devido a insucesso relatado em experimento *in vivo* (LERONDELLE; POUTREL, 1980), os testes *in vitro* são realizados para avaliar a atividade de fago em bactérias provenientes de mastite bovina O primeiro teste *in vitro* foi realizado com a cepa Newbould 305 de *S. aureus*, comumente usada em ensaios de mastite bovina, e com o fago K (BARKEMA et al., 1999). Este fago é um membro lítico da família *Myoviridae* e atua contra uma variedade de cepas de *S. aureus* (O'FLAHERTY et al., 2004). As origens do fago K não são precisamente conhecidas, mas provavelmente foi originalmente isolado por André Gratia no início dos anos 1920 (BURNET; LUSH, 1934; ROUNTREE 1949).

Sendo assim, diversas pesquisas têm investigado o emprego de fagos no controle de mastite bovina, onde os resultados apresentam-se variáveis dependendo do fago e dos isolados bacterianos testados (BASDEW; LAING, 2011). No entanto, em relação aos fagos de *Staphylococcus* spp. a maioria dos fagos apresenta-se altamente lítico e hospedeiro – específico (KWIATEK et al., 2012; TITZE et al., 2020; ZHANG et al., 2014). Pesquisas também reportam fagos para outras bactérias provenientes de mastites bovinas como *Escherichia coli* (PORTER et al., 2016) e *Streptococcus agalactiae* (BAI et al., 2013).

Em relação às enzimas líticas, lisinas de fagos de *Staphylococcus* spp. de leite bovino, demonstram atividade lítica em testes *in vivo* e *in vitro,* bem como, apresentam atividade lítica contra outras espécies do mesmo gênero (MISHRA et al., 2014; SCHMELCHER; POWELL; et al., 2012; VERBREE et al., 2017). Adicionalmente, lisinas de fagos de *Streptococcus* spp. quando testados *in vitro* contra *Streptococcus dysgalactiae, Streptococcus uberis* e *S. agalactiae* de mastites bovinas, demonstraram alta atividade lítica e uma forte ação sinérgica SCHMELCHER et al., 2016. Portanto, as lisinas, também, têm sido sugeridas como potenciais antimicrobianos para o tratamento da mastite bovina (VERBREE et al., 2017).

Além disso, estudos demonstraram a inibição por bacteriófagos na formação de biofilmes de *Trueperella pyogenes* oriunda de mastite bovina. Posteriormente, tais enzimas foram capazes de controlar biofilmes formados por *Staphylococcus* spp. de alimentos (DUARTE et al., 2018; CHA; SON; RYU, 2019). Portanto, os fagos e suas enzimas líticas constituem alternativas promissoras na terapêutica e controle de infecções bacterianas como, as infecções intramamárias bovinas, e também podem ser estudados como agentes de controle de biofilmes bacterianos.

3. **CAPÍTULO 1**

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In vitro **lytic efficiency of** *Staphylococcus aureus* **bacteriophages in bacteria from bovine mastitis: a meta-analysis**

Eficiência lítica *in vitro* **de bacteriófagos de** *Staphylococcus aureus* **em bactérias de mastites bovina: uma meta-análise**

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ABSTRACT

Bacteriophages have been investigated as alternative to the treatment of bacterial infections, including bovine mastitis, in production animals. In this meta-analysis, we evaluated *in vitro* efficiency of phages *Staphylococcus aureus* against *S. aureus*, which is involved in the etiology of bovine mastitis. Seventeen studies were included and the bacterial lytic activity was extracted using proportion analysis. The lytic efficiency of phages was obtained in this meta-analysis using a random-effects model [significant difference ($p<0.05$)]. Forest plots were used to graphically represent the efficiency of phages on bacterial isolates. Most phages (e.g., CS1, DW2, ΦSA011, ΦSA012, ΦSA022, ΦSA023, ΦSA024, ΦSA025, ΦSA037, ΦSA038, ΦSA039, ΦSA041, ΦSA042, ΦSA043, ΦSA044, MSA6, Ufv-aur2 to Ufv-aur11, SAH-1, SPW, vB_SauM_JS25, SaPh1 to SaPh6, SA, SANF, SA2, ΦSA012, ΦSA039, phi11, phiIPLA88, phiIPLA35, phiIPLA-RODI, phiIPLA-C1C, SAJK-IND, vBSP-A1, vBSP-A2, STA1.ST29, EB1.ST11, EB1.ST27, Remus, and ISP) of *S. aureus* were efficiently lytics or infected most of the *S. aureus* isolates, demonstrating 80% ($p<0.05$) lytic efficiency. The phages SA, SANF and SA2, also demonstrated lytic activity or infected the non-*Staphylococcus aureus* and *Macrococcus caseolyticus* isolates. In this meta-analysis, we compared and demonstrated the *in vitro* efficiency and host range of bacteriophages in *S. aureus*. Additionally, the phages represent an alternative to be researched to treat bovine mastitis in dairy cattle caused by the prevalent microorganism, *S. aureus.*

Keywords: bacteriophages, bovine mastitis, phages, *Staphylococcus aureus*.

RESUMO

Os bacteriófagos têm sido investigados como alternativa ao tratamento de infecções bacterianas em animais de produção, incluindo as mastites bovina. Nesta meta-análise, avaliamos a eficiência *in vitro* de fagos de *Staphylococcus aureus* contra *S. aureus* envolvidas na etiologia da mastite bovina. Dezessete estudos foram incluídos e a atividade lítica bacteriana foi extrída usando análise de proporção. A eficiência lítica dos fagos foi obtida nesta meta-análise usando um modelo de efeitos aleatórios [diferença significativa ($p < 0,05$)] . Os gráficos de Forest plots usados para representar graficamente a eficiência dos fagos em isolados bacterianos. Os fagos avaliados, na sua grande maioria, (por exemplo, CS1, DW2, ΦSA011, ΦSA012, ΦSA022, ΦSA023, ΦSA024, ΦSA025, ΦSA037, ΦSA038, ΦSA039, ΦSA041, ΦSA042, ΦSA043, ΦSAf e U0f, ua04 SPW, vB_SauM_JS25, SaPh1 a SaPh6, SA, SANF, SA2, ΦSA012, ΦSA039, phi11, phiIPLA88, phiIPLA35, phiIPLA-RODI, phiIPLA-C1C, SAJK-IND, vBSP-A1, vBSP-A2, STA1.ST29, EB1.ST11, EB1.ST27, Remus, e ISP) foram eficientemente lítico ou infectaram os isolados de *S. aureus*, demonstrando 80% ($p < 0,05$) de eficiência lítica. Os fagos SA, SANF e SA2 também demonstraram atividade lítica ou infectaram os isolados *Staphylococcus* não-*aureus* e *Macrococcus caseolyticus*. Nesta meta-análise, comparamos e demonstramos a eficiência *in vitro* e gama de hospedeiros de bacteriófagos de *S. aureus*. Adicionalmente, os fagos representam uma alternativa a ser pesquisada para o tratamento da mastite bovina em gado leiteiro causada pelo microrganismo prevalente, ou seja *S. aureus*.

Palavras-chave: bacteriófagos, mastite bovina, fagos, *Staphylococcus aureus*.

INTRODUCTION

Bovine mastitis is defined as an inflammation of the mammary gland and the use of antimicrobials to treat of this disease in dairy cows has increased (GRAVE *et al.*, 1999; MITCHELL *et al.*, 1998; SHARMA; SINGH; BHADWAL, 2011). Among the etiological agents involved in the occurrence of mastitis of bacterial origin, *S. aureus* has been reported as the most prevalent microorganism globally (BARKEMA; SCHUKKEN; ZADOKS, 2006; SOUZA, *et al.*, 2016; SEYOUM *et al.*, 2017; SULEIMAN; KARIMURIBO; MDEGELA, 2018; MESQUITA, *et al.*, 2019). Furthermore, an increase in penicillin resistance has been reported, as well as methicillin-resistant *S. aureus* (MRSA) in bovine milk (AQIB *et al.*, 2017;

SILVA *et al*., 2020). Once, the resistance to penicillin is related to indiscriminate use of antibiotics with the absence of laboratory diagnosis and the sensitivity test to antimicrobials in the bovine mastitis, mainly caused by *S. aureus* (BEURON et al., 2014).

Therefore, bacterial resistance to available antimicrobials has increased, including in the veterinary field (PALMA; TILOCCA; RONCADA, 2020). Thus, the discovery of alternatives to control bovine mammary gland infections is critical and therapeutic alternatives to treat bacterial infections, including the use of bacteriophages or phages, have been studied all around the world (BASDEW & LAING, 2011; ROHDE; WITTMANN; KUTTER, 2018).

Bacteriophages infect bacteria, causing cell lysis; consequently, they are considered potential therapeutic agents for more than a century (D'HERELLE, 1931). These viruses have received attention from many research groups (SULAKVELIDZE, 2004; MONK *et al.*, 2010; SILLANKORVA; AZEREDO, 2014), owing to their characteristics, including host specificity, lytic activity in pathogenic bacteria, absence of harm to humans and animals, and rapid application to combat the emergence of new bacterial threats (SULAKVELIDZE& ZEMPHIRA; JR, 2001).

The mechanism of action of phages is completely different from those of antimicrobials and they are effective against multi-drug-resistant bacteria. Moreover, owing to their environmental presence, it is easier to find new bacteriophages than to develop new drugs or immunobiologicals (VEIGA-CRESPO; BARROS-VELÀZQUEZ; VILLA, 2010). Therefore, phage therapy is considered a promising alternative compared to the use of antimicrobials (PELFRENE et al., 2016), making this study remarkably relevant.

In vitro studies have investigated the use of phages in the biological control of bovine mastitis; however, variable results have been reported (SON et al., 2010; BASDEW & LAING, 2011; IWANO et al., 2018). Therefore, these studies were selected for this meta-analysis, as well as standardized by the eligibility criteria to homogenize the samples. In addition, in this study, it was not possible to include *in vivo* tests because they did not meet our eligibility criteria. Thus, in this study, we performed a meta-analysis of *in vitro* efficiency of *S. aureus* phages against isolates *S. aureus* involved in bovine mammary-gland infections.

MATERIALS AND METHODS

Search strategies

A systematic review of the literature was performed, identifying studies that tested the lytic activity of phages of *S. aureus* isolates from bovine mastitis. The investigation was

developed by pairs, focusing on studies that determine the host range of the phages. This review was conducted in the following four stages: identification, selection, eligibility assessment, and inclusion, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (MOHER et al., 2009).

Selection of studies

Bibliographic research was performed using data from scientific journals indexed in the following databases: PubMed, Web of Science and ScienceDirect, using the search terms: phage OR phages OR bacteriophage OR bacteriophages AND mastitis NOT humans. Only publications in the English language were considered for this analysis.

During the selection phase, duplicate publications were removed. Subsequently, the studies were evaluated based on the title and abstract. All studies related to tests using the lytic enzymes were excluded, as well as literature reviews.

The articles included in this meta-analysis were comprehensively assessed through the complete reading of the selected texts. The selected papers fulfilled the following eligibility criteria: *S. aureus* isolated from bovine mastitis, host-specific phages of *S. aureus* of bovine milk, determination of the range hosts, methodology that employed the use of a single phage, no association with other phages, and the performance of *in vitro* analyses. Additionally, the plaque assay, spot test and double-layered agar methods were included.

Notably, we excluded studies that reported the concomitant use of other molecules with antimicrobial potential, as well as those that included intracellular bacteria or other analyses that did not allow the visualization of lysis. These exclusions were necessary to ensure the homogeneity of samples to compare studies that were included in this meta-analysis.

The references cited in the articles were also analyzed and included in the present study based on the eligibility criteria described above. Thus, the information from the articles was systematized in a spreadsheet (Microsoft Excel). The bacterial lytic activity was extracted using proportion analysis.

Statistical Analysis

Initially, a logit transformation of the proportion data was performed based on total the number of bacterial isolates (total) *versus* the number of lysed bacterial isolates (events). Additionally, the same procedure it was made for each phage isolated and tested from each study. The lytic efficiency of *S. aureus* phages was obtained using random-effects model [significant difference ($p<0.05$)]. Forest plots were used to graphically represent the meta-

analysis results. In particular, the meta-analysis was conducted using the "Metaprop" package of R v3.4.2 software.

RESULTS

Selection of studies

A total of 564 articles were found, including 122 studies from PubMed, 95 from Web of Science and 347 from ScienceDirect. Next, the articles were screened, and 21 were chosen and read completely, after which the eligibility criteria were evaluated. Nine articles met all eligibility criteria for this meta-analysis. The bibliographic references of the selected studies were reviewed and two other scientific publications were included. We performed a general search using the same indexers and other websites for studies that were not reviewed, as well as recently published studies to update the references. Thus, eight studies were included, resulting in a total of 17 articles that were analyzed in this current study (Figure 1). The period of publication of the selected articles ranged from June 2005 to September 2020. Information regarding the following aspects was obtained from each article: phage isolates, phage family, bacteria isolated from bovine milk, the presence of antimicrobial resistance or resistance genes, and techniques for analyzing the occurrence of bacterial lysis (Table 1 and Table S1).

Occurrence of lytic activity and host range

The total number of bacterial isolates analyzed in the studies was 603, of which 92.37% (557/603) were *S. aureus*, which included 47.21% (263/557) of isolates that were resistant and 52.78% (294/557) of isolates that were non-resistant to antimicrobials. Based on the analysis of the Forest plots (Figure 2 and Figure 3), it was evident that the phages lyzed the bacteria isolated from bovine milk samples. However, the lytic activity presented was variable in relation to the different phages and bacterial isolates tested. Therefore, most *S. aureus* phages (e.g. CS1, DW2, ΦSA011, ΦSA012, ΦSA022, ΦSA023, ΦSA024, ΦSA025, ΦSA037, ΦSA038, ΦSA039, ΦSA041, ΦSA042, ΦSA043, ΦSA044, MSA6, Ufv-aur2 to Ufv-aur11, SAH-1, SPW, vB_SauM_JS25, SaPh1 to SaPh6, SA, SANF, SA2, ΦSA012, ΦSA039, phi11, phiIPLA88, phiIPLA35, phiIPLA-RODI, phiIPLA-C1C, SAJK-IND, vBSP-A1, vBSP-A2, STA1.ST29, EB1.ST11, EB1.ST27, Remus, and ISP) were efficiently lytic or infected most of *S. aureus* isolates tested, except phages ΦA72, ΦH5, ΦL7, ΦL13, ΦA8, ΦG7, ΦSA003, ΦSA004, ΦSA026, SAP-1, SAP-3, MSP, Romulus, and DSM105264, which presented low lytic efficiency or infected a low number of *S. aureus* isolates. The bacteriophages demonstrated

80% ($p<0.05$) lytic efficiency against *S. aureus* isolates (Figure 2). Additionally, the lytic activity of phages is species-specific, that is, these viruses are only able to destroy their bacterial host. Conversely, the phages SANF, SA2 and SA also demonstrated lytic activity for isolates of non-*S. aureus* bacteria (e.g. *Staphylococcus chromogenes, Staphylococcus saprophyticus, Staphylococcus xylosus, Staphylococcus sciuri,* and *Staphylococcus succinus)* and other species (e.g. *Macrococcus caseolyticus)* (Figure 3). Therefore, the lytic efficiency was 30% for non-*S. aureus* bacteria and 16% for other bacterial species (e.g. *Streptococcus agalactiae, Streptococcus uberis, Streptococcus parasanguinis, Arcanobacterium pyogenes, Kocuria rosea, Enterococcus faecalis*, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Lactobacillus plantarum, Citrobacter koseri,* and *Macrococcus caseolyticus*) ($p<0.05$) (Figure 3).

DISCUSSION

Data analysis and review revealed that most phages of *S. aureus*, specifically in the family *Myoviridae*, presented prominent and specific activity in the 603 bacterial isolates from bovine mastitis. These findings demonstrate that these phages could be the subject of research in the development of therapeutic alternatives for bovine mastitis and could be used to target infections involving *S. aureus.*

Additionally, the efficiency of the phages analyzed in several bacterial isolates permitted us to verify their probability in infecting and causing bacterial death in other isolates responsible for causing bovine mastitis, but principally those of *S. aureus*. As seen in Figure 2, the lytic efficiency was 80% infect other bacterial isolates of *S. aureus*. With this meta-analysis, it was also verified the possibility of the analyzed phages to infect and be used in other bacterial species in bovine mastitis.

It was observed that phages SA, SA2 and SANF demonstrated lytic activity against *Staphylococcus saprophyticus, Staphylococcus xylosus, Staphylococcus sciuri, Staphylococcus succinus,* and *Macrococcus caseolyticus*. Although the main characteristic of bacteriophages is their host specificity, some phages can infect different bacterial genera and species (BOHANNAN & LENSKI, 1997). Consequently, these phages should be tested in more isolates to confirm their possible host amplitude. Thus, if there is no resistance displayed by a specific bacterium, the phage does not need to find another host cell to infect, but if resistance is exhibited, the bacteriophage seeks diversification of the species host to continue its infectious cycle (HOLMFELDT et al., 2007; RODRIGUEZ-VALERA et al., 2009).

The high efficiency of cell lysis by phages is based on the specificity of bacterial receptors and cell disintegration after a short viral life cycle, leading to the rapid elimination of the target cell (LECLERC et al., 2000; MATSUZAKI et al., 2005). In Gram-positive bacteria, such as *S. aureus*, receptors are present on peptidoglycans (or mureins) and teichoic acids in the bacterial cell wall (SHAW & CHATTERJEE, 1971; KANEKO et al., 2009; XIA et al., 2011). Phages are bound to these compounds present in the cell wall to be adsorbed into the cell.

In this study, some phages (SA, SANF and SA2) were lytic in other *Staphylococcus* species and *Macrococcos caseolyticus*. Importantly, the genus *Macrococcos* belongs to the same family as *S. aureus* and is also a Gram-positive bacterium. This suggests that these microorganisms share similar receptors for the phages.

The methodology used allowed greater precision in establishing the effects of the treatments, regulating data heterogeneity, and facilitating the verification of a greater number of results (LOVATTO et al., 2007). *In vitro* lytic activity tests of studies presented variability; thus, the eligibility criteria conferred the best homogeneity of samples, allowing comparisons and analyses of data.

Phage SA (HAMZA et al., 2016) was excluded from this meta-analysis because of a contradiction in the results of the phage lytic activity against *K. pneumoniae* isolates. In addition, studies using bacterial isolates from humans and other species of animals were also excluded, since the aim of the present study was to focus on phages used for the prevention and treatment of bovine mastitis.

Notably, there is a scarcity of studies evaluating *in vivo* lytic activity of *S. aureus* phages in cows (LERONDELLE & POUTREL, 1980; GILL et al., 2006a) and mice (ALDOORI et al., 2015; BREYNE et al., 2017; IWANO et al., 2018; GENG et al., 2019). However, we opted to exclude these studies to homogenize the data for this meta-analysis.

Phage therapy against mastitis presents many important challenges. Some of the problems encountered are phage stability, inhibitory effects on the cow's immune system and certain thermolabile proteins are present in raw milk that affect phage-cell interaction (O'FLAHERTY et al., 2005; GILL et al., 2006b; TANJI et al., 2015; BARI et al., 2017).

Another issue is the possibility of bacteria developing phage resistance. However, the use of a cocktail containing multiple phages could be indicated to circumvent this phenomenon, since the bacterium must develop simultaneous resistance mechanisms against multiple phages. This phenomenon is less likely to occur when compared to a single phage used to lysis a particular bacterium (OECHSLIN, 2018). A limitation for the use of phages for mastitis therapy

would be the need to perform laboratory diagnosis and testing the susceptibility of the agent to phages. However, this fact is also a current limitation for antimicrobial use.

