UNIVERSIDADE ESTADUAL DE FEIRA DE SANTANA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

DANITZA XIOMARA ROMERO CALLE

Isolamento e caracterização de óleos essenciais e bacteriófagos como tratamentos de controle biológico para *Salmonella enterica*

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia, da Universidade Estadual de Feira de Santana como requisito parcial para obtenção do título de Doutor em Biotecnologia.

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FEIRA DE SANTANA – BA

2022

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Feira de Santana – BA

2022

Ficha Catalográfica - Biblioteca Central Julieta Carteado - UEFS

C161

Calle, Danitza Xiomara Romero

Isolamento e caracterização de óleos essenciais e bacteriófagos como tratamentos de controle biológico para Salmonella entérica / Danitza Xiomara Romero Calle. – 2022.

166 f.: il.

Orientador: Aristóteles Góes Neto.

Coorientador: Craig Billington.

Tese (doutorado) – Universidade Estadual de Feira de Santana, Programa de Pós-graduação em Biotecnologia, Feira de Santana, 2022.

1. Bacteriófagos. 2. Biotecnologia. 3. Salmonella enterica. I. Título. II. Góes Neto, Aristóteles, orient. III. Billington, Craig, coorient. IV. Universidade Estadual de Feira de Santana.

CDU: 576.58.9

Renata Aline Souza Silva - Bibliotecária - CRB-5/1702

AGRADECIMIENTOS

Agradeço a Deus quem guia o meu caminho, a minha família: meus pais Lidia Calle Mamani e Hugo Romero Chuca, a minhas irmãs Lizeth e Gabriela, meus irmãos Hugo e Miguel e a minhas sobrinhas Zaciel e Zoe.

Fico muito grata aos meus orientadores, professores: Atistóteles Góes Neto, sem ele eu não teria feito o doutorado nem a tese e o Craig Billington, sem ele não seria possível desenvolver a pesquisa em bacteriófagos e focar nos objetivos. Eles são admiráveis e excelentes profissionais:

Aos professores que me orientaram e me deram dicas na parte metodológica da tese: Gabriel Magno, Maria Angelica Luchesse, Raquel Guimarães Benevides e Alexandro Branco.

Aos meus amigos que foram como uma família para mim: Edgar, Luz Helena, Isabel, Fabiola, Edjane, Yareliz, Claudiana, Katty, Jesús, Fabian e Gustavo.

Aos meus colegas dos grupos de pesquisa, onde aprendi muito: LAPEM, LBMCF e o Laboratório de Fitoquímica da UEFS, tem tanta gente boa que não gostaria de esquecer ninguém.

Meus agradecimentos também para a coordenadora do Programa de Pós Graduação em Biotecnologia, professora Soraya Castro Trinidade e o secretário Alberto Vicente Silva.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

RESUMO

Antecedentes: As doenças transmitidas por alimentos são um problema de saúde pública global, 1 de cada 10 pessoas adoecendo após ingerir alimentos contaminados todos os anos. No Brasil, *Salmonella* enterica sorovar Enteritidis é uma ameaça significativa na saúde publica. Bacteriófagos (fagos; vírus de bactérias), têm potencial para serem usados como métodos antimicrobianos naturais para controlar patógenos bacterianos como *Salmonella* spp.

Objetivos: A tese tem cinco capítulos, os objetivos de cada capítulo foram: 1) Desenvolver uma revisão narrativa sobre as aplicações da terapia fágica para várias doenças infecciosas, farmacologia dos fagos, respostas imunológicas aos fagos, preocupações legais e os potenciais benefícios e desvantagens deste novo tratamento. 2) Desenvolver uma revisão sistemática e meta-análise que avaliou a eficiência de fagos patenteados como controle biológico de patógenos de origem alimentar e determinou as características físico-químicas do efeito antimicrobiano. 3) Determinar a atividade antimicrobiana de dezessete extratos vegetais, óleos essenciais comerciais, carvacrol e timol contra *Salmonella* ATCC 14028. 4) Descrever a caracterização fenotípica e genômica do isolado da cepa *Salmonella* SE3 do Rio Subaé, Santo Amaro, BA-Brasil. 5) Descrever a caracterização fenotípica e genômica do fago SF1, isolado do Rio Subaé, Santo Amaro, BA-Brasil. Determinamos a gama de hospedeiros do fago SF1 contra cepas de *Salmonella* e outras espécies de bactérias.

Resultados: A revisão sistemática foi desenvolvida utilizando bases de artigos científicos e de patentes com critérios de inclusão e exclusão aplicados por processos automáticos e manuais. Uma meta-análise de efeitos aleatórios foi realizada e revelou: (i) efeito antimicrobiano significativo de fagos de *Listeria* em maçã, suco de maçã, pêra e suco de pêra (ii) efeito antimicrobiano significativo de fagos de *Salmonella* em ovos, maçã e frango pronto para cozinhar, (iii) nenhuma heterogeneidade foi identificada em qualquer meta-análise, (iv) viés de publicação foi detectado em fagos de Listeria, mas não em fagos de *Salmonella.* (v) Os fagos ListShield e Felix01 apresentaram o melhor resultado para o controle biológico de *Listeria* e *Salmonella*, respectivamente, (vi) concentração de fago e bactéria, tempo e alimento tiveram efeito significativo no

controle biológico de *Listeria*, (vii) temperatura e tempo tiveram efeito significativo sobre a atividade antimicrobiana de fagos de *Salmonella*.

Além disso, a atividade antibacteriana de 17 plantas da região semiárida do nordeste do Brasil, os extratos foram macerados usando hexano, acetato de etila e etanol para produzir 51 extratos. Foram avaliados seis óleos essenciais produzidos comercialmente e os óleos essenciais de *Croton heliotropiifolius* (obtido por hidrodestilação), timol e carvacrol contra *Salmonella* por meio de abordagens *in vitro*. O óleo essencial de botão de cravo e o timol mostraram atividade contra *Salmonella* na concentração de 1mg/ml.

Salmonella SE3 foi isolada do solo do Rio Subaé em Santo Amaro, Brasil, região contaminada com metais pesados e resíduos orgânicos. A montagem de sequenciamento híbrido de novo de Salmonella SE3 empregando o sequenciamento Illumina HiSeq e o sequenciamento de genoma inteiro ONT MinION rendeu 10 contigs e mostrou 99,98% de identidade com *Salmonella enterica* subsp. *enterica* sorovar Enteritidis OLF-SE2-98984-6. Doze ilhas patogênicas de *Salmonella*, múltiplos genes de virulência, múltiplos genes de resistência antimicrobiana, sete sistemas de defesa, sete profagos e um gene de resistência a metais pesados (*arsC*) foram identificados. A análise do pangenoma do clado *S. enterica*, incluindo SE3, revelou um pangenoma aberto, com um genoma central de 2.137 genes. O genoma acessório compreendeu 3.390 shared genes e 69.352 singletons genes.

Além disso, o fago SF1 foi isolado e caracterizado, o genoma dele foi sequenciado nas plataformas ONT MinION e Illumina Hiseq, três montagens genômicas a partir das sequências do MinION, Hiseq e MinION + Hiseq (montagem híbrido) foi obtidas, os genomas foram anotados e analisados, e seus genomas foram comparados com o fago Salmonella de referência. A montagem do MinION apresentou os melhores resultados. Além disso, não foram identificados genes de ciclo lisogênico, resistência antimicrobiana e virulência em nosso trabalho. O fago SF1 mostrou atividade contra vinte e sete cepas: *Salmonella* var. Enteritidis, *Salmonella* var. Typhimurium, *Salmonella* var. Minnesota, *Shigella flexneri, Escherichia coli, Escherichia cloacae, Escherichia fergusonii, Citrobacter europeus, Citrobacter freundii, Corynebacterium pseudotuberculosis, Corynebacterium striatum, Glutamicibacter creatinolyticus, Klebsiella oxytoca, Listeria monocytogenes* e *Rodococos iaqui.*

Conclusões: Em resumo, avaliamos a eficiência de fagos previamente patenteados como controle biológico de frutas e hortaliças e carnes. A maioria dos extratos de

produtos naturais testados neste estudo não apresentou atividade antimicrobiana significativa contra *Salmonella* enterica subsp. Typhimurium ATCC 14028. No entanto, o óleo essencial de botão de cravo e o timol mostraram atividade contra *Salmonella* na concentração de 1mg/ml. Por outro lado, mostramos a eficácia de uma abordagem de montagem híbrida (HiSeq e MinION) para análise do genoma de Salmonella SE3. A montagem do genoma híbrido permitiu a identificação de genes de virulência e resistência, elementos genéticos móveis e análise de pangenoma. No entanto, a plataforma MinION apresentou a melhor montagem para o fago SF1. Dois receptores foram identificados: receptor de proteína da cauda. A gama de hospedeiros do fago SF1 mostrou atividade contra 27 cepas. O fago SF1 mostrou ser um fago polivalente.

ABSTRACT

Background: Foodborne diseases are a global public health issue with 1 in 10 people falling ill after eating contaminated food every year. In Brazil, *Salmonella enterica* serovar Enteritidis is a significant health threat. Bacteriophages (phages; bacteria viruses), have potential to be used as natural antimicrobial methods to control bacterial pathogens such as *Salmonella* spp.

Objetives: The thesis has five chapters, the aims of every chapter were: 1) Develop a narrative review about the applications of phage therapy for various infectious diseases, phage pharmacology, immunological responses to phages, legal concerns, and the potential benefits and disadvantages of this novel treatment. 2) Develop a systematic review and meta-analysis that evaluated the efficiency of phages patented as a biological control for foodborne pathogens and determined the physical-chemical characteristics of the antimicrobial effect. 3) Determine the antimicrobial activity of seventeen plant extracts, commercial essential oils, carvacrol and thymol against *Salmonella* ATCC 14028. 4) Describe the fenotipic and genomic characterization of *Salmonella* SE3 strain isolate from Subaé River, Santo Amaro, BA-Brazil. 5) Describe the fenotipic and genomic characterization of SF1 phage, isolate from Subaé River, Santo Amaro, BA-Brazil. Determine the host range of SF1 phage against Salmonella strains and other species of bacteria.

Results: The systematic review was developed using scientific article and patent databases with inclusion and exclusion criteria applied by automatic and manual processes. A random-effects meta-analysis was carried out and revealed: (i) significant antimicrobial effect of *Listeria* phages in apple, apple juice, pear, and pear juice (ii) significant antimicrobial effect of *Salmonella* phages in eggs, apple and ready to cook chicken, (iii) no heterogeneity was identified in either meta-analysis, (iv) publication bias was detected in *Listeria* phages but not in *Salmonella* phages. (v) ListShield and Felix01 phages showed the best result for *Listeria* and *Salmonella* biological control, respectively, (vi) concentration of phage and bacteria, time and food had significant effect in the biological control of *Listeria*, (vii) temperature and time had a significant effect on the antimicrobial activity of *Salmonella* phages.

Moreover, the antibacterial activity of Seventeen plants extracts from the semi-arid region of the northeast Brazil were macerated using hexane, ethyl acetate and ethanol to produce 51 extracts. Six commercially produced essential oils and the essential oils of *Croton heliotropiifolius* (obtained by hydrodistillation), thymol and carvacrol against *Salmonella* using *in vitro* approaches were evaluated. Clove bud essential oil and thymol showed activity against *Salmonella* at a concentration of 1mg/ml.

Salmonella SE3 was isolated from soil at the Subaé River in Santo Amaro, Brazil, a region contaminated with heavy metals and organic waste. *De novo* hybrid sequencing assembly of *Salmonella* SE3 from Illumina HiSeq and ONT MinION whole genome sequencing yielded 10 contigs and showed 99.98% of identity with *Salmonella enterica* subsp. *enterica* serovar Enteritidis OLF-SE2-98984-6. Twelve *Salmonella* pathogenic islands, multiple virulence genes, multiple antimicrobial gene resistance genes, seven defense systems, seven prophages and a heavy metal resistance gene (*arsC*) were identified. Pangenome analysis of the *S. enterica* clade, including SE3, revealed an open pangenome, with a core genome of 2,137 genes. The accessory genome comprised 3,390 shell genes and 69,352 cloud genes.

Furthermore, SF1 phage was isolated and characterized, Furthermore, phage genome were sequenced by ONT MiION and Illumina Hiseq sequencing, three genomes assemblies, no Hybrid (MinION and Hiseq) and hybrid (MinION + Hiseq), was tested, the genomes were annotated and analyzed, and their genomes were compared with the reference Salmonella phage. MiION assembly showed the best results. Besides, no lysogenic cycle, antimicrobial resistance and virulence genes were identified in our work. SF1 phage showed activity against twenty seven strains: *Salmonella* var. Enteritidis, *Salmonella* var. Typhimurium, *Salmonella* var. Minnesota, *Shigella flexneri*, *Escherichia coli*, *Escherichia cloacae*, *Escherichia fergusonii*, *Citrobacter europeus*, *Citrobacter freundii*, *Corynebacterium pseudotuberculosis*, *Corynebacterium striatum*, *Glutamicibacter creatinolíticus*, *Klebsiella oxytoca*, *Listeria monocytogenes* and *Rodococos iaqui*.

Conclusions: In summary, we evaluated the efficiency of phages previously patented as a biological control for fruits and vegetables, and meat. Most of the natural products extracts tested in this study did not show significant antimicrobial activity against *Salmonella enterica* subsp. Typhimurium ATCC 14028. However, Clove bud essential oil and thymol showed activity against *Salmonella* at a concentration of 1mg/ml. On the

other hand, we showed the effectiveness of a hybrid sequence assembly approach for environmental *Salmonella* genome analysis using HiSeq and MinION data. The hybrid genome assembly enabled identification of virulence and resistance genes, mobile genetic elements and pangenome analysis. No obstant, MinION platform showed the best assembly for SF1 phage. The host range of SF1 phage showed activity against twenty seven strains. SF1 phage showed to be a polyvalent phage. Two receptors were identified: receptor b and tail tube protein.

Key-words: *Salmonella* spp., bacteriophages, MinION, Illumina and polyvalent.

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LISTA DE ABREVIATURAS E SIGLAS

FDA Administração de Alimentos e Medicamentos

MOI Multiplicidade ideal de infecção

OMS Organização Mundial da Saúde

PFU Unidade Formadora de Placas

UFC Unidade Formadora de colônias

NTS Salmonella não tifóide

SUMARIO

1. JUSTIFICATIVA

1.1- INTRODUÇÃO

A doença diarreica é a segunda principal causa de morte em crianças menores de cinco anos e foi responsável pela morte de 370.000 crianças em 2019 (https://www.who.int/health-topics/diarrhoea#tab=tab_1). A salmonelose, uma das principais causas de infecções alimentares resultantes de bactérias enteropatogênicas gram-negativas, *Salmonella* spp. é uma ameaça global para à saúde humana (HERNANDEZ-REYES, et al., 2013). A *Salmonella* tifóidea causa febre entérica em humanos, enquanto a *Salmonella* não tifóide (NTS) resulta em gastroenterite aguda e crônica. Anualmente, estima-se que a NTS seja responsável de aproximadamente 93,8 milhões de infecções e ~155.000 mortes (MAJOWICZ et al., 2010).

As infecções por NTS causam diarreia e uma doença febril inespecífica que é clinicamente indistinguível de outras doenças febris (GBDN-TSID Collaborators, 2019). *Salmonella enterica* subespécie enterica possui mais de 2600 sorovares de acordo com fórmulas antigênicas somáticas (O) e flagelares (H) únicas (DAS, et al., 2018; Saleh et al., 2019). *Salmonella enterica* serovar Typhimurium e *Salmonella enterica* serovar Enteritidis são os principais patógenos responsáveis por causar gastroenterite em humanos (RABSCH et al., 2002; CARDEN et al., 2015).

Para evitar a ocorrência dos principais sorovares de *Salmonella* em todo o mundo, diversas medidas de prevenção e controle são adotadas em fazendas produtoras e indústrias de processamento de alimentos. No Brasil, a infecção de rebanhos por Salmonella e a transmissão para alimentos derivados de aves é uma importante via de transmissão para o patógeno. *Salmonella* é rotineiramente identificadanas granjas por vacinação de aves e testes laboratoriais (Disponível online: https://www.gov.br/agricultura/pt-br/assuntos/sanidade-animal-e-vegetal/saude-animal/programas-desaude- animal/pnsa/2003_78.INconsolidada.pdf). No entanto, nas últimas décadas, várias doenças avícolas e surtos de *Salmonella* de origem alimentar foram relatados no Brasil (KIPPER et al., 2022).

O sequenciamento de todo o genoma (WGS) é útil em investigações de surtos de origem alimentar e vigilância de patógenos (ALLARD et al., 2016). A combinação de leituras curtas para precisão de base e leituras longas para integridade estrutural foi recentemente desenvolvida como uma abordagem de montagem híbrida para fechar genomas completos, empregando os pipelines Unicycler e SPAdes (ANTIPOV et al., 2016 ; WICK et al., 2017). Essas tecnologias podem ser úteis para a análise genômica de *Salmonella* e fagos de Salmonella.

O uso extensivo de antibióticos na indústria de alimentos contra patógenos de origem alimentar ou modelos de alimentos resultou em resistência adicional aos antibióticos à *Salmonella*, o que se tornou um assunto de grande preocupação para a saúde pública. Tem havido uma preocupação crescente em todo o mundo sobre os valores terapêuticos dos produtos naturais. A natureza apresentou à humanidade a dádiva de vastos agentes terapêuticos antimicrobianos de origem vegetal. Existem inúmeras substâncias bioativas potencialmente úteis a serem derivadas de plantas (BAJPAI et al., 2012).

Os níveis crescentes de resistência a antibióticos em muitos patógenos nosocomiais bacterianos acrecentaram o interesse na exploração de bacteriófagos como agentes terapêuticos e de biocontrole e no estudo dos mecanismos moleculares subjacentes à infecção produtiva (JASSIM & LIMOGES, 2014; MAHONY et al., 2011; DE SMET et al., 2017; TUNER et al., 2021). Da mesma forma, nosso entendimento de que os profagos podem influenciar na aptidão, no fenótipo e no metabolismo global do hospedeiro requer uma identificação e caracterização genômica cuidadosa de bacteriófagos. Em comparação com a plataforma Illumina, existem relativamente poucos estudos de fagos sequenciados por sequenciamento ONT ou PacBio (TURNER et al., 2021). Além disso, não há um estudo sobre o uso de montagem híbrida de bacteriófagos de Salmonella. A detecção da interação da gama de hospedeiros é importante para detectar a especificidade de bacteriófagos (GAMBINO et al. 2020).

Considerando a salmonelose como um problema de saúde pública, o objetivo deste trabalho foi isolar e realizar a caracterização fenotípica e genômica de linhagens de *Salmonella* de um ambiente natural, do Rio Subaé, Santo Amaro, BA-Brazil, empregando duas montagens de genoma híbrido usando as plataformas Ilumina Hiseq e MinION, no hibridas empregando as plataformas Illumina Hiseq ou MinION.

Além disso, nesse trabalho procurou-se 17 plantas (*Artemisia absinthium Linné, Calendula officinalis, Cecropia Hololeuca Miquel, Commiphora leptophloeos, Costus spicatus Swartz, Cuphea ingrate, Jacarandá semiserrata Cham, Laurus nobilis, Miconia albicans, Mikania hirsutíssima, Momordica charantia Linné, Pereskia anéata, Salvia officinalis, Thuja Occidentalis Linné, Tilia cordata, Zea mays Linné* e *Croton heliotropiifolius*) extratos do semiárido do nordeste Brasileiro, seis óleos essenciais produzidos comercialmente (*Larus nobilis, Salvia officinalis, Rosmarinus officinalis*, *Cymbopogon, Orégano Selvagem* e *Clove bud*), dois produzidos comercialmente metabólitos secundários (timol e carvacrol) e um óleo essencial (*Croton*

heliotropiifolius) obtido por hidrodestilação neste estudo, contra Salmonella usando abordagens *in vitro.*

Duas revisões bibliográficas foram feitas; uma foi uma revisão narrativa do uso de bacteriófagos como alternativas aos antibióticos na prática clínica e uma revisão sistemática e metanálise de bacteriófagos patenteados para aplicação no uso de controle biológico de alimentos. Nesse sentido o bacteriófago de Salmonella denominado fago SF1 foi isolado do Rio Subaé, Santo Amaro, BA-Brazil, a caracterização fenotípica e genômica foram feitas, da mesma maneira que no analise da *Salmonella* para melhorar a caracterização genômica do fago o montagem hibrido e não hibrido foi avaliado, posteriormente determinou-se a atividade antimicrobiana frente a linhagens de *Salmonella* e outras espécies bacterianas.

1.2- JUSTIFICAÇÃO

As doenças diarréicas estão associadas a uma estimativa de 1,3 milhão de mortes anualmente (MOKOMANE *et al*., 2018). A morbimortalidade mundial causada por doenças diarreicas continua sendo um problema de saúde significativo. A Organização Mundial da Saúde (OMS) estima que, globalmente, existem quase 1,7 bilhões de casos de doenças diarreicas na infância em que 525.000 crianças com menos de cinco anos de idade todos os anos (OMS, 2018).

Enquanto as doenças por *Salmonella enterica* (180 milhões) são excedidas em frequência pelas causadas por norovírus (685 milhões), *E. coli* enterotoxigênica (241 milhões), *Shigella* spp. (190 milhões) e *Giardia* spp. (183 milhões), um número desproporcional de mortes (298.000, ou 41% de todas as mortes associadas à doença diarreica) pode ser atribuído à Salmonella. Os sorotipos causadores não são distribuídos uniformemente pelo mundo; *Salmonella* não tifóide (NTS) é mais comum na África, enquanto Salmonella tifóide (por exemplo, sorotipos Typhi e Paratyphi A) são mais comuns no sudeste da Ásia (WHO, 2016; BESSER, 2017).

A OMS divulgou os primeiros dados de vigilância sobre resistência a antibióticos e revelou altos níveis de resistência a várias infecções bacterianas graves em países de alta e baixa renda. (OMS, 2018). De acordo com o Sistema Global de Vigilância Antimicrobiana (GLASS, 2016-2017), há uma ocorrência generalizada de resistência a antibióticos entre 500.000 pessoas suspeitas de infecção bacteriana em 22 países. As bactérias resistentes mais comumente relatadas foram *Escherichia coli*,

Klebsiella pneumoniae, *Staphylococcus aureus*, *Streptococcus pneumoniae*, seguidas por *Salmonella* spp. (WHO, 2018).

As infecções resistentes a antibióticos também estão associadas a maior morbimortalidade, o que aumenta os custos com saúde. Em países de baixa renda, a acessibilidade de medicamentos de segunda linha e acesso reduzido aos cuidados de saúde pode restringir o uso de antibióticos de amplo espectro mais recentes, como resultado do aumento da morbidade e mortalidade por infecções resistentes os antibióticos nesses países (BRYCE *et al*., 2016).

Segundo dados do Ministério da Saúde do Brasil, no período entre 2000 e 2011, entre 3487 agentes etiológicos, *Salmonella* spp. foi a principal causa de doença de origem alimentar (42,27%), seguida por *Staphylococcus aureus* (20,34%) e *Escherichia coli* (10,46) (SVS, 2011; Tondo & Ritter, 2012). Várias estratégias estão sendo adotadas para reduzir os níveis de resistência a antibióticos.

1.3 Objetivos

1.3.1- Objetivo geral

Isolar e caracterizar óleos essenciais e bacteriófago como tratamentos de controle biológico para *Salmonella enterica*.

1.3.2- Objetivos específicos

1. Realizar uma revisão narrativa do uso de bacteriófagos como alternativa aos antibióticos no atendimento clínico.
2. Realizar uma revisão sistemática e metanálise de bacteriófagos patenteados com aplicação no controle biológico em alimentos.
3. Isolar e caracterizar extratos vegetais, óleos essenciais, carvacrol e timol.
4. Determinar a atividade antimicrobiana de extratos vegetais, óleos essenciais, carvacrol e timol frente à *Salmonella enterica*.
5. Isolar e caracterizar linhagens de *Salmonella* de solo Rio Subaé, Santo Amaro, SA-Brasil.
6. Isolar, caracterizar bacteriófagos de *Salmonella* de solo Rio Subaé, Santo Amaro, SA-Brasil.

7. Determinar a atividade antimicrobiana do fago SF1 frente a linhagens de *Salmonella* e outras espécies bacterianas.

2. REVISÃO BIBLIOGRÁFICA

2.1- Definição de diarreia

A diarreia aguda é descrita como um aumento no número de fezes e / ou uma diminuição em sua consistência, de início rápido. Além disso, podem ocorrer sinais e sintomas como náusea, vômito, febre ou dor abdominal. A causa mais frequente é a infecção gastrointestinal, que produz gastroenterite ou inflamação da mucosa gástrica e intestinal. Devido a isso, o termo diarreia aguda é praticamente sinônimo de gastroenterite aguda de causa infecciosa.

Existem três tipos clínicos de diarreia:

- Diarreia aquosa aguda - persiste várias horas ou dias e inclui cólera;
- Diarreia com sangue aguda - também chamada disenteria;
- Diarreia persistente - persiste 14 dias ou mais.

2.2- Etiologia

A diarreia é causada por uma série de organismos bacterianos, virais e parasitários, a maioria dos quais se espalha pela água contaminada com fezes. Os dois agentes etiológicos mais comuns da diarreia moderados a grave em países de baixa renda são o Rotavirus e *Escherichia coli*. Além de outros patógenos, como as espécies *Cryptosporidium*, *Shigella* e *Salmonella*, a desnutrição, a contaminação da água com fezes humanas, condições higiênicas e doenças transmitidas por alimentos podem causar episódios diarreicos (OMS, 2018).

2.3- Epidemiologia

De acordo com o relatório da Organização Mundial da Saúde (2017), a diarreia é a segunda principal causa de morte em crianças com menos de cinco anos e aproximadamente 525.000 crianças morrem a cada ano. Por outro lado, o Sistema Global de Vigilância Antimicrobiana (GLASS) relatou ocorrências generalizadas de resistência aos antibióticos em torno de 500.000 pessoas envolvidas com infecção

bacteriana em 22 países. As bactérias resistentes aos antimicrobianas mais comumente relatadas foram *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* e *Streptococcus pneumoniae*, seguidas por *Salmonella* spp.

Segundo dados oficiais da Divisão de Vigilância Sanitária do Estado do RS (DV / RS), desde 1993, *Salmonella* spp. Tornou-se a principal causa de doenças transmitidas por alimentos no sul do Brasil. Entre 3487 agentes etiológicos identificados em doenças transmitidas por alimentos, *Salmonella* spp. foram identificados em 42,27% dos surtos, seguidos por *S. aureus* (20,34%) e *E. coli* (10,46%) (Tondo & Ritter, 2012).

2.4- Características de *Salmonella* spp.

Salmonella é um bacilo, bactéria Gram-negativa da família Enterobacteriaceae. As suas espécies são enterobactérias predominantemente móveis, não formadoras de esporos, e possuem as seguintes características: comprimentos de 2 a 5 µm, diâmetro entre 0,7 e 1,5 µm e flagelos peritríticos. *Salmonella enterica* e *Salmonella bongori* são as duas espécies de Salmonella. *S. enterica* possui seis subespécies e mais de 2.500 sorotipos, de acordo com a sequência 16S rRNA.

O gênero Salmonella é composto por duas espécies, *S. enterica* e *S. bongori*. *S. enterica* possui subespécies designadas I, II, IIIa, IIIb, IV, VI e VII, comxistem mais de 2.500 sorovares das subespécies II, IIIa, IIIb, IV, VI e VII das espécies *S. bongori* e *S. enterica*. Subespécies de S. enterica I são reservatórios de espécies de mamíferos, aves e répteis (GARAI *et al*., 2012; BÄUMLER & FANG, 2013).

Para os sorovares tifoides de Salmonella, o único reservatório conhecido são os seres humanos e assentados em quatro linhagens clonais filogeneticamente não relacionadas, associados à gastroenterite (SELANDER *et al*., 1990). Uma das linhagens clonais, *S. enterica* serovar Typhi (*S.* Typhi relatou ter entre 10.000 e 71.000 anos) (ROUMAGNAC *et al*., 2006). *S. enterica* serovares Paratyphi C (*S.* Paratyphi C), Paratyphi B (*S.* Paratyphi B), *S. enterica* serovars Paratyphi A (*S.* ParatyphiA) e Sendai (*S.* Sendai) causam febre paratifóide, então diferentes linhagens de *Salmonella* afeitam a diferentes hospedeiros, Figura 1 (BÄUMLER & FANG., 2013).

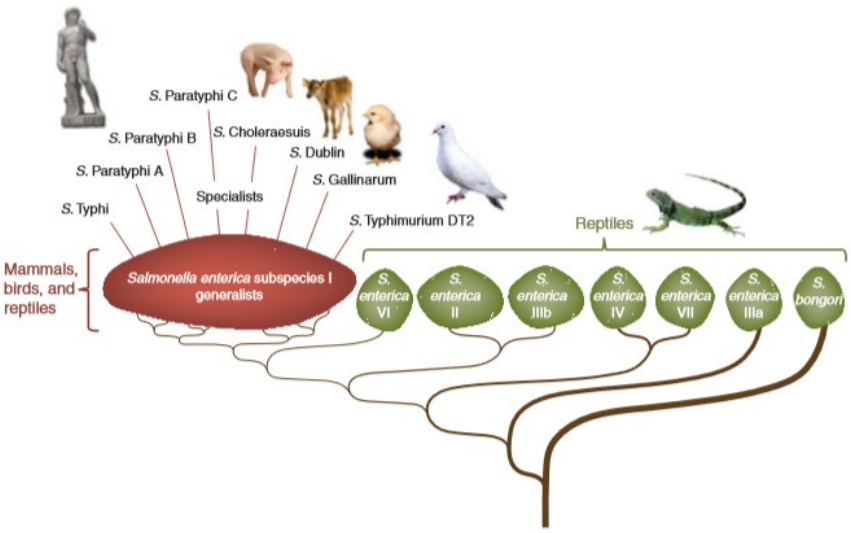

Figura 1- Intervalo de hospedeiros de membros do gênero *Salmonella*

Fonte: BÄUMLER & FANG, 2013.

Existem várias diferenças entre a febre entérica causada por sorovares tifoide e a gastroenterite associada à Salmonella não tifoide (NTS). A apresentação clínica não pode ser distinguida entre infecções causadas por diversos sorovares tifoides (por exemplo, Typhi e Paratyphi A); a tabela 1 descreve algumas características entre o NTS e os sorovares tifoides (GAL-MOR, *et al*. 2014).

Tabela 1. Resumo das diferenças entre NTS e sorovares tifoides associados à doença em humanos.

	Serotipos NTS	**Serotipos tifoideos**
Serotipos	*Typhimurium e Enteritidis, ~1500* outros serovares de *S. enterica* ssp.	Typhi, Paratyphi, e Sendai.
Espectro de atividade	Amplo.	Restrito a humanos.
Hospedeiros	Animais de fazenda, produtos e animais de granja.	Transmissão não humana a humana
Manifestaçõe	Gastroenterite autolimitada	Doença invasiva, sistêmica,

s clínicas	em indivíduos imunocompetentes (diarreia, vômito, cãibras) Em pacientes imunocomprometidos (deficiência do sistema IL-12 / IL-23 e HIV), a doença está associada a infecções intestinais invasivas.	em indivíduos imunocompetentes (febre, calafrios, dor abdominal, erupção cutânea, náusea, anorexia, hepatoesplenomegalia, diarreia ou constipação, dor de cabeça, tosse seca).
Curso da doença	Curto período de incubação (6-24 h) Breve duração dos sintomas (menos de 10 dias).	Longo período de incubação (7 a 21 dias) Duração prolongada dos sintomas (até 3 semanas).
Resposta imune humana	Inflamação intestinal robusta, recrutamento de neutrófilos, resposta Th1	Inflamação intestinal mínima, leucopenia, resposta Th1
Base genética das diferenças de doenças e especificidade do hospedeiro	Baixo grau de inativação do genoma. Capaz de usar aceptores de elétrons terminais para respiração anaeróbica no intestino inflamado. Fatores únicos de virulência (por exemplo, fímbrias, ilha de patogenicidade (SPI) -14).	~ 5% do genoma é inativado (por exemplo, genes metabólicos e fator de virulência inativados). Fatores únicos de virulência e ilhas de patogenicidade (por exemplo, antígeno Vi, SPIs 7, 15, 17 e 18).
Vacinação	Nenhuma vacina disponível para humanos.	(I) vacina parenteral de células inteiras mortas, (II) vacina oral viva atenuada (Ty21a), (III) vacina baseada em cápsula de polissacarídeo VI.

Fonte: adaptado de GAL-MOR *et al.*, 2014.

2.5- Bacteriófago

2.5.1- Taxonomia de bacteriófagos

Embora as estruturas dos bacteriófagos da ordem caudovirales sejam diversas, os bacteriófagos são compostos de capsídeo, pescoço e cauda. Várias proteínas encapsulam um genoma de DNA ou RNA que varia de 5 a 500 Kpb em um capsídeo, a altura é de 20 a 200 nm. Uma das principais diferenças é a presença ou ausência de estruturas da cauda.

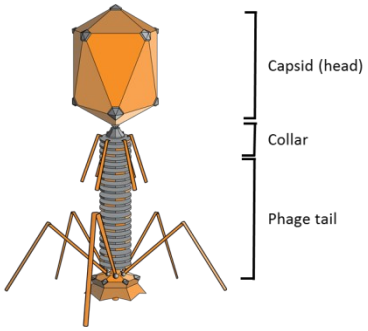

Figura 2- Estrutura do primeiro bacteriófago descoberto chamado bacteriófago λ (lambda).
De acordo com sua for

ma, os fagos são de cauda, de simetria cúbica ou helicoidal ou pleomórfica, e contêm DNA ou RNA de fita dupla ou fita simples.
Fonte: https://commons.wikimedia.org/wiki/File:PhageExterior.svg

Os fagos são classificados por morfotipo e hospedeiro. Embora os fagos dos anos tenham sido divididos e subdivididos de acordo com o formato da cabeça, o que é útil para na microscopia eletrônica, no entanto, não tem resolução taxonômica suficiente. Em 1967, os seis primeiros morfotipos de fagos, famílias de fagos Myoviridae, Siphoviridae, Podoviridae, Inoviridae e Leviviridae foram classificados de acordo com características como morfologia e natureza de seu genoma, além de 13 famílias de fagos descritas na tabela 3 e na figura 3 (ACKERMANN, 2001).

Tabela 2. Morfotipos e propriedades básicas das famílias de fagos

Morfotipo	Forma	Ácido nucleico	Família	Dados
A1 - A3	Cauda	DNA, 2, L	Myoviridae	Cauda contrátil
B1 - B3			Siphoviridae	Cauda longa, não contrátil
C1 - C3			Podoviridae	Cauda curta
D1	Poliédrica	DNA, 1, C	Microviridae	Capsômeros conspícuos
D3		2, C, S	Corticoviridae	Compleco capsídeo complexo, lipídios
D4		2, L	Tectiviridae	Vesícula lipídica, pseudo-cauda
E1		RNA, 1, L	Leviviridae	
E2		2, L, seg.	Cystoviridae	Envelope, lipídios
F1	Filamentoso	DNA, 1, C	Inoviridae	Filamentos longos
F2				Hastes curtas
F3		2, L	Lipothrixviridae	Envelope, lipídios
F4		2, L	Rudiviridae	TMV-like
G1	Pleomórfico	DNA, 2, C, S	Plasmavirida	Envelope,

		e	lipídios, sem capsídeo
G2	2, C, S	Fuselloviridae	O mesmo, em forma de limão

C circular; L linear; S superhelical; seg. segmentado; 1 fita simples; 2 fita dupla

Fonte: adaptado de ACKERMANN, 2001.

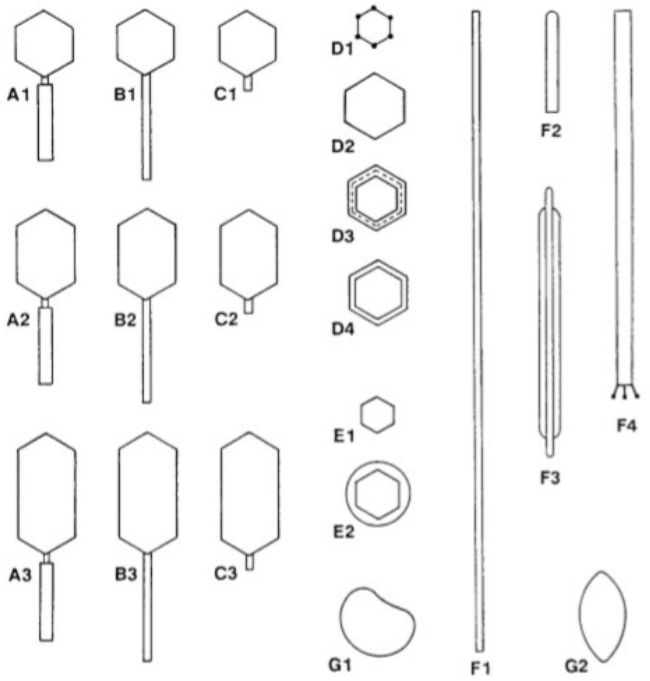

Figura 3- Morfotipos de bacteriófagos. Veja a Tabela 2 para explicação.

Fonte: ACKERMANN, 2001**.**

Novos morfotipos e famílias de fagos foram usados ao longo dos anos. A classificação do Comitê Internacional de Taxonomia de Vírus (ICTV) inclui uma ordem: os Caudovirais ou Fagos de Cauda, 13 famílias e o Salterprovírus do gênero flutuante. As três famílias caudovirais: Myoviridae, Podoviridae e Siphoviridae, Myoviridae, que possuem caudas contráteis que agem como uma seringa perfurando para penetrar e injetar o genoma do fago na célula hospedeira; os Siphoviridae com caudas longas não contráteis; e os Podoviridae, com caudas curtas não contráteis, têm seis subfamílias, 80 gêneros e 441 espécies (ACKERMANN, 2001, MAYNERIS-PERXACHS et al. 2022).

ACKERMANN, 2001 relacionou mais de 5.100 observados pelo microscópio eletrônico dos 96% que foram contados, 3,6% foram filamentos ou pleomórficos, entre outros, como micoplasmas e arqueobactérias. Os fagos com cauda representados em Myoviridae (25,1%); Siphoviridae (60,8%) e Podoviridae (14,1%), alguns deles têm cabeças alongadas (15%). Aproximadamente 150 novos fagos são registrados por ano. Vários fagos arqueobacterianos filamentosos e pleomórficos.

Fagos encontrados em 10 gêneros de archaea e 144 de eubacteria, 14 gêneros de enterobacteria (figura 4). De acordo com as exposições existem em enterobactérias (906 fagos), *Lactococcus* (700), *Bacillus* (380) e *Streptococcus* (290). Seu alto número de lactococos e enterobactérias se deve à prevalência de fagos dos gêneros c2 e T4. Além disso, o número de bactérias de Lactococcus e *S. thermophilus* mostra interesse por pesquisas em bactérias de leite (ACKERMANN, 2007).

Além disso, é provável que a disponibilidade de técnicas e meios bacteriológicos para a divisão bacteriana, e não para os táxons na natureza. A maioria inclui proteobactérias e bactérias gram-positivas do ramo de baixo G + C (e. g. bactérias de bacilos e ácido lático). Os sifovírus estão presentes nas bactérias gram-positivas com ramo de G + C alto (actinobactérias). Bactérias gram-positivas com alto G + C ramificado antes da Gram negativas podem ser elucidadas pela evolução (figura 4).

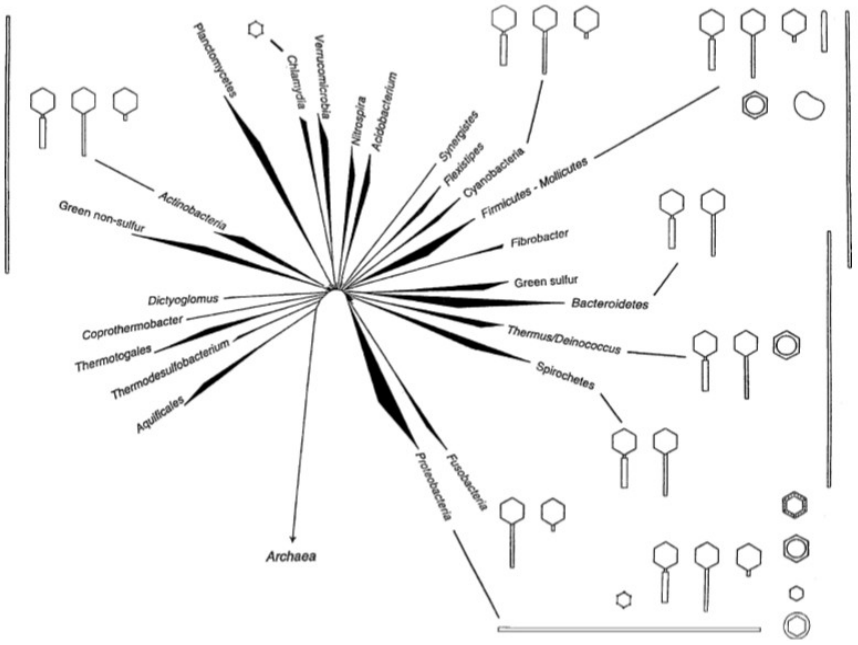

Figura 4- Observações de morfotipos de fagos em filos eubacterianos.

Fonte: ACKERMANN, 2001.

Vários grupos filogenéticos bacterianos foram classificados através do sequenciamento de 16S rRNA, até agora 3.356.809 sequências procarióticas de 16S rRNAs foram publicadas no RDP (Ribosomal Database Project) (KRUPOVIC *et al.*, 2016).

A classificação de fagos é considerando um processo altamente desafiador e esmagador, a menos que uma taxonomia genômica para vírus esteja incluída. Mesmo assim, a análise da sequência do genoma pode ser uma ferramenta útil para a taxonomia, por si só pode ter falhas devido a rearranjos genômicos desenfreados em vírus, é a única solução escalável (KRUPOVIC *et al.,* 2016).

2.5.2 Os ciclos de vida dos bacteriófagos

Os bacteriófagos têm dois ciclos de vida distintos: o ciclo lítico consiste na síntese de novas partículas de fago e o ciclo lisogênico ou estágio silencioso em que o genoma do fago é integrado ao cromossomo hospedeiro, figura 5.

a) O ciclo lítico

Inicialmente, o bacteriófago entra em contato com uma célula bacteriana que codifica um receptor, complementar ao anti-receptor do fago, quando o contato celular é estabelecido, o bacteriófago entra na célula bacteriana e começa a se replicar. Os bacteriófagos usam o mecanismo das células bacterianas para expressar seus próprios genes e replicar seus próprios genomas; eles devem, portanto, sequestrar essa capacidade do maquinário da célula hospedeira para continuar a síntese de proteínas e a montagem da estrutura dos bacteriófagos. Depois que as partículas filhas são totalmente montadas, a célula hospedeira é rompida (lise) por enzimas codificadas em fagos e as partículas de bacteriófagos são liberadas na área circundante, prontas para infectar um novo hospedeiro.

b) O ciclo lisogênico

O ciclo lisogênico ocorre se, após a infecção, o genoma do bacteriófago se integra ao cromossomo hospedeiro como profago em um estado de lisogenia; nesse sentido, o bacteriófago pode permanecer em seus hospedeiros por muitas gerações. A expressão gênica deve ser estimulada para fazer a transição do ciclo lisogênico para o

ciclo lítico. O modelo mais estudado de expressão gênica e transição lisogênico-lítica foi realizado com o bacteriófago lambda, figura 5.

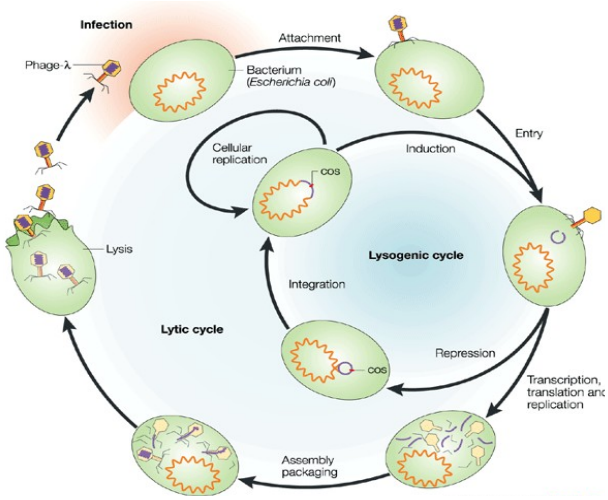

Figura 5- Ciclos de vida líticos e lisogênicos do fago λ.

Fonte: CAMPBELL, 2003.

2.5.3- Terapia com bacteriófagos

A atividade antibacteriana do fago foi descoberta na segunda metade do século 20, que deu origem aos primeiros testes terapêuticos em humanos. Vale ressaltar que em alguns dos ensaios iniciais, bacteriófagos foram utilizados no tratamento de infecções por S. aureus. No entanto, por várias razões, incluindo pouco conhecimento sobre a biologia dos fagos, muitos ensaios realizados durante a primeira metade do século XX não tiveram êxito e a terapia com fagos não obteve aceitação na medicina ocidental. Portanto, não surpreende que o interesse neles tenha diminuído com a introdução dos primeiros antibióticos (SKURNIK E STRAUCH, 2006).