The increase in multi-resistant bacteria, including *S. aureus*, the principal microorganism involved in the etiology of bovine mastitis, has necessitated the search for alternative treatments for this disease (FERNÁNDEZ et al., 2018). Thus, the use of phages may be recommended in cases where mastitis is caused by antimicrobial-resistant bacteria (SULAKVELIDZE; ZEMPHIRA; JR, 2001), since bacteriophages infect and eliminate bacteria through mechanisms distinct from those of antimicrobials (MONK et al., 2010).

CONCLUSIONS

This meta-analysis described *S. aureus* phages tested *in vitro* to infect bacterial cells, demonstrating the lytic potential of these viruses. Thus, this study evaluated the efficiency of *S. aureus* phages in bacterial isolates revealing high specificity and lytic activity, particularly against *S. aureus* isolates, including MRSA.

In addition, we suggest that among the evaluated phages, those with high lytic efficiency could be selected to produce a cocktail to be tested *in vivo* for the treatment and/or prevention of mastitis caused by *S. aureus*. Other investigations using bacteriophages as an alternative treatment method, should be performed to ensure the health of the dairy cattle using environmentally sustainable technologies, thereby preserving the efficacy of antimicrobials.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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Figure 1: Flowchart of process of identification and selection of articles.

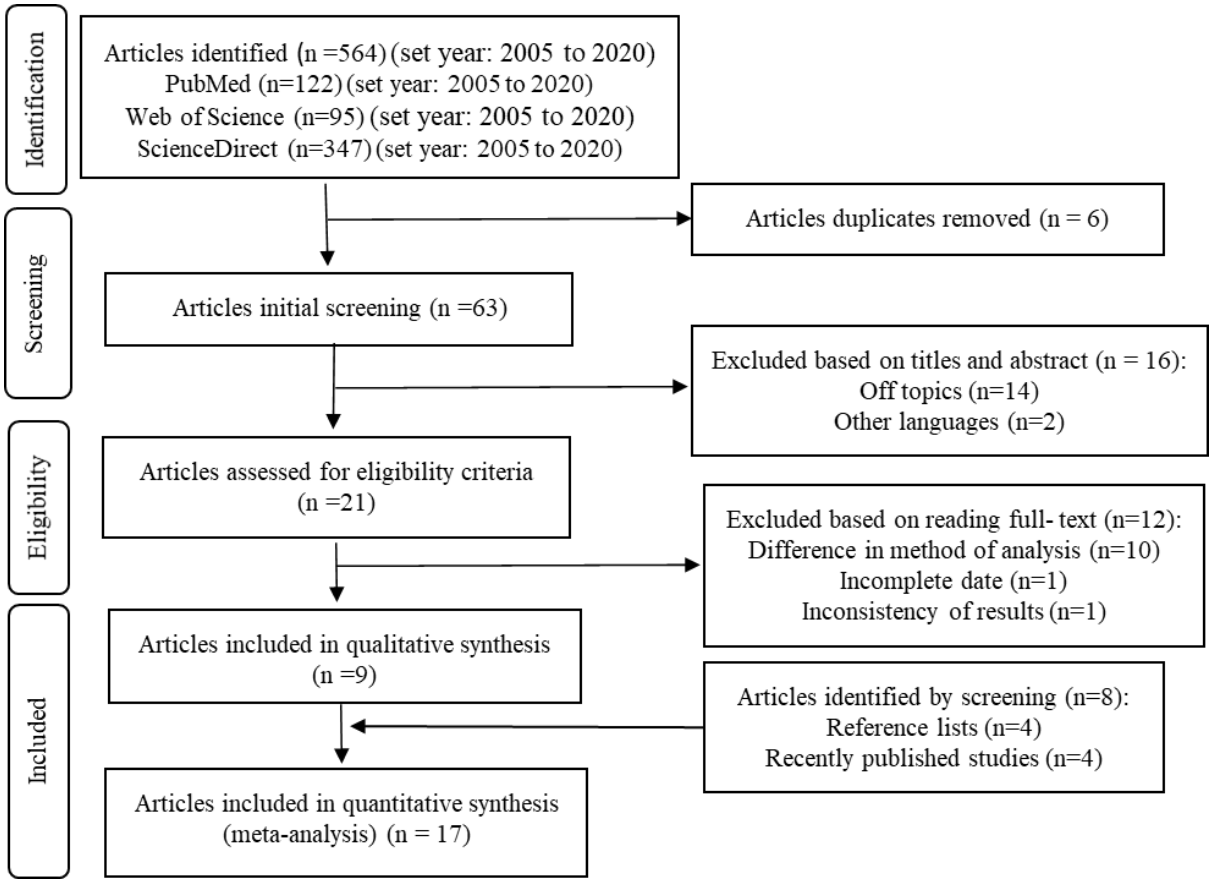

Table 1: Main characteristics of the 17 studies that were selected starting systematic review and included in the meta-analysis database.

Author	Country	Phages family	Phages isolates	Analysis method	Bacterial isolated	N°**	Antimicrobial resistance
O'Flaherty et al., (2005)	Ireland	*Siphoviridae*	CS1 DW2	Spot test	*S. aureus*	3	
Synnott et al., (2009)	Japan	*Myoviridae*	ΦSA003 ΦSA004 ΦSA011 ΦSA012 ΦSA022 to ΦSA026 ΦSA037 to ΦSA039 ΦSA041 to ΦSA044	Double-layered agar	*S. aureus*	16	
García et al., (2009)	Spain		ΦA72 ΦH5 ΦL7 ΦL13 ΦA8 ΦG7	Double-layered agar	*S. aureus*	14	
Son et al., (2010)	Korea	*Myoviridae*	SAP-1 SAP-3	Double-layered agar	*S. aureus; S. epidermidis; S. agalactie; S. uberis; E. faecalis; E. coli; L. plantarum; C. koseri; S. haemolyticus*	20	Nal, Str, Gen, Ami, Ct, Cfl, Nor, Eri, Oxa
Kwiatek et al., (2012)	Poland	*Myoviridae*	MSA6	Double-layered agar	*S. aureus; S epidermidis; S. saprophyticus*	35	MRSA, VRSA
Dias et al., (2013)	Brazil	*Myoviridae*	Ufv-aur2 to Ufv-aur11	Double-layered agar	*S. aureus*	20	Amp, Gen, Pen, Tet, Eri, Rif, Ami, Clo, Cro, Oxa, Cfl, Cli, Cip, Van, Sut
Han et al. (2013)	Korea	*Myoviridae*	SAH-1	Double-layered agar	*S. aureus; E. faecalis*	47	MRSA
Li; Zhang (2014)	China	*Myoviridae*	SPW	Spot test	*S. aureus; E. coli*	05	MRSA

MRSA: methicillin-resistant *S. aureus.* VRSA: vancomycin-resistant *S. aureus.* Nal: nalidixic acid. Str: streptomycin. Ct: colistin. Gen: gentamycin. Ami: amicacin. Cfl: cephalothin. Nor: norfloxacin. Eri: erythromycin. Oxa: oxacillin. Amp: ampicillin. Cfo: cefoxitin. Van: vancomycin. Dc: dicloxacillin. Pef: pefloxacin. Cxm: cefuroxime. Ctx: cefotaxime. Sxt: sulfamethoxazole-trimethoprim. Caz: ceftazidime. Met: methicillin. Pen: penicillin. Pen G: penicilina G. A/F: amoxycillin/ fucloxacillin. Ob: oxytetracycline. Tr: trimethopim. Tet: tetracycline. Rif: rifampicin. Clo: chloramphenicol. Cli: clindamycin. Cip: ciprofloxacin. Sut: sulfazotrim. **Number of isolates bacterial from each study.

Table 1: Main characteristics of the 17 studies that were selected starting systematic review and included in the meta-analysis database (continued).

Author	Country	Phages family	Phages isolates	Analysis method	Bacterial isolated	Nº**	Antimicrobial resistance
Zhang et al. (2014)	China	*Myoviridae*	vB_SauM_JS25	Spot test	*S. aureus*	52	
Basdew; Laing (2015)	South Africa	*Myovoridae*	SaPh1 to SaPh6	Spot test	*S. aureus*	4	Amp, Pen G, Met, A/F, Eri, Str, Tet, Ob, Van, Tr
Tahir et al. (2017)	Pakistan		SA SANF SA2	Spot test	*S. aureus; S. xylosus; M. caseolyticus; S. saprophyticusS. succinus; S. sciuri*	11	
Iwano et al. (2018)	Japan	*Myoviridae*	ΦSA012 ΦSA039	Spot test	*S. aureus*	93	
Varela-ortiz et al. (2018)	México	*Siphoviridae Myoviridae*	phi11 phiIPLA88 phiIPLA35 phiIPLA-RODI phiIPLA-C1C	Spot test	*S. aureus*	27	Pen, Dc, Amp, Tet, Ctx, Cfl, Pef, Gen, Eri, Stx, Caz, Cxm
Ganaie et al. (2018)	India	*Myoviridae Podoviridae*	SAJK-IND MSP	Spot test	*S. aureus; E. coli; S. agalactie; K. pneumonie; P. aureuginosa*	125	MRSA
Geng et al. (2019)	China	*Myoviridae Podoviridae*	vBSM-A1 vBSP-A2	Double-layered agar	*S. aureus; S. chromogens; K. pneumoniae; S. parasanguinis A. pyogenes; S. agalactie; E. coli*	29	
Titze et al. (2020)	Germany	*Myoviridae Podoviridae*	STA1.ST29 EB1.ST11 EB1.ST27	Spot test	*S. aureus*	92	
Ngassam-Tchamba et al. (2020)	Belgium		Romulus Remus ISP DSM105264	Spot test	*S. aureus*	10	Cfo, Amp, Tet, Amo, Cip, Cli, Ct, Eri, Ff, Gen, Lzd, Pen, Tr, Sulfatrim, Sulfamycin,

MRSA: methicillin-resistant *S. aureus*. VRSA: vancomycin-resistant *S. aureus*. Nal: nalidixic acid. Str: streptomycin. Ct: colistin. Gen: gentamycin. Ami: amicacin. Cfl: cephalothin. Nor: norfloxacin. Eri: erythromycin. Oxa: oxacillin. Amp: ampicillin. Cfo: cefoxitin. Van: vancomycin. Dc: dicloxacillin. Pef: pefloxacin. Cxm: cefuroxime. Ctx: cefotaxime. Sxt: sulfamethoxazole-trimethoprim. Caz: ceftazidime. Met: methicillin. Pen: penicillin. Pen G: penicilina G. A/F: amoxycillin/ fucloxacillin. Ob: oxytetracycline. Tr: trimethopim. Tet: tetracycline. Rif: rifampicin. Clo: chloramphenicol. Cli: clindamycin. Cip: ciprofloxacin. Sut: sulfazotrim. Ff: Florfenicol. Lzd: Lizenoid. **Number of isolates bacterial from each study

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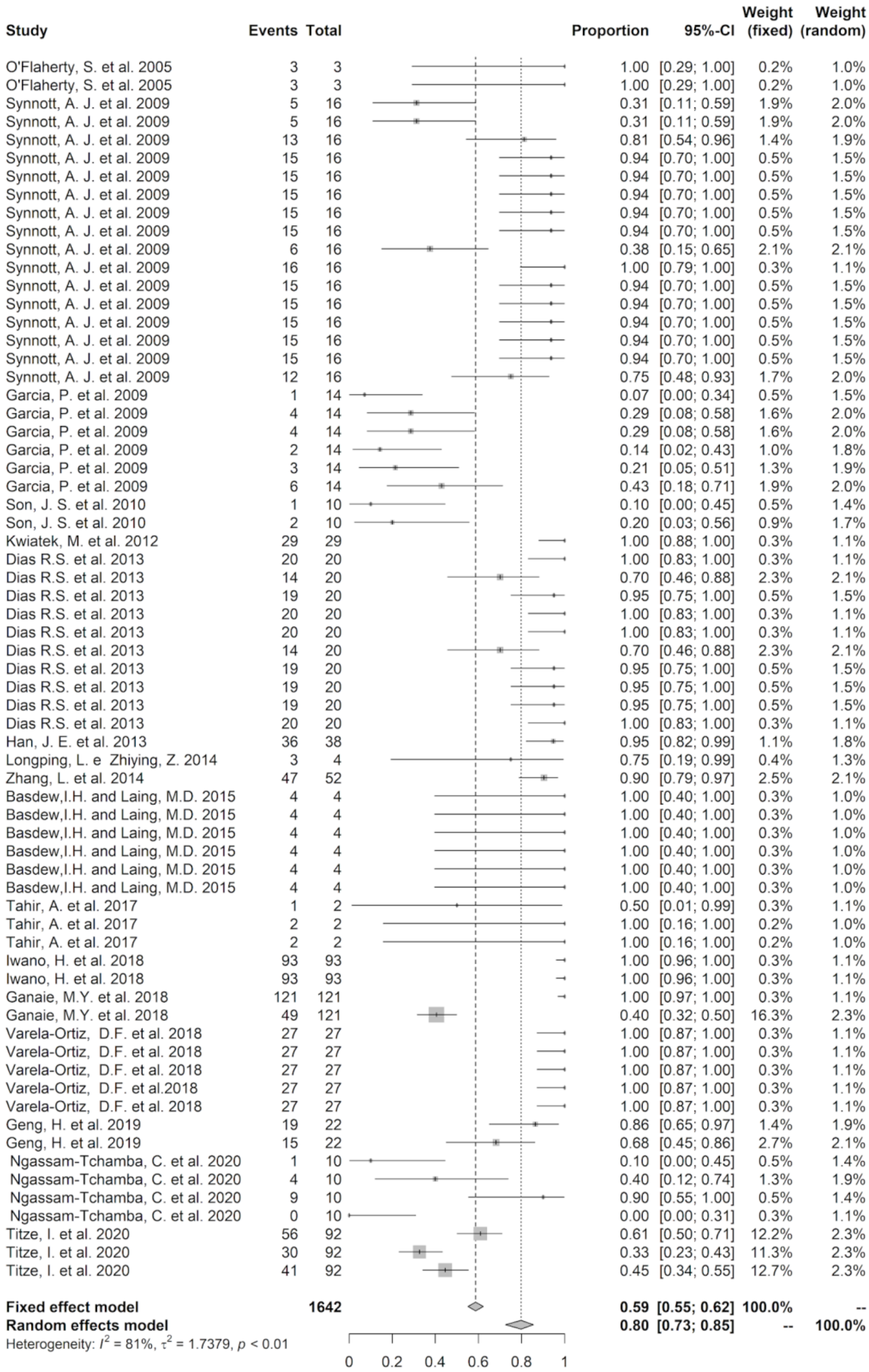
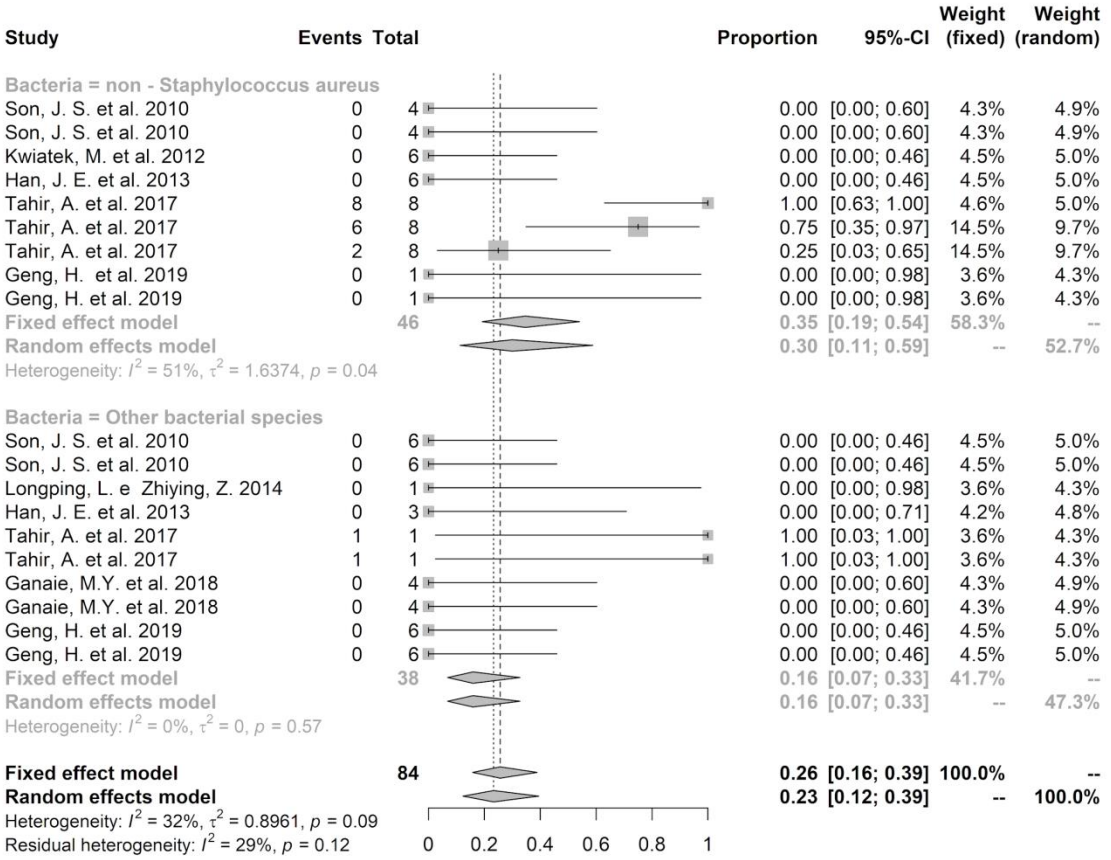
Figure 2: Forest plots of efficiency from *Staphylococcus aureus* phages in bacterial isolates *Staphylococcus aureus.*



Total: number of bacterial isolates from bovine mastitis. Events: number of bacterial isolates demonstrated lytic efficiency (i.e. proportion analysis). Analysis through random-effects model (p<0.05).

Figure 3: Forest plots of efficiency from *Staphylococcus aureus* phages in bacterial isolates non-*Staphylococcus aureus* and other bacterial species.



Total: number of bacterial isolates from bovine mastitis. Events: number of bacterial isolates demonstrated lytic efficiency (i.e. proportion analysis). Analysis through random-effects model ($p<0.05$).

Supplementary Material:

Table S1 Phages of *S. aureus* tested in isolated from bovine mastitis.