Alguns exemplos de terapia com bacteriófagos incluem o uso do bacteriófago CEV1, isolado de ovelhas resistentes à colonização por *Escherichia coli* O157: H7. In vitro, CEV1 infectou eficientemente *E. coli* O157: H7 crescida aerobicamente e anaerobicamente. In vivo, ovelhas que receberam uma dose oral única de CEV1 mostraram uma redução de 2 unidades logarítmicas nos níveis intestinais de *E. coli* O157: H7 em 2 dias em comparação aos níveis nos controles (RAYA *et al*., 2006).

HUDSON *et al*, 2013 relataram que o controle de *E. coli* O157 *in vitro* e na carne do fago FAHEc1 pertencia à família Myoviridae, que classificou 28 de 30

isolados de *E. coli* O157, um sorotipo não de *E. coli* O157 (O162: H7) e nenhum das outras 13 espécies bacterianas testadas. A 5 ° C, o fago FAHEc1 a> 10^7 PFU / ml inativou 4 log10 de *E. coli* O157: H7 em caldo *in vitro*. Em pedaços de carne em fatias finas, 3,2 x 10^7 PFU/4 cm^2 de pedaço de carne reduziram >2,7 log10 *E. coli* O157: H7 incubados a 37 ° C. Assim, foram necessárias concentrações de fago de 10^7 e10^8 PFU por peça para resultar em boas reduções da concentração do hospedeiro *E. coli* O157: H7.

2.5.4- Impacto do biocontrole do fagos nos microrganismos ambientais

Na segunda metade do século, as terapias fágicas foram usadas de maneira eficaz e em larga escala na Europa Oriental, especialmente na Polônia, Geórgia e Rússia. Nos últimos anos, a resistência generalizada aos antibióticos, agora aparente entre as bactérias, tem renovado o interesse em empregar bacteriófagos como agentes antibacterianos (DIAZ & KOSKELLA, 2014).

As aplicações de bacteriófagos se espalharam para a indústria de alimentos, a empresa Intralytix, por exemplo, possui dois produtos aprovados pela FDA para uso em produtos de consumo (o ListShield agindo especificamente contra *Listeria monocytogenes* e o EcoShield ativo contra *Escherichia coli*). Da mesma forma, a MICREOS BV, empresa holandesa envolvida na pesquisa e desenvolvimento da fagoterapia, também possui produtos fágicos para o controle da contaminação de alimentos com *L. monocytogenes* que foram aprovados pelo FDA para uso em peixes e queijos. Para uso em alimentos, existem vários aspectos a serem considerados entre eles: alguns bacteriófagos podem transportar genes para resistência a antibióticos e virulência de bactérias (LU & KOERIS, 2011).

Critérios a serem considerados para seleção de fagos: Fagos obrigatoriamente líticos, não transdutores, ampla gama de hospedeiros, reconhecimento de fatores de virulência, genoma, toxicidade, esterilidade e estabilidade. Embora os fagos sejam compostos de proteínas e DNA, eles têm poucos efeitos alérgicos ou tóxicos, não foram observados efeitos em 200 pessoas, que foram administradas com fagos usando um teste de função imune (WEDGWOOD *et al.*, 1975).

Os bacteriófagos são imunogênicos e uma resposta imune foi relatada durante alguns ensaios de terapia fágica (CLARK & MARCH, 2006). Por outro lado, estudos

anteriores de MIWDZYBRODZKI *et al*., 2017 mostraram o efeito imunossupressor do fago T4 na artrite reumatóide, modelo de camundongo, além de existir a hipótese de que os fagos intestinais poderiam desempenhar um papel importante não na eliminação de bactérias, inibição de reações inflamatórias e imunológicas locais, a fim de manter a homeostase imune (GÓRSKY *et al.*, 2005; GÓRSKY *et al*., 2017).

Também os bacteriófagos estão envolvidos em vários processos durante o processo de infecção de bactérias patogênicas, como adesão bacteriana, colonização, invasão e disseminação através dos tecidos humanos, resistência às defesas imunológicas e produção de exotoxina, que é o principal mecanismo patogênico das bactérias (WAGNER & WALDOR, 2002). Os fagos podem transformar uma cepa não patogênica em um patógeno através da aquisição do gene da toxina após a integração de um genoma do fago no cromossomo hospedeiro.

Muitos estudos amostram que os níveis de endotoxina reduziram em 10 a 30 vezes para diferentes métodos de purificação de bacteriófagos, como centrifugação em gradiente de CsCl, precipitação com sulfato de amônio, ultrafiltração, cromatografia em duas etapas, entre outros (BORATYNSKI *et al*., 2004; MERRIL *et al*., 2006), no entanto 97-99% de bacteriófagos foram perdidos durante o procedimento (SKURNIK *et al*., 2007).

Os fagos portadores de genes de virulência em seus genomas produzem um risco de transferência das propriedades patogênicas para as bactérias saprofitas do ser humano são um dos fatores que devem ser considerados na terapia fágica. Sequenciar é um método útil para identificar genes de virulência; no entanto, há um grande número de ORFs não identificadas nos genomas dos fagos (SKURNIK *et al*., 2007). No entanto, essa abordagem é restrita pelo grande número de ORFs não identificadas nos genomas de fagos (MERRIL *et al*., 2006). Fagos temperados como Gifsy-1, Gifsy-2, Gifsy-3, SopE / e Fels-1 de *Salmonella enterica* serovar O typhimurium produz genes envolvidos na produção de vários fatores de virulência, como dismutases, neuraminidase e múltiplas proteínas efetoras translocadas do tipo III (FIGUEROA *et al*., 2001; HO *et al*., 2002). Assim, as propriedades patogênicas de Salmonella podem ser atribuídas à presença do profago.

Por outro lado, poucos estudos relataram fagos de DNA com atividade mutagênica. Por exemplo, o genoma dos fagos PBS1 e PBS2 *Bacillus subtilis* possuía

enzimas para a síntese ou modificação de seu genoma, que contêm uracil em vez de citosina (TAKAHASHI & MARMUR, 1963), enzima fágica que inibe a degradação do DNA-U, DNA-glicosilase, aumento da frequência de mutação em células humanas (RADANY *et al*. 2000). A mesma composição genômica foi descrita em yersiniophage/R1–37 (KILJUNEN *et al*., 2005). Apesar dos riscos listados acima, os fagos podem ser considerados seguros (SKURNIK *et al*., 2005).

O sequenciamento do genoma pode garantir que o fago não carregue genes de virulência, resistência a antibióticos ou lisogenia, aumentando a prontidão, segurança, eficácia e avaliação na terapia fágica. Além disso, o sequenciamento de genoma é uma ferramenta útil para descobrir e projetar novas estratégias de resistência a fagos (STURINO & KLAENHAMMER, 2006). A sequência de fagos funcionais, fagos remanescentes e profágios mostrou que os fagos caudas estão agrupados em um grande grupo evolutivo, caso o pool genético possa proporcionar evolução horizontal. Além disso, não há correlação entre a semelhança de fagos e a origem geográfica; fagos semelhantes são amplamente dispersos geograficamente (HATFULL *et al*., 2006).

De qualquer forma, alternativas terapêuticas, como partículas intactas de bacteriófagos, endolisinas de bacteriófagos, são úteis no controle biológico. Essas enzimas dos bacteriófagos digerem o peptidoglicano, o principal componente da parede celular bacteriana, que induz a lise da célula bacteriana na conclusão do ciclo replicativo do fago. Embora essa capacidade de lise tenha sido relatada pela primeira vez em 1959, não foi até 2001 que as endolisinas recombinantes provaram serem agentes antibacterianos eficazes. Como os fagos, as lisinas podem destruir bactérias resistentes a antibióticos (SCHMELCHER E LOESSNER, 2016).

2.7 Produtos naturais e atividade antimicrobiana

Os compostos derivados de plantas com atividade antimicrobiana são principalmente metabólitos secundários, a maioria dos quais são fenóis ou seus derivados substituídos por oxigênio. Esses metabólitos secundários têm vários benefícios, incluindo propriedades antimicrobianas contra microorganismos patogênicos (ROSA *et al*., 2003). Vários compostos e seus mecanismos de ação sobre os microrganismos são descritos na Tabela 3, incluindo os principais mecanismos de ação dos antimicrobianos vegetais de acordo com os grupos já mencionados, Tabela 3.

Tabela 3: Principais grupos de metabólitos secundários de plantas com atividade antimicrobiana

Metabólitos Secundários	Mecanismo
Fenólico	Privação de substrato.
Catecol	Ruptura da membrana.
Epicatequina	Ligação das adesinas, complexo com a parede celular, Inative as enzimas.
Hipericina e Crisina	
Abissinona	Enzimas inativas inibem a transcriptase reversa do HIV.
Elagitaninos	Ligação das adesinas, inibição enzimática e privação de substrato, complexo com a parede celular, ruptura da membrana e tez do íon metálico.
Varfarina	Interação com DNA eucariótico.
Terpenóides	
Capsaicina	Ruptura da membrana.
Alcalóides	
Berberina, piperina	Intercalação na célula e/ou DNA.
Lectinas e polipeptídeos	
Fabatina de aglutinina específica para manose	Bloqueia a fusão ou adsorção viral das pontes dissulfeto.

Fonte: adaptado de MURPHY, 1999.

Produtos naturais, principalmente óleos essenciais (OEs), têm demonstrado altos níveis de atividade antimicrobiana *in vitro* e *in vivo* contra diferentes tipos de bactérias

independentemente da presença de resistência a antibióticos, incluindo atividade documentada contra (*Staphylococcus aureus* resistente à meticilina) (MRSA), *Enterococcus resistente* à vancomicina (VRE) e outras bactérias de resistência a múltiplas drogas (MDR) (KON & RAI, 2012). OEs de folhas de orégano, tomilho, manjericão, manjerona, capim-limão, rizomas de gengibre mostraram atividade antimicrobiana contra *Listeria monocytogenes*, *S. aureus, E. coli* e *Salmonella enteritidis*.

COSTA *et al*., 2008 determinaram a capacidade inibitória de óleos essencias de folhas de *Croton zehntneri* (canela selvagem) contra *Shigella flexneri, Salmonella* Typhimurium, *E. coli, S. aureus* e linhagems de *Streptococcus* β-hemolyticus, atividade antimicrobiana contra todas as bactérias foi detectada, exceto *Salmonella*. A atividade antibacteriana de óleos essenciais de orégano (*Origanum vulgare*) contra bactérias multirresistentes, incluindo *E. coli*, *E. faecalis*, *Acinetobacter baumannii*, *K. pneumoniae*, *P. aeruginosa* e MRSA. Em outro trabalho, *A. sativum* (bulbos), *Z. officinale* (rizomas), *Caryophyllus aromaticus* (botões de flores), *C. citratus* (folhas), *P. guajava* (folhas) e *M. glomerata* (folhas) foram testados contra *Enterococcus* sp., *E. coli, S. aureus* e *Salmonella* sp. Os extratos de alho (*A. sativum*) e gengibre (*Z. officinale*) apresentaram a atividade mais intensa contra bactérias gram-negativas para o alho, as concentrações variaram de 1,38 a 1,61 mg/mL enquanto para o gengibre foi de 6,97 (SILVA & FERNANDEZ, 2010).

Por outro lado, extratos hexânicos de *Amansia multifida* mostraram atividade antimicrobiana contra cepas Gram-negativas entéricas como *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* Typhi, *S.* Choleraesuis, *Serratia marcescens, Vibrio cholerae* e as bactérias Gram-positivas *Bacillus subtilis* e *Staphylococcus aureus* (LIMA-FILHO *et al*., 2002).

a) Combinações de bacteriófagos e produtos naturais

Efeitos benéficos significativos foram demonstrados nas interações entre bacteriófagos e antibióticos, e entre bacteriófagos e nanopartículas metálicas (YOU et al. 2011). No entanto, faltam estudos sobre interações entre bacteriófagos e EOS. Os óleos essenciais (OES) possuem uma composição química multicomponente e mecanismos de ação alternativos, incluindo a capacidade de afetar muitas estruturas bacterianas simultaneamente, o que possibilita sua atividade tanto contra isolados sensíveis como resistentes a antibióticos. A Figura 6 mostra o possível mecanismo de ação dos OEs, (A) desintegração da membrana citoplasmática e interação com proteínas de membrana (ATPases e outras), (B) distúrbio do membrana externa de bactérias Gram-negativas com descarga de lipopolissacarídeos, (C) desestabilização da força próton-motriz com vazamento de íons, (D) coagulação do conteúdo celular, (E) inibição da síntese enzimática e (F) dano ao membrana celular bacteriana causada por OEs, facilita a penetração de bacteriófagos com subsequente replicação dentro da célula bacteriana e sua lise, ou é suportada pela ação simultânea de bacteriófagos e OEs nas membranas celulares. BF: Bacteriófago; OE: Óleo essencial (KON & RAI2012).

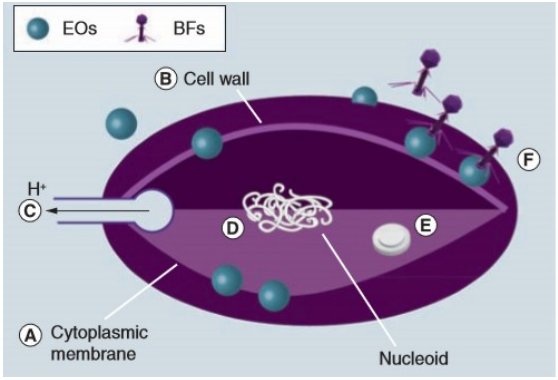

Figura 6- Possíveis mecanismos de ação antibacteriana de OEs em combinação com bacteriófagos.

Fonte: KON & RAI, 2012

Capítulo 1

Bacteriophages as Alternatives to Antibiotics in Clinical Care

antibiotics

MDPI

Bacteriophages as Alternatives to Antibiotics in Clinical Care

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Received: 2 August 2019; Accepted: 3 September; Published: date

Abstract: Antimicrobial resistance is increasing despite new treatments being employed. With a decrease in the discovery rate of novel antibiotics, this threatens to take humankind back to a "pre-antibiotic era" of clinical care. Bacteriophages (phages) are one of the most promising alternatives to antibiotics for clinical use. Although more than a century of mostly ad-hoc phage therapy has involved substantial clinical experimentation, a lack of both regulatory guidance standards and effective execution of clinical trials has meant that therapy for infectious bacterial diseases has yet to be widely adopted. However, several recent case studies and clinical trials show promise in addressing these concerns. With the antibiotic resistance crisis and urgent search for alternative clinical treatments for bacterial infections, phage therapy may soon fulfill its long-held promise. This review reports on the applications of phage therapy for various infectious diseases, phage pharmacology, immunological responses to phages, legal concerns, and the potential benefits and disadvantages of this novel treatment.

Keywords: bacteriophages; clinical trials; antibiotic resistance; infectious disease; phage therapy

1. Introduction

There are approximately 10^{30-31} bacteriophages (phages) in the biosphere [1,2], which is estimated to be 10-fold higher than the total number of bacterial cells [3]. Phages are also an inherent part of the human microbiome, and so are usually well-tolerated when used in phage therapy [4–6]. Phages are one of the most promising alternatives to antibiotics, which can be used for medicine, agriculture, and related fields [7]. The evolution of multidrug-resistant and pan-drug-resistant bacteria poses a real threat to the control of infectious diseases globally, so it is urgent to have new therapeutic tools available. The United States National Institutes of Health have stated that phages are promising tools for combatting microbial resistance [8].

A post-antibiotic era in which minor injuries and common infections can kill because of the lack of drugs or their ineffectiveness is nowadays not an apocalyptic fantasy, but a real 21st-century threat. For example, ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp.) are extremely resistant to multiple antimicrobial agents [9] and are a serious challenge in medicine today. On the other hand, there historically has been no fit for purpose regulatory framework to deal with novel flexible and sustainable therapeutic approaches such as phages. For phages, this

includes oversight of the setup and approval of adequate clinical trials, so as a result, there is no standard protocol for phage therapy.

In this review, we summarize the phage therapy clinical trials that have shown promising results in patients. We cover several diseases, immunological responses to phages, phage pharmacology, legal concerns about phage therapy, phage genetic modification, and a description of the advantages and disadvantages of phage therapy when compared to conventional treatments with antibiotics.

2. Phage Biology

Viruses that infect bacteria and *Achaea* are called phages, which have no machinery for generating energy and no ribosomes for making proteins. They are obligate bacterial parasites that carry all the genetic information required to undertake their reproduction in an appropriate host. The genome size of phages varies from a few thousand base pairs up to 498 kilobase pairs in phage G, which is the largest phage sequenced to date [10]. Most phages have a high level of host specificity (though some are broad in range), high durability in natural systems, and the inherent potential to reproduce rapidly in an appropriate host. They can be found associated with a great diversity of bacterial species in any natural ecosystem [11].

Phages can be characterized by their size and shape into three general groups: icosahedron, filamentous, and complex. Members of these groups may contain nucleic acid of various types including single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA), or double-stranded RNA (dsRNA). Phages can be further classified with respect to their actions that follow infection of the bacterial cell. Virulent bacteriophages reproduce immediately and induce lysis of the cell to enable progeny release, whereas temperate phages insert their genetic material into the host genome or accessory elements, where they reproduce with the host until triggered to enter the lytic pathway as observed for virulent phages [12].

Virulent tailed phages of the Caudovirales order have been the best described for phage therapeutic applications. Within this group, the Myoviridae have a large capsid head and contractile tail, the Siphoviridae have a relatively small capsid and a long flexible non-contractile tail, and the Podoviridae have a small capsid head and short tail [13]. The virulent tailed phages follow a lytic cycle that begins with the specific attachment of phage anti-receptors to host cell surface receptor molecules. This interaction is often two-step, with an initial reversible phase and then irreversible phase. Once irreversibly bound, enzymes degrade the cell wall and the genetic material is ejected into the cell with (usually) the assistance of processive host enzymes. Once transcribed, the phage genome begins to redirect the host cell metabolism including DNA replication and protein biosynthesis to the reproduction of viral nucleic acid and proteins. Often, the host genome is degraded during this process. Once complete daughter viral particles are assembled, cell lysis is initiated to release the particles. Bacterial lysis is triggered by late encoded phage proteins including holins (to permeabilize the inner cell membrane) and endolysins (to degrade the peptidoglycan) with the loss of cell wall integrity causing lysis due to osmotic differential [14].

2.1. Specificity

Host specificity (range) of phages is variable, with some phages infecting multiple species and others only growing on one known isolate. However, their specificity is much higher than that of antibiotics. The phage host cell surface receptors and antiviral defense mechanisms (genetic and physical) are the main properties that determine specificity. For some highly conserved species, a single phage can kill the majority of strains (e.g., phage P100 infects >90% *Listeria monocytogenes* isolates tested [15]). Phages that propagate on species with high clonal diversity (e.g., *Pseudomonas aeruginosa*) typically only kill a small cohort of strains [16].

Establishment of phage banks or training (*in vitro* evolution) of phages to become more active and to elicit less bacterial resistance against the infecting bacterial strain can be valid strategies to overcome limited host specificity for targeted phage therapy [16]. This strategy

likely works best for chronic infections where the target bacterium is well characterized. In order to treat acute infections, phage cocktails including phages that together span the whole spectrum of potential strains are proposed. However, the research and resources needed for the production of suitable and stable multi-component cocktails are disadvantages of this approach. An alternative approach is to use phage lytic enzymes (endolysins), which show broader host specificity at the genus and species level. Endolysins have been the subject of a recent review by our group [17], so are not discussed further here.

Antibiotics typically kill a broad-spectrum of either Gram-positive and Gram-negative bacteria including benign flora, which is increasingly considered to be non-desirable due to their adverse effects on the whole microbiota and potential to spread antibiotic resistance [18,19]. Phage therapy meets these challenges by its superior specificity and ability to treat drug-resistant isolates.

3. Phage Pharmacology

The pharmacology of phages necessitates the study of interactions between phages and bacteria as well as interactions between phages and body tissues [14]. Successful and safe phage therapy involves the effective control of phage–host interactions involving two fundamental components: pharmacodynamics and pharmacokinetics [20].

3.1. Pharmacodynamics

Pharmacodynamics is the study of the interaction of drugs with their receptors, the transduction systems to which they are related, and the changes in cells, organs, and the whole organism. The drugs' impact on the body can either be positive, thus maintaining or restoring health, or negative such as causing toxic side effects [20].

Phages can be applied via active or passive therapeutic strategies. In active treatment regimes, phages are introduced at low concentrations relative to the bacteria concentration and therapy relies on the production and release of progeny phages to infect all bacteria. Active treatments with phages are considered to have features of automated dosing and to mimic the bodies' homeostatic mechanism better than standard pharmaceuticals through the targeted killing of bacteria and phage production at actual sites of infection rather than systemically [21]. In contrast, passive phage treatment relies on single, or multiple rounds of sufficient phage concentrations to infect all target bacteria.

Compared to antibiotics, only a single phage is required to kill a single bacterium and so fewer units are required per treatment. Phages also do not dissociate from bacterial targets once irreversibly adsorbed. However, multiple phages may adsorb to individual bacteria. For these reasons, it is important to understand the concepts of multiplicity of infection (MOI), which is the ratio of phage infections per bacteria, and MOI, which is the number of phages that are administered per cell. The killing titer is another concept that can be used to guide phage therapy and is the number of effective bactericidal phage particles delivered (c.f. the number of plaque-based phage counts) [14,20–23]. Failure to recognize the special requirements of phage pharmacodynamics could result in compromises to phage therapy efficacy [20].

The degradation of phages by antibodies and other aspects of the immune system do not lead to the production and accumulation of toxic by-products. The low toxicity of phages is a consequence of their composition which, for tailed phages, is entirely protein and nucleic acid. As a result, phage therapy can be considered comparatively physiologically benign when compared to standard antibiotic therapies.

3.2. Pharmacokinetics

Pharmacokinetics describes the absorption, distribution, metabolism, and excretion of a drug. Absorption and distribution of the drug require its movement throughout the body, at first to the blood and then beyond the blood into specific tissues or compartments where the

drug may accumulate at different densities [20]. Phage pharmacokinetics are also influenced by decay and proliferation as a result of the self-replication of bacteriophages.

The route of administration for phages will also affect in situ pharmacokinetics. In clinical cases, phages are frequently delivered by parenteral administration with oral dosing, topical application, and aerosolization also common. Data on the relative effectiveness of these approaches is largely drawn from animal studies. For instance, intramuscular, intraperitoneal, and subcutaneous injection of a phage cocktail were compared for efficacy in treating a *P. aeruginosa* in a murine burn model where intraperitoneal injection was found to be the most effective, most likely due to the delivery of higher numbers of phages more quickly and for a greater sustained period than other routes [24]. When using oral phage dosing in mice, the addition of 0.025% $CaCO_3$ was found to effectively protect the phage from stomach acids and deliver the phage to the upper and lower gastrointestinal tract where they reduced numbers of the targeted *E. coli* O157:H7 [25]. When treating *Burkholderia* infections induced in mice, the aerosolization of phages was found to be superior to intraperitoneal injection [26]. Some advantages and disadvantages of the administration routes are shown in Table 1.

In vitro studies of phage pharmacokinetics using mathematical models do not necessarily reflect the *in vivo* phage kinetics observed. For instance, phage T4 was reported to not replicate *in vitro* at host concentrations below 10^4 per mL, but evidence suggests that this is possible in murine models [27]. Phage feeding experiments in animals and humans frequently report irregular shedding and the passage of high percentages (up to 90% administered) of phages in feces [27]. The failure of many phage therapy experiments has been related to a poor understanding of phage pharmacokinetics, for instance, when dosing relies too much on the self-replicating nature of phages [20].

Phage lytic enzymes (endolysins) can also be used for therapy, but their kinetics are more similar to conventional treatments. For example, Jun *et al*. [28] determined that a *Staphylococcus aureus* specific endolysin had a half-life between 0.04 and 0.38 h after intravenous administration in healthy volunteers. The decay kinetics of this endolysin is likely explained by the presence of plasma proteases. Other endolysins have demonstrated a longer half-life such as 11.3 h for CF-301 and 5.2–5.6 h (for 30 and 60 mg/kg, respectively) for P128 [29,30].

Toxin (e.g., endotoxin) release due to significant bacterial cell lysis could potentially trigger septic shock during phage therapy. However, antibiotics like amikacin, cefoxitin, and imipenem have been shown to induce higher amounts of released endotoxin than coliphages [31]. The increase in endotoxin produced after 180 min incubation of *E. coli* LM33 was 3.8-fold with phage LM33_P1, 5.5-fold with amikacin, 8.7-fold with cefoxitin, and 30-fold with imipenem. With *E. coli* strain 536, there was a 19.8-fold increase in endotoxin with amikacin, 29.9-fold with phage 536_P1, 53.7-fold with imipenem, and 125.1-fold with ceftriaxone [31].

So, whilst less of an issue than for most conventional antibiotics, high fragmentation of the cell wall must be minimized with either phage or phage endolysin therapies to prevent an increase in pro-inflammatory cytokines [19,32]. To address this potential issue, several groups have proposed genetically engineering phages to prevent or reduce cell lysis, whilst still causing cell death by mechanisms such as degrading the host genome (see Section 7 and [33]).

Table 1. Routes of administration for phage therapy.

Delivery Route	Advantages	Disadvantages	Mitigations to Hurdles
Intraperitoneal	Higher dosage volumes possible. Diffusion to other sites.	Extent of diffusion to other sites may be overestimated in humans (most data from small animals).	Multiple delivery sites.
Intramuscular	Phages delivered at infection site.	Slower diffusion of phages (possibly).	Multi-dose courses.
		Lower dosage volumes.	Multi-dose courses.
Subcutaneous	Localized and systemic diffusion.	Lower dosage volumes.	Multi-dose courses.
Intravenous	Rapid systemic	Rapid clearing of phages by the	*In vivo* selection of low-

	diffusion.	immune system.	immunogenic phages may be possible.
Topical	High dose of phages delivered at infection site.	Run-off from target site if phages suspended in liquid.	Incorporate phages into gels and dressings.
Suppository	Slow, stable release of phages over long time.	Limited applications/sites. Risk of insufficient dosing. Technically challenging to manufacture.	Careful consideration of phage kinetics required.
Oral	Ease of delivery. Higher dosage volumes possible.	Stomach acid reduces phage titer. Non-specific adherence of phages to stomach contents and other microflora.	Add calcium carbonate to buffer pH. Microencapsulation to deliver phages to target area.
Aerosol	Relative ease of delivery. Can reach poorly perfused regions of infected lungs.	High proportion of phages lost. Delivery can be impaired by mucus and biofilms	Use of depolymerases to reduce mucus.

4. Role of the Immune Response in Phage Therapy

Phages can potentially trigger innate and adaptive immune cells that may influence the success of phage therapy. Three major fields of phage-immune interaction can be discerned. First, involving immune recognition via pattern recognition receptor (PRR), which is a means for the recruitment of phagocytes to the infection site [34]. Phages can mediate the activation of innate immune cells when PRR recognizes phage-derived DNA and RNA. The extent of immune activation will differ depending on the phage type, the phage dose, and in vivo nucleic acid synthetic activity.

Second, promoted phage-neutralizing antibodies can hamper therapeutic success and this effect can increase with repeated administration [35]. Antibody induction against phages is considered to be highly variable, thus immunogenicity should be considered during phage screening prior to phage therapy. There are several externally presenting proteins on phages such as Hoc, which can potentially induce such an immune response [36–38]. Strategies to avoid phage-induced neutralizing antibody formation include refining dose concentrations, the use of low-multi-dose regimes, or low-dose passive therapy approaches.

Third, the inhibitory effect of humoral (adaptive) immunity and anti-phage antibody production on phages in the mammalian system is broadly known. Effects seem dose-dependent, with only high doses for long periods inducing specific responses. For instance, Majewska [39] developed a long-term study of antibody induction (IgM, IgG, secretory IgA) in mice fed T4 phage orally at high doses (10^9 PFU/mL drinking water). No effect was noted in the first two weeks, then in weeks 3–5, there was an increase in blood serum IgG. IgM did not increase until IgG began increasing, while IgA did not increase until days 63–79, but when it reached its maximum, no phage was found in the mouse feces. Increased IgA concentrations antagonized the gut transit of active phage and phage resistant hosts dominated the gut flora by day 92. However, IgA was rapidly cleared after phage withdrawal [39]. A similar study determined the immunological response of *Pseudomonas* phage F8 and T4 treatment in a murine systemic inflammatory response syndrome (SIR) model. The primary (IgM) and the secondary (IgG) responses inhibited the phages, and phage concentration in the spleen was significantly decreased [40].

Human trials in 26 patients with immunodeficiency diseases were undertaken to evaluate immunologic responses to phage ϕX174. An intravenous dose of 10^9 PFU/kg body weight was given, and the phage titer measured in blood. No antibody response was detected in eight cases of infantile X-linked agammaglobulinemia with circulating phages present for up to 11 days. The other 18 patients produced antibodies and phages were cleared from circulation within four days. Ten of these patients showed the IgM antibody, and eight patients produced both

IgM and IgG [41]. Other work using ϕX174 [42] has demonstrated that repeated (up to quaternary) dosing of phages does not lead to serious adverse reactions.

It is currently not well understood if anti-phage antibodies could prevent bacterial resistance development to phages and if the pre-existing immunity to natural phages could affect phage therapy. Furthermore, there is no clear information about the impact of phage-specific factors on phage clearance mechanisms. There are also gaps in our understanding of the clinical relevance of the phage immune interaction. Nevertheless, the immunogenicity of phages itself does not seem to represent a significant safety risk for patients. Reports about immune effects in clinical studies using virulent phages are limited. The introduction of validated in vitro and in vivo methods to determine the comparability of immune effects of different phages and phage combinations would be indispensable. This would allow for valid conclusions on the value of immune-based parameters for the selection of phages, identification of responsive patient populations, exchangeability of phages, and the importance of individualized phage cocktails [33]. The engineering of phages to make them less immunogenic is also an area of active research (see section 7).

5. Resistance to Phages

An important consideration for phage therapy is the potential for bacterial resistance. Phage-resistant bacteria have been noted in up to 80% of studies targeting the intestines and 50% of studies using sepsis models, with phage-resistant variants also observed in human studies [43].

As with resistance to classical antibiotics, spontaneous resistance to phages may occur through a number of mechanisms. For example, the cell surface target receptor(s) may not be expressed or become mutated, thus causing a complete loss of adsorption or decreased adsorption. This is a limitation of both phage and conventional antibiotic therapy. For both approaches, knowing the receptor site(s), their stability, and conservation across strains will help with the mitigation of resistance.

Acquired resistance is another area that requires investigation for both therapeutic approaches. Accessory genetic elements such as plasmids, temperate phages, and mobile genetic islands can carry genes coding for resistance to antibiotics. For phages, acquired resistance can encompass CRISPR-Cas systems [33], immunity proteins produced by temperate phages (though rare) and the acquisition of DNA restriction-modification systems.

A key advantage of phage therapy over conventional treatments for the avoidance of resistance development is the deployment of phage cocktails. The use of several phages, each targeting different receptors and each of a diverse genetic clade will enhance the ability to mitigate against the loss of adsorption or host genetic protection mechanisms. Genetic engineering may also provide a means to improve the diversity and targeting efficiency of phages for the avoidance of resistance (see Section 7). Another consideration is that bacterial mutations that confer phage-resistance often result in fitness costs to the resistant bacterium. Therefore, understanding and exploiting the fitness costs to resistant pathogens during therapy is a potentially promising research avenue [43].

6. Phage Therapy Clinical Trials in Humans

To date, human phage therapy trials have largely been empirical, with routine use limited to Georgia, Poland, and Russia [44]. In particular, the George Eliava Institute in Georgia has longstanding experience with the selection, isolation, and preparation of monophage and phage cocktails against a variety of bacterial pathogens for phage therapy. The therapeutic application of phages has also been undertaken for several decades at the Institute of Immunology and Experimental Therapy in Poland [45]. However, the experimental clinical data published in Russian and Polish journals are difficult to access due to security and language barriers.

Although the reporting and assessment of phage therapy need to improve, particularly with regard to efficacy and tolerability and the use of adequate patient numbers, several successful case reports have been published. The reports do provide some evidence that the

development of phage therapy is a promising alternative to combat bacterial resistance to antibiotics.

In France, the national health regulator has authorized the first treatment of patients with extremely drug-resistant and difficult to treat infections using phage therapy. Since then, six cases with various bacterial infections have been successfully treated [44]. Even though several treatments were not conducted using clinical standards suitable for drug approval in the Western world, they showed therapeutic potential for phages and how phages can be applied [45].

New therapeutic products must usually go through a long and comprehensive process involving preclinical and clinical trials to gain regulatory approval for market access. In the US, the average time for the approval of a new drug from preclinical testing is 12 years and the costs run into millions of dollars due to the length, size, and complexity of human clinical trials. For these reasons, the number of formal phage therapy clinical trials (as listed on www.ClinicalTrials.gov or https://globalclinicaltrialdata.com/) is very limited [45]. However, some of the human phage therapy clinical trials underway are summarized in Figure 1 and are described in the following case studies.

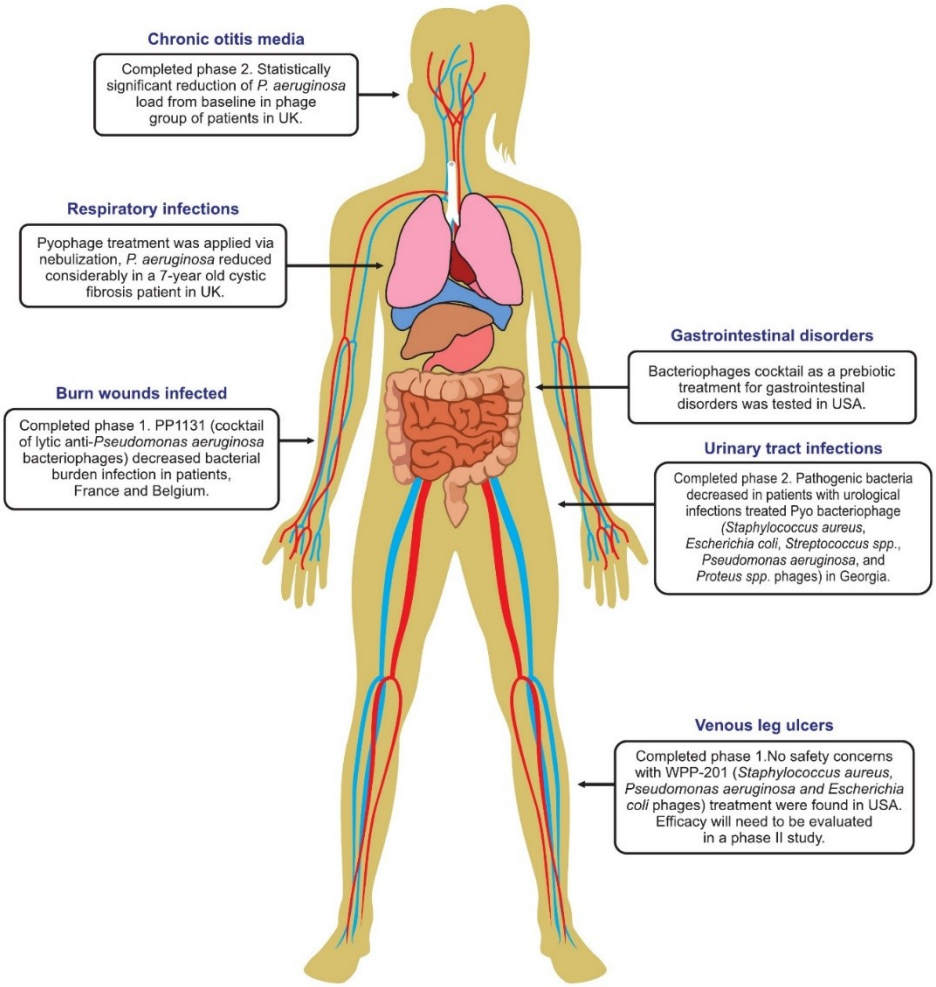

Figure 1. Human phage therapy trials and the range of target sites/infections. Image adapted from Furfaro et. al. [46].

6.1. Phage Treatment of Burns

Phage therapy was applied in wound infections in 27 patients from hospitals in France and Belgium using a cocktail of virulent anti-*Pseudomonas aeruginosa* bacteriophages. Participants were randomly assigned (1:1) to a cocktail of 12 natural virulent anti-*P. aeruginosa*

bacteriophages (10^6 plaque-forming units [PFU] per mL) or standard of care (1% sulfadiazine silver emulsion cream), and the route of administration was topical for seven days, with 14 days of follow-up [44].

The median of the primary endpoint was 144 h in the phage treatment group and 47 h in the standard of care group. Three (23%) of the 13 analyzable participants showed adverse events in the phage treatment group when compared with seven (54%) out of 13 in the standard care group. Bacteria isolated from patients of the failed phage treatment were resistant to low phage doses [44].

This study showed that phage treatment decreased bacterial burden in burn wounds in more time than the standard treatment. In this regard, studies increasing the phage concentration and the use of "phagograms" (as used for antibiograms) with more patients are warranted.

6.2. Treatment of A Septicemia Patient with Acute Kidney Damage

A man in his sixties was hospitalized for *Enterobacter cloacae* peritonitis and severe abdominal sepsis, dispersed intravascular coagulation, herniation, and bowel strangulation. Following prolonged treatment for these ailments, the patient developed gangrene and pressure sores colonized by drug-resistant *P. aeruginosa*. The infection developed to septicemia and colistin treatment (the only drug sensitivity) was carried out, however, acute kidney damage was detected, and the treatment was suspended. Subsequently, phage therapy against *P. aeruginosa* was conducted using a mixture of two phages active against the isolate in vitro, under the umbrella of Article 37 (Unproven Interventions in Clinical Practice) of the Declaration of Helsinki [46]. Following phage therapy, the patient showed improved kidney function, which returned to normal function after a few days, and blood cultures were negative.

However, the patient's pressure sores remained infected with *P. aeruginosa* and other species and four months later, the patient developed a refractory cardiac arrest due to blood culture-confirmed *Klebsiella pneumoniae* sepsis and the patient died. In vivo studies revealed that a *K. pneumoniae* strain isolated from the patient was sensitive to the antibiotics. According to historical reports, the use of phages by intravenous route in typhoid fever and *Staphylococcus aureus* [47] bacteremia were efficacious, nevertheless, this is the first contemporary report using phage monotherapy against *P. aeruginosa* septicemia in humans through the intravenous route [48].

6.3. Engineered Phages for Treatment of Mycobacteria in A Cystic Fibrosis Patient

Therapeutic phage treatment for mycobacteria has been explored in several animal models [49,50], but until recently had not been successfully used for mycobacterial infections in humans. A 15-year-old patient with cystic fibrosis and extensive comorbidities was referred for lung transplant with a disseminated infection of *Mycobacterium abscessus*. Following bilateral lung transplantation and persistent *M. abscessus* infections, phage genome engineering and forward genetics were used to engineer phages to target and kill the infectious *M. abscessus* strain. Therapy was conducted using an intravenous three-phage cocktail of 10^9 PFU of each phage every 12 h for 32 weeks [51].

Intravenous phage treatment was well tolerated, clinical improvement including sternal wound closure, improved liver and lung function, and substantial resolution of infected skin nodules were detected in the six months following therapy. No evidence of phage neutralization was detected in sera, although weak antibody responses to phage proteins were identified. Weak cytokine responses were reported for interferon-γ, interleukin-6, interleukin-10, and tumor necrosis factor-α [51]. Some evidence was presented that indicated active in vivo phage replication was taking place. Despite the apparent success of this therapy, the authors did caution that there was significant variation in *M. abscessus* phage susceptibility, so the treatment of similar patients will require more work to be undertaken to understand the science underlying this observation.

6.4. Phage Therapy for Respiratory Infections

There have been several pre-clinical studies describing the use of phage therapy against chronic bacterial lung infections using murine models. Pabary et. al. [52] determined that phage treatment reduced the infective burden and inflammatory response in the murine lung. All phage-treated mice cleared *P. aeruginosa* infection at 24 h, whereas infection persisted in all of the control mice. Phage also reduced infection and inflammation in bronchoalveolar lavage fluid when administered prophylactically. Another study showed that intranasal treatment with phage rescued mice from *Acinetobacter baumannii*-mediated pneumonia. Microcomputed tomography also indicated a reduction in lung inflammation in mice given phage [53]. In a study using a biofilm-associated murine model of chronic lung infection, phage therapy was effective seven days post-infection. Additionally, these studies established the potential for phage therapy in established and recalcitrant chronic respiratory tract infections [54].

Notwithstanding the reported treatment of *Mycobacterium* described in Section 5.3, reports of phage therapy of human bacterial respiratory infections are rare. In a 2011 case study from Georgia, a seven-year-old cystic fibrosis (CF) patient presented with chronic colonization of *P. aeruginosa* and *S. aureus* with antibiotic treatments having limited effect. Phage therapy was undertaken using a "Pyo phage" phage cocktail produced by the Eliava Institute, which reportedly contains phages active against *S. aureus*, *Streptococcus*, *Proteus*, *P. aeruginosa*, and *E. coli* [55]. The Pyo phage cocktail was delivered to the patient via nebulization at four-to-six week intervals for nine rounds of treatment. The *P. aeruginosa* numbers reduced considerably, however, the treatment was not effective against *S. aureus*. Consequently, Sb-1 phage (a phage targeting *S. aureus)* was added to the Pyo phage cocktail and administered five times with a nebulizer. This treatment reduced the concentration of *S. aureus* significantly. No adverse effects were detected in the patient upon Sb-1 phage treatment. [55].

Recent advances in the spray drying of phages that have achieve increased numbers of phages delivered to the lungs (up to 10^8 PFU/aspiration) may considerably improve clinical outcomes for respiratory infections such as these [56]. Work has also shown that a cocktail of ten phages significantly decreased *P. aeruginosa* numbers in sputum samples from 58 CF patients collected from hospitals in Paris [45,57]. Forty-eight of 58 samples were positive for *P. aeruginosa* and the addition of phages significantly decreased the concentrations of *P. aeruginosa* in the sputum. An increase in the number of bacteriophages in 45.8% of these samples was also detected, demonstrating the potential for active phage therapy of respiratory infections in vivo.

6.5. Phage Therapy for Urinary Tract Infections

Therapy for treating urinary tract infections (UTIs) is one of the most promising applications for phages and one of the few that have been studied in a multi-stage clinical trial. In the first stage of the trial, 130 patients planned for transurethral resection of the prostate were screened for UTIs and 118 patients enrolled [58]. Criteria for inclusion in the trial were having $\geq 10^4$ cfu/mL of the pathogens *S. aureus*, *E. coli*, *Streptococcus*, *P. aeruginosa*, or *Proteus mirabilis* in their urine culture. Initial in vitro screening of these cultures against the Pyo phage cocktail, a commercial product produced by the Eliava Institute, revealed that the sensitivity was 41% (48/118). Directed evolution experiments were applied to the cocktail to select for expanded host range phages, and the sensitivity was improved to 75% (88/118).

In the second stage, nine patients who had infections caused by bacteria sensitive to the Pyo cocktail underwent non-blinded phage therapy. Administration of 20 mL 10^7–10^9 PFU/mL phages was via a suprapubic catheter twice every 24 h for seven days, starting the first day after surgery. Urine from the patients was subsequently cultured seven days after surgery or at the time of adverse indications. Prior to therapy, the patients' urine screening revealed the presence of *E. coli* in four cases, *Enterococcus* in two cases, *Streptococcus* in two cases, and *P. aeruginosa* in one case. Following therapy, titers of the pathogens decreased by 1–5 log cfu/mL in six out of nine patients. One of the four *E. coli* cases had no detectable pathogen, one of two *Streptococcus* cases had no detectable pathogen, one of the *Enterococcus* cases had no pathogens, but the other case detected *E. coli*. The patient with the *P. aeruginosa* infection required antibiotic therapy

following a spike in fever and became asymptomatic; however, *P. aeruginosa* was detected in his urine. No adverse effects of phage therapy were detected. The study authors hope to further progress this work to full randomized and blinded control studies in the future.

6.6. Phage Therapy for Diarrhea

Whilst no longer an active partnership, the Nestlé Research Centre in Switzerland and the International Centre for Diarrhoeal Diseases Research in Bangladesh have undertaken joint research projects over a number of years that have explored the efficacy of phage therapy for the treatment of diarrheal diseases. In one of the studies, 120 Bangladeshi male children (6–24 months) presenting with acute bacterial diarrhea were given either 3.6×10^8 PFU of a T4-like coliphage cocktail (39 children), 1.4×10^9 PFU of a commercial coliphage preparation (Coliproteus from Microgen, 40 children), or a placebo (0.9% NaCl, 41 children) suspended in oral rehydration solution.