Author	Phages	Family
O'Flaherty et al., (2005)	CS1	*Siphoviridae*
O'Flaherty et al., (2005)	DW2	*Siphoviridae*
Synnott et al., (2009)	ΦSA003	*Myoviridae*
Synnott et al., (2009)	ΦSA004	*Myoviridae*
Synnott et al., (2009)	ΦSA011	*Myoviridae*
Synnott et al., (2009)	ΦSA012	*Myoviridae*
Synnott et al., (2009)	ΦSA022 to ΦSA026	*Myoviridae*
Synnott et al., (2009)	ΦSA037 to ΦSA039	*Myoviridae*
Synnott et al., (2009)	ΦSA041 to ΦSA044	*Myoviridae*
García et al., (2009)	ΦA72	-
García et al., (2009)	ΦH5	-
García et al., (2009)	ΦL7	-
García et al., (2009)	ΦL13	-
García et al., (2009)	ΦA8	-
García et al., (2009)	ΦG7	-
Son et al., (2010)	SAP-1	*Myoviridae*
Son et al., (2010)	SAP-3	*Myoviridae*
Kwiatek et al., (2012)	MSA6	*Myoviridae*
Dias et al., (2013)	Ufv-aur2 to Ufv-aur11	*Myoviridae*
Han et al. (2013)	SAH-1	*Myoviridae*
Li; Zhang (2014)	SPW	*Myoviridae*
Zhang et al. (2014)	vB_SauM_JS25	*Myoviridae*
Basdew; Laing (2015)	SaPh1 to SaPh6	*Myoviridae*
Tahir et al. (2017)	SA	*Myoviridae*
Tahir et al. (2017)	SANF	*Myoviridae*
Tahir et al. (2017)	SA2	*Myoviridae*
Iwano et al. (2018)	ΦSA012	*Myoviridae*
Iwano et al. (2018)	ΦSA039	*Myoviridae*
Varela-ortiz et al. (2018)	phi11	*Siphoviridae*
Varela-ortiz et al. (2018)	phiIPLA88	*Siphoviridae*
Varela-ortiz et al. (2018)	phiIPLA35	*Siphoviridae*
Varela-ortiz et al. (2018)	phiIPLA –RODI	*Myoviridae*
Varela-ortiz et al. (2018)	phiIPLA-C1C	*Myoviridae*
Ganaie et al. (2018)	SAJK-IND	*Myoviridae*
Ganaie et al. (2018)	MSP	*Podoviridae*
Geng et al. (2019)	vBSM-A1	*Myoviridae*
Geng et al. (2019)	vBSP-A2	*Podoviridae*
Titze et al. (2020)	STA1.ST29	*Myoviridae*
Titze et al. (2020)	EB1.ST11	*Podoviridae*
Titze et al. (2020)	EB1.ST27	*Podoviridae*
Ngassam-Tchamba et al. (2020)	Romulus	-
Ngassam-Tchamba et al. (2020)	Remus	-
Ngassam-Tchamba et al. (2020)	ISP	-
Ngassam-Tchamba et al. (2020)	DSM105264	-

4 . CAPÍTULO 2

(Manuscrito submetido para publicação no periódico *Virus gene*)

Characterization of new temperate phages of *Staphylococcus aureus* isolated from bovine milk

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Abstract

Bovine mastitis is an important disease in dairy cows, and *Staphylococcus aureus* is the most prevalent microorganism involved in this infection. Bacteriophages are considered an alternative to treat bacterial infections. In this study, we isolated and characterized new temperate phages of *S. aureus*, namely B_UFSM4 and B_UFSM5, from bovine milk. Their genomes were sequenced and analyzed, and their morphology and host range were also determined. Phage's activities were relatively stable at pH 3-11; however, at temperatures of 50°C, 60ºC, 70ºC for 60 min, the phages were completely inactivated. The complete genomes of B_UFSM4 and B_UFSM5 have 41.396 bp and 41.829 bp, with GC-content 33.97% and

33.98%, respectively. Both phages'genome contain 61 putative ORFs. The viruses have double stranded DNA and linear architecture. Phylogenic similarity was observed by proteome with *Staphylococcus* phage phiPV83 (45,536 nt), CN125 (44,492 nt) and JS01 (43,458 nt). Based in the analysis, the phages were classified in the family *Siphoviridae,* genus *Biseptimavirus* and order *Caudovirales*. Host range testing revealed that B_UFSM4 and B_UFSM5 had lytic activity of 51.8% and 59.2%, respectively, inclusive on isolates from *Pseudomonas aeruginosa*, *Staphylococcus* spp., *Staphylococcus sciuri,* and *Rothia terrae.* Additionally, they had a high EOP in *R. terrae.* Thus, in this study *S. aureus* temperate phages (B_UFSM4 and B_UFSM5) were isolated and characterized, these phages reveal similarities each other; however, they are distinct from other *S. aureus* phages of the family *Siphoviridae*. Additionally, the basic data are provided and further studies of the interaction mechanism between phages and hosts will be performed.

Keywords: bacteriophages; phages; *S. aureus*; bovine mastitis.

1. Introduction

Mastitis is an important disease in dairy cows, caused by several pathogens and results in high economic losses due to reduced milk quality and production, and high treatment costs [1–3]. *Staphylococcus aureus* is the most prevalent agent among the microorganisms that cause mastitis [4–6] and its main characteristic is to persist in mammary glands causing subclinical infection [7,8].

Antimicrobial therapy is a major advance in the control of bovine mastitis; however, it is less effective in infection caused by *S. aureus* because of the virulence of the microorganism [9–11], as the bacterial biofilms production. Furthermore, the excessive use of antimicrobials contributes to increased antimicrobial resistance as observed in *S. aureus* isolates from bovine mastitis worldwide [2,12,13]. Therefore, other alternatives to control this disease include the use of silver nanoparticles, cytokines, natural compounds, and the bacteriophages are being studied [14–16].

Bacteriophages (or phages) are viruses able to infect and kill bacteria, and are considered as potential antimicrobial agents [17]. They may be important tools in controlling antimicrobial resistance and multi-resistant bacteria [18]. The tailed phages may be virulent or temperate. The virulent phages only develop lytic cycle in bacterium. However, the temperate phages can insert their genetic content (the prophage) into the chromosomes of the bacterium, where they

can replicate as part of the bacterial chromosome, thereby developing the lysogenic cycle or the lytic cycle and cause host cell lysis [19,20]. Therefore, due to their characteristics, dynamic and numerous natures, phages must be characterized and evaluated for phage therapy.

Phages of *S. aureus* have been isolated and tested for bovine mastitis, and their efficiency and specificity are described [21–23], however, the results are variable. In this study, we isolate and characterize phages of *S. aureus* from bovine milk, and investigate their lytic efficiency in bacteria isolated from bovine mastitis.

2. Material and methods

2.1. Bacteriological analysis

Initially, *California Mastitis Test* (CMT) was performed to identify subclinical mastitis in cows of a dairy farm from Southern Brazil. Positive mammary glands were disinfected with cotton soaked in 70% (v/v) ethyl alcohol. Thereafter, 10-15 mL of milk samples were collected in sterile tubes and immediately transported to the laboratory under refrigeration. The samples were inoculated in 5% sheep blood and MacConkey Agar and incubated at 37°C for 24h. The bacterial isolates were identified by colony morphology and Gram staining. Next, colonies of Gram - positive cocci, were subjected to catalase and coagulase tests to identify coagulase positive *Staphylococcus* (CPS). The bacterial species of host isolate was confirmed by PCR and DNA sequencing using *16S ribosomal RNA (16S rRNA)* region.

2.2. Isolation of bacteriophages

Bacteria identified as CPS were cultivated in 5 mL Tryptic Soy Broth (TSB) and incubated under agitation (150 rpm) overnight at 37°C. Both were used as host bacteria for virus isolation. Thus, these were mixed in the bovine milk samples, which comprised samples collected for three consecutive days from CPS positive mammary glands.

A milk pool (100 mL) and 50 µL of each bacterium isolate were added in 100 mL TSB 2× and incubated at 37ºC for 24 h under agitation (160 rpm). The cultures were centrifuged at 4000 rpm for 20 min and filtered through 0.22 µm syringe filter. Thereafter,100 µL of bacterial culture (in log phase) of each isolate was mixed with 5 mL de molten soft agar (TSB with 0.6 % agar), spread on TSA plates (double-layer agar plate method), and incubated at 37°C for 18h [24]. The plates were then screened for presence of cleared zones on bacterial lawn, which indicated the presence of lytic phages.

2.3. Phage purification, propagation and titration

After isolation, five cleared zones were randomly selected and pealed thrice on six pre-prepared TSA plates (double-layer agar) containing bacteria host for purification. For propagation, the method described previously [25] with some modifications, was used. Briefly, cleared zones were streaked on various pre-prepared plates and incubated overnight at 37ºC. Afterward, salt magnesium buffer (SM; 100 mM NaCl, 8 mM MgSO4, 50 mM Tris-HCl; 2% gelatin; pH 7.5) was added in the plates and incubated at 4ºC overnight under agitation (50 rpm). Next, the solution was centrifuged at 8000×g at 4ºC for 10 min and thereafter, 0.584g NaCl and 1g polyethylene glycol (PEG, M.W. 8,000) was added to 10 mL of this solution. Subsequently, the solution was incubated at 4ºC under agitation (150rpm), for 12-18h (overnight), and then the solution was centrifuged at 8000×g at 4°C for 10 min. Furthermore, 1mL chloroform was added in the pellet and subjected to strong agitation for 1 min. Then, 4 mL of SM was added and centrifugated at 8000×g at 4°C for 10 min. The supernatant was collected and filtered using syringe filter 0.22 μm and stored at 4°C for further analyses. The phages titer was assessed using the conventional double-layer agar method previously described [24] and experiments were performed in triplicate.

2.4. Multiplicity of infection (MOI) assay

CPS isolate (bacteria host) was pre-cultivated in TSB and added to 15 mL TSB (OD_{600} 0.05), and was then incubated at 37°C under agitation (160 rpm) until 1×10^6 colony forming units (CFU). Phage suspensions were added into the cultures at three different ratios (MOI = 0.01, 0.1 and 1). Solutions were incubated at 37°C for 3.5h. Thereafter, each solution was centrifuged (10.000×g at 4°C) for 3 min. The supernatants were filtered (0.22 μm), diluted (10^{-1} to 10^{-8}) and seeded into double-layer agar containing CPS. Cell culture with CPS only were used as negative control. All assays were performed in triplicate [26].

2.5. One-step growth

This assay was performed with some modifications based on the previous protocols [21,26]. Briefly, CPS isolate (bacteria host) was cultivated (OD_{600} 0.1) and mixed in phage suspensions at MOI = 1, and then incubated at 37ºC for 30 min under agitation (160 rpm). Thereafter, it was centrifuged at 5.000×g/10 min and the pellets containing infected cells were resuspended with 2 mL of TSB, incubated at 37ºC under agitation. Next, 100 μL was collected at 10 min intervals up to 2h and each aliquot was immediately subjected to titration by the double-layer agar assay. This experiment was performed in triplicate.

2.6. Adsorption

This experiment was based on previous protocols, [22,27] with some modifications. CPS (bacteria host) (1×10^6 CFU/mL) was infected with a phage's suspensions (MOI = 1) and incubated at 30ºC under agitation (160 rpm). Aliquots of 100µL were collected at 5, 10, 15, 20, 30, and 40 min and diluted in 0.9 mL of TSB. Following centrifugation (12.000×g, 5 min), the supernatants were titrated (10^{-1} to 10^{-8}) by the double-layer agar assay. This experiment was performed in triplicate.

2.7. Thermal and pH stability

Thermal and pH stability tests were performed based on previous protocol [21] and pH stability test with adaptation. To perform the pH stability assay, the phage suspensions [10^6 Plate Forming Units (PFU) / mL] were added in a series of tubes containing SM buffer with different pH values (3, 5, 7, 9, and 11) and incubated overnight at 37ºC. Posteriorly, the solutions were quantified by the double-layer agar plate method. All experiments were carried out in triplicate.

2.8. Bacteriolytic activity

These tests were carried out according with previous protocol [22] and the following modifications: a suspension of CPS isolate (bacteria host) was adjusted to OD_{600} 0.05 and, thereafter the phages were added at an MOI = 1 for at 37ºC for 6h under agitation (160 rpm). In parallel, an aliquot of the same suspension, but without phage, was used as a control. The bacteriological activity of the phages was evaluated by monitoring the cell absorbance of the culture solution (OD_{600}) at 1h intervals up to 6h after phage infection.

2.9. Phage host range

The susceptibility levels were detected using two methods, spot test [28] and efficiency of plating (EOP) [29]. In total, 27 bacterial isolates from milk bovine and three strains were used: *S. aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12225, *Pseudomonas aeruginosa* ATCC 25853, *S. aureus*, CPS, *S. sciuri, S. hominis, S. cromogenes*, Coagulase - negative *Staphylococcus* (CNS), *Rothia terrae*, and *P. aeruginosa* in the concentration of 10^8 bacterial cells. For the spot test, 10^8 PFU/mL of phages solutions (propagation without chloroform) were used and 100 µL of a serial dilution of phages solutions for EOP. The relative

EOP was calculated as the titer of the phage (PFU/mL) for each isolate divided by the titer for the relevant propagating host.

All samples belong to Laboratory Bacteriology (LABAC) at Universidade Federal de Santa Maria (UFSM), Rio Grande of Sul State, Brazil. All bacterial isolates are registered in the Brazilian platform "Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado" (SISGEN) with the code A7085CE. Furthermore, the bacterial isolates of *Staphylococcus* spp. and *P. aeruginosa* were subjected to molecular analysis for identification by DNA sequencing of *16S ribosomal RNA (16S rRNA)* region.

2.10. Transmission electron microscopy (TEM)

After performance purification and propagation, as previously described, the viral particles were ultracentrifugated at 27.000 rpm at 4°C for 4h, at Sorvall™ WX, Ultracentrifuge series, Thermo Scientific™, rotor T-865. The pellet was dissolved in 300 µL of SM buffer. Negative staining was performed from one drop of viral suspension mixed with phosphotungstic acid 2% in grids of 100 mesh and was observed using a transmission electron microscope 120 keV, Jeol, JEM-1400, coupled with EDS microprobe of Centro de Microscopia da Zona Sul - FURG (CEME-SUL) at Universidade Federal de Rio Grande (FURG), Brazil.

2.11. Viral DNA isolation, sequencing and assembly

After, purification and propagation of phages as described above, 1.25 µL of DNAse I 100U (1/µL) and RNAse A (20 mg/mL) (Invitrogen®) were added in 1 mL phage suspension and incubated at 37ºC for 1h. Thus, the DNA extraction protocol was performed [30], with some modifications. The viral DNAs were dissolved in 50 µL of sterile Milli-Q water. The DNAs quality were assessed on the spectrophotometry using Picodrop™. The DNA integrity was verified by agarose gel electrophoresis. The viral DNA was stored at 4ºC.

Whole-genome sequencing was performed in Illumina MiSeq® platform, with v2 500-cycle kit (paired-end). DNA libraries were prepared with Nextera XT sample preparation kit following the manufacturer's instructions (Illumina™).

The quality of generated sequences was evaluated using FastQC tool [31]. Low-quality sequences were trimmed with the aid of FastQ Toolkit V.2.2.0. The paired-end sequence reads were assembled into contigs with SPAdes genome assembler v.3.9.0. All assemblies were confirmed by mapping reads to contigs [32] Geneious Software (version R9). Additionally, the ResFinder Server [33] and Virulence Factor Predictor [34] were used to identify in the determinant's antibiotic resistance and virulence genes on genomes.

2.12. Genomic annotation and phylogenetic analyses

Genomes annotation was carried by web serve PHASTER (Phage Search Tool Enhanced Release) [35]. In addition, when necessary, ORFs were examined to search for similarities in known sequences (*nr* database) using blastN and blastX software.

To understand the evolutionary relationships between B_UFSM4 and B_UFSM5 phages and other *Siphoviridae* species, two different approaches were applied to reconstruct the phylogenetic tree. First, we used an analysis based on the principle of phage proteomic tree reconstruction (10.1093/bioinformatics/btx157); all encoded proteins were extracted and concatenated and further used to generate a phylogenetic tree (generated by BIONJ based on the genomic distances).

Second, representative sequences from major capsid protein (MCP) and terminase gene were obtained from GenBank (October 2020) and then aligned with the sequences identified in this study using MAFFT software [32] which was optimized for accurate global alignment (option "G-INS-i"). Alignment was used to generate maximum-likelihood (ML) phylogenetic trees with PhyML, using the best fit substitution models determined by Smart Model Selection [36]. Statistical significance analyses of tree topologies were performed with the approximate likelihood branch support test (aLRT) [37].

2.13 Nucleotide Sequence Accession Number

Nucleotide sequence data reported are available in the GenBank database under the accession number MW147366 for phage B_UFSM4 and MW192778 for B_UFSM5.

2.14 Statistical Analysis

One-step growth assay, adsorption, bacteriolytic activity, and the stability analysis data were presented as the mean±standard deviation (SD) and analyzed using GraphPad Prism version 8.4.3. (GraphPad Software, USA).

3. Results

3.1. Phage isolation, purification, propagation, and titration

Initially, phages were isolated from bovine milk of cattle presenting subclinical mastitis caused by CPS. Cleared zones, suggestive of phage replication were observed; only in one CPS isolate (SBP 01/19 Gaivota PE), which was subjected to DNA sequencing of 16S rRNA

confirming *S. aureus.* Thereafter, it was used as the host bacteria for all laboratory assays. Thus, new phages, named *Staphylococcus* phage B_UFSM4 (B_UFSM4) and *Staphylococcus* phage B_UFSM5 (B_UFSM5) were isolated and characterize. After propagation, phages reached the following titers: B_UFSM4 9×10^{11} PFU/mL and B_UFSM5 22×10^{12} PFU/mL. The host bacteria and phages were registered in SISGEN platform (AAA70C4).

3.2. Determination of optimal multiplicity of infection (MOI) and one-step growth

The optimal MOI of phages was determined to be 1, because at this MOI, higher multiplication was observed, with both phages (B_UFSM4: 9×10^7 PFU/mL, B_UFSM5: 1.2×10^7 PFU/mL). Based on the MOI 1, the one - step growth curve was determined. Both phages presented a constant increase in their multiplication until 3.6×10^7 PFU/mL and 1.7×10^9 PFU/mL for B_UFSM4 and B_UFSM5, respectively (Fig 1).

3.3. Adsorption, thermal and pH stability

Initial phage adsorption was detected at 10-20 min for B_UFSM4 and at 20-30 min for B_UFSM5, according to virus reduction in the supernatant (Fig 2a). Phage's activities were relatively stable at pH 3-11. However, the B_UFSM5 decreased activity at pH 11 (Fig 2b). Phage's activity remained at the same higher level when heated at 37°C and completely disappeared when heated at 50°C, 60ºC or 70ºC for 60 min (Fig 2c and d).

3.4. Bacteriolytic activity

Bacteriolytic activity of both phages was evaluated by absorbance measurement (OD_{600}) of a CPS culture incubated with each phage. Bacteria culture absorbance remained stable and increased considerably after 3-4 h of incubation. However, after 5h, the host cell growth decreased, reaching 0.03 OD_{600} at 6 h in the cultures incubated with both phages. In comparison, the control (only CPS culture) reached OD_{600} 1.250 (Fig 3).

3.4. Host range

Spot test and EOP were performed to determine the lytic efficacy (host range) of phages in different bacterial isolates recovered from milk samples (n=27) and ATCC strains (n=3) (Table 1). Additionally, in host cell (SBP 01/19 PE) demonstrated in spot test high lytic (+++) and in EOP high score (> 10%; $3x10^8$ PFU/mL). By spot test, the phage B_UFSM4 exhibited ability to produce plaques in 14 (51.8%, 14/27) bacteria isolates and B_UFSM5 in 16 (59.2%, 16/27) isolates. Both phages did not replicate in the ATCC strains. Furthermore, the phages had

a high EOP in one out of 13 isolates from bovine mastitis tested. Notably, beyond isolates of *S. aureus,* plaques were visualized in CPS, CNS, *Staphylococcus sciuri*, *Rothia terrae,* and *P. aeruginosa,* including antimicrobial resistant and/or multiresistant isolates and had a high EOP in *R. terrae* (Table 1 and Fig S1).

3.5. Phage morphology

Transmission electron microscopy (TEM) revealed that both phages have an icosahedral head with a long tail (Fig 4a, b). The tails are ±120 nm and ± 150 nm in diameter in B_UFSM4 and B_UFSM5 respectively, and are non-contractile and longer than the head diameter. Thus, no long tail fibers and contractile sheath were observed. Based on these morphological characteristics, phages were assigned to the family *Siphoviridae* based on the classification systems [38,39].

3.6. Genomic characterization and phylogenetic analyses

The genomes of B_UFSM4 and B_UFSM5 have a size of 41.396 bp and 41.829 bp, respectively, in a double stranded DNA and linear architecture. The GC-content of the phage B_UFSM4 and B_UFSM5 is 33.97% and 33.98%, respectively. The phages present nucleotide identity of 98.91% between them. In both phages, 61 protein-coding genes were identified (Fig 5a, b; Table S1 and S2). Of the 61 coding ORFs, only 1 protein coded distinctly; particularly on ORF 14.