Results of this randomized blind trial indicated no adverse effects of oral phage treatment of the children. The phage survived the gastric passage, but there was no strong evidence of intestinal replication occurring in patients. Neither the T4-like nor the Microgen coliphage cocktail showed a significant clinical effect when compared to the control group for stool output or frequency, or rehydration. Likely reasons for the lack of significant effects were the lower than expected incidence of *E. coli* (60%) and the incidence of mixed species infections, the presence of non-susceptible coliforms (phage cocktail was not optimized for local isolates), and insufficient phage titer [59,60].

6.7. Treatment of Peri-Prosthetic Joint Infection

In this case study [61], an 80-year-old female patient with obesity and a history of relapsing prosthetic joint infection of the right hip presented with a *S. aureus* postoperative infection and was treated with debridement, antibiotics, and implant retention (DAIR). Four years later, another DAIR was performed for fluoroquinolone-resistant *E. coli,* following reimplantation surgery in the prior year. Then, due to a relapse including positive *E. coli* cultures, another DAIR was performed three weeks later. Antibacterial therapy with ceftriaxone was started; however, there were further signs of relapse and antibiotic treatment was stopped. Multidrug-resistant *P. aeruginosa* and penicillin-resistant *S. aureus* were identified in swabs of the wound discharge.

To undertake phage therapy, three phages targeted against the *P. aeruginosa* isolate were first prepared by Pherecydes Pharma (France). The *S. aureus* isolate was lost, so three phages from the Pherecydes Pharma phage bank were used. Phages were produced in a research environment with the manufacture overseen by The French National Agency for Medicines and Health Products Safety (ANSM). The final formulation of the *P. aeruginosa* and *S. aureus* phages were undertaken by the hospital pharmacy by mixing equal volumes of 10^{10} PFU/mL phage stocks. During the following DAIR, 20 mL of the phage cocktail was injected into the joint region. Co-therapy with antibiotics (daptomycin, amoxicillin, and clindamycin) was followed for the next six months without signs of *P. aeruginosa* or *S. aureus* infection. The patient later had a *Citrobacter* infection, which required DAIR, but once this was treated with Ciprofloxacin, no further infection was found in the joint (18 months post-phage therapy).

The bespoke use of phage and antibiotic combinations to treat a patient's infection has the potential to be utilized to create personalized therapy for deep and persistent tissue infections such as those found associated with peri-prosthetic joints (Figure 2).

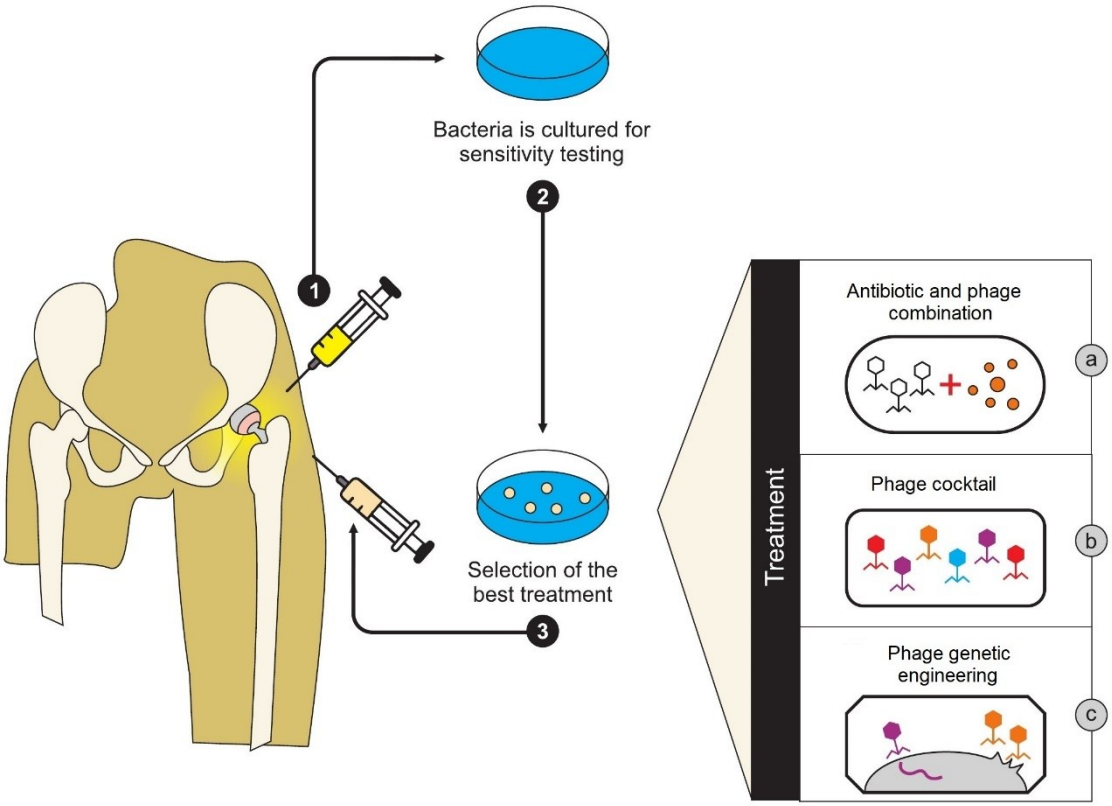

Figure 2. Personalized combinatorial phage therapy**.** Image adapted from Akanda et al. [62].

6.8. Treatment of Leg Ulcers

A Phase I trial of phage therapy with 42 patients with chronic venous leg ulcers has been undertaken [63]. Ulcers were treated for 12 weeks with a phage cocktail (WPP-201; 8×10^7 PFU/mL) or a control (saline). The phage cocktail targeted *P. aeruginosa*, *S. aureus*, and *E. coli*. Patient follow-up continued until week 24 and no adverse events were attributed to the phage treatment. There was no significant difference between the phage therapy group and the control group for the rate or frequency of ulcer healing. Efficacy of the preparation will need to be evaluated in a phase II efficacy study.

6.9. Therapy of Drug-Resistant Craniectomy Infection

A previously healthy 77-year-old male who suffered assault, subdural hematoma, and traumatic brain injury underwent a craniectomy, which was complicated by postoperative intracranial infection with multidrug-resistant *A. baumannii*. The isolate was resistant to all antibiotics; however, some isolates were sensitive to colistin [64]. An emergency investigational new drug application to use phage therapy on the patient was approved by the US Food and Drug Administration. Phages from the Naval Medical Research Center-Frederick were screened against the isolate and the most active phage prepared.

The phage (2.1×10^7 PFU/mL) was administered intravenously through a central catheter line every 2 h for eight days, with 98 total doses given. Following phage treatment, the patient initially seemed more alert, but continued to be unresponsive. The craniotomy site and skin flap healed well, though fevers and leukocytosis continued. There were no further signs of infection at the craniotomy site after surgical debridement. However, bacterial cultures obtained prior to phage administration were negative, therefore it was not possible to directly measure phage efficacy. Before the receipt of a second phage cocktail, the patient's family decided to withdraw care and the patient died.

The authors concluded that administration of phages through the surgical drain would likely have had more benefit than parenteral administration, less targeted phages with broader activity may have been more efficacious, and a better outcome might have been possible if personalized phage therapy had been developed more quickly and administered earlier in the course of infection.

6.10. Therapy of Ear Infections

A Phase I/II research trial was conducted in the UK to test the efficacy and safety of phages for the treatment of chronic ear infections (otitis media), where the infection is known to harbor antibiotic-resistant *P. aeruginosa* [65]. In this randomized double-blinded study, a cocktail of six phages produced by Biocontrol Limited (BiophagePA, 6×10^5 PFU), or placebo (glycerol-PBS solution) were administered to the ear canal of 24 patients. The follow-up to treatment was at 7, 21, and 43 days and revealed a statistically significant improvement in both clinical condition and patient-reported indicators for the phage treated group when compared to the control. No adverse reactions were noted in the phage-treated group.

In vivo replication of phages in the patients was evident for up to 23 days, with the mean recovery of phages during the trial sampling points being 200 times the input concentration. Clearance of phages was noted when the *P. aeruginosa* infection was resolved in patients. Reductions in overall *P. aeruginosa* numbers in the phage treatment group, whilst statistically significant, were generally modest, but measurement was likely to be compromised by the lack of access to deep parts of the ear canal. When *P. aeruginosa* was not completely cleared by phage therapy, there was an increase in clinical scores for some patients. The study authors suggest that repeated phage therapy after three to four weeks may be beneficial to these types of patients in any future work.

7. Engineering and Other Genetic Technologies for Phage Therapy

The advent of whole-genome sequencing and metagenomics have rapidly increased the number of phage genomes sequenced and is unlocking new insights into phage genetics. The use of this new knowledge for phage engineering holds great potential to increase the utility of phages for therapy, however, there are additional considerations such as ethical, safety, and regulatory, which need to be accounted for above that of 'natural' phage therapy. Engineering can be used to produce new variants of phages with expanded host range, decreasing the number of phage strains needed to cover bacterial diversity, and generating patentable phage variants [66–68]. For example, the host specificity of the *E. coli* K12-specific phage T2 was able to be changed by swapping gene products expressed at the tip of the long tail fiber with those of the PP01 phage, which is an *E. coli* O157: H7-specific phage [66]. The recombinant phage was able to infect *E. coli* O157: H7 and related strains, but could not infect *E. coli* K12 or its derivatives. Similarly, homologous recombination was used to replace the long tail fiber genes (genes 37 and 38) from the genome of T2 with those of the IP008 phage. The recombinant T2 phage had a host range identical to that of IP008 [67]. Lin et. al. [68] were also able to modify the *E. coli* female-specific T7 phage to overcome male exclusion by recombination with phage T3. The recombinant phages of T3 and T7 carried altered tail fibers and had better adsorption efficiency than T3.

Genetic engineering of phage permits the addition of novel functionality such as bacteriocins, enzybiotics, quorum sensing inhibitors, CRISPRs, and biofilm degrading enzymes that can enhance their killing potential [69–73]. Phages can be modified using the RNA-guided nuclease Cas9 to create sequence-specific antimicrobials. Cas9 was reprogrammed to target virulence genes and killed virulent, but not avirulent, strains of *S. aureus* in a mouse skin colonization model [65]. Another study used CRISPR-Cas technology to create RNA-guided nucleases delivered by phages to target specific DNA sequences in carbapenem-resistant *Enterobacteriaceae* and enterohemorrhagic *E. coli* [70]. Delivery of the nucleases improved the survival in a *Galleria mellonella* infection model. Phage-borne CRISPR-Cas systems can also be

used to enable site-specific cleavage to induce cytotoxicity, activate toxin-antitoxin systems, re-sensitize bacterial populations to antibiotics, and modulate bacterial consortia [70].

Biofilms are the major cause of persistent infections in clinical settings, thus phage treatment to lyse bacteria in biofilms has attracted growing interest. An engineered T7 phage was constructed to encode a lactonase enzyme with broad-range activity for the quenching of quorum sensing molecules necessary for biofilm formation. The T7 phage incorporating the AHL lactonase *aiiA* gene from *Bacillus anthracis* degraded AHLs from diverse bacteria and caused the inhibition of a mixed-species biofilm composed of *P. aeruginosa* and *E. coli* [71]. In another approach using the T7 phage, a biofilm-degrading enzyme, DspB, produced by *Actinobacillus actinomycetemcomitans*, was inserted into the T7 genome and the resultant phage reduced *E. coli* biofilm cell counts by an additional 2 log when compared to the unmodified T7 [72].

As described in Section 4, components of the innate immune system can remove a significant proportion of administered phage. Studies have shown that long-circulating phage mutants can be isolated to address this issue. Vitiello et. al. [73] determined that a single specific substitution in the major phage capsid (E) protein of the lambda Argo phage was enough to confer a long-circulating phenotype that enhanced phage survival in the mouse circulatory system by more than a 1000-fold. Merril et. al. [74] used a serial passage selection method to isolate phage mutants with a greater capacity to remain in the circulatory system of the mouse. Lambda phage mutants with 13,000–16,000-fold better capacity to stay in the mouse circulatory system for 24 h after intraperitoneal injection were isolated.

Many antibiotics, as well as phage therapy, can present side effects due to endotoxin release from Gram-negative bacteria. To address this, genetic engineering was used to generate non-replicating non-lytic phage targeting *P. aeruginosa*. An export protein gene of the *P. aeruginosa* filamentous phage Pf3 was replaced with a restriction endonuclease gene and the variant (Pf3R) was non-replicative and prevented the release of phage from the target cell. Endotoxin release was kept to a minimum and the Pf3R phage efficiently killed a wild-type host in vitro. Phage therapy using Pf3R showed comparable or increased survival rates (depending on dose) when compared to Pf3 upon challenge in the mice model. Higher survival rates were correlated with a reduced inflammatory response when using Pf3R treatment [75]. Matsuda et. al. [76] also produced lysis-deficient T4 phages for this purpose. Mutant t amber A3 T4 phages were compared to wild-type T4 in mouse bacterial peritonitis model. Survival was significantly higher in mice treated with the lysis deficient phage when compared to the wild-type, and enterotoxin levels were significantly lower in the t A3 T4-treated mice at 12 hours after infection [76].

8. The Medicinal Regulatory Status of Phages

Phages are not specifically classified as living or chemical agents in any national medicinal legislation (as far as we are aware). This considerably complicates the regulation of human phage therapy clinical trials and commercialization of phage products as well-established safety, good manufacturing practice, and efficacy benchmarks are lacking [77]. Another barrier is that in order to prove the efficiency of phage preparations, their effectiveness and host range toward currently circulating pathogenic strains must be constantly monitored. This is most likely why the Russian Federation and Georgia approved phage preparations are continuously updated to target newly emerging pathogenic strains [78]. Therefore, any specific legislation regarding phage products would ideally permit these formulation updates as required to avoid repeated registration procedures.

A breakthrough for the regulation of phage therapy occurred in 2016, when the Belgian Minister of Social Affairs and Public Health defined the status of therapeutic phage preparations as industrially-prepared medicinal products (subjected to constraints related to marketing authorization) or as magistral (compounded) preparations prepared in the pharmacies' officinal [79]. Natural phages and their products can be processed by a pharmacist as raw materials (active ingredients) in magistral preparations, providing there is compliance

with several provisions of the European Directive requirements for medicinal products for human use [78].

Several jurisdictions also permit the use of phages on compassionate grounds, where all other therapies have failed, and the condition is immediately life-threatening. These include the US FDA Expanded Access Program (www.fda.gov/news-events/public-health-focus/expanded-access) and Investigational Drug Program (https://www.fda.gov/drugs/types-applications/investigational-new-drug-ind-application) and the European Medicines Agency (https://www.ema.europa.eu/en/human-regulatory/research-development/compassionate-use).

9. Advantages and Disadvantages of Phage Therapy

Compared to conventional antibiotic therapy for bacterial infections, phage therapy has both a number of great advantages, but also some disadvantages. Some of these have been summarized in Table 2 and some aspects are discussed in more detail in the following subsections.

Table 2. Advantages and disadvantages of phage vs. antibiotic therapy for the treatment of bacterial infections.

Consideration	Antibiotic therapy	Phage therapy
Specificity	Low	High
Development costs	High	Low-moderate
Side effects	Moderate-high	Usually low, but yet to be fully established
Resistance	Increasing incidence of multi-drug resistant isolates.	Can treat multi-drug-resistant isolates. Phage resistant isolates generally lack fitness.
Delivery to target	Moderate	Moderate to good. Can penetrate the blood-brain barrier.
Formulation	Fixed	Fixed or variable
Regulation	Well established	Underdeveloped
Kinetics	Single hit	Single hit or self-amplifying
Immunogenicity	Variable	Likely low, but not well established
Clinical validation	Many trial studies	Relatively few trial studies

9.1. Key Advantages

Phage therapy has several key advantages that make it an attractive alternative to antibiotics. First, phages have high specificity to their hosts and unlike antibiotics, which have a much wider spectrum, are unlikely to cause dysbiosis and secondary infections (e.g., fungal infections). To date, phages have also not shown any significant side effects or risks of toxicity on mammalian cells [79]. Moreover, the process of isolation and selection of new phages is less expensive, in terms of time and costs, than the development process required for antibiotics: it typically takes millions of dollars and numerous years to develop an effective antibiotic drug [81].

The development of the resistance of bacteria to phage therapy is likely less significant than for antibiotics because of the ability to adapt phage cocktails by the substitution of phages, applying in vitro evolutionary pressure, or by genetic engineering. The variant resistant mutants are also generally of lower fitness. Phages are also able to successfully treat multi-drug-resistant bacteria as they use different mechanisms for targeting cells.

The ability of phages to widely spread through the body when applied by systemic administration, along with self-replication in the presence of the host, are qualities that most antibiotics do not have. Unlike most antibiotics, phages can also pass through the blood–brain barrier [82]. Some phages can also infiltrate and disrupt the biofilms that many pathogens naturally inhabit [82,83].

For patients with allergies to antibiotics, their treatment options can be restricted. About 1% of hospitalized patients have an allergy to penicillin-group drugs, the most common antibiotic allergy, followed by sulfonamides and tetracyclines [84]. Cross-reaction of penicillin allergies to next-generation cephalosporins and carbapenems has also been reported, but this remains controversial [85]. Phage therapy may be a valuable option for patients with antibiotic allergies, but reports are rare. For example, 12 patients with inflammatory soft tissue shotgun wounds and allergy to antibiotics (not specified) were reported to have been treated by polyvalent phage therapy (*Staphylococcus*, *Streptococcus*, *Proteus vulgaris*, *Proteus mirabilis*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae*) for 15 days [86]. The concentrations of bacteria and the areas of wound healing were similar in the phage treatment group when compared to a control group of 35 patients receiving antibiotics. The authors concluded that phages were a reliable method for reducing microbial infection and that treatment led to a rapid epithelialization of the wound site [86].

9.2. Key Disadvantages

There are currently some key disadvantages of using phages as alternatives for antibiotics. However, these are predominantly due to gaps in knowledge and regulations, which may be resolved in the future. Critically, there is a lack of depth of information about the clinical application of phages for controlling bacterial infections. Much experimental clinical data published in Russian and Polish journals are difficult to access due to security and language barriers. There are also many more challenges for scientists in obtaining regulatory approval for phage-based therapeutic applications when compared to conventional therapies [80].

There is a lack of common established and validated protocols for the routes of administration, dose, frequency, and duration of phage treatment, which hampers inter-study comparison [87]. Often, the purity and stability of phage preparations used for clinical trials are also uncertain, with insufficient quality control data presented.

The concentration of phages may be reduced significantly during therapy by the reticuloendothelial system or be neutralized by antibodies, thus inhibiting their antimicrobial activity [39,88]. However, the effect of phage-neutralizing antibodies can be mitigated by refining dosing regimens and breeding phages to evade the immune system.

The genetic biosafety of phages is complex to assess. Phages used for therapy must not contain toxin or virulence genes, antibiotic resistance genes, or be able to horizontally transfer genes in the human microflora. Whilst whole-genome sequencing is a powerful tool to assist with these analyses, there is still an incomplete understanding of the functions of all encoded phage genes. Genetic engineering of phages will also likely invite greater scrutiny of safety which practitioners will need to address before application.

10. Conclusions

Antimicrobial resistance is increasing globally, and new treatments are urgently needed to meet this challenge in medical care. Whilst phage therapy for bacterial infections has been around for more than a century, the antibiotic-resistance crisis is providing renewed impetus for phage therapy to deliver on its long-held promise as a clinical treatment. As described here, there is an increasing number of well-executed Phase I/II clinical trials describing the safety and efficacy of phage therapy. There is an improved understanding of the pharmacology, immunology, safety, and potential for bacterial resistance. Technologies such as genetic engineering, whole-genome sequencing, and metagenomics also provide new tools to optimize phage therapeutic strategies. However, there are still data gaps on its efficacy and a lack of standardization and suitable regulatory frameworks that need to be resolved before phage therapy can take its place in mainstream medicine. Given the renewed interest and impetus in the field of phage therapy, there are reasons to be optimistic that these challenges can be met in the coming years.

Author Contributions: Conceptualization, D.R-C. and C.B.; methodology, D.R-C.; writing—original draft preparation, D.R-C. and C.B.; writing—review and editing, D.R-C., R.G.-B., A.G.-N. and C.B.; visualization, D.R-C.; supervision, R.G.-B., A.G.-N. and C.B.; project administration, C.B.; funding acquisition, C.B.

Funding: This research was funded by an ESR Strategic Science Investment Fund grant to CB.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Capítulo 2

Systematic review and meta-analysis as tools to improve application of bacteriophages patented for biocontrol of foodborne pathogens

Systematic review and meta-analysis as tools to improve application of bacteriophages patented for biocontrol of foodborne pathogens

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Abstract

Foodborne diseases are a global public health issue with 1 in 10 people falling ill after eating contaminated food every year. In response, the food industry has implemented several new pathogen control strategies, such as biotechnological tools using the direct application of bacteriophages for biological control. We undertook a systematic review and meta-analysis that evaluated the efficiency of phages patented as a biological control for foodborne pathogens and determined the physical-chemical characteristics of the antimicrobial effect.

The systematic review was developed using scientific article and patent databases with inclusion and exclusion criteria applied by automatic and manual processes. A random-effects meta-analysis was carried out and revealed: (i) significant antimicrobial effect of *Listeria* phages in apple, apple juice, pear, and pear juice (p-val= < 0.0001), (ii) significant antimicrobial effect of *Salmonella* phages in eggs, apple and ready to cook chicken (p-val= 0.0001), (iii) no heterogeneity (I2= 0%, tau^2=0) was identified in either meta-analysis, (iv) publication bias was detected in *Listeria* phages but not in *Salmonella* phages. (v) ListShield and Felix01 phages showed the best result for *Listeria* and *Salmonella* biological control, respectively, (vi) concentration of phage and bacteria (p-val=$1.05x10^5$ and < $2x10^{-16}$), time ($1.44x10^{-9}$) and food ($8.16x10^{-5}$) had significant effect in the biological control of *Listeria*, (vii) temperature (p-val= 0.00825) and time (p-val=0.00374) had a significant effect on the antimicrobial activity of *Salmonella* phages.

In summary, we evaluated the efficiency of phages previously patented as a biological control for fruits and vegetables, and meat.

Keywords: biocontrol, bacteriophages, foodborne disease and patent.

1. Introduction

Foodborne diseases are a significant public health issue, causing pressure on healthcare systems, lost productivity due to worker illness, and they harm tourism and impact trade. For the foodborne diseases causing diarrhea, a disproportionate burden falls on children under five years old and those living in low- and middle-income countries. In addition to environmental contamination, pollution in water, soil and air, food processing and unsafe food storage are factors in illness development (WHO, 2020).

Outbreaks of listeriosis, salmonellosis, campylobacteriosis, hemorrhagic colitis and hemolytic uremic syndrome are still commonly associated with the consumption of processed and raw foods. In designing effective interventions to mitigate these outbreaks, consequences such as antibiotic resistance, gut microbiota disturbances, and residual effects on human health and the environment must be avoided. The increasing popularity of more natural and organic foods, changing consumer preferences, and large-scale production of food animals, are also driving the need for new interventions (Brauer *et al*., 2019).

One alternative to control foodborne pathogens in foods is the use of bacteriophages (phages). Phages are viruses that infect bacteria and Archaea, which have no machinery for generating energy, and no ribosomes for making proteins. Phages are very specific in targeting and infecting the host bacteria or Archaea species (2015; Brauer *et al*., 2019; Romero *et al*., 2019).

The therapeutic potential of phages was recognized immediately after the discovery by d'Herelle and Twort at the beginning of the 20th century (Twort, 1915; D'Herelle and Roux, 2007; Holtappels *et al*., 2019). Nonetheless, after the discovery and successful application of antibiotics, phage therapy was virtually forgotten in the

Western countries (Americas, Western, Europe), although, phage therapy was routinely carried out in the former Soviet Union and Eastern Europe (Holtappels *et al*., 2019).

Due to the rise of multidrug resistance in bacteria and the scarcity of new antibiotics in the drug development pipelines (WHO, 2020), the interest in phage therapy has been rekindled for use in human health (2012; Romero *et al*., 2019), veterinary medicine (Fenton *et al*., 2010), agriculture (Buttimer *et al*., 2017, Premaratne *et al*., 2021), aquaculture (Richards, 2014), and food safety (Pires *et al*., 2016, Goodridge *et al*., 2018).

Several products based on phages have been approved as food processing aids: LISTEX (effective against *Listeria monocytogenes*), SALMONELLEX (effective against *Salmonella enterica*), both from the company Micreos, and ListShield™ (effective against *Listeria monocytogenes*), Ecoshield™ (effective against *Escherichia coli*) and SalmoFresh™ (effective against *Salmonella enterica*) from the company Intralytix,

Patent applications in the life sciences are the basis for the commercialization of new life-science and healthcare-related technologies as well as the critical metric of innovation (Smith *et al*., 2017). Here we describe a systematic review of patented phage approaches for biocontrol of bacterial pathogens on food with the aim of discovering the key factors for efficacy, so that future applications of phage biotechnology in foods can be optimally deployed.

2. Materials and methods

The systematic review and meta-analysis were conducted in five stages: planning, bibliographic search, initial selection, final selection, quality data selection and quantitative data selection, a summary of data and results. All these steps were

performed based on the bibliographic search protocols developed by Moher *et al.*, 2009. Indexing databases for the bibliographic search (Scopus, Web of Science (WoS) and PubMed (Medline) were addressed by an automated script written in Python 3 Python Core Team (2015) and deposited on GitHub (https://github.com/glenjasper). Subsequently, a manual review of outputs was performed by three independent reviewers (Figure 1).

2.1.2 Selection of articles and documents

First keyword screening: search in titles, abstracts and keywords sections

Scopus, WoS, and PubMed (Medline) databases were searched with the following search string: ((phage) OR (bacteriophage) OR (phage therapy) OR (biocontrol) OR (biosanitization) OR (biopreservation) AND (foodborne pathogens) OR (food safety)) in titles, abstracts and keywords of the publications. Database searches included documents published from 1960 for Scopus and from 1945 for WoS, to October 2021 it was not possible to use data restriction for PubMed. Duplicate records were filtered with the Digital Object Identifier (DOI) using a ***format_input.py*** *script* (https://github.com/glenjasper/format-input) and removed with the ***remove_duplicates.py*** script (https://github.com/glenjasper/remove-duplicates), the documents were downloaded using a script that engaged the UFMG (Federal University of Minas Gerais, Brazil) network. PDF file format were transformed into TXT files using the script ***pdf2txt.py*** (HTTP: // github.com/glenjasper/pdf2txt). The script internally uses the XpdfReader program (http://www.xpdfreader.com).

Second keyword screening: search in the materials and methods section

Keywords were searched in the materials and methods section of the publications with the following string: "phages, bacteriophages, biocontrol, the multiplicity of

infection, MOI, PFU, *Streptococcus, Staphylococcus, Campylobacter, Shigella, Bacillus, Clostridium, Listeria, Salmonella, Enterobacter, Yersinia, Aeromonas, Pseudomonas, Escherichia" using the script* ***search_keywords.py*** (https://github.com/glenjasper/search-keywords).

Manual document review. A full-text manual review was conducted by three independent reviewers according to inclusion and exclusion criteria.

2.1.2 Selection of patent files

Patent databases (WIPO, ESPACENET, UPSTO, LATIPAT and INPI) were used to identify the phage patents according to the following strings: WIPO *Keywords:* (phage OR bacteriophage) AND (biocontrol) AND (foodborne pathogens OR food safety) not (*Bacillus subtilis* or *Paenibacillus*). ESPACENET *Keywords:* (phages OR bacteriophages) AND (Food safety OR foodborne pathogens). USPTO *Keywords*: (Phages or Bacteriophages) AND (biocontrol) AND (foodborne), LATIPAT *Keywords*: (bacteriófagos) AND (alimentos) and INPI *Keywords*: (bacteriófagos) AND (alimentos).

The databases WIPO, ESPACENT, and UPSTO databases were filtered, then the duplicate patent files were deleted, and unique documents were selected using a script (a programming language for a special run-time environment that automates the execution of tasks). For LATIPAT and INPI databases, full-text manual reviews were conducted by three independent reviewers according to the inclusion and exclusion criteria.

After that, the documents were selected according to exclusion and inclusion criteria all these processes were carried out by three independent reviewers. A detailed PRISMA

flow diagram guideline for systematic review and meta-analysis steps is described in Figure 1.

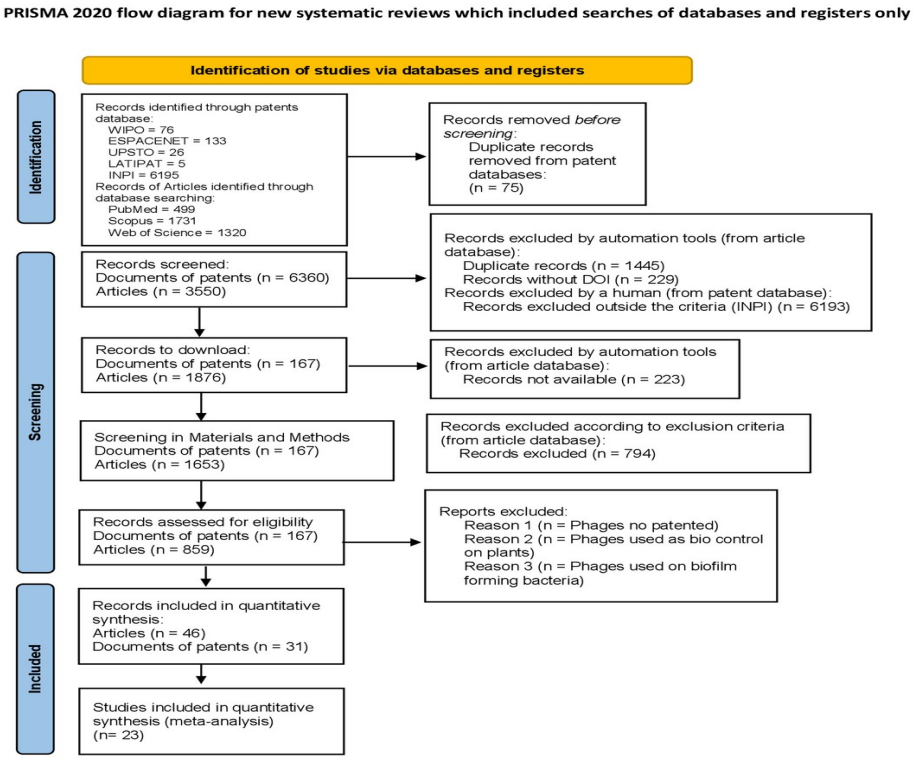

Figure 1. PRISMA work-flow applied to the study

2.3 Selection criteria

Eligibility criteria were based on the PICO approach, study design, and date, certain features that were described in excluded criteria (Section 2.3.1) and included criteria (Section 2.3.2) and undertaken by three reviewers to avoid bias in the systematic review.

2.3.1 Included criteria

Phage patent files with an application in biocontrol on food, and scientific articles and book chapters that used phages patented for food biological control.

2.3.2 Excluded criteria

Patent documents, scientific articles, and book chapters that included phage therapy in humans, animals, and biocontrol on plants but did not have an application on food were not considered in the study.

Studies (patents files, book chapters, and scientific articles) of phage biocontrol of pathogenic bacteria biofilm on food and did not evaluate the phage biocontrol on the planktonic stage of pathogenic bacteria on food. Unrelated, duplicated, unavailable full texts or abstract papers were not considered for the study.

2.4 *Analysis of data*

For visualization of the data, VOSviewer® software was used, and the displayed network depicts the maps of authors and keywords (Van Eck and Waltman, 2009). VOSviewer combines visualization and clustering techniques, enhancing the analyses while bypassing unnecessary technical complications. This tool was designed for articles and chapter of book analyses, but not for patent documents.

Mapping of phage patents visualization was performed using Leaflet, an open-source JavaScript library for mobile-friendly interactive maps using the *script* (https://glenjasper.github.io/leaflet-phage-map/). This information was collected from patent documents, scientific articles, and book chapters; table 6 describes the name of the applicant, country, code, patent, bacteria target, food, and reference, the table 6 was also uploaded on the website.

2.5 Statistical analysis

Twenty-three scientific articles were selected for meta-analysis, the efficacy of *Salmonella* phages in Cantaloupe melons, Chicken, Chicken breast, eggs, ground turkey, honeydew melons, lettuce, milk, mung bean sprouts, pasteurized milk cheese, pigskin, precooked sliced turkey, raw cheese milk, raw meat, raw tuna, ready to cook chicken, red apples, romaine lettuce, Sausage and sprout, and for *Listeria* phages in fresh sausage, apple, apple juice, beef, cabbage, catfish fillets, cheese, chocolate milk, honeydew melons, hot dogs, Iceberg lettuce, lettuce, melon, melon juice, mixed seafood, mozzarella cheese brine, pear, pear juice, precooked sliced turkey, raw salmon fillet tissue, red smear soft cheese, sliced cooked turkey breast, smoked salmon and white mold soft cheese.

The efficacy and the heterogeneity of one hundred ninety-two (192) experiments of ten studies were for *Salmonella* phages and four hundred eighty-four (484) experiments of ten studies for *Listeria* phages were evaluated by meta-analyses using the random-effect model (statistical synthesis of trials that examine the same or similar research question under the assumption that the underlying true effects differ across trials), standardized mean difference (SMD) was determined from a cross-over trial divides the mean difference by the standard deviation of measurements (and not by the

standard deviation of the differences). A SMD can be calculated by pooled intervention-specific standard deviations

The effect size was calculated by default by Meta package. A Principal Components Analysis (PCA) and meta-regression of some physical-chemical characteristics involved in the phage antimicrobial effect are temperatures, time, initial concentration of phages and bacteria were evaluated. ANOVA of the phages with respect to the log reduction of bacteria was measured. The map was built using ggplot2 and scatterpie packages, an interactive map is available (https://glenjasper.github.io/leaflet-phage-map), made with leaflet 1.6, in which more details can be found of patents, phages, food matrices and patent institutions, all these analyses were carried out in R Core Team (2021). The keywords selection map was done using Nvivo software.

3. Results

3.1 Systematic review

Identification of relevant documents relating to the use of phage for food biocontrol, first began by keyword searching of scientific articles using the terms described in materials and methods. This search identified 3550 records, including 499 records in PubMed, 1731 records in Scopus, and 1320 records in WoS. In total, 1653 unique records were identified, with 1897 records either duplicated or not available download which were excluded. Records without DOI were also excluded. All the PDFs were transformed into TXT files for subsequent analysis. In the second keyword screening, 859 documents were identified. Subsequently, 45 documents in English and 1 in Polish were selected manually for further analysis by three independent reviewers. For patent screening, 6360 documents were identified in patent databases, comprising

6139 duplicate documents or not available to download, as a result, 167 unique patents were chosen. In addition, 31 patents were manually selected by three reviewers.

3.1.1 Space-time analysis of phage

A map visualization of applications for phage patents that made claims for phage biocontrol in foodborne diseases was made using Leaflet software (Figure 2). The geographic distribution showed 41 phages patented applied to food, 46.34% from Europe, 29.27% from North America, 21.95% from Asia and 2.44 % from South America. There were no identified phage patents for food biological control in Africa and Oceania, (Supplementary material, Table 1s). Overall, 58.54% of phage patents belonged to specific foods and 41.46% to general food (Figure 2).

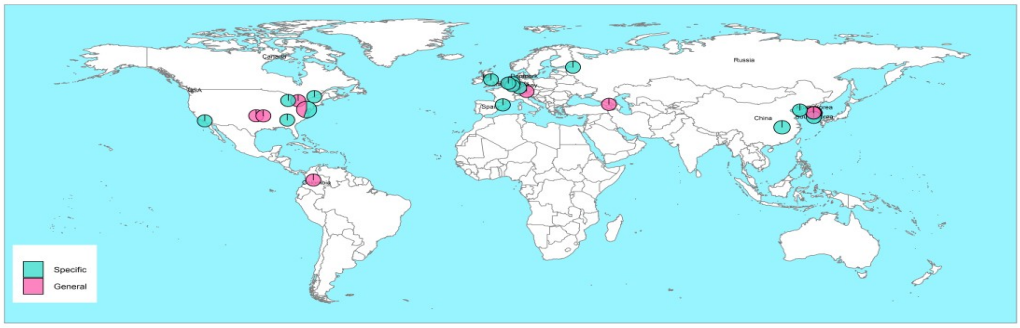

Figure 2. Map visualization of phage patents used for food biocontrol pink circles: for general food matrix, and sky-blue circles: for specific foods, the size of the circles represents the number of phage patents (https://glenjasper.github.io/leaflet-phage-map/).

Temporal analysis of the number of institutions submitting phage patents with time revealed relatively infrequent patent activity from 1995 to 2006. Then the most applications in a single year were made in 2007. Following 2007, there was increased patent activity with several applications and publications of granted patents per year.

Two applications, and three publications, of phage patents were revealed in the last year analysed (2019) (Figure 3). Although several phage patents were later withdrawn or not granted, an increasing interest in phages as antimicrobial agents in the food industry is evident.

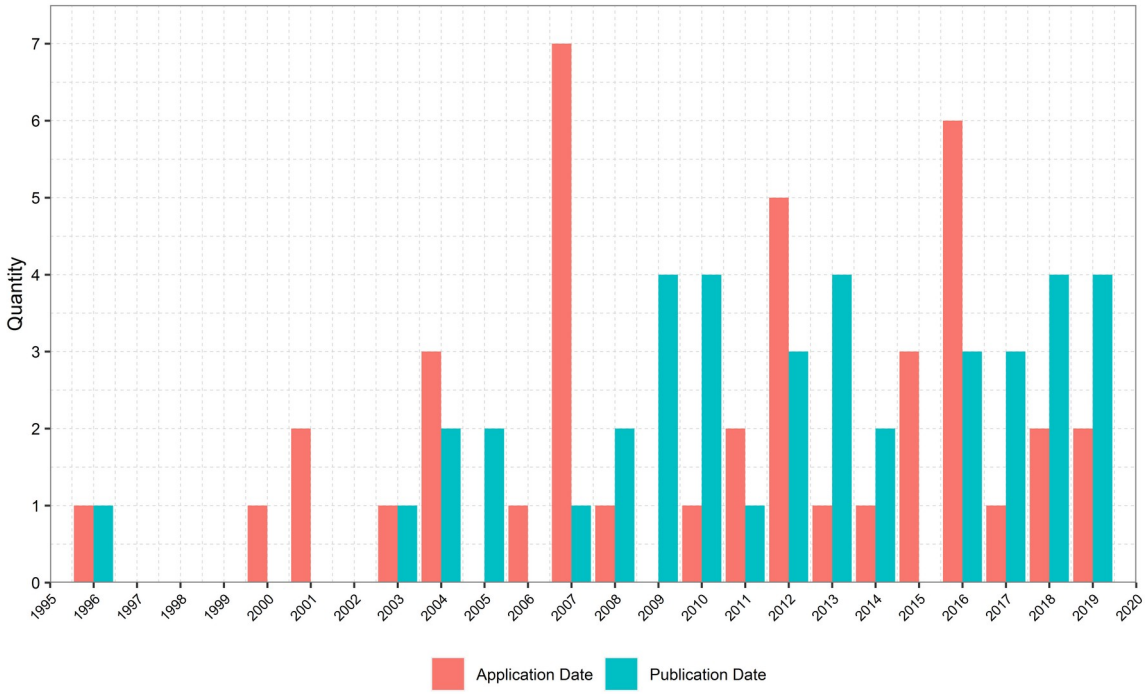

Figure 3. Number of phage for food biocontrol patents with respect to application and publication dates.

3.1.2 Phage patent description for biocontrol usage

Among the 41 phages patented for biocontrol in food, the minority of applicants (29.26%) were from Universities and the majority (70.74%) were from private companies. Target bacteria included *L. monocytogenes, Salmonella* sp., *E. coli, Pseudomonas* sp., *Shigella* sp., *Staphylococcus* sp., *Clostridium sp., Campylobacter* sp., and *Staphylococcus* sp. The most frequent targets for biological control of foodborne diseases in patents were *L. monocytogenes* and *Salmonella* sp. Foods including dairy

products, fruits, vegetables, meats, and fish were used as a matrix to test the biological control potential of the phage patented (supplementary material, table 1s).

3.2 Scientific article analysis

An analysis of the selected scientific articles was undertaken to determine the most frequent terms used in these food biocontrol studies. The results are shown as a word cloud (Figure 4). In total, 100 keywords were identified, including several words related to foodborne diseases. The most frequent keywords associated with this category were: phage (1.26%) *Listeria monocytogenes* (0.94%), food (0.85%), P100 (0.73%), CFU (0.53%), bacteriophage (0.43%) *Salmonella* (0.42%); and others (94.84%).

Figure 4. Word map reflecting the most cited terms for all the evaluated articles used in the review processes.

The scientific article data were analyzed to identify the connections between the most relevant keywords in title and abstracts fields using the VOSviewer software. The association strength method was used for normalizing the strength of the links between items. As a result, five clusters were identified (Figure 5). The authors most cited in

each of these clusters were Leverentz *et al*., (2001), Guenter *et al*., (2009), Goodridge (2001) and Hooton *et al*., (2001) and Lone (2016).

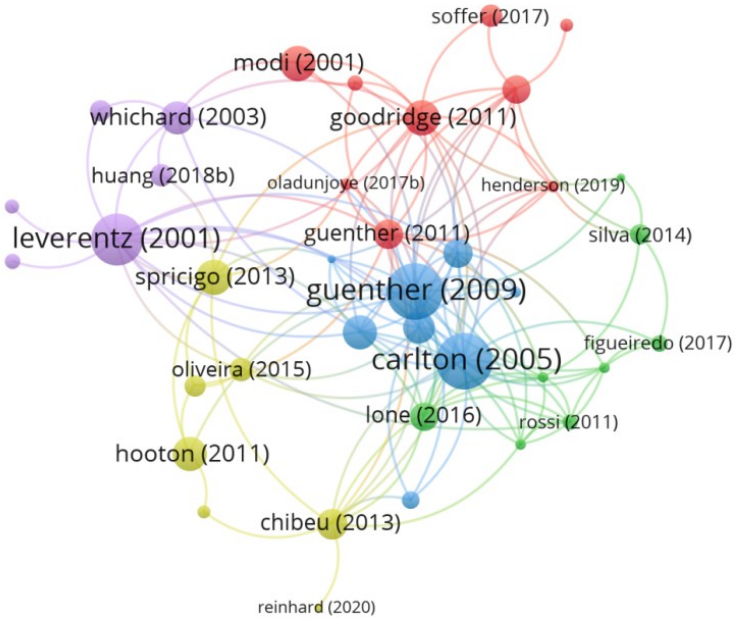

Figure 5. Network visualization of association between the author and citations, each colour represents a cluster

3.3 Meta-analysis

The systematic analysis identified 77 documents, 46 scientific articles and 31 documents of patents (Figure 1), *Listeria monocytogenes* and *Salmonella* sp. were the majority of targets identified in the screening, so we focused on these strains to do the meta-analysis, 484 and 192 experiments for *Listeria* and *Salmonella* phages for quantitative data analysis of these materials revealed *L. monocytogenes* and *Salmonella* sp. had a higher frequency of keywords in scientific articles (Figure 4).

3.3.1 *Listeria* and *Salmonella* phage activity

To identify the antimicrobial activity of different phages, ANOVA of log reduction of bacteria and phages was determined. Four phages of *Listeria* (two single phages and two cocktails) and nine *Salmonella* phages (five single phages and four cocktail phages) were identified. *Listeria* and *Salmonella* phage data showed non-parametric distribution (Shannon index, p-val= $< 2.2x10^{-16}$ and $4.53x10^{-9}$, respectively). Furthermore, significant differences in bacteria log reduction achieved were identified for *Listeria* and *Salmonella* phages (Kruskal-Wallis, p-val= $< 2.2x10^{-16}$ and $2.67x10^{-6}$). ListShield™ phages had the lowest median log reduction of *Listeria* on foods ($0.10\log_{10}$ CFU/sample), whereas the A511 phage had the highest median reduction ($2.7\log_{10}$ CFU/sample; Figure 6A). Furthermore, A511, LM 103 and LMP 102 cocktail, and A511 phages showed outliers (Figure 6A). For *Salmonella* applications, Felix 01 phage showed the lowest median log reduction of bacteria on foods ($0.35\log_{10}$ CFU/sample), and SJ2 had the highest median reductions ($2.0\log_{10}$ CFU/sample, Figure 6B). Felix 01 phage, LPST10 phage, SalmoFresh, SalmoLyse, and SJ2 phage showed outliers (Figure 6B).