Fifty-four ORFs were located on the minus strand and only seven ORFs were presented on the plus strand. Both contain functional modules, such as phage structure, host lysis, lysogeny, phage DNA packaging, and replication. Four proteins were involved in phage structure, namely putative major tail protein (ORF52), putative phage head tail adapter (ORF55), tail length tape measure protein (ORF48), and capsid protein (ORF58). The host lysis proteins identified were holin (ORF42) and amidase (ORF41). Ten proteins were identified to be involved in lysogenic, DNA packaging, modification, replication, and transcription, including DNA packaging protein (ORF56), single strand DNA binding protein (ORF21), dUTP nucleotidohydrolase (ORF7), transcriptional activator RinB (ORF5), integrase (ORF38), portal protein (ORF60), repressor (ORF34), anti- repressor (ORF29), cro (ORF33), and putative restriction-modification protein (ORF40).

Nevertheless, majority of proteins verified (n=29) are hypothetical proteins (47.5%); of these 16(55.2%) are similar to the *Staphylococcus* prophage phiPV83 (GenBank accession no. NC_002486). Additionally, the proteins including single strand DNA binding, cro, repressor,

and integrase, also are similar to the prophage phiPV83 proteins. The putative major tail protein is similar to *S. aureus* phage JS01 protein (GenBank accession no. NC_021773), as well as four hypotheticals. One of the hypothetical proteins is similar to *Staphylococcus* phage tp310_1 (GenBank accession no. NC_009761), and also to, the proteins of capsid, portal and putative phage head tail adapter. Notably, the proteins lysis (holin and amidase) is similar to phage phiJB (GenBank accession no. NC_028669). Other proteins are the tail length tape measure is similar to *Staphylococcus* phage 13 (GenBank accession no.NC_004617), DNA packaging to *Staphylococcus* phage CN125 (GenBank accession no. NC_012784), anti- repressor to *Staphylococcus* phage DW2 (GenBank accession no. NC_024391), and putative restriction-modification to *Acinetobacter* phage B1251 (GenBank accession no. NC_019541).

A proteomic phylogenetic tree was reconstructed using ViPTree (Fig 6). Phages B_UFSM4 and B_UFSM5 are closely related *Staphylococcus* prophage phiPV83 (45,536 nt). These sequences form a sister clade with phages isolated from *Staphylococcus* species: *Staphylococcu*s phage JS01 (43,458 nt), *Staphylococcus* phage 13(42,722 nt), *Staphylococcus* phage CN125 (44,492 nt), and *Staphylococcus* phage tp310_1(42,232 nt). These phage sequences belong to family *Siphoviridae*, genus *Biseptimavirus* and order *Caudovirales.* Thus, the phages B_UFSM4 and B_UFSM5 can be considered as new species in the family *Siphoviridae.*

In addition, the phylogenetic tree was reconstructed using the MCP and Terminase coding regions (Fig S2). The phages B_UFSM4 and B_UFSM5, in both phylogenies, grouped with *Staphylococcus* phages tp310_1 and 13. In analyses with the Terminase, both the phages, also are grouped in a sister clade with *Staphylococcus* phages phiPV83 and JS01. Therefore, the tree topologies are very similar, in the analysis of the proteome, MCP and Terminase.

4. Discussion

Staphylococcus aureus is a significant microorganism in udder infections in dairy cows, and the reported cure rates for mastitis caused by *S. aureus* vary considerably. Another concern related to *S. aureus* is antimicrobial resistance, which contributes to lower cure rate [9]. Considering the increasing global resistance of bacteria to antimicrobials, the use of phages is an alternative to control bacterial infections [40,41].

In this study, novel *S. aureus* temperate phages (B_UFSM4 and B_UFSM5) were isolated. These viruses belong to the family *Siphoviridae*, order *Caudovirales*, based on their

genomic and morphological aspects and, the phylogenetic data verified that the phages belong to the genus *Biseptimavirus*.

In the studied phages, three tail proteins were found: putative major tail protein, putative phage head tail adapter and tail length tape measure. The structural proteins of the tail determine the tail length and form a channel to transmit DNA into the host cell [42]. Also, the portal protein was found, form a specialized machinery of order *Caudovirales,* that are involved in replication, such as virion assembly, DNA packaging and DNA delivery [43]. Notably, the proteins holin and amidase were identified, and are responsible for the lysis of bacterial cell. Amidase is principally related to lysis, and holin is involved in amidase activation [27,44].

Moreover, the integrase protein was identified and their presence indicates that both phages can insert their DNA into the host. Thus, beyond lytic cycle, temperate phages also have a lysogenic cycle, and incorporate their genomes into the host chromosome; these phages can be as considered natural vectors for transmission genes virulence and resistance among bacteria [45–47], however, no homologs of virulence transfer or lysogenic genes were found in the genomes of phages B_UFSM4 and B_UFSM5. Additionally, the phages integrase protein of this study is similar to the phage phiPV83, therefore, belongs to group Sa5, serogroup Fb, besides the holin belongs to group 255a, according to the classification of Staphylococcus prophages [48].

Besides the aforementioned genes, several hypothetical proteins were identified, but due to the insufficient database information about the functional genes of *S. aureus* phages genomes, we were unable to verify their functionality [49].

In regards to the phylogenetic analyses, the isolated phages have similarity with *Staphylococcus* prophage phiPV83 (GenBank accession no. NC_002486) and *S. aureus* phage JS01 (GenBank accession no. NC_021773). The prophage phiPV83 genome has 45,636 bp and 64 ORFs, including two extra operons, lukM-lukF-PV. And, also presents the proteins: integrase, repressor, cro, and anti-repressor, thus demonstrate lysogeny similar mechanisms, as well as replication proteins (transcriptional activator RinB and single strand DNA binding). Moreover, it presents other proteins such as Ntpase (replication protein), Cos (packaging protein) and protease (head protein). Futhermore, the identified genes lukM and lukF-PV encode the leukocidin toxin Panton-Valentine (PVL), a virulence factor found in strains of *S. aureus* [50].

S. aureus phage JS01 was isolated from the milk of cattle affected by mastitis and also presents proteins similar to putative major tail and hypothetical proteins [51]. Thus, the

similarity was 45,536 nt with *Staphylococcus* prophage phiPV83 and 43,458 nt with *S. aureus* phage JS01.

It was verified that high temperature (50ºC-70ºC) and extreme pH (3-11) do not affect the bacteriophages B_UFSM4 and B_UFSM5 stability, which is similar to studies conducted with other phages [21,52]. The bacteriological activity was considerably reduced in the prokaryotic cells after 5h of virus incubation. Similarly, other surveys showed this reduction between 3h-4h after the virus incubation [26,52].

Here, both phages also demonstrated lytic efficiency mainly against *S. aureus* and CPS isolates. Additionally, we detected their wide host amplitude included *S. sciuri*, CNS, *R. terrae,* and *P. aeruginosa*. Although most phages are highly host specific, some could infect different bacterial genera and species [53], as observed in the case *S. aureus* phages SA, SANF and SA2, which demonstrated lytic activity against others species of *Staphylococcus* and *M. caseolyticus* isolates from bovine mastitis [54]. Another study demonstrated host amplitude of *P. aeruginosa* phage PA1Ø infecting Gram-positive bacteria, such as *S. aureus*, in which this ability to infect other genera is related to production of lytic enzymes of phages[55].

Additionally, the wide range of hosts may be attributed to the ability of the phage to adsorb to a host cell by different receptor-binding proteins (RBPs). RBPs have endless adaptation cycles; therefore, some phages can use various RBPs for adsorbing to the bacterial host cell [56]. In this study, lytic activity against other genera and *Staphylococcus* species was observed. Therefore, presumably these genera share similar viral protein receptors.

In this study, novel temperate phages of *S. aureus* were isolated from bovine milk samples. Their genomes were similar to each other; however, they are distinct from other *S. aureus* phages of the family *Siphoviridae*. Thus, our results indicate that these phages infect bacterial isolates from their host specie and also present host amplitude, therefore, the basic data are provided and further studies of the interaction mechanism between phages and hosts will be performed.

Nucleotide Sequence Accession Numbers

Nucleotide sequence data reported are available in the GenBank database under the accession number MW147366 for phage B_UFSM4 and MW192778 for B_UFSM5.

Author Contributions

Conceptualization: BMB; SAB. Methodology: BMB; ESP; DIBP; JFC; SAB. Formal analysis and investigation: BMB; GFG; DIBP; SC; APMV; FQM; SAB. Writing - original draft preparation: BMB; SAB.

Writing - review and editing: BMB; ESP; DIBP; SC; APMV; FQM; GFG; LAS; JFC; SAB. Funding acquisition: SAB. Resources: FQM; JFC; SAB. Supervision: SAB.

Declarations

Funding

Not applicable.

Conflict of interest

There are no conflicts of interest.

Ethics approval

Not applicable.

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Legends to Figures and Tables

Fig 1 One-step growth curve of phages B_UFSM4 and B_UFSM5 on *S. aureus* (SBP 01/19 Gaivota PE) with standard deviations. The multiplication of B_UFSM4 was 7.56 PFU/mL (Log 10) and B_UFSM5 was 9.3 PFU/ mL (Log 10) after 2h growth.

Fig 2 Adsorption (a), pH stability(b) and thermostability (c, d) of phages B_UFSM4 and B_UFSM5 on *S. aureus* (SBP 01/19 Gaivota PE) with standard deviations.

Fig 3 Bacteriolytic activity of phages B_UFSM4 and B_UFSM5 on *S. aureus* (SBP 01/19 Gaivota PE). After 5h incubation, both cultivations decreased to 0.030 (OD_{600}) and the control reached 1.250 (OD_{600}).

Fig 4 Transmission electron microscopy (TEM) of *Staphylococcus* phage B_UFSM4 (a). TEM of *Staphylococcus* phage B_UFSM5 (b). Bar corresponds to 100 nm.

Fig 5 The graphical representation of protein-coding genes of the phage B_UFSM4 (a) and B_UFSM5 (b). The annotation of the genomes was carried by web serve PHASTER.

Fig 6 Graphical representation of phylogeny of the phages B_UFSM4 and B_UFSM5, using MAFFT software with alignment using PhyML - ViPTree.

Table 1 [a]Spot test: (-) without lytic activity; (+) low lytic activity; (++) moderate lytic activity; and (+++) high lytic activity. [b]EOP: High, low, lysis from without (LFW), and negative, representing score >10, 0.1–1, <0.1%, and 0, respectively. N/A (Non-applicable). Gen: Gentamycin. PolB: Polymyxin B. Sut: sulfazotrim. Pen:

penicillin. Amp: ampicillin. Tet: Tetracycline. Oxa: oxacillin. Ero: enrofloxacin. Neo: neomycin. Cfc: cefaclor. Cfe: Cephalexin. Cef: ceftiofur. Nit: nitrofurantoin.

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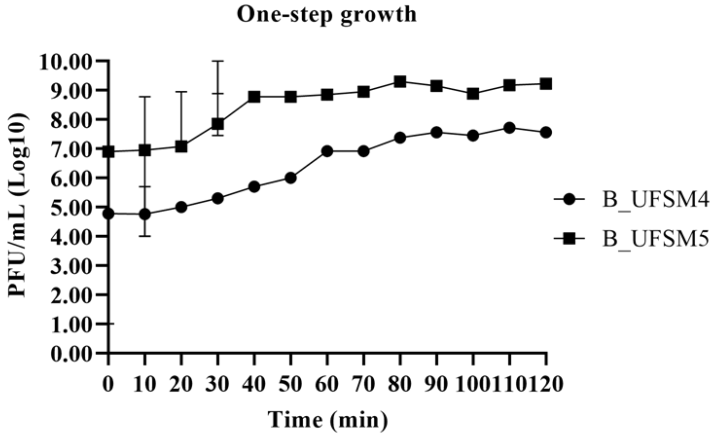
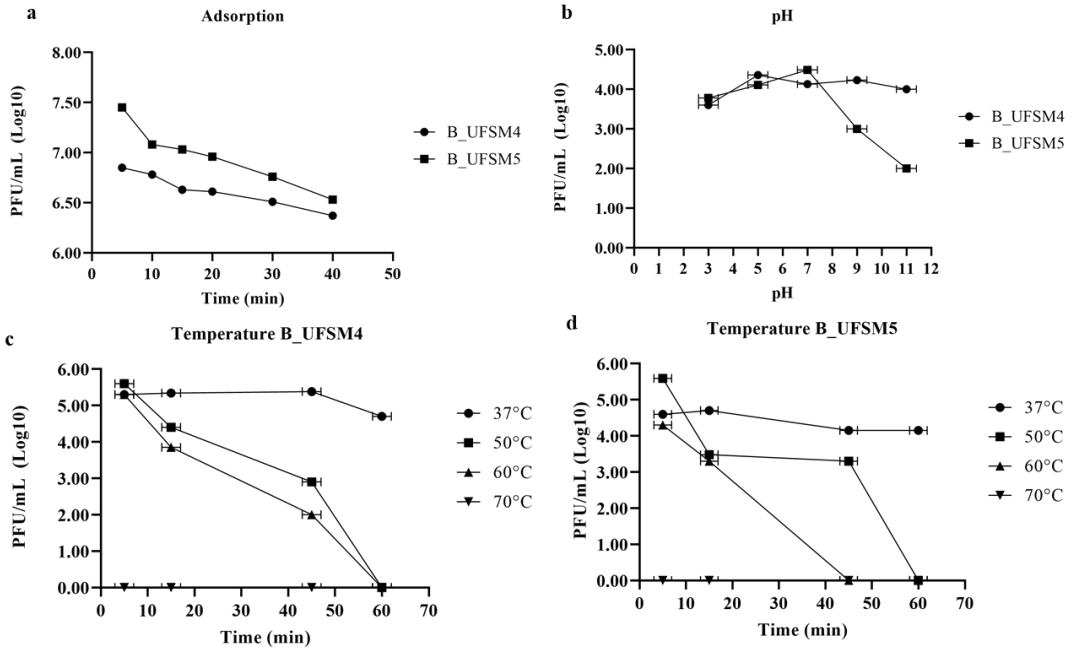
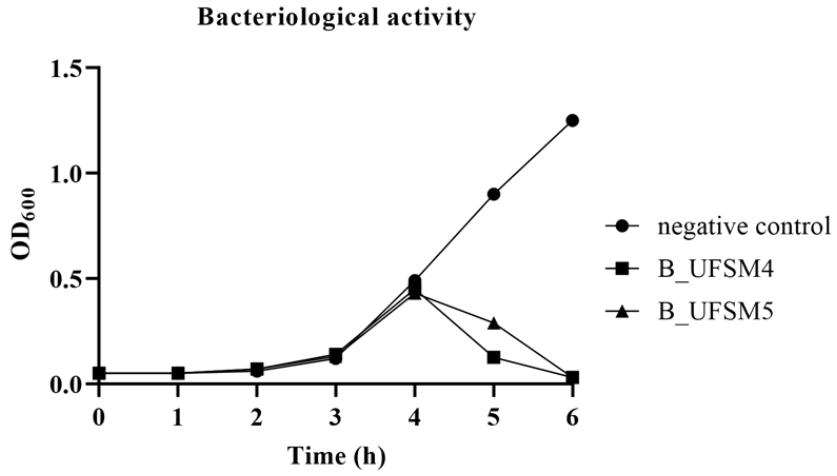
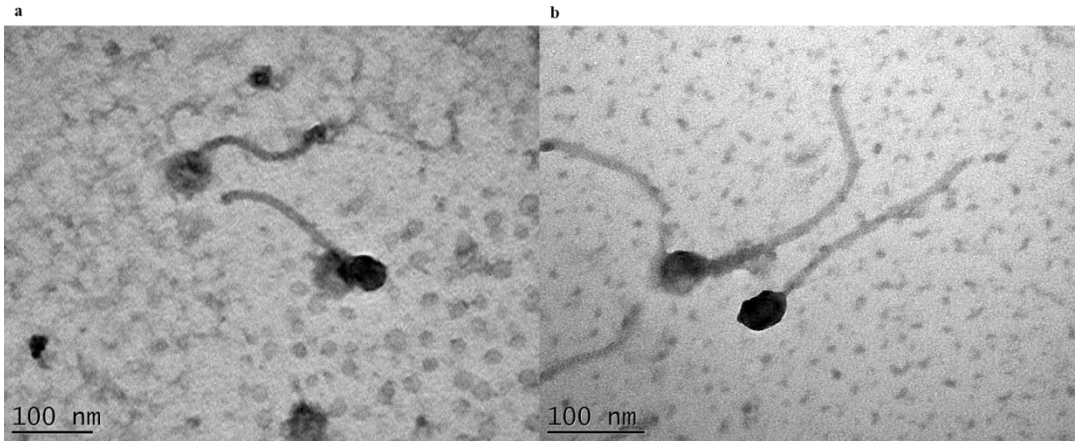
Table 1 Host range against bacterial isolates of bovine mastitis and strains.