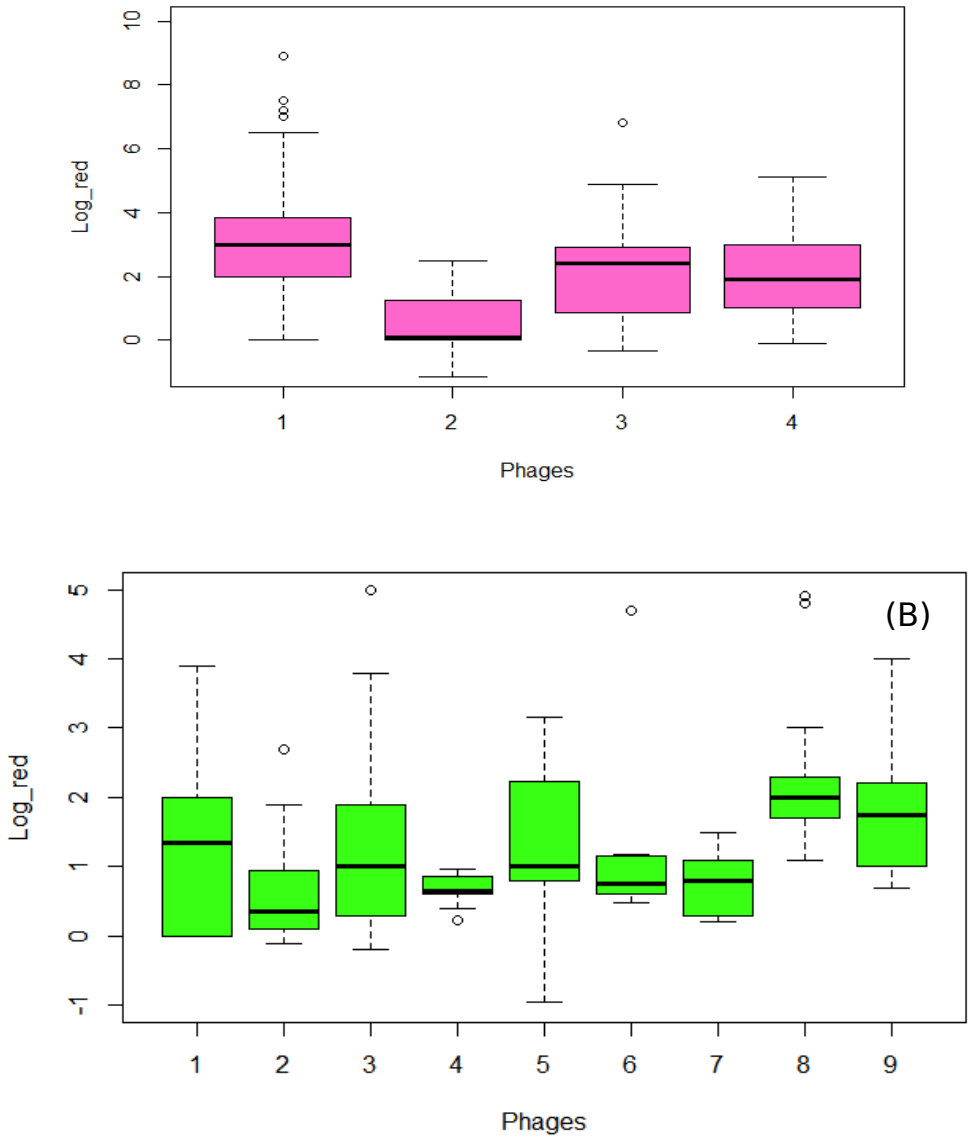

Figure 6. Boxplots of $\log_{10}$ CFU reductions of *Listeria monocytogenes* and *Salmonella* by different phages

(A.) *Listeria* (1= A511, 2= ListShield, 3=LM103 and LMP102, 4=P100) and (B.) *Salmonella* (1= SCPLX1, 2= Felix O1, 3= LPST10, 4= Felix O1, ΦSH17, ΦSH18 and ΦSH19, 5= SalmoFresh, 6= SalmoLyse, 7= P7, 8= SJ2 and 9= UABPhi20, UABPhi78 and UABPhi87).

3.3.2 Principal Component Analyses

Principal Components Analysis (PCA) was carried out for *Listeria* and *Salmonella* phage biocontrol variables (Figure 7). For *Listeria* phages, phage concentration was positively associated with temperature in Principal Component 1 (PC1), and bacteria log reduction is associated positively with food in Principal Component 2 (PC2), with bacteria log reduction having the most contribution to the PCA (Figure 7A). For *Salmonella* phages, bacteria concentration was positively associated with temperature in PC1, and bacteria log reduction was associated positively with food, the concentration of phages and time in PC2, and time had the most contribution to PCA (Figure 7B).

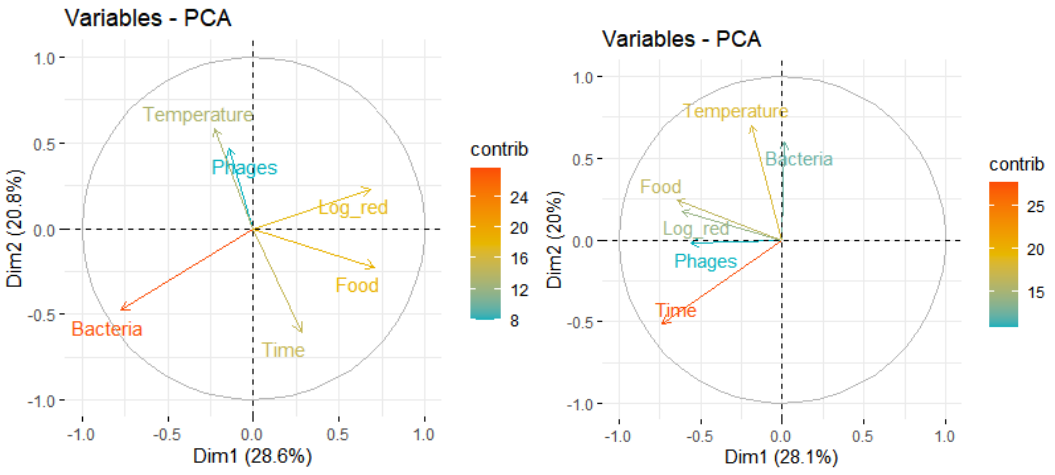

Figure 7. Principal Components Analysis on food biocontrol variables for *Listeria* and *Salmonella* phages.

(A) *Listeria* phages PCA (49.4%) and (B) *Salmonella* phages PCA (48.1%).

3.3.3 Meta-regression of physicochemical parameters

Significant correlation was identified between the log reductions of *Listeria* and initial concentration of phages (p-val=1.05x10^{-5}), initial concentration of bacteria (< 2x10^{-16} ***), time (1.44x10^{-9} ***) and food type (8.16x10^{-5} ***). An overall positive

and significant correlation was found (adjusted R-square = 0.2764, p-val = $2x10^{-16}$, intercept = 7.69) for the physicochemical factors described and log reduction by *Listeria* phages.

For *Salmonella* phages, temperature (p-val = 0.00825 **) and time (p-val = 0.00374 **) showed positive and significant correlation with log reduction of bacteria. But overall, there was no significant correlation for physicochemical factors with log reduction bacteria (adjusted R-square = 0.09095, p-val = 0.0003457, intercept = 0.46).

3.3.4 Meta-analysis of *Listeria* and *Salmonella* phages in different food matrices

We investigated the effect of the food matrix on the biological control efficacy of *Listeria* and *Salmonella* phages. A meta-analysis of the antimicrobial effect of *Listeria* phages on vegetables, meat, and dairy products was undertaken on 484 experiments from the literature. In these experiments, a significant antimicrobial effect was found (p-val < 0.0001), however there was high heterogeneity (I^2 = 82.0% [80.5%; 83.4%] and tau^2 =12.94 [<0.00; <0.00]). Further analysis of the standardized mean difference (SMD = -3.15; 95%-CI= -3.90 and -2.40) revealed there was a significant antimicrobial activity effect of *Listeria* phages (a lower bacteria concentration in the treated group) and a high effect size (z= -8.24).

To better understand these data, a subgroup meta-analysis was undertaken for 23 foods individually (Table 1). There were significant differences found both between and within groups (p-val < 0.0001). The biggest effect of *Listeria* phages was found in Mozzarella cheese brine (SMD = -377.34) and the least effect of the phages was found in apple juice (SMD = -0.02). Results from experiments with hot dogs, apple juice, iceberg lettuce, melon, melon juice, mixed seafood, mozzarella cheese brine, pear, pear

juice, red smear soft cheese, and sliced cooked turkey breast showed no heterogeneity (0 for I^2 and tau^2). Fresh sausage, apple and other lettuce experiments showed moderate heterogeneity (I^2<50). Experiments with cabbage, catfish fillets, cheese, chocolate milk, honeydew melons, precooked sliced turkey, raw salmon fillet tissue, smoked salmon and white mold soft cheese showed high heterogeneity (I^2<75) (Table 1).

Table 1. Meta-analysis of food subgroups with *Listeria* phages

Food	K	SMD	95%-CI	tau^2	I^2 (%)
Fresh sausage	05	-11.00	[-15.24; -05.70]	11.35	41.70
Apple	06	-00.27	[-01.30; 00.75]	00.82	43.70
Apple juice	03	-00.02	[-00.73; 00.68]	00.00	00.00
Cabbage	28	-236.16	[-299.82; -172.49]	11009.61	65.90
Catfish fillets	19	-179.82	[-237.25; -122.38]	12064.15	89.80
Cheese	112	-00.82	[-01.32; -00.30]	03.46	79.30
Chocolate milk	33	-201.00	[-256.79; -144.94]	5691.72	69.50
Honeydew melons	50	-500.00	[-59.87; -40.20]	197.9	92.80
Hot dogs	37	-313.00	[-365.19; -261.62]	00.00	00.00
Iceberg lettuce	08	-244.15	[-328.16; -160.13]	0.00	00.00
Lettuce	02	-75.00	[-134.20; -15.20]	620.15	25.40
Melon	03	-173.00	[-282.78; -63.70]	00.00	00.00
Melon juice	03	-307.14	[-403.05; -211.22]	00.00	00.00
Mixed seafood	12	-289.17	[-372.06; -206.28]	00.00	00.00
Mozzarella cheese brine	17	-377.34	[-468.33; -286.34]	00.00	00.00
Pear	03	-01.31	[-2.55; -0.07]	00.00	00.00
Pear juice	03	-0.06	[-0.76; 0.63]	00.00	00.00
Precooked sliced turkey	22	-07.57	[-11.33; -3.81]	20.04	85.50
Raw salmon fillet tissue	17	-208.63	[-280.39; -136.87]	7860.9	73.00
Red smear soft cheese	48	-333.36	[-384.34; -282.3]	00.00	00.00
Sliced cooked turkey breast	08	-138.70	[-187.83; -89.58]	00.00	00.00
Smoked salmon	14	-03.29	[-6.93; 00.35]	10.76	71.00
White mold soft cheese	24	-59.98	[-86.06; -33.90]	441.59	73.70

K=number of studies, SMD (Standardized Mean Difference; phage treated vs. control) and 95%-CI (95 % confidence interval), tau^2 = variance of the distribution of true effect sizes, I^2 = residual heterogeneity /unaccounted variability.

A meta-analysis of the antimicrobial effect of *Salmonella* phages on vegetables, meat, and dairy products was undertaken using 192 experiments. In these experiments, a significant antimicrobial effect was found (p-val< 0.0001), again with high indexes of heterogeneity (I^2 = 89.6% [88.4%; 90.7%] and tau^2= 30.4226 [1131.28; 2752.20]). There was a significant antimicrobial activity effect of *Salmonella* phages when comparing the standardized mean difference of treated and untreated groups (SMD= -11.21; 95%-CI= -12.79 and -9.62) and a high effect size (z= -13.89). As with *Listeria* phages, a subgroup analysis was undertaken to explore the effect of *Salmonella* phages on each different food type (Table 2).

Nineteen foods were analysed as subgroups, and significant differences between and within groups were detected (p < 0.0001). The biggest effect of *Salmonella* phages was found in ground turkey (SMD = -654.71) and the least effect of the phages was found in apples (SMD = -0.38). Experiments with chicken, cooked meat, eggs, ground turkey, raw cheese milk, raw meat, raw tuna, and ready to cook chicken showed no heterogeneity (I^2=0). Honeydew melon experiments showed moderate heterogeneity (I^2<50). Experiments with apples, cantaloupe melons, chicken breast, lettuce, milk, mung bean sprouts, pasteurized milk cheese, pigskin, precooked sliced turkey, romaine lettuce and sausage showed higher heterogeneity (I^2<75; Table 2).

Table 2. Meta-analysis of food subgroups with *Salmonella* phages

Food	K	SMD	95%-CI	tau^2	I^2 (%)
Apples	13	-00.38	[-1.42; 0.65]	02.21	81.30
Cantaloupe melons	03	-73.80	[-165.56; 17.95]	76.30	91.70
Chicken	03	-69.45	[-92.21; -46.69]	-69.45	00.00
Chicken breast	08	-5.52	[-07.65; -03.39]	05.08	61.90
Cooked meat	03	-97.89	[-136.77; -59.00]	-97.89	00.00
Eggs	02	-01.02	[-01.82; -00.21]	-01.02	00.00
Ground turkey	02	-654.71	[-916.89; -392.53]	00.00	00.00
Honeydew melons	12	-259.85	[-310.60; -209.09]	3336.40	45.00
Lettuce	17	-43.10	[-60.37; -25.83]	417.08	81.60
Milk	08	-70.63	[-104.24; -37.01]	1140.32	87.90
Mung bean sprouts	17	-164.34	[-197.91; -130.77]	2808.13	64.80
Pasteurized milk cheese	07	-309.94	[-423.93; -195.96]	13232.78	63.50
Pig Skin	22	-10.03	[-13.77; -6.28]	24.62	87.20
Precooked sliced turkey	26	-11.24	[-15.16; -7.33]	33.05	85.20
Raw cheese milk	02	-206.67	[-252.95; -160.38]	00.00	00.00
Raw meat	06	-26.78	[-37.57; -15.99]	00.00	00.00
Raw tuna	02	-114.39	[-160.84; -67.94]	00.00	00.00
Ready to cook chicken	03	-3.98	[-07.60; -00.37]	00.00	00.00
Romaine lettuce	23	-33.83	[-46.23; -21.43]	251.16	92.60
Sausage	08	-87.35	[-131.95; -42.75]	3183.30	93.40

K = number of studies, SMD (Standardized Mean Difference; phage treated vs. control) and 95%-CI (95 % confidence interval), tau^2 = variance of the distribution of true effect sizes, I^2 = residual heterogeneity /unaccounted variability.

3.3.4 Bias error detection of meta-analysis of *Listeria* and *Salmonella* phages

A regression test using funnel plot asymmetry showed significant systematic error (p-val= 0.0003643), and high size effect (z= 3.5) for *Listeria* phage patents, and no significant error was detected in *Salmonella* phages patents (p-value = 0.58) and low effect size (z = 0.55) (Figure 8).

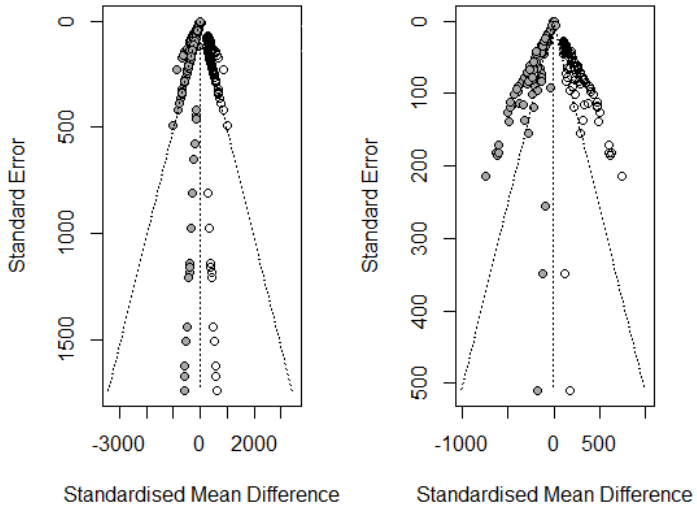

Figure 8. Funnel plot of antimicrobial effect of *Listeria* and *Salmonella* phage on foods.

(A) Effect of *Listeria* phages and (B) *Salmonella* phages

4. Discussion

There is scarce literature on using systematic review and meta-analysis methods to evaluate and improve the application of interventions for foodborne pathogens, such as phage biocontrol. The most similar approaches we could find were in the medical field (Clark *et al*., 2020). The systematic review and meta-analyses of phage applications in food could be important tools to evaluate the state of phage biotechnology development, establish evidence-based application and acceptance in the food industry, and guide future research.

4.1 **Systematic review**

4.1.1. Space-time analysis of phage patents

The geographical distribution of both of the patent documents and scientific articles on phage biocontrol in foods reported in this work was mainly (>80%) in North America and Europe, with Asia and South America only minor contributors, detail of patent (https://glenjasper.github.io/leaflet-phage-map/). This contrasts with patent

applications for other food sectors, for instance, food crops patents are predominantly (43%) filed in Asia (Holtappels *et al.*, 2019). This may be explained by the longer association of phage research with European and North American laboratories dating back to the work of Twort (UK) and d'Herelle (France/Canada) at the turn of the 19th century.

The patents review showed sporadic filings from the late 1990s and then a notable increase in 2007, followed by the publication of these filings in 2009 and 2010. This three-year period coincides with the first regulatory approvals and release of phage products to the market for foodborne pathogen biocontrol of *Listeria* (McIntyre *et al.*, 2007). From then on applications and publication of phage patents for food use have been increasing steadily. This trend of patent applications has also been reported for the use of phages in crop plant protection (McIntyre *et al*., 2007).

4.1.2 Phage patent description for biological control usage

Most patent applicants for biocontrol in foods (73.18 %) were from private companies, and the minority of applicants were from Universities (26.82%). Intralytix Inc. has more patents than any other company with 41% of the total patents in this field. In contrast, patents for phage biocontrol of plant pathogens have been mostly filed by academia (56%), with a minority (37%) linked to industry (without joint applicants), and 7% were joint applicants (Holtappels *et al.,* 2019).

In recent years, several studies have been published for biocontrol of phages in bacterial plant pathogens, like *Dickeya*, *Ralstonia*, *Xanthomonas* and *Pseudomonas*, with promising results (Holtappels *et al*., 2019). The infection properties of a given phage may appear to have great potential with *in vitro* studies, this does not necessarily translate into biocontrol potential in the field, so field or greenhouse trials are very

important for this research. Just a few biopesticides made it to the market. Agriphage from USA-based company OmniLytics was registered in 2006, these two phages are specific against *Xanthomonas campestris* pathovar vesicatoria or *Pseudomonas syringae* pathovar tomato and prevent and control bacterial spot or speck of tomato and pepper plants. A Hungarian company Enviroinvest was the second company to receive registration for their pesticide. Erwiniaphage controls fire blight of apple trees and is specific for *Erwinia amylovora* (Holtappels *et al*., 2019). However, in the food industry, there may be greater potential for phage biocontrol: from the decontamination of livestock to the sanitation of equipment and contact surfaces on farms and industry (Goodridge & Bisha, 2011). This could be a reason for increased commercial interest in phages for food safety instead of crops.

4.1.3 Scientific article analysis

The word map showed that the most frequent keywords of the systematic review were associated with phage biocontrol of *L. monocytogenes* and *Salmonella* in food. A network analysis of these keywords revealed three clusters of literature, and within each cluster, the article most cited in these papers was identified. Notably, the more cited article by Guenther (2009) studied the *Listeria* phage P100 which is one of the most widely studied phages and was the key active ingredient in the first phage product (Listex™ P100) approved by the USDA (GRAS notice GRN 000198) for use in foods. The European Food Safety Authority (2016) had also evaluated the safety and efficacy of Listex™ P100 during the processing of three ready-to-eat (RTE) product categories (meat and poultry, fish and shellfish, and dairy products). The early studies which described phages applied to food such as Leverentz (2001) and Carlton (2005) were also widely cited.

4.2 *Meta-analysis*

More than 500 phages specifically infecting *Listeria* sp. have been identified, however, the majority of known phages are temperate (Hagens & Loessner, 2014; Klumpp & Loessner, 2013), as a result, most of them are not very useful for inhibition of *L. monocytogenes* in food products and food processing plants. There were many experiments using bacteriophages for biocontrol of *L. monocytogenes* in food products, such as raw meat, smoked fish, fermented fish, milk, cheeses, fresh-cut fruits, vegetables and various ready to eat products. In the majority of trials, authors succeeded with the reduction or even eradication of *L. monocytogenes* from food products. Most of the trials were performed with P100 phage, then PhageGuard Listex cocktail bacteriophage, ListShield cocktail bacteriophage and only a few attempts were performed with other bacteriophages (Kawacka, 2020).

In our meta-analysis of *Listeria* phages, ListShield™ phages had the best performance for biocontrol for *L. monocytogenes* in apples, cheese, lettuce, and smoked salmon. ListShield™ (formerly LMP-102) is produced by Intralytix Inc, is a cocktail of 6 distinct lytic phages: LIST-36 (ATCC # PTA-5376), LMSP-25 (ATCC # PTA-8353), LMTA-34 (ATCC # PTA-8354), LMTA-57 (ATCC # PTA-8355), LMTA-94 (ATCC # PTA-8356), LMTA-148 (ATCC # PTA-8357) (Perera *et al*., 2015, Sadekuzzaman *et al*., 2017; Yang *et al*., 2017 and Kawacka *et al*., 2020).

For *Salmonella*, Felix O1 had the best antimicrobial effect on food. According to Cristobal *et al*., (2021), the company Micreos Food Safety has developed the brand Phageguard S based on phages Felix-O1a and S16 against *Salmonella enterica*. This product was able to kill all *Salmonella* serovars including those that are resistant to antibiotics and the 20 most virulent *Salmonella* strains according to the United States Department of Agriculture (USDA) (Cristobal *et al*., 2021), Phageguard S can reduce

the bacterial population by 1–3 $\log_{10}$ CFU/mL without affecting taste, odor or texture of foods. It is effective from 0 to 35 °C and its use is recommended as a final treatment in spray or directly immersing food into the phage solution (Cristobal *et al*., 2021),

Furthermore, phage LPSEYT demonstrated potential efficiency as a biological control agent against *Salmonella* in a variety of food matrices, including milk at a MOI of 1000, the viable *Salmonella* count was reduced by 2.07$\log_{10}$ CFU/mL at 4 °C and 3.67 $\log_{10}$ CFU/mL at 25 °C, and lettuce, at MOI = 10,000 produced a reduction in the viable count of *Salmonella* by 2.2$\log_{10}$ CFU/sample at 4 °C and 2.34$\log_{10}$ CFU/sample at 25°C for 6 hours of incubation (Yang *et al*., 2020). Wang *et al*. (2017) showed phage fmb-p1 reduced *Salmonella* on duck meat by 0.57$\log_{10}$ CFU/cm^2. Bao *et al.* (2015) tested two lytic phages, vB_SenM-PA13076 (PA13076) and vB_SenM-PC2184 (PC2184), in chicken breast, pasteurized milk and Chinese cabbage, PA13076 was able to infect 222 strains (71.4%) and PC2184 infected 298 strains (95.8%) out of 311 isolates tested.