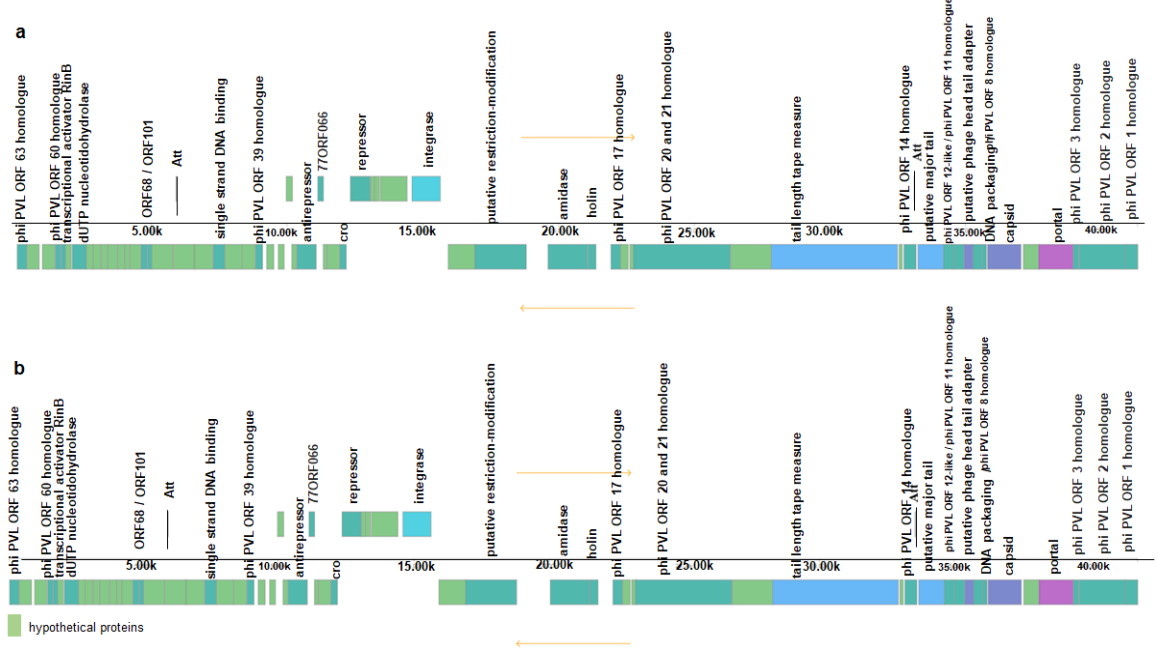
Bacterial species	Isolates/ strains	Antimicrobial resistence	[a]Spot test		[b]EOP	
			B_UFSM4	B_UFSM5	B_UFSM4	B_UFSM5
S. aureus	SB 93/16 340 PE	Gen, PolB	-	-	0	0
	MRSA 2		+	+	<0.1%	<0.1%
	MRSA 3		+	++	<0.1%	<0.1%
	SB 113/06 162 PE	PolB, Sut, Cfc, Amp, Oxa, Pen	+++	+++	0.1%	<0.1%
	SBP 120/19 Nevasca PD		-	+	N/A	N/A
	ATCC 25923		-	-	N/A	N/A
CPS	SB 101/16	PolB	+	+	0.1%	0.1%
	SB 53/17 20 AD	PolB	-	-	N/A	N/A
	SBP 17/20 342 PE		-	-	N/A	N/A
	SBP 17/20 312 AD	Pen, Ero	+	+	<0.1%	<0.1%
	SBP 17/20 79 PE	Cfe	-	-	N/A	N/A
	SBP 112/12 Chata AE		+	+	N/A	N/A
	SBP 01/13 33 PD		++	+++	0.1%	<0.1%
	SB 208/18		+++	+++	N/A	N/A
S. sciuri	SB 57/17 49 PD		+	+	0	0
S. hominis	SB 08/12	Pen, Amp	-	-	N/A	N/A
S. cromogenes	SB 47/12 252 AD		-	-	N/A	N/A
S. epidermidis	ATCC 12225		-	-	N/A	N/A
CNS	SB 99/17 70 PE		-	-	N/A	N/A
	SB 99/17 25 PE		-	-	N/A	N/A
	SB 52/17 PD	Pen, Amp	-	+	N/A	N/A
	SB 105/94 Pintada PD	Pen, Nit	+	+	0	0
	SB 90/12		-	-	N/A	N/A
	SB 57/16		-	-	N/A	N/A
	SB 52/12 Baia AD		-	-	N/A	N/A
Rothia terrae	SB 39/10 008 PE		+++	+++	>10	>10
P. aureuginosa	SB 120/19 Frida PD	Cfe, Amp, Pen, Oxa, Sut, Ero, Cef, PolB, Tet	+	+	0	0
	SB120/19 Frida AD		+	+	0	0
	SB 134/16	Sut, Pen, Amp, Cfe, Tet, Cef, Oxa, Neo, Ero	+	+	0	0
	ATCC 25853		-	-	N/A	N/A

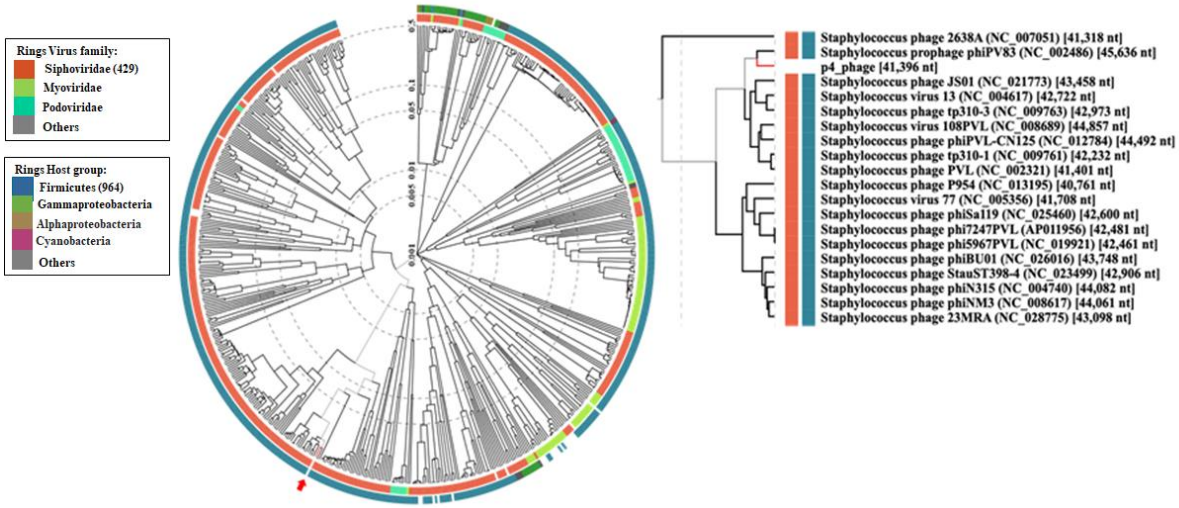

One-step growth
PFU/mL (Log10)
Time (min)
B_UFSM4
B_UFSM5


a
Adsorption
PFU/mL (Log10)
Time (min)
B_UFSM4
B_UFSM5
b
pH
PFU/mL (Log10)
pH
B_UFSM4
B_UFSM5
c
Temperature B_UFSM4
PFU/mL (Log10)
Time (min)
37°C
50°C
60°C
70°C
d
Temperature B_UFSM5
PFU/mL (Log10)
Time (min)
37°C
50°C
60°C
70°C


Bacteriological activity
1.5
1.0
0.5
0.0
OD600
0
1
2
3
4
5
6
Time (h)
negative control
B_UFSM4
B_UFSM5


a
b
100 nm
100 nm


a
phi PVL ORF 63 homologue
phi PVL ORF 60 homologue
transcriptional activator RinB
dUTP nucleotidohydrolase
ORF68 / ORF101
Att
single strand DNA binding
phi PVL ORF 39 homologue
antirepressor
77ORF066
cro
repressor
integrase
putative restriction-modification
amidase
holin
phi PVL ORF 17 homologue
phi PVL ORF 20 and 21 homologue
tail length tape measure
phi PVL ORF 14 homologue
Att
putative major tail
phi PVL ORF 12-like / phi PVL ORF 11 homologue
putative phage head tail adapter
DNA packaging/phi PVL ORF 8 homologue
capsid
portal
phi PVL ORF 3 homologue
phi PVL ORF 2 homologue
phi PVL ORF 1 homologue
5.00k
10.00k
15.00k
20.00k
25.00k
30.00k
35.00k
40.00k
b
hypothetical proteins


Rings Virus family:
Siphoviridae (429)
Myoviridae
Podoviridae
Others
Rings Host group:
Firmicutes (964)
Gammaproteobacteria
Alphaproteobacteria
Cyanobacteria
Others
Staphylococcus phage 2638A (NC_007051) [41,318 nt]
Staphylococcus prophage phiPV83 (NC_002486) [45,636 nt]
p4_phage [41,396 nt]
Staphylococcus phage JS01 (NC_021773) [43,458 nt]
Staphylococcus virus 13 (NC_004617) [42,722 nt]
Staphylococcus phage tp310-3 (NC_009763) [42,973 nt]
Staphylococcus virus 108PVL (NC_008689) [44,857 nt]
Staphylococcus phage phiPVL-CN125 (NC_012784) [44,492 nt]
Staphylococcus phage tp310-1 (NC_009761) [42,232 nt]
Staphylococcus phage PVL (NC_002321) [41,401 nt]
Staphylococcus phage P954 (NC_013195) [40,761 nt]
Staphylococcus virus 77 (NC_005356) [41,708 nt]
Staphylococcus phage phiSa119 (NC_025460) [42,600 nt]
Staphylococcus phage phi7247PVL (AP011956) [42,481 nt]
Staphylococcus phage phi5967PVL (NC_019921) [42,461 nt]
Staphylococcus phage phiBU01 (NC_026016) [43,748 nt]
Staphylococcus phage StauST398-4 (NC_023499) [42,906 nt]
Staphylococcus phage phiN315 (NC_004740) [44,082 nt]
Staphylococcus phage phiNM3 (NC_008617) [44,061 nt]
Staphylococcus phage 23MRA (NC_028775) [43,098 nt]

Supplementary Material:

Table S1 Identification of phage B_UFSM4 proteins.

ORF	Strand	Start	End	Identified proteins functions and best match	Similarity	E-value	GenBank accession
01	-	1	354	phi PVL ORF 63 homologue [Staphylococcus phage phiPV83]	45,636 nt	1.45e-79	NC_002486.1
02	-	361	813	hypothetical protein [Staphylococcus phage tp310_1]	42,232 nt	6.51e-91	NC_009761.3
03	-	928	1389	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	1.00e-104	NC_021773.2
04	-	1412	1600	phi PVL ORF 60 homologue [Staphylococcus phage phiPV83]	45,636 nt	1.06e-36	NC_002486.1
05	-	1612	1785	transcriptional activator RinB [Staphylococcus phage B236]	43,228 nt	2.34e-34	NC_028915.1
06	-	1782	1988	hypothetical protein [Staphylococcus phage StauST398_3]	41,392 nt	5.62e-40	NC_021332.1
07	-	2025	2558	dUTP nucleotidohydrolase [Staphylococcus phage phiETA2]	43,081 nt	3.17e-114	NC_008798.1
08	-	2551	2799	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	6.17e-51	NC_021773.2
09	-	2792	3076	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	6.09e-58	NC_002486.1
10	-	3091	3333	hypothetical protein [Staphylococcus phage phiMR25]	44,342 nt	5.74e-51	NC_010808.1
11	-	3337	3693	hypothetical protein [Staphylococcus phage SA97]	40,592 nt	2.76e-79	NC_029010.1
12	-	3705	3962	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	1.43e-55	NC_002486.1
13	-	3963	4148	hypothetical protein [Staphylococcus phage Ipla88]	42,526 nt	7.12e-35	NC_011614.1
14	-	4153	4557	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	6.92e-94	NC_002486.1
15	-	4568	4789	ORF068 [Staphylococcus phage 52A]	41,690 nt	2.35e-47	NC_007062.1
16	-	4802	4963	ORF101 [Staphylococcus phage 69]	42,732 nt	4.77e-31	NC_007048.1
17	-	4957	5730	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
18	-	5740	6543	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
19				attL			
20	-	6515	7210	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.91e-175	NC_002486.1
21	-	7224	7652	single strand DNA binding protein [Staphylococcus phage phiPV83]	45,636 nt	7.60e-100	NC_002486.1
22	-	7652	8290	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	1.90e-156	NC_002486.1
23	-	8290	8769	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	3.30e-110	NC_002486.1
24	-	8784	9044	phi PVL ORF 39 homologue [Staphylococcus phage phiPV83]	45,636 nt	5.00e-57	NC_002486.1
25	-	9194	9457	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	9.94e-57	NC_002486.1
26	-	9618	9839	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.13e-45	NC_002486.1
27	+	9911	10141	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	9.66e-49	NC_002486.1
28	-	10116	10292	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	7.06e-35	NC_002486.1
29	-	10305	11018	Antirepressor [Staphylococcus phage DW2]	41,941 nt	5.06e-174	NC_024391.1
30	+	11075	11284	77ORF066 [Staphylococcus phage 77]	41,708 nt	5.09e-42	NC_005356.1
31	-	11277	11417	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	3.23e-24	NC_002486.1
32	-	11432	11875	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	9.78e-103	NC_002486.1
33	-	11888	12127	Cro [Staphylococcus phage phiPV83]	45,636 nt	3.31e-53	NC_002486.1
34	+	12278	13009	Repressor [Staphylococcus phage phiPV83]	45,636 nt	4.13e-173	NC_002486.1
35	+	13021	13167	hypothetical protein [Staphylococcus phage YMC/09/04/R1988]	44,459 nt	1.67e-26	NC_022758.1

36	+	13164	13349	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.57e-37	NC_002486.1
37	+	13385	14368	hypothetical protein [Staphylococcus phage StB20_like]	40,670 nt	0.0	NC_028821.1
38	+	14547	15593	integrase [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
39	-	15881	16882	hypothetical		N/A	
40	-	16866	18758	putative restriction-modification protein [Acinetobacter phage B1251]		4.24e-18	NC_019541.1
41	-	19560	21014	amidase [Staphylococcus phage phiJB]	43,012 nt	0.0	NC_028669.1
42	-	21025	21327	holin [Staphylococcus phage phiJB]	43,012 nt	8.28e-65	NC_028669.1
43	-	21877	22233	phi PVL ORF 17 homologue [Staphylococcus phage phiPV83]	45,636 nt	3.23e-63	NC_002486.1
44	-	22230	22517	hypothetical protein [Staphylococcus phage StauST398_4]	42,906 nt	5.58e-58	NC_023499.1
45	-	22569	22721	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	1.54e-27	NC_002486.1
46	-	22708	26277	phi PVL ORF 20 and 21 homologue [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
47	-	26293	27783	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	0.0	NC_021773.2
48	-	27783	32435	tail length tape measure protein [Staphylococcus phage 13]	42,722 nt	0.0	NC_004617.1
49	-	32491	32613	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	3.20e-21	NC_021773.2
50	-	32673	33119	phi PVL ORF 14 homologue [Staphylococcus phage phiPV83]	45,636 nt	1.19e-97	NC_002486.1
51				attR			
52	-	33183	34136	putative major tail protein [Staphylococcus phage JS01]	43,458 nt	0.0	NC_021773.2
53	-	34129	34518	phi PVL orf 12-like protein [Staphylococcus phage JS01]	43,458 nt	9.96e-69	NC_021773.2
54	-	34515	34892	phi PVL ORF 11 homologue [Staphylococcus phage phiPV83]	45,636 nt	2.37e-75	NC_002486.1
55	-	34892	35224	putative phage head tail adapter [Staphylococcus phage tp310_1]	42,232 nt	2.60e-69	NC_009761.3
56	-	35214	35546	DNA packaging protein [Staphylococcus phage CN125]	44,492 nt	4.49e-58	NC_012784.1
57	-	35555	35713	phi PVL ORF 8 homologue [Staphylococcus phage phiPV83]	45,636 nt	2.70e-26	NC_002486.1
58	-	35750	36985	capsid protein [Staphylococcus phage tp310_1]	42,232 nt	0.0	NC_009761.3
59	-	37073	37657	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.82e-138	NC_002486.1
60	-	37632	38906	portal protein [Staphylococcus phage tp310_1]	42,232 nt	0.0	NC_009761.3
61	-	38909	39112	phi PVL ORF 3 homologue [Staphylococcus phage phiPV83]	45,636 nt	5.69e-26	NC_002486.1
62	-	39126	40820	phi PVL ORF 2 homologue [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
63	-	40820	41290	phi PVL ORF 1 homologue [Staphylococcus phage phiPV83]	45,636 nt	2.94e-106	NC_002486.1

Table S2. Identification of phage B_UFSM5 proteins.

ORF	Strand	Start	End	Identified proteins functions and best match	Similarity	E-value	GenBank accession
01	-	1	354	phi PVL ORF 63 homologue [Staphylococcus phage phiPV83]	45,636 nt	1.45e-79	NC_002486.1
02	-	361	813	hypothetical protein [Staphylococcus phage tp310_1]	42,232 nt	6.51e-91	NC_009761.3
03	-	928	1389	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	1.00e-104	NC_021773.2
04	-	1412	1600	phi PVL ORF 60 homologue [Staphylococcus phage phiPV83]	45,636 nt	1.06e-36	NC_002486.1
05	-	1612	1785	transcriptional activator RinB [Staphylococcus phage B236]	43,228 nt	2.34e-34	NC_028915.1
06	-	1782	1988	hypothetical protein [Staphylococcus phage StauST398_3]	41,392 nt	5.62e-40	NC_021332.1
07	-	2025	2558	dUTP nucleotidohydrolase [Staphylococcus phage phiETA2]	43,081 nt	3.17e-114	NC_008798.1
08	-	2551	2799	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	6.17e-51	NC_021773.2
09	-	2792	3076	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	6.09e-58	NC_002486.1
10	-	3091	3333	hypothetical protein [Staphylococcus phage phiMR25]	44,342 nt	5.74e-51	NC_010808.1
11	-	3337	3693	hypothetical protein [Staphylococcus phage SA97]	40,592 nt	2.76e-79	NC_029010.1
12	-	3705	3962	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	1.43e-55	NC_002486.1
13	-	3963	4148	hypothetical protein [Staphylococcus phage Ipla88]	42,526 nt	7.12e-35	NC_011614.1
14	-	4153	4557	hypothetical protein [Staphylococcus phage 80]	42,140 nt	3.59e-94	NC_030652.1
15	-	4568	4789	ORF068 [Staphylococcus phage 52A]	41,690 nt	2.35e-47	NC_007062.1
16	-	4802	4963	ORF101 [Staphylococcus phage 69]	42,732 nt	4.77e-31	NC_007048.1
17	-	4957	5730	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
18	-	5740	6543	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
19				attL			
20	-	6515	7210	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.91e-175	NC_002486.1
21	-	7224	7652	single strand DNA binding protein [Staphylococcus phage phiPV83]	45,636 nt	7.60e-100	NC_002486.1
22	-	7652	8290	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	1.90e-156	NC_002486.1
23	-	8290	8769	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	3.30e-110	NC_002486.1
24	-	8784	9044	phi PVL ORF 39 homologue [Staphylococcus phage phiPV83]	45,636 nt	5.00e-57	NC_002486.1
25	-	9194	9457	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	9.94e-57	NC_002486.1
26	-	9618	9839	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.13e-45	NC_002486.1
27	+	9911	10141	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	9.66e-49	NC_002486.1
28	-	10116	10292	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	7.06e-35	NC_002486.1
29	-	10305	11018	Antirepressor [Staphylococcus phage DW2]	41,941 nt	5.06e-174	NC_024391.1
30	+	11075	11284	77ORF066 [Staphylococcus phage 77]	41,708 nt	5.09e-42	NC_005356.1
31	-	11277	11417	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	3.23e-24	NC_002486.1
32	-	11432	11875	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	9.78e-103	NC_002486.1
33	-	11888	12127	Cro [Staphylococcus phage phiPV83]	45,636 nt	3.31e-53	NC_002486.1
34	+	12278	13009	Repressor [Staphylococcus phage phiPV83]	45,636 nt	4.13e-173	NC_002486.1
35	+	13021	13167	hypothetical protein [Staphylococcus phage YMC/09/04/R1988]	44,459 nt	1.67e-26	NC_022758.1
36	+	13164	13349	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.57e-37	NC_002486.1

37	+	13385	14368	hypothetical protein [Staphylococcus phage StB20_like]	40,670 nt	0.0	NC_028821.1
38	+	14547	15593	integrase [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
39	-	15881	16882	hypothetical		N/A	
40	-	16866	18758	putative restriction-modification protein [Acinetobacter phage B1251]		4.24e-18	NC_019541.1
41	-	19560	21014	amidase [Staphylococcus phage phiJB]	43,012 nt	0.0	NC_028669.1
42	-	21025	21327	holin [Staphylococcus phage phiJB]	43,012 nt	8.28e-65	NC_028669.1
43	-	21877	22233	phi PVL ORF 17 homologue [Staphylococcus phage phiPV83]	45,636 nt	3.23e-63	NC_002486.1
44	-	22230	22517	hypothetical protein [Staphylococcus phage StauST398_4]	42,906 nt	5.58e-58	NC_023499.1
45	-	22569	22721	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	1.54e-27	NC_002486.1
46	-	22708	26277	phi PVL ORF 20 and 21 homologue [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
47	-	26293	27783	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	0.0	NC_021773.2
48	-	27783	32435	tail length tape measure protein [Staphylococcus phage 13]	42,722 nt	0.0	NC_004617.1
49	-	32491	32613	hypothetical protein [Staphylococcus phage JS01]	43,458 nt	3.20e-21	NC_021773.2
50	-	32673	33119	phi PVL ORF 14 homologue [Staphylococcus phage phiPV83]	45,636 nt	1.19e-97	NC_002486.1
51				attR			
52	-	33183	34136	putative major tail protein [Staphylococcus phage JS01]	43,458 nt	0.0	NC_021773.2
53	-	34129	34518	phi PVL orf 12-like protein [Staphylococcus phage JS01]	43,458 nt	9.96e-69	NC_021773.2
54	-	34515	34892	phi PVL ORF 11 homologue [Staphylococcus phage phiPV83]	45,636 nt	2.37e-75	NC_002486.1
55	-	34892	35224	putative phage head tail adapter [Staphylococcus phage tp310_1]	42,232 nt	2.60e-69	NC_009761.3
56	-	35214	35546	DNA packaging protein [Staphylococcus phage CN125]	44,492 nt	4.49e-58	NC_012784.1
57	-	35555	35713	phi PVL ORF 8 homologue [Staphylococcus phage phiPV83]	45,636 nt	2.70e-26	NC_002486.1
58	-	35750	36985	capsid protein [Staphylococcus phage tp310_1]	42,232 nt	0.0	NC_009761.3
59	-	37073	37657	hypothetical protein [Staphylococcus phage phiPV83]	45,636 nt	2.82e-138	NC_002486.1
60	-	37632	38906	portal protein [Staphylococcus phage tp310_1]	42,232 nt	0.0	NC_009761.3
61	-	38909	39112	phi PVL ORF 3 homologue [Staphylococcus phage phiPV83]	45,636 nt	5.69e-26	NC_002486.1
62	-	39126	40820	phi PVL ORF 2 homologue [Staphylococcus phage phiPV83]	45,636 nt	0.0	NC_002486.1
63	-	40820	41290	phi PVL ORF 1 homologue [Staphylococcus phage phiPV83]	45,636 nt	2.94e-106	NC_002486.1

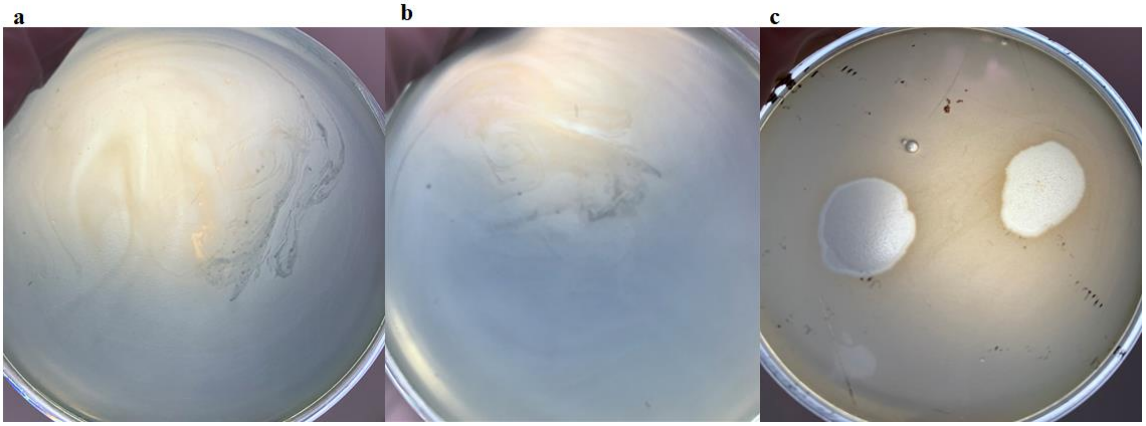
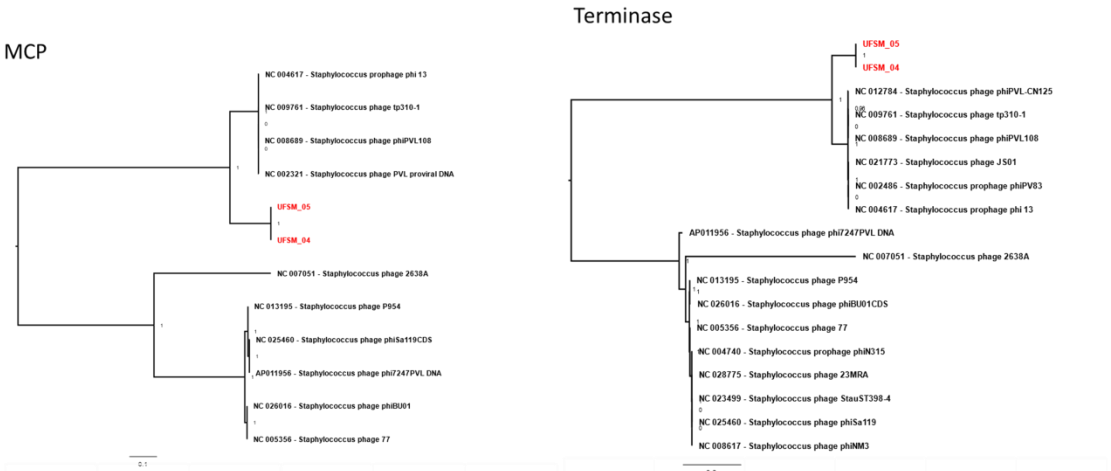


Fig S1 Spot test of the phage B_UFSM4 and isolate SB 134/16 of *P. aeruginosa* (a). Spot test of the phage B_UFSM5 and isolate SB 134/16 of *P. aeruginosa* (b). Spot test of the phages B_UFSM4 and B_UFSM5 in isolate SB 39/10 008 PE of *R. terrae*.



Model	Decoration	K	Llk	AIC	BIC	Model	Decoration	K	Llk	AIC	BIC
GTR	+G	30	-5587,48465	11234,96930	11391,58051	GTR	+G	42	-7867,22596	15818,45192	16048,17739

Fig S2 Phylogenetic tree of the phages B_UFSM4 and B_UFSM5 reconstructed using the MCP and Terminase coding regions.

5. CAPÍTULO 3

(Manuscrito submetido para publicação no periódico *Folia microbiologica*)

In vitro antibiofilm activity of *Staphylococcus aureus* bacteriophages in bacterial isolates from bovine mastitis

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Abstract

This study investigated the capacity of four *Staphylococcus aureus* phages (B_UFSM1, B_UFSM3, B_UFSM4 and B_UFSM5) and two phage cocktails were prepared: cocktail A (composed of phages B_UFSM4 and B_UFSM5); and cocktail B (composed of all four phages) to inhibit and remove biofilm of *Staphylococcus* spp. and *Pseudomonas aeruginosa* isolates from bovine mastitis. The phages and cocktails, inhibited biofilms most *Staphylococcus* spp. isolates and strain, except the phages B_UFSM3 and B_UFSM4 ($P \leq 0.0001$; $P \leq 0.001$ and $P \leq 0.01$). In *P. aeruginosa* isolates and strain, only the phage B_UFSM4 ($P \leq 0.05$) did not inhibit the biofilms. In removing biofilms from *Staphylococcus* spp. after 24h incubation, the phage B_UFSM1 was effective mainly in MRSA2, SB113/06 162 PE and ATCC 25923 and, the phages B_UFSM 3, B_UFSM4 and B_UFSM5 removed the biofilms in *P. aeruginosa* ATCC 25853**.** The phages B_UFSM1, B_UFSM3, B_UFSM4, B_UFSM5, and their cocktails, presented activity in *Staphylococcus* spp. and *P. aeruginosa* bacterial biofilms.

Keywords: phages; bacteriophages; *Staphylococcus aureus*; *Pseudomonas aeruginosa*; bovine mastitis; biofilms.

Introduction

Bovine mastitis is the main disease affecting dairy cattle and *Staphylococcus aureus* is one of the main causative agents (Rysanek et al. 2009). This pathogen can persist in the udders of cows and has numerous resistance mechanisms, which may be related to the ability of *S. aureus* forming biofilm (Grunert et al. 2018). Studies have demonstrated the biofilm formation of several *S. aureus* isolates from bovine mastitis and the possible relationship with recurrent mastitis, antimicrobial resistance, and the animal's immune system (Aslantaş and Demir 2016; Gomes et al. 2016; Marques et al. 2017; Notcovich et al. 2018). Other members of *Staphylococcus*

genus, as coagulase - negative *Staphylococcus* (CNS) isolated from bovine mastitis, can also form biofilms (Darwish and Asfour 2013).

Besides *S. aureus*, a wide variety of bacteria are related to mastitis in cattle and one important causative agent of environmental mastitis is *Pseudomonas aeruginosa*, and its occurrence is reported as outbreaks in dairy farms (Coats 1998; Fernandes et al. 2009). In addition, *P. aeruginosa* is a strong biofilm producer that also can reduce antimicrobials effectiveness and lead to chronic mastitis(Melchior et al. 2006; Aguayo et al. 2020).

Biofilms are microbial communities attached to a surface and protected by a self-produced polymeric matrix. Bacterial colonization in biofilms increases bacterial tolerance to antimicrobial treatment and disinfectants (Mah and Toole 2001). Since the ability of the bacteria to produce biofilm may reduce the effectiveness of antimicrobials and disinfectants, it is necessary to seek effective alternatives against theses bacteria that can form biofilms as *Staphylococcus* spp. and *Pseudomonas* spp. involved in bovine mastitis (Weber et al. 2019). In this sense, bacteriophages or phages, could be important tools for the control of bovine mastitis, since lytic phages have the ability to reach bacterial cells in its planktonic and sessile form (Sillankorva and Azeredo 2014).

Therefore, considering the importance of bacterial biofilms in the persistence and difficulty in controlling infections caused by bacteria with this potential, this study investigated the capacity of four *Staphylococcus aureus* phages and cocktails to inhibit and remove biofilm of *Staphylococcus* spp. and *P. aeruginosa* isolated from bovine mastitis.

Materials and methods

Bacterial and bacteriophage selection

Fifteen isolates of *Staphylococcus* spp. were used in the biofilm assays, being seven coagulase-positive *Staphylococcus* (CPS), seven coagulase-negative *Staphylococcus* (CNS) (SISGEN A7085CE) and one *S. aureus* standard strain (ATCC 25923). In addition, three *P. aeruginosa* isolates (SISGEN A7085CE) and one *P. aeruginosa* standard strain (ATCC 25853) were used, totaling 19 samples. The isolates belong to the Laboratório de Bacteriologia (LABAC) at Universidade Federal de Santa Maria (UFSM), Rio Grande do Sul State, Brazil (Table1).

Four *S. aureus* phages temperate (SISGEN AAA70C4) belonging to family *Siphoviridae*, genus *Biseptimavirus* and order *Caudovirales* were isolated from bovine milk in southern Brazil and named *Staphylococcus* phage B_UFSM1, *Staphylococcus* phage B_UFSM3, *Staphylococcus* phage B_UFSM4, and *Staphylococcus* phage B_UFSM5, GenBank accession numbers MW650841, MW627293, MW147366, and MW192778, respectively. The genomes of B_UFSM1, B_UFSM3, B_UFSM4 and B_UFSM5 have a size of 42.099 pb (34% GC), 42.952 bp (34.1% GC), 41.396 bp (33.97% GC), and 41.829 bp (33.98% GC), respectively, in a double stranded DNA and linear architecture. The phages present nucleotide identity of 94.8 - 99.4% between them, being the phage B_UFSM3 is most distinct among the phages.

Additionally, two phage cocktails were prepared: cocktail A (composed of equal parts of 10^6 PFU ml^{-1} of phages B_UFSM4 and B_UFSM5); and cocktail B (composed of all four phages, 1/4 of 106 PFU ml-1 of each virus). The spot test was performed to verify the infectivity of the phages against the isolates and strains (Table 1).

Biofilm formation assay

The biofilm assay was performed following (Christensen et al. 1985) with modifications. Biofilm formation was performed in 96-well polystyrene microplates (Kasvi^{TM}) to yield cell attachment, and Trypticase Soy Broth (TSB) (Kasvi^{TM}) supplemented with 1% d-(+)-glucose (TSBg) was used to perform the assay. First, all isolates and bacterial strains were grown at 37ºC overnight in 5 ml of TSBg. Then, each inoculum of *Staphylococcus* spp. and *P. aeruginosa* was adjusted to a titer of 10^8 CFU ml^{-1}, 100 µL was added to microplate wells, and 100 µL of TSBg was added to a set of wells as the negative control. The microplates were then incubated at 37°C for 24h for biofilm formation. All assays were performed in triplicate.

After incubation, the medium was removed and the wells were carefully washed twice with sterile saline solution (0.9% NaCl) to remove any planktonic cells. Microplates were allowed to dry, and were then stained with 100 µL of 0.1% crystal violet, left for 5 min, washed twice with sterile saline solution (0.9% NaCl) and 33% acetic acid added. Afterward, the biofilm was determined using a plate reader with the optical density at 550 nm (OD_{550}). Thus, the OD_{550} values obtained from the triplicates were tabulated and the mean was calculated for each sample.

Biofilm formation inhibition assay

Biofilm formation inhibition assay was performed according to (Kelly et al. 2012), with modifications. For *Staphylococcus* spp. and *P. aeruginosa* isolates, the inoculum of 100 µL of TSBg (10^8 CFU ml^{-1}) was dispensed in triplicate in the 96-well polystyrene plates with each of the isolates tested. In addition, 50 µL (10^6 PFU ml^{-1}) of each phage suspension (*Staphylococcus* phage B_UFSM1, *Staphylococcus* phage B_UFSM3, *Staphylococcus* phage B_UFSM4, *Staphylococcus* phage B_UFSM5, and both cocktails) were added and the microplates were incubated at 37°C for 24h. For the negative control, 100 µL of sterile TSBg and 50 µL of the phage suspension were added to the wells.

After incubation, the medium was removed and the wells were carefully washed twice with sterile saline (0.9% NaCl) to remove planktonic cells and remain only the sessile cells. The microplates were allowed to dry and were then stained with 100 µL of 0.1% crystal violet for 5 min., the dye was removed, and the plates were carefully washed twice with sterile saline. The material was resuspended with 100 µL of 33% acetic acid. Biofilm determination was performed on a spectrophotometer by determining the optical density at 550 nm (OD_{550}). Thus, the OD_{550} values obtained from the triplicates were tabulated and the mean was calculated for each sample. Afterward the data were calculated as follow: biofilm inhibition = mean of the treatment ÷ mean of the positive control.

Biofilm removal assay

The removal assay was performed according to (Kelly et al. 2012), with modifications. For *Staphylococcus* spp. and *P. aeruginosa*, 100 µL of TSBg (10^8 CFU ml^{-1}) were inoculated in triplicate in the 96-well polystyrene plates with each of the isolates tested. Thus, the microplates were incubated at 37°C for 24h for biofilm formation.

Following incubation, the medium was removed and the wells were carefully washed twice with sterile saline solution (0.9% NaCl) to remove the planktonic cells. Microplates were allowed to dry, and the phage suspension (10^6 PFU ml^{-1}) was added to each of the wells, being 100 µL of *Staphylococcus* phage B_UFSM1, *Staphylococcus* phage B_UFSM3, *Staphylococcus* phage B_UFSM4, *Staphylococcus* phage B_UFSM5, and both cocktails. The microplates were incubated at 37°C for 24h. The optical density at 550 nm (OD_{550}) was measured using a plate reader at 0, 2, 14, and 24h.

For the negative control, 100 µL of sterile TSBg was added to the wells. After incubation, the content was removed and 100 µL of the phage suspension was added for OD measured. In addition, the values obtained in triplicate were calculated and expressed as the mean ± standard deviations and positive controls.

Scanning electron microscopy (SEM)

Material preparation for scanning electron microscopy (SEM) was performed according to (Sillankorva et al. 2008), with adaptations. For biofilm inhibition the cocktail B with 100µL of each phage (B_UFSM1, B_UFSM3, B_UFSM 4, and B_UFSM5) were added with *S. aureus* ATCC 25923 before incubation and for biofilm removal after 24h of incubation in ATCC 25923. For the control of biofilm formation, 100 µL of a suspension containing *S. aureus* ATCC 25923was added, 24h before the microscope protocol. Subsequently, an aliquot of 10 µL was removed and transferred to a coverslip. Then, the samples were dried at room temperature and fixed with 2% glutaraldehyde for 2h. Dehydration of the material was performed in increasing ethanol concentrations, ranging from 30% to 100% for 10 min at each concentration. The coverslips were then dried in an oven. After the described process, the coverslips with biofilm were submitted to metallization by gold bath (approximately 15 nm thick). The samples were visualized under an electron microscope (JEOL, JSM 6360).

Statistical analysis

Comparisons between the values of the positive control and biofilm inhibition were performed using Kruskal-Wallis Test. The P value of ≤ 0.0001, ≤ 0.001 and ≤ 0.01 were considered statistically significant for *Staphylococcus* ssp., while the P value of <0.05 was considered statistically significant for *P. aeruginosa* between isolates and standard strain.

Results

Formation and inhibition of biofilm of *Staphylococcus spp.* and *P. aeruginosa* by *Staphylococcus aureus* phages

The biofilm formation and inhibition on *Staphylococcus* spp. and *P. aeruginosa* isolates and strains by the phages and both cocktails are shown in Tables 2 and 3. Statistically, the phages B_UFSM3 and B_UFSM4 did not promote the inhibition of biofilm formation in *Staphylococcus* spp. isolates and ATCC 25923 ($P \leq 0.0001$, $P \leq 0.001$ and $P \leq 0.01$). Additionally, only B_UFSM4 did not demonstrated inhibition in *P. aeruginosa* isolates and ATCC 25853 ($P<0.05$).

Removal of consolidated biofilms of *Staphylococcus spp.* by *S. aureus* phages

In *Staphylococcus* spp. biofilms, the phages B_UFSM1, B_UFSM3 and B_UFSM4 promoted decrease in the mean optical density values (OD_{550}) in bacterial isolates and ATCC 25923, after 24h of incubation (Fig 1). Although there was no significant difference in the values of optical density, it was possible to observe that the phage B_UFSM1 reduced the mean optical density values in most *Staphylococcus* spp. isolates (8/14) and ATCC 25923 (1/1) tested, especially in isolate MRSA2 (0.893 to 0.666), ATCC 25923 (0.829 to 0.700) and SB113/06 162 PE (0.539 to 0.413). However, the phage B_UFSM3 reduced the mean optical density only in isolate SB 235/08 (0.217 to 0.210) and phage B_UFSM4 in isolates SB 99/17 70 PE (0.423 to 0.392) and MRSA2 (0.724 to 0.688). The results for all isolates are shown in Figure 1.

The phage cocktails also reduced the mean optical density values (OD_{550}) in the biofilms produced by some *Staphylococcus* spp. isolates (Fig 2). In addition, the decrease in optical density was observed in the isolates SB 235/08 86 (0.461 to 0.319), MRSA 2 (0.711 to 0.658) and MRSA 3 (0.736 to 0.584) after 24h of incubation with cocktail A. The results for all isolates are shown in Figure 2.

Removal of consolidated biofilms of *Pseudomonas aeruginosa* by *S. aureus* phages

In *P. aeruginosa*, *S. aureus* phages B_UFSM3, B_UFSM4 and B_UFSM5 reduced the mean optical density (OD_{550}) in the biofilm of ATCC 25853. Using phage B_UFSM3 a slight reduction of 0.586 to 0.567, phage B_UFSM4 caused a reduction of 0.512 to 0.476, and phage B_UFSM5 generated a 0.588 to 0.457 reduction after 24h of incubation (Fig 3). In addition, cocktail A reduced the optical density of isolate SB 134/16 (0.763 to 0.733) (Fig 4).

Scanning electron microscopy (SEM)

In the SEM, it was possible to observe the biofilm formation and the effect of the treatments with cocktail B (B_UFSM1, B_UFSM3, B_UFSM4, and B_UFSM5) in *S. aureus* ATCC 25923 (Figure 5). The results observed in the SEM are consistent with that found in the microplate biofilm formation assays. Treatments with cocktail B promoted efficient activity both for inhibition and removal of the biofilm produced by *S. aureus* ATCC 25923, and consequently, SEM was performed on these representative samples, containing a mixture of phages and a standard strain. Notably, treatment with the phage cocktail had more effect on inhibiting biofilm than removing the biofilm produced by *S. aureus* ATCC 25923.

Discussion

Bovine mastitis is the most prevalent bacterial infection in livestock, consequently causing serious problems for the dairy industry. Prevetion and treatment of mastitis is based on antimicrobials, although increasing antimicrobial resistance has affected the efficiency of these conventional drugs (Aguayo et al. 2020). In addition, *S. aureus* and *P. aeruginosa* are important bacteria involved in the etiology of bovine mastitis, and both produce biofilms, making treatment difficult and contributing to the chronicity of disease (Notcovich et al. 2018; Aguayo

et al. 2020). Moreover, CNS is a relevant group of agents isolated from subclinical mastitis (Darwish and Asfour 2013).

In the biofilm inhibition assay, *S. aureus* phages (B_UFSM1 and B_UFSM5) and both cocktails showed activity to inhibit biofilms formation of *Staphylococcus* spp. isolates ($P \leq 0.0001$; $P \leq 0.001$ and $P \leq 0.01$) for up to 24h of inoculation. Our results are similar to data reported previously by who evaluated the prevention of *Staphylococcus* spp. biofilm formation using a phage K mixture and did not observe growth of *S. aureus* isolated from bovine mastitis. Another study demonstrated the inhibition of bacterial growth with the treatment of a single phage K and phage mixture (phage K and a new phage DRA88). In general, the phage mixture proved to be more effective than a single phage (Alves et al. 2014).

In our study, biofilm removal was observed based on the visualization of the optical density (OD_{550}) in *Staphylococcus* spp. isolates and strain after 24h of treatment. The phage B_UFSM1 reduced mean optical density values in eight isolates and an ATCC. However, it was observed that both phages, B_UFSM3 and B_UFSM4, and cocktails slightly reduced the OD values in removing biofilms consolidated in a few bacterial isolates evaluated. In addition, both phages and cocktails showed no activity to remove biofilms produced by standard strain used in the assays.

Alves et al. (2014) also evaluated the removal of consolidated biofilms through biofilm density measurement (OD_{590}) using crystal violet and after 48h incubation, the authors verified a clear reduction of three *S. aureus* isolates after *S. aureus* phage K inoculation compared to untreated control. In another study, (Kelly et al. 2012) observed that biofilm produced by *S. aureus* (OD_{590}), reduced when submitted to phage cocktail treatment for more than 36h although the main reduction in OD_{590} occurred 15-20h after cocktail inoculation.

In the biofilm inhibition assay, all *P. aeruginosa* biofilms were inhibited ($P<0.05$) after almost all phage treatments. Only phage B_UFSM4 did not have action against *P. aeruginosa* biofilms. In the assay removing the consolidated *P. aeruginosa* biofilm, the phages B_UFSM3, B_UFSM4 and B_UFSM5 promoted a reduction in the optical density of ATCC 25853 and isolate SB136/16 when the phages were applied individually. Cocktail A also promoted biofilm reduction of isolate SB136/16, but cocktail B did not promote biofilm reduction. Recent studies demonstrated that treatments with *P. aeruginosa* phages reduced the biomass of bacterial biofilms in 24 and 74h compared to the control (Fong et al. 2017; Adnan et al. 2020).

In the SEM, it was possible to confirm the biofilm production by *S. aureus* ATCC 25923 and the activity of the cocktail B in inhibiting biofilm formation and removing the consolidated biofilms, thus corroborating Song et al. (2021). As shown in Figure 5, there was a reduction of bacterial aggregates of *S. aureus* and presence of cellular debris after treatment with the phage cocktail in inhibiting biofilm formation (Fig 5b) and removing the consolidated biofilms (Fig 5c) compared to the cellular organization observed in the biofilm without treatment (Fig 5a). These samples were chosen for this assay due to a greater representativeness, both of the strong biofilm formation by the ATCC and of the antibiofilm activity of the evaluated phages.

The phages presented properties that make biofilms susceptible to their action, since high quantities of bacteria in biofilms facilitate the action of phages, allowing rapid and efficient infection of the host and consequent amplification of the phage, which produces enzymes that degrade the extracellular matrix. Furthermore, the phages can infect persistent cells and remain dormant inside them, reactivating after becoming metabolically active (Harper et al. 2014).

In this study, with exception of phage B_UFSM4, the phages and cocktails demonstrated antibiofilm activity, mainly promoting biofilm formation inhibition in isolates from bovine mastitis and *P. aeruginosa* and *Staphylococcus* spp. and standard strains. The potential of using phages to prevent and control bacterial biofilms still requires further studies, as there is little data on the effects of phages on biofilms, especially in biofilms by multispecies, since the phages through their lysis mechanisms do not require bacterial specificity in biofilms (Geredew Kifelew et al. 2019). Additionally, the biofilm formation inhibition by phages, can prevent bacterial resistance, thus avoiding multidrug-resistant strains (Di Somma et al. 2020).

Therefore, the phages (B UFSM1, B UFSM3 and B UFSM5) and cocktails should be further analyzed to verify their applicability in inhibiting biofilm formation by *Staphylococcus* spp. and *P. aeruginosa*. Moreover, the phages B_UFSM4 and B_UFSM5 presented little expressive results when compared to the other phages, therefore, we tried to join these phages in a cocktail to evaluate if there would be an increase in their anti-biofilm activity it was verified a better efficiency in inhibiting biofilms when the two phages were used together (cocktail A). Notably, *S. aureus* phages included in this study, individually or in combination, were effective in inhibiting biofilms produced by a Gram-negative bacterium. Therefore, demonstrating broad antibiofilm activity and a possible expansion of bacterial host.

In conclusion, *Staphylococcus aureus* phages tested in this study presented activity against *Staphylococcus* spp. and *P. aeruginosa* bacterial biofilms. *S. aureus* phages, individually or in combination, inhibit the formation of consolidated biofilms. Thus, this study demonstrates the possibility of using these phages to inhibit biofilms. However, as these phages are temperate, future studies must be performed to verify their applicability in bacterial biofilms, as well as in the removal of consolidated biofilm should be performed.

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Declarations

Conflict of interest

There are no conflicts of interest.

Ethics approval

Not applicable.

Captions

Fig 1 Biofilm removal (OD_{550}) of *Staphylococcus* spp. isolates and standard strain (ATCC 25923) by *S. aureus* phages from bovine mastitis. Biofilm removal by phage B_UFSM1 (a); B_UFSM3 phage (b); B_UFSM4 phage (c); and B_UFSM5 phage (d).

Fig 2 Biofilm removal (OD_{550}) of *Staphylococcus* spp. isolates and standard strain (ATCC 25923) by *S. aureus* phages from bovine mastitis. Removal biofilm by cocktail A (phages B_UFSM4 and B_UFSM5) (a) and cocktail B (phages B_UFSM1; B_UFSM3; B_UFSM4 and B_UFSM5) (b).

Fig 3 Biofilm removal (OD_{550}) of *P. aeruginosa* isolates and standard strain (ATCC 25853) by *S. aureus* phages from bovine mastitis. Biofilm removal by phage B_UFSM1 (a); B_UFSM3 phage (b); B_UFSM4 phage (c); and B_UFSM5 phage (d).

Fig 4 Biofilm removal (OD_{550}) of *P. aeruginosa* isolates and standard strain (ATCC 25853) by *S. aureus* phages from bovine mastitis. Removal biofilm by cocktail A (phages B_UFSM4 and B_UFSM5) (a) and cocktail B (phages B_UFSM1; B_UFSM3; B_UFSM4 and B_UFSM5) (b).

Fig 5 Scanning electron microscopy of *S. aureus* ATCC 25923 biofilms grown under static conditions at 37°C for 24h. Control untreated (a). Biofilm removal with cocktail B for 24h (b) and inhibition with cocktail B for 24h(c).

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Table1 Isolates and strains used in the tests and analyze of the phage's infectivity.

Bacterial species	**Isolates /strain**	**Lysis***
Coagulase Positive *Staphylococcus* (CPS)	SB 93/16 340 PE	-
	MRSA 2	+
	MRSA 3	+
	SB 113/06 162 PE	+
	SB 235/08 86	+
	SBP 17/20 312 AD	+
	SBP 01/19 Gaivota PD	+
S. aureus	ATCC 25923	-
Coagulase Negative *Staphylococcus* (CNS)	SB 57/17 49 PD	+
	SB 47/12 252 AD	-
	SB 99/17 70 PE	-
	SB 52/17 PD	_
	SB 105/94 Pintada PD	+
	SB 99/17 30 PD	_
	SB 39/10 008 PE	+
P. aureuginosa	SBP 120/19 Frida PD	+
	SBP120/19 Frida AD	+
	SB 134/16	+
	ATCC 25853	-

*Bacterial isolates and strains lysates and susceptible of the phage's infectivity by spot test; (-) absense and (+) presence of lysis.

Table 2 Values of *Staphylococcus* spp. biofilm formation and *Staphylococcus* spp. biofilm inhibition with the addition of each of the phages and cocktails.

Bacterium	Biofilm Formation[#]	Phages/Cocktails					
		B_UFSM1	B_UFSM3	B_UFSM4	B_UFSM5	Cocktail A[1]	Cocktail B[2]
		Biofilm Inhibition[##]					
ATCC 25923	39.97	3.50*	12.20	33.34	14.40***	6.49**	2.14*
SBP 01/19 Gaivota PD	9.72	1.04*	2.43	12.22	4.91***	2.15**	0.88*
SB 113/06 162 PE	5.00	0.40*	5.73	9.57	4.07***	1.56**	0.86*
MRSA 2	12.25	0.68*	19.72	7.86	6.94***	2.11**	1.01*
MRSA 3	5.92	2.20*	20.86	10.61	4.81***	1.82**	1.27*
SB 47/12 252 AD	10.87	1.56*	6.08	11.49	7.61***	3.10**	0.80*
SB 93/16 340 PE	8.62	3.74*	19.63	6.17	3.85***	5.12**	1.85*
SB 57/17 49 PD	36.37	6.15*	35.61	14.66	18.53***	11.11**	5.86*
SB 39/10 008 PE	6.72	0.14*	2.75	3.50	5.88***	3.46**	0.20*
SB 105/94 Pintada PD	6.17	1.94*	2.64	9.09	3.32***	2.36**	0.56*
SB 99/17 70PD	25.72	0.89*	5.03	5.37	4.75***	20.43**	0.60*
SB 235/08 86	5.87	4.56*	6.68	3.18	3.94***	4.28	1.04*
SB 52/17 PD	8.01	3.54*	3.76	12.10	8.91***	4.75	1.58*
SBP 17/20 312 AD	13.23	3.33*	1.94	6.98	3.07***	2.27	0.98*
SB 99/17 30PD	14.71	4.43*	4.04	9.77	8.79***	3.85	1.76*

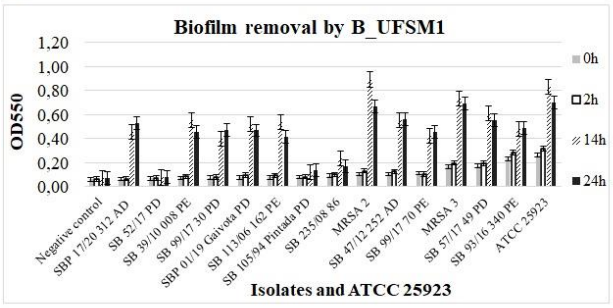
[#]For the values of biofilm formation, the optical density readings were averaged at 550 nm (OD_{550}), in triplicates, for each bacterium. [##]To obtain the biofilm inhibition values, the following calculation was performed: treatment average ÷ positive control average. [1]Cocktail A (B_UFSM4 and B_UFSM5). [2]Cocktail B (composed of all four phages). *P≤ 0.0001; **P≤ 0.001 and ***P≤ 0.01.

Table 3 Values of *P. aeruginosa* biofilm formation and *P. aeruginosa* biofilm inhibition with the addition of each of the phages and cocktails.

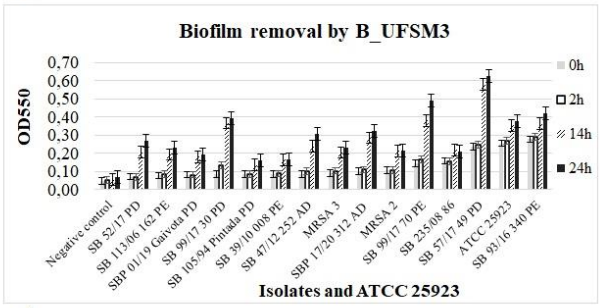
Bacterium	**Biofilm fomation[#]**	**Phages/Cocktails**					
		B_UFSM1	**B_UFSM3**	**B_UFSM4**	**B_UFSM5**	**Cocktail A[1]**	**Cocktail B[2]**
		Biofilm Inhibition [##]					
ATCC 25853	10.18	2.40*	6.66*	11.39	7.17*	5.78*	2.79*
SBP 120/19 Frida AD	9.99	1.24*	2.09*	2.76	5.03*	2.72*	1.28*
SBP 120/19 Frida PD	9.04	1.44*	1.39*	3.13	4.12*	3.15*	2.28*
SB 136/16	8.92	0.94*	2.15*	1.98	6.70*	3.02*	1.68*

[#]For the values of biofilm formation, the optical density readings were averaged at 550 nm (OD_{550}), in triplicates, for each bacterium. [##]To obtain the biofilm inhibition values, the following calculation was performed: treatment average ÷ positive control average. [1]Cocktail A (B_UFSM4 and B_UFSM5). [2]Cocktail B (composed of all four phages). *$P \leq 0.05$.

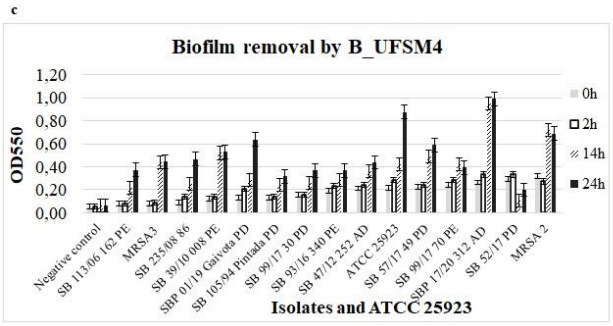
a


Biofilm removal by B_UFSM1
OD550
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0h
2h
14h
24h
Negative control
SBP 17/20 312 AD
SB 52/17 PD
SB 39/10 008 PE
SB 99/17 30 PD
SBP 01/19 Gaivota PD
SB 113/06 162 PE
SB 105/94 Pintada PD
SB 235/08 86
MRSA 2
SB 47/12 252 AD
SB 99/17 70 PE
MRSA 3
SB 57/17 49 PD
SB 93/16 340 PE
ATCC 25923
Isolates and ATCC 25923

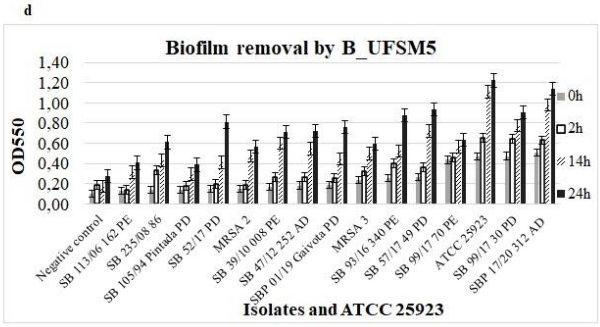
b


Biofilm removal by B_UFSM3
OD550
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
0h
2h
14h
24h
Negative control
SB 52/17 PD
SB 113/06 162 PE
SBP 01/19 Gaivota PD
SB 99/17 30 PD
SB 105/94 Pintada PD
SB 39/10 008 PE
SB 47/12 252 AD
MRSA 3
SBP 17/20 312 AD
MRSA 2
SB 99/17 70 PE
SB 235/08 86
SB 57/17 49 PD
ATCC 25923
SB 93/16 340 PE
Isolates and ATCC 25923

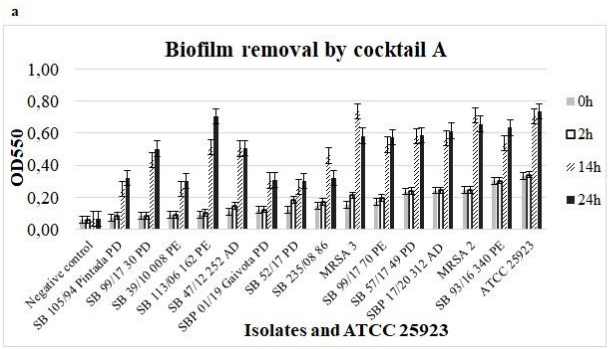
c


Biofilm removal by B_UFSM4
OD550
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0h
2h
14h
24h
Negative control
SB 113/06 162 PE
MRSA3
SB 235/08 86
SB 39/10 008 PE
SBP 01/19 Gaivota PD
SB 105/94 Pintada PD
SB 99/17 30 PD
SB 93/16 340 PE
SB 47/12 252 AD
ATCC 25923
SB 57/17 49 PD
SB 99/17 70 PE
SBP 17/20 312 AD
SB 52/17 PD
MRSA 2
Isolates and ATCC 25923

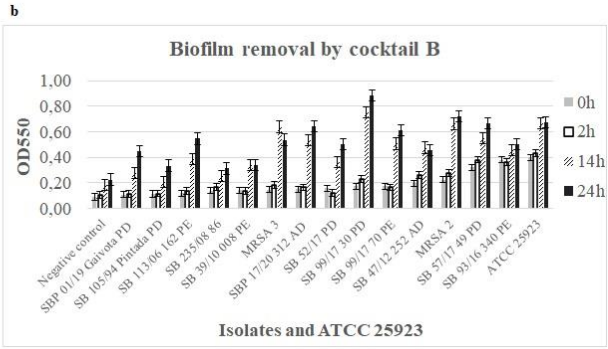
d


Biofilm removal by B_UFSM5
OD550
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0h
2h
14h
24h
Negative control
SB 113/06 162 PE
SB 235/08 86
SB 105/94 Pintada PD
SB 52/17 PD
MRSA 2
SB 39/10 008 PE
SB 47/12 252 AD
SBP 01/19 Gaivota PD
MRSA 3
SB 93/16 340 PE
SB 57/17 49 PD
SB 99/17 70 PE
ATCC 25923
SB 99/17 30 PD
SBP 17/20 312 AD
Isolates and ATCC 25923

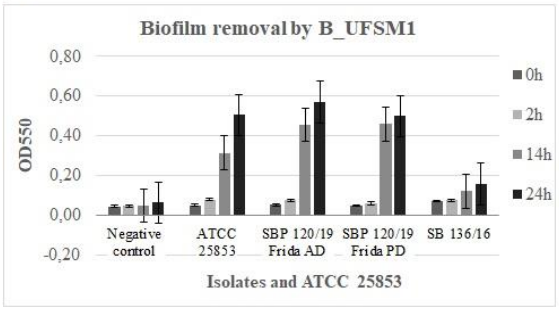
a


Biofilm removal by cocktail A
OD550
1,00
0,80
0,60
0,40
0,20
0,00
0h
2h
14h
24h
Negative control
SB 105/94 Pintada PD
SB 99/17 30 PD
SB 39/10 008 PE
SB 113/06 162 PE
SB 47/12 252 AD
SBP 01/19 Gaivota PD
SB 52/17 PD
SB 235/08 86
MRSA 3
SB 99/17 70 PE
SB 57/17 49 PD
SB 17/20 312 AD
MRSA 2
SB 93/16 340 PE
ATCC 25923
Isolates and ATCC 25923

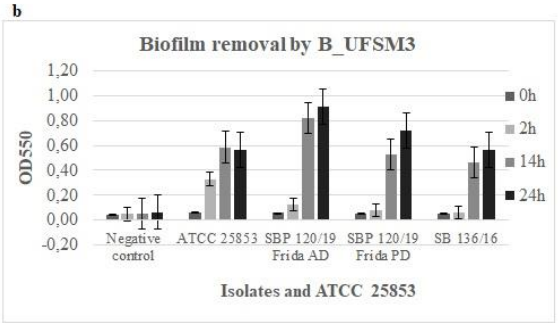
b


Biofilm removal by cocktail B
OD550
1,00
0,80
0,60
0,40
0,20
0,00
0h
2h
14h
24h
Negative control
SBP 01/19 Gaivota PD
SB 105/94 Pintada PD
SB 113/06 162 PE
SB 235/08 86
SB 39/10 008 PE
MRSA 3
SBP 17/20 312 AD
SB 52/17 PD
SB 99/17 30 PD
SB 99/17 70 PE
SB 47/12 252 AD
MRSA 2
SB 57/17 49 PD
SB 93/16 340 PE
ATCC 25923
Isolates and ATCC 25923

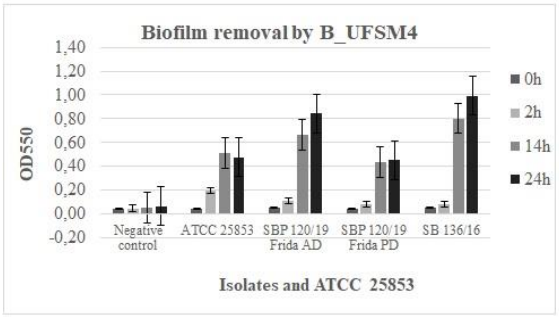
a


Biofilm removal by B_UFSM1
OD550
0,80
0,60
0,40
0,20
0,00
-0,20
0h
2h
14h
24h
Negative control
ATCC 25853
SBP 120/19 Frida AD
SBP 120/19 Frida PD
SB 136/16
Isolates and ATCC 25853

b


Biofilm removal by B_UFSM3
OD550
1,20
1,00
0,80
0,60
0,40
0,20
0,00
-0,20
0h
2h
14h
24h
Negative control
ATCC 25853
SBP 120/19 Frida AD
SBP 120/19 Frida PD
SB 136/16
Isolates and ATCC 25853

c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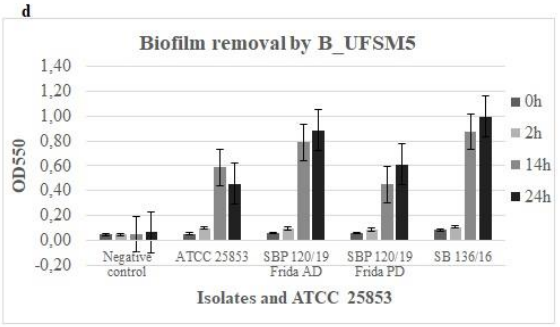
1,20
1,00
0,80
0,60
0,40
0,20
0,00
-0,20
0h
2h
14h
24h
Negative control
ATCC 25853
SBP 120/19 Frida AD
SBP 120/19 Frida PD
SB 136/16
Isolates and ATCC 25853

d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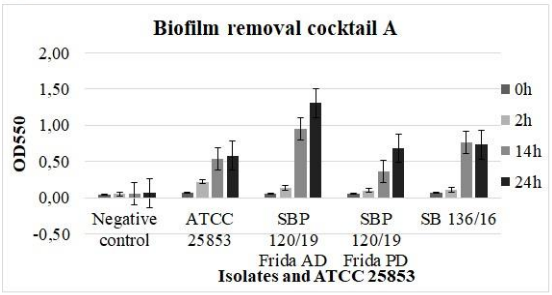
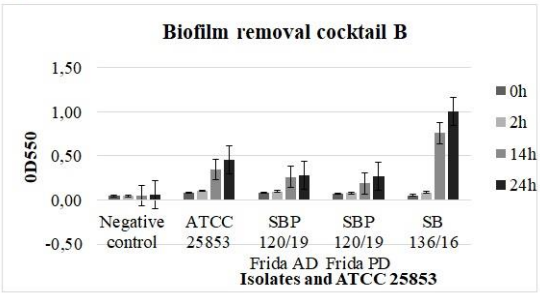
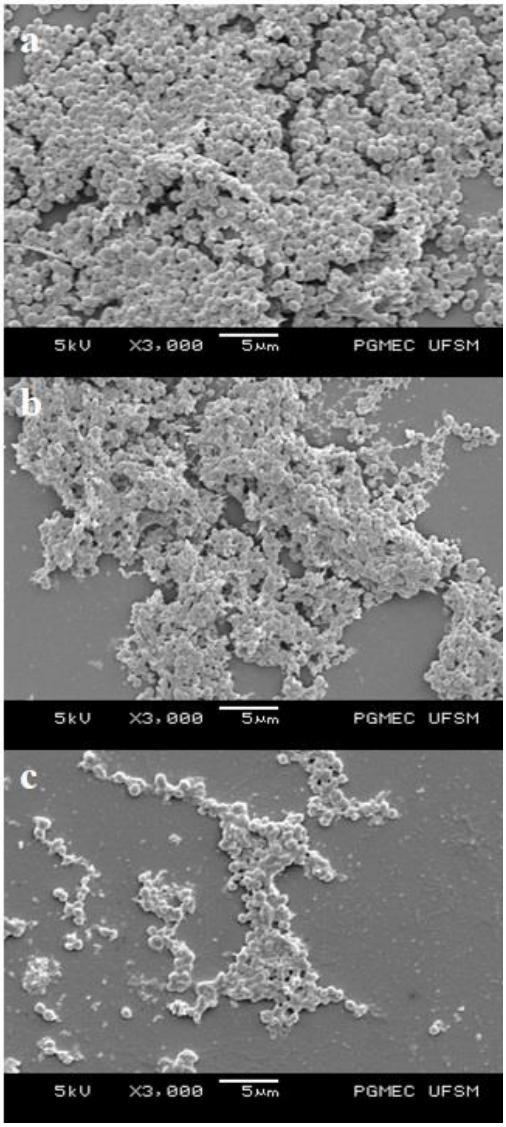
OD550
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
-0,20
0h
2h
14h
24h
Negative control
ATCC 25853
SBP 120/19 Frida AD
SBP 120/19 Frida PD
SB 136/16
Isolates and ATCC 25853

a


Biofilm removal cocktail A
OD550
2,00
1,50
1,00
0,50
0,00
-0,50
0h
2h
14h
24h
Negative control
ATCC 25853
SBP 120/19 Frida AD
SBP 120/19 Frida PD
SB 136/16
Isolates and ATCC 25853

b


Biofilm removal cocktail B
OD550
1,50
1,00
0,50
0,00
-0,50
0h
2h
14h
24h
Negative control
ATCC 25853
SBP 120/19 Frida AD
SBP 120/19 Frida PD
SB 136/16
Isolates and ATCC 25853


a
5kV X3,000 5µm PGMEC UFSM
b
5kV X3,000 5µm PGMEC UFSM
c
5kV X3,000 5µm PGMEC UFSM

6. DISCUSSÃO

O conceito de terapia fágica para tratar infecções bacterianas surgiu logo após a descoberta dos fagos, há mais de um século. Entretanto, com a descoberta dos antibióticos, o interesse na terapia fágica diminuiu ao longo dos anos. Todavia, a pesquisa e o uso dos fagos foram se reestabelecendo com o passar do tempo, através do uso de fagos para o controle biológico na área da agricultura e indústria de alimentos, e o uso de fagos como vetores virais na biologia molecular (MULUGETA; TESFAYE; TESFAYE, 2018) e, principalmente, por meio do *phage display*, que é uma tecnologia usada para sintetizar polipeptídeos (GP, 1985; SIDHU, 2000).

Diante disso, os pesquisadores estão trabalhando no intuito de reviver a técnica centenária do uso de fago para a terapêutica de infecções bacterianas e como uma opção para contornar a resistência aos antimicrobianos convencionais (MULUGETA; TESFAYE; TESFAYE, 2018). Adicionalmente, a terapia fágica em infecções bacterianas em humanos é utilizada em países do Leste Europeu como a Polônia, na Rússia e Geórgia (SALMOND; FINERAN, 2015).

A literatura científica contém informações a respeito de diversos fagos isolados de mastite bovina. Isto indica que o problema não está relacionado em encontrar fagos isolados contra bactérias específicas, mas em selecionar os prováveis fagos para fins terapêuticos. Isso inclui fagos líticos que têm alta eficácia e ampla atividade em isolados bacterianos clínicos de importantes enfermidades, como os agentes de mastite bovina. Na seleção de fagos terapêuticos, também é relevante avaliar a capacidade dos fagos lisarem isolados bacterianos mutantes que apresentaram resistência a outros fagos (OECHSLIN, 2018). Para resolver este problema, o uso de coquetéis multi-fagos específicos para um único patógeno tem sido estudado para minimizar o desenvolvimento de mutantes resistentes aos fagos (SHARMA, 2013).

Desta forma, nesta tese avaliou-se a eficiência e a especificidade dos fagos de *S. aureus*, isolados e caracterizados, provenientes de fazendas leiteiras empregando-se uma meta-análise, descrita no Capítulo 1. Dentre todos os fagos avaliados no estudo (n=603), verificou-se que a maioria dos fagos de *S. aureus* são específicos e possuem uma alta eficiência (80%) contra os isolados de *S. aureus* oriundos de mastites bovinas. Sendo que, alguns fagos apresentavam a capacidade de infectar outros gêneros e espécies de bactérias Gram-positivas. Adicionalmente, todos os fagos eram de cauda e pertecentes às famílias *Myoviridae* e *Siphoviridae*. Todavia, a maioria dos estudos que integraram a meta-análise não demonstravam a análise completa do genoma dos fagos; portanto, não foi possível averiguar se todos os fagos analisados eram

temperados ou líticos, exceto para os fagos vB_SauM_JS25, MSA6, SAJK- IND e MSP os quais foram descritos como líticos. Sendo assim, sugere-se que esses bacteriófagos poderiam ser utilizados futuramente em testes *in vivo* para verificar a sua aplicabilidade na terapêutica da mastite bovina, uma vez que, não existem muitos estudos analisando a eficácia dos fagos nesta enfermidade.

Antes de serem empregados como agentes terapêuticos, os fagos devem ser amplamente avaliados, incluindo estudos de identificação, atividade antibacteriana e investigação da atividade lítica (TURNER; DRAGHI; WILPISZESKI, 2012). Além disso, deve-se pesquisar as características fenotípicas e genotípicas de cada fago a ser empregado no combate de infecções, especialmente as causadas pelas bactérias resistentes aos antimicrobianos. Sendo assim, neste estudo, foi possível isolar quatro novos fagos de *S. aureus* a partir de leite bovino positivo para mastite (B_UFSM1, B_UFSM3, B_UFSM4 e B_UFSM5). Desta forma, foi descrito na seção referente ao Capítulo 2, o isolamento de dois novos bacteriófagos de *S. aureus*, B_UFSM4 e B_UFSM5, que foram amplamente caracterizados, e evidenciou-se a eficiência destes frente aos isolados hospedeiro-específico, bem como sua capacidade de infectar outros gêneros bacterianos como, *P. aeruginosa* e *R. terrae.* Os vírus isolados também foram capazes de lisar bactérias de outras espécies de *Staphylococcus* incluindo *S. sciuri*. Entretanto, através do teste de eficiência de plaqueamento (EOP), teste com maior sensibilidade, verificou-se alta eficiência de ambos os fagos sobre o isolado de *R. terrae*. Portanto, os fagos caracterizados demonstram amplitude de hospedeiro bacteriano. Sendo assim, é interessante obter um fago com amplitude de hospedeiro que atue sobre várias bactérias patogênicas e não apresentasse atividade contra as bactérias pertencentes à microbiota. Além disso, se um determinado fago for altamente específico a uma espécie bacteriana, o seu uso se restringiria ao grupo pequeno de bactérias (NILSSON, 2014; MAPES et al., 2016). Então, para verificar a aplicabilidade de determinado fago no controle de determinada enfermidade, seria necessário um diagnóstico mais especifico.

Adicionalmente, pela caracterização genômica, foi possível verificar que os fagos B_UFSM4 e B_UFSM5 possuem o gene codificador da integrase. Sendo assim, é possível inferir que ambos os vírus possuem a capacidade de inserir seu material genético no genoma do hospedeiro, realizando o ciclo lisogênico. Todavia, não foi identificado nenhum gene de resistência e/ou virulência em ambos os genomas. Desta forma, os fagos B_UFSM4 e B_UFSM5 isolados e caracterizados neste estudo, são fagos temperados. Embora um fago lítico seja mais adequado como um fago terapêutico do que os temperados, devido às complicações relacionadas à lisogenia (HOSHIBA et al., 2010), não se deve descartar o uso de fagos

temperados na terapêutica clínica, pois dado o árduo processo de isolamento e carcaterização de bacteriófagos, principalmente, os de bactérias Gram-positivas.

Devido à pandemia COVID-19, ocorreram atrasos nos prazos estabelecidos para a finalização da avaliação e caracterização morfológica e molecular, dos fagos B_UFSM1 e B_UFSM3; sendo assim, estes resultados não puderam ser incluídos nesta tese. Entretanto, os mesmos estão sob análises visando futuras publicações científicas para enriquecer o arsenal de conhecimento gerado com a pesquisa sobre os fagos oriundos de mastite bovina.

Estudos têm apontado que os fagos também podem ser considerados como alternativas promissoras para o controle de biofilmes bacterianos, uma vez que, demonstraram alta eficiência na inibição e remoção de biofilmes bacterianos (FERRIOL-GONZÁLEZ; DOMINGO-CALAP, 2020). Portanto, neste estudo, também foi analisada a capacidade dos fagos temperados isolados e duas combinações de fagos, os coquetéis: A e B em inibir e remover biofilmes produzidos por isolados de *Staphylococcus* spp. e *P. aureginosa* provenientes de mastite bovina. Os resultados desta pesquisa, apresentados no Manuscrito 2, apontaram que principalmente o fago B_UFSM1 e o coquetel B, contendo a mistura dos quatro fagos isolados, foram capazes de inibir eficientemente a maioria dos isolados de *Staphylococcus* spp. de mastites bovinas, bem como os fagos B_UFSM4 e B_UFSM5 foram capazes de inibir a ATCC 25853 de *P. aureginosa*. Contudo, o fago B_UFSM4 sozinho, não demonstrou efetividade na inibição dos biofilmes das bactérias testadas.

Em relação a remoção dos biofilmes das bactérias testadas, os fagos B_UFSM3, B_UFSM4, B_UFSM5 e coquetel A, demonstram a capacidade de remover biofilmes produzidos pela cepa bacteriana de *P. aureginosa*. Por outro lado, os fagos B_UFSM1 e B_UFSM4 removeram os biofilmes produzidos por isolados de *Staphylococcus* spp. de mastite bovina. Ambas as combinações de fagos foram eficientes na remoção dos biofilmes produzidos por *Staphylococcus* spp. de alguns isolados clínicos testados. Sendo assim, os fagos estudados nessa pesquisa demonstraram capacidade de remover biofilmes produzidos por bactérias envolvidas na mastite bovina. Isto é importante, uma vez que as maiorias dos antimicrobianos não possuem efeito nos biofilmes; entretanto, os fagos são capazes de infectar a matriz extracelular e promover a remoção dos biofilmes bacterianos.

De acordo com Sillamkorva et al. (2011), além da capacidade de fagos de removerem efetivamente os hospedeiros do substrato, podem também, causar danos parciais em hospedeiro não suscetível, ou seja, hospedeiro de outro gênero bacteriano. Portanto, neste estudo foi averiguada a capacidade dos fagos temperados de *S. aureus* de inibir biofilmes bacterianos de

sua espécie hospedeira e, também, de outro gênero bacteriano, demonstrando possível perda da especificidade de hospedeiro no biofilme bacteriano.

Nesta pesquisa foi possível isolar e avaliar fagos temperados de *S. aureus* como alternativa no controle de biofilmes bacterianos, bem como, os fagos como potenciais opções a serem estudados para a terapêutica das infecções intramamárias bovinas, isoladamente ou em combinações. Adicionalmente, ressalta-se que os resultados obtidos indicam que ambos os fagos temperados isolados e caracterizados (B_UFSM4 e B_UFSM5) podem ser uma alternativa antimicrobiana a ser futuramente analisada e explorada para o tratamento da mastite nas fêmeas bovinas ocasionadas por *S. aureus*.

7. **CONCLUSÕES**

- Na meta-análise avaliou-se vários estudos sobre isolamento de fagos de *Staphylococcus aureus*, bem como testes *in vitro* de eficácia ou infectividade contra isolados bacterianos provenientes de mastites bovinas. A maioria dos fagos de *S. aureus* foram específicos e demonstram alta atividade lítica contra seu hospedeiro alvo. Todavia, a maioria dos estudos não demonstrou intervalo ou amplitude de hospedeiro. De forma geral, todos os fagos descritos na meta-análise são eficientes e podem ser utilizados em futuras pesquisas *in vivo* com mastites bovinas causadas por *S. aureus*.
- Na meta-análise, pode-se observar que poucos são os estudos *in vivo* avaliando a eficiência destes vírus. Sendo assim, pesquisas desta natureza são requeridas a fim de avaliar o uso de fagos em infecções intramamárias bovinas.
- Nessa pesquisa, obteve-se os bacteriófagos temperados B_UFSM1, B_UFSM3, B_UFSM4 e, B_UFSM5 de origem de leite bovino com mastite subclínica oriundos da região central do Rio Grande do Sul, Brasil.
- Os fagos temperados de *S. aureus* (B_UFSM4 e B_UFSM5) isolados foram amplamente caracterizados fenotípica e genotipicamente e, pertecem a ordem *Caudovirales*, família *Siphoviridae* e genêro *Biseptimavirus*. Adicionalmente, verificou-se que os novos fagos temperados B_UFSM4 e B_UFSM5 são altamente similares entre si e através das análises filogenéticas, apresentam similaridade com os fagos de *Staphylococcus* tp310_1, 13, phiPV83 e JS01.
- Os fagos B_UFSM4 e B_UFSM5, demonstraram atividade lítica contra isolados bacterianos de mastites bovinas, principalmente, contra isolados de *Staphylococcus* coagulase positiva. Adicionalmente, os dois fagos possuem a capacidade de infectar outras espécies bacterianas, como *Rothiae terrae*, evidenciada pelo teste de eficiência de plaqueamento (EOP). No entanto, os vírus demonstram baixa capacidade de infectividade frente aos isolados de *Pseudomonas aureginosa* proveniente de mastite bovina. Notavelmente, os fagos demonstram infectividade em isolados clínicos com resistência antimicrobiana.
- Em relação a capacidade de inibir os biofilmes de *Staphylococcus* spp. e *P. aureginosa*, os fagos B_UFSM1, B_UFSM3 e B_UFSM5 e o coquetel A (B_UFSM1, B_UFSM3, B_UFSM4, B_UFSM5) são os mais eficientes. Contudo, o fago B_UFSM4 não demonstra atividade antibiofilme nos isolados de *Staphylococcus* spp. e *P. aeuroginosa* provenientes de mastite bovina.

- Com o desenvolvimento desta pesquisa, ressalta-se as perspectivas da fagoterapia como alternativa promissora no controle da mastite bovina, especialmente, causadas por *S. aureus* resistentes aos antimicrobianos comumente empregados na rotina veterinária. Adicionalmente, demonstra-se que os fagos podem ser empregados para o controle de biofilmes bacterianos produzidos por *S. aureus* provenientes de mastites bovinas. Contudo, pesquisas adicionais *in vitro* e *in vivo* envolvendo os fagos temperados, isolados nesse estudo, devem ser conduzidas a fim de averiguar a utilização desses fagos na terapêutica da mastite bovina por *S. aureus.*

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