When examining the methods of phage application in the selected articles, phage cocktails were used in 50% of the *Listeria* studies and 44.44% of the *Salmonella* studies. For most applications, cocktails of phages are likely required to achieve good coverage of all strains as most phages are intrinsically narrow in host range (Ross *et al*., 2016), however, there are some exceptions such as P100 which can infect ~95% of *L. monocytogenes* strains in serovars 1/2 and 4 (Guenther *et al*., 2009).

With respect to the physicochemical parameters, the initial concentration of phages and bacteria, time of storage, and food type had a significant correlation with the log reduction of bacteria for *Listeria* phages. In general, increasing the initial phage: host ratio has been found to enhance the efficacy of the phage in reducing bacterial populations (Hudson *et al*., 2015, Kawacka *et al*., 2020). Guenther *et al*. (2009)

suggested that phages suspended in liquid foods can diffuse almost freely and thus their distribution and potential contact with their host cells does not appear to be a problem. While, on solid foods such as hot dogs, salad leaves and so forth that have an uneven surface, where the surface properties or total surface area accessibility are limited, the parameters may be of great importance.

Using Listex™ P100, a commercially available phage against *Listeria monocytogenes* has been widely applied to several foods, concentration of Listex™ P100 showed a greater reduction in tuna using an MOI of 10^2 than an MOI of 0.1 (Miguéis *et al*., 2017). The greatest effect was seen with a lower starting concentration of *L. monocytogenes* combined with a higher concentration of Listex™ P100 (Lewis & Hill. 2020).

Another study tested the efficacy of phage-based products as biopreservatives, dry-cured ham samples were experimentally contaminated with 10^5, 10^4 and 10^3 CFU/cm^2 of *L. monocytogenes* S2 and treated with ListShield™ (10^7 PFU/cm^2) or Listex™ P100 (10^9 PFU/cm^2). Samples were stored at 4 °C or 12 °C for 14 days. Notably, Listex™ P100 reduced the viable counts below the detection limit (<10 CFU/cm^2) after one day of treatment at all the assayed inoculum levels. In contrast, ListShield™ turned out to be less effective in the most contaminated samples (Gutiérrez *et al.*, 2017).

Oliviera *et al*. (2014) found that the effectiveness of reducing *L. monocytogenes* with P100 phage in fruit juices was higher than on the fruit slices.

Studies show that the time of application of the phages is also an important factor for both *Listeria* and *Salmonella* phage biocontrol. Leverentz *et al.* (2003) performed an experiment where a *Listeria* phage cocktail was applied to honeydew melon pieces at 1, 0.5 and 0 h before contamination with *Listeria monocytogenes* and

0.5, 1, 2 and 4 h after contamination. The phage treatment was most successful when applied not earlier than 1 h before contamination.

Some phages are naturally resistant to high physiochemical environmental influences, such as temperature, pH, salinity and disinfectants making them potential biocontrol agents for use in food processing or on-farm to improve food safety (Binetti *et al*., 2002, Tomat *et al.*, 2014).

Phage LPSTLL remained stable over a pH range of 3.0 to 12.0 and at temperatures up to 60 °C for 60 min (Guo *et al*., 2021). Compared with Salmonella phage reported previously, phage LPSTLL showed higher tolerance to harsh environments (Fong *et al*., 2017, Jung *et al*., 2017, Krasowska *et al*., 2015).

Thung *et al*. (2017) reported the antimicrobial effect of bacteriophage SE07, isolated from retail meat samples, against *S. enterica* serovar Enteritidis on different food matrices, such as fruit juice, fresh egg, beef and chicken meat, the reduction of the bacteria population in all of them was significant at 12 h ($2.05\log_{10}$ CFU/mL, $1.98\log_{10}$ CFU/mL, $1.79\log_{10}$ CFU/mL, and $1.83\log_{10}$ CFU/mL, respectively), and after that time there was no further significant reduction.

In 2018, Phongtang et al. evaluated the effect of P22 phage (ATCC 97541) against *S. enterica* serovar Typhimurium in milk. This phage showed an inhibitory effect of more than $3\log_{10}$ CFU/mL reduction after 4 h.

For *Salmonella* phage treatments, temperature and time also had a significant effect. In work with melon slices stored at 5°C, the SCLPX-1 phage mixture reduced *Salmonella* populations by approximately $3.5\log_{10}$ CFU/g compared with the control at 120 h of incubation (Leverentz, 2001). Temperature has also been found to affect phage biocontrol of *E. coli* O157 on cooked and raw beef (Hudson 2015), where it was suggested that lower temperatures prevent the host from overgrowing the phage,

improving biocontrol efficiency. Given the relatedness of *E. coli* and *Salmonella*, these likely accounts for the positive results of Leverentz et al. (2001) and others in the meta-study at low temperatures with *Salmonella*.

On the other hand, vB_SenM-PA13076 (PA13076) and vB_SenM-PC2184 (PC2184) phages were rapidly inactivated at temperatures above 60 °C (PA13076) or 70 °C (PC2184), PA13076 reduced Salmonella population in chicken breast, pasteurized milk and Chinese cabbage by $2\log_{10}$, $2\log_{10}$ and $2.5\log_{10}$ CFU/mL, respectively, whereas PC2184 reduced bacteria population in chicken breast, pasteurized milk and Chinese cabbage by $3\log_{10}$, 4 $\log_{10}$ and $3.5\log_{10}$ CFU/mL, respectively (Bao *et al*., 2015).

The meta-analysis showed that phages specific for foodborne pathogens *Salmonella* spp. and *L. monocytogenes* significantly reduced pathogens on food, but high heterogeneity was detected. This heterogeneity could be explained by subgroup analyses of individual food types in both cases. Sabitova *et al*., (2020) reported that a meta-analysis ideally combines the results of several studies that are highly comparable in design, intervention, and patient population. However, in real life, meta-analyses frequently contain multiple, relatively small studies that differ in many respects (Sabitova *et al*., 2020), hence subgroup analysis is warranted. When examining 23 food subgroups tested with *Listeria* phages, 11 subgroups reduced the heterogeneity to 0, and 3 subgroups reduced the heterogeneity to moderate, which represents 60% of studies. It was notable that subgroups with a number of samples higher than 14 showed more heterogeneity. Similar to *Listeria* phages, in *Salmonella* phages, the heterogeneity was reduced among the 20 food subgroups, 8 reduced to 0, 1 to moderate, so 45% of the subgroups had reduced heterogeneity. Like the *Listeria* phages, subgroups where the number of samples was higher than 13 showed more heterogeneity. The increasing data

heterogeneity with increasing sample number for both groups of phage experiments is likely due to the natural physical heterogeneity of food products when tested across studies undertaken in different countries, climates and with different varieties of foods.

For the smaller subgroup analyses, it may not be possible to estimate heterogeneity with much precision as I^2 has a substantial bias when the number of studies is small (Von Hippel, 2015). In small meta-analyses, confidence intervals should supplement or replace the point estimate I^2 (Von Hippel, 2015).

5. Conclusions

In summary, we evaluated the efficiency of phages previously patented as a biological control for fruits, vegetables and meat. Our meta-analyses revealed that initial concentration of phage and bacteria, time and food were associated with an antimicrobial effect on *Listeria.* Temperature and time were associated with an antimicrobial effect on *Salmonella*. ListShield and Felix01 phages showed the best result for *Listeria* and *Salmonella* biological control, respectively.

The use of phages has much promise to control bacterial pathogens in food industries and other applications. It is evident that the application of phages to each food system and pathogen needs to be optimized, and that some food matrices are more challenging for phage use than others. A systematic approach such as we have used here will help inform future applications of phages to foodborne bacterial pathogens and highlights the need to improve the comparability of results to give the best confidence in the conclusions of such studies.

Acknowledgements

This research was supported by the Coordination for the Improvement of Higher Education (CAPES), the National Council for Scientific and Technological Development (CNPq) and also was funded by an ESR Strategic Science Investment Fund.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, selection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Author Contributions: Conceptualization, D.R.-C., A.G.-N. and C.B. methodology, D.R.-C.; G. Y.-G, V. P. S writing—original draft preparation, D.R.-C. G. Y.-G, A.G.-N. and C.B.; writing—review and editing, D.R.-C., R.G.-B., A.G.-N. and C.B.; visualization, G. Y.-G, D.R.-C., V. P. S; supervision, R.G.-B., C.B and. A.G.-N.

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Capítulo 3

Antimicrobial activity of vegetable extracts, essential oils, carvacrol and thymol against *Salmonella* spp.

Antimicrobial activity of vegetable extracts, essential oils, carvacrol and thymol against *Salmonella* spp.

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Abstract

The incidence of bacterial antimicrobial resistance is increasing despite new treatments being employed, so novel strategies are required to ensure that bacterial infections remain treatable.

The aim of this study was to determine the antimicrobial activity of seventeen plant extracts from the semi-arid region of the northeast Brazil commercial essential oils, carvacrol and thymol against Salmonella ATCC 14028.

In this study, the antibacterial activity of Seventeen plants (*Artemisia absinthium* Linné, *Calendula officinalis*, *Cecropia Hololeuca* Miquel, *Commiphora leptophloeos*, *Costus spicatus Swartz*, *Cuphea ingrate*, *Jacarandá semiserrata* Cham, *Laurus nobilis*, *Miconia albicans*, *Mikania hirsutíssima*, *Momordica charantia* Linné, *Pereskia aculeata*, *Salvia officinalis*, *Thuja Occidentalis* Linné, *Tilia cordata*, *Zea mays* Linné and *Croton heliotropiifolius*) extracts from the semi-arid region of the northeast Brazil were macerated using hexane, ethyl acetate and ethanol to produce 51 extracts. Six

commercially produced essential oils (*Larus nobilis, Salvia officinalis, Rosmarinus officinalis, Cymbopogon, Oregano Selvagem* and Clove bud) and the essential oils of *Croton heliotropiifolius* (obtained by hydrodistillation), thymol and carvacrol against *Salmonella* using *in vitro* approaches were evaluated.

Most of the natural products extracts tested in this study did not show significant antimicrobial activity against *Salmonella enterica* subsp. Typhimurium ATCC 14028. However, Clove bud essential oil and thymol showed activity against *Salmonella* at a concentration of 1mg/ml.

Keywords: Essential oils, plant extracts, *Salmonella spp.* and antimicrobial activity.

1. Introduction

Infections caused by resistant pathogens such as *Candida* spp., *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus* spp., *Enterococcus* sp. *Salmonella* spp. *Escherichia coli*, among others, became more frequent and a helath care problem around the world, in this sense, new antibiotics with new mechanisms of action are needed (ROSA *et al*., 2003). The increase in the level and type of resistance that exists due to the overuse and inappropriate use of antibiotics in humans and animals cannot be underestimated and has shifted the focus to new strategies to combat multidrug-resistant infections, including reducing antibiotic consumption and development of new therapies (BROWN & WRIGHT, 2016).

Salmonella resistant to commercial antibiotic drugs has emerged as a great health concern to the consumers. Extensive use of antibiotics in food industry against foodborne pathogens or food models has resulted in additional antibiotic resistance to *Salmonella* which has become a matter of great concern to the public health. There has been an increasing concern worldwide on therapeutic values of natural products. Nature has presented to humanity the gift of vast therapeutic antimicrobial agents of plant origins. There are multitudes of potential useful bioactive substances to be derived from plants (BAJPAI *et al*., 2012).

The plant volatiles or plant essential oils (PEOs) are plant secondary metabolites which are biosynthesized in glandular structures of a plant cell. PEOs are known to work as potential antimicrobial agents having the ability to control foodborne

pathogenic bacteria (BAJPAI & KANG, 2008; BURT, 2004, OUSSALAH *et al*., 2007; BAJPAI *et al*., 2020).

In general, the biological efficacy of PEOs could be attributed to their chemical components that include phenolics or the components from terpene origin (Conner, 1993, Didry *et al*., 1993). While, almost all the PEOs have been shown to exert antimicrobial efficacy, a number of variations have been reported in their chemical nature and the amount of their volatiles reason being variations in the collection time of sample, abundance and/or lack of mineral components, distribution, changes in genetic levels, environmental conditions and the portion of the plant used for distillation (Salgueiro *et al*., 1997, Venskutonis, 1996). The antimicrobial efficacy of PEOs has been credited to the components present in higher amount, as well as, the components present in lower amount, they have been shown to exert synergistic effect with the major components of the oil (Paster *et al*., 1995; BAJPAI *et al*., 2020).

On the other hand, the utilization of plant extracts as antimicrobial agents for food preservation (Nasar-Abbas & Kadir, 2004, HARA-KUDO *et al*., 2004, MATHABE *et al*., 2005) was also successfully employed, Plant extract are considered as natural sources of antimicrobial agents, regarded as nutritionally safe and easily degradable (COWAN, 1999, DUFFY & POWER, 2001, BERAHOU *et al*., 2007, OGBULIE *et al*., 2007). The antimicrobial activity exhibited by plant extracts against food poisoning bacteria has been demonstrated by several researchers (DELGADO *et al*., 2004, ALZOREKY & NAKAHARA, 2003, VERMA *et al*., 2012, AKINPELU *et al*., 2015).

The objective of this work was to determine the antimicrobial activity of Seventeen plants (*Artemisia absinthium* Linné, *Calendula officinalis, Cecropia Hololeuca* Miquel, *Commiphora leptophloeos, Costus spicatus Swartz, Cuphea ingrate, Jacarandá semiserrata* Cham, *Laurus nobilis, Miconia albicans, Mikania hirsutíssima, Momordica charantia* Linné, *Pereskia aculeata, Salvia officinalis, Thuja Occidentalis* Linné, *Tilia cordata*, *Zea mays* Linné and *Croton heliotropiifolius*) extracts from the semi-arid region of the northeast Brazil, six commercially produced essential oils (*Larus nobilis, Salvia officinalis, Rosmarinus officinalis, Cymbopogon, Oregano Selvagem* and Clove bud), two commercially produced secondary metabolites (thymol

and carvacrol) and one essential oil (*C. heliotropiifolius*) obtained by hydrodistillation in this study, against *Salmonella* using *in vitro* approaches.

2. Material and methods

2.1 Plant material

Seventeen plants (*Artemisia absinthium Linné, Calendula officinalis, Cecropia Hololeuca Miquel, Commiphora leptophloeos, Costus spicatus Swartz, Cuphea ingrate, Jacarandá semiserrata Cham, Laurus nobilis, Miconia albicans, Mikania hirsutíssima, Momordica charantia Linné, Pereskia aculeata, Salvia officinalis, Thuja Occidentalis Linné, Tilia cordata, Zea mays Linné* and *Croton heliotropiifolius*) were bought from Palmar e Herbal Essences store in Salvador de Bahia, the semi-arid region from the northeast Brazil.

Besides, *Croton heliotropiifolius* Kunth were collected from the Feira de Santana State University (UEFS) campus, Feira de Santana City, Bahia state (12°00'00.0"S 39°00'00.0"W). A voucher specimen was deposited in the HUEFS 247936 herbarium from UEFS. Leaves were collected in February (summer). orig.: [lat: -12.25 long: -38.966667 WGS84].

2.2 Plant extracts maceration

Seventeen plants were used, 150 grams of aerial parts of the plant were macerated with hexane, acetyl acetate and ethanol as solvents for maceration for three days at room temperature, after which the extract will be filtered through filter paper (Whatman, 10334352), pore size 7-12 µm and cotton. The solvent will be removed by evaporation under vacuum at reduced pressure at 40-45°C using a rotary evaporator. Ethanol and ethyl acetate extracts were dissolved in dimethylsulfoxide (DMSO). Hexanic extracts were dissolved in ethanol, DMSO, Tween (final concentration of 0.05%) and water.

2.3 Plant extracts fractionation

Five hexane extracts: *Laurus nobilis*, *Thuja occidentalis*, *Costus spicatus*, *Savila occidentalis* and *Momordica charantia*, were fractionated in hexane, ethyl acetate and ethanol. Fractions were dissolved in ethanol and Tween (final concentration of 0.05%).

Secondary metabolites of *Croton heliotropiifolius* were extracted using maceration using different solvents as water, ethanol, acetyl acetate and hexane. In addition, essential oil was also extracted.

2.4 Essential oil extraction

The essential oil of *Croton heliotropiifolius* was obtained by hydrodistillation in a Clevenger-type apparatus adapted to a 2000-mL round-bottom flask. Aerial parts (leaves) of the fresh material (200 g) were immersed in distilled water. The extraction time was set at 360 min (Alencar *et al*., 2017).

Furthermore, six essential Clove bud (Sigma Aldrich), *Larus nobilis* (Oshadhi), *Salvia officinalis* (Oshadhi), *Rosmarinus officinalis* (Samia), *Cymbopogon* (Samia), *Oregano Selvagem* (Oshadhi) and two chemical products as Thymol (Sigma Aldrich), and carvacrol (Sigma Aldrich) were bought.

2.5 Antimicrobial activity

Broth dilution method was used in order to determine the antimicrobial activity against *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (CLSI 2012) in the aforementioned preliminary trial. Tests were performed in Müeller-Hinton broth. The aqueous extract was re-suspended in the water, while the methanol extract and other fractions were re-suspended in 25% of dimethyl-sulfoxide (DMSO). Serial dilutions of 100 mg/mL at 0.0488 mg/mL were prepared from the extracts and fractions on sterile 96-well microtitration plates. Each well then received 10 µL of the test microorganism suspension (1.5×10^5 UFC/mL per well).

The plates were incubated at 37 °C for 24 h. A purity verification of the suspension was performed by subculture of a corresponding aliquot on MHB plate for simultaneous incubation. After incubation, 30 µL of aqueous Resazurin solution at 0.015 % were added to each of the wells and the microplates were reincubated for three more hours, as reported by Elshikh *et al.* (2016), at 37°C for qualitative assessment of microbial growth. Chloramphenicol dilutions (6 at 0.0488 mg/mL) were used as the

controls for data comparison between the independent experiments and as indicators for relative evaluation of the inhibition level of the samples tested. Controls were also prepared for viability assessment of the test microorganisms and sterility assessment of the culture medium and the solvent used for dissolution of extracts and fractions, for verification of any possible effects on microorganisms. All the tests were performed in triplicate (Hughes *et al*., 2013).

The activity pattern used for interpretation of minimum inhibitory concentrations was based on the values established by Aligiannis *et al*. (2001): strong inhibition, MIC lower than 0.5 mg/mL; moderate inhibition, MIC between 0.6 and 1.5 mg/mL; and poor inhibition, MIC higher than 1.5 mg/mL.

3. Results

The yield of the sixteen plants was evaluated; *Commiphora leptophloeos* plant showed the highest yield in the three solvents used: hexane (10.823%), ethanol (6.288%), and Ethyl acetate (5.475%), following of *Laurus nobilis* ethanol extract (5.360%) and *Salvia officinalis* ethyl acetate extract (5.183%). Besides, *Cuphea ingrata* hexane extract showed the the lowest yield (0.311%) (Table1 and Figure1).

Table 1: Yield plant extract using hexane, ethyl acetate and ethanol as solvents, obtained in 100 x g extract/g dry plant material (%)

	Plant	Hexane p/p (%)	Ethyl acetate p/p (%)	Ethanol p/p (%)
1	*Artemisia absinthium Linné*	1.067	2.339	4.901
2	*Calendula officinalis*	3.007	2.515	4.639
3	*Cecropia Hololeuca Miquel*	1.251	0.691	1.551
4	*Commiphora leptophloeos*	10.823	5.475	6.288
5	*Costus spicatus Swartz*	0.743	0.712	3.018
6	*Cuphea ingrate*	0.311	0.568	2.547
7	*Jacarandá semiserrata Cham*	1.177	4.135	4.511
9	*Laurus nobilis*	1.263	0.744	5.360
8	*Miconia albicans*	0.867	1.925	2.255
10	*Mikania hirsutíssima*	0.835	3.365	2.162
11	*Momordica charantia Linné*	0.402	1.21	1.933

12	*Pereskia aculeate*	0.911	1.234	2.460
13	*Salvia officinalis*	1.083	5.183	2.640
14	*Thuja Occidentalis Linné*	1.101	3.190	2.600
15	*Tilia cordata*	0.643	0.978	1.098
16	*Zea mays Linné*	0.368	0.411	1.403

Source: own authorship

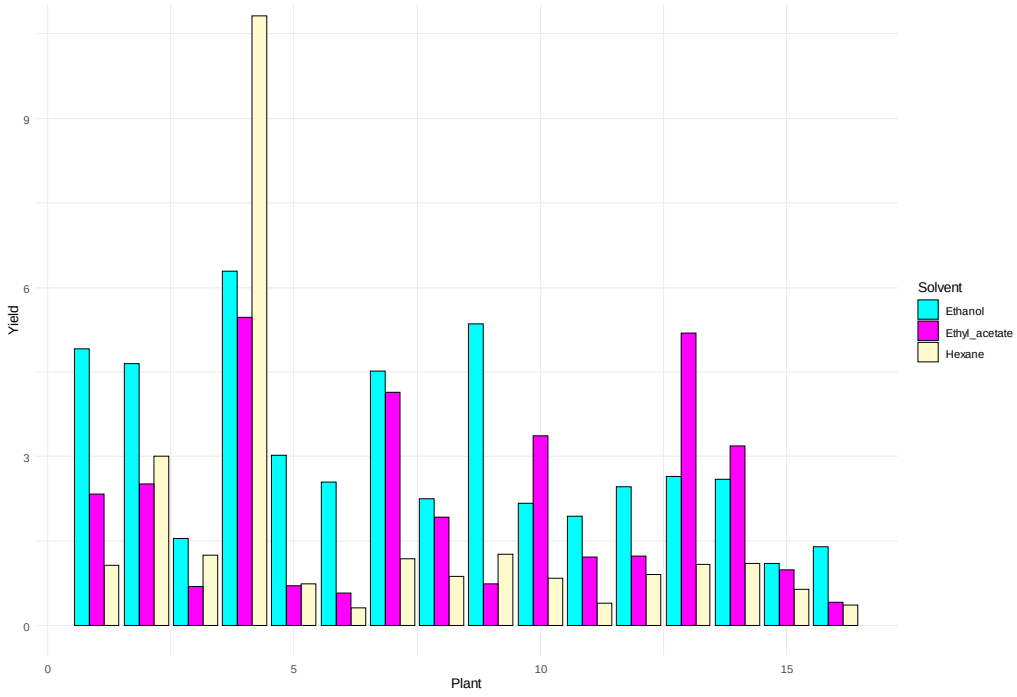

Figure 1: Yield plant extract using hexane, ethyl acetate and ethanol as solvents, obtained in 100 x g extract/g dry plant material (%).

Source: Own authorship

Regarding to *Croton heliotropiifolius*, the highest yield was obtained using hexane maceration and the lowest using essential oil extraction.

Table 2: Yield of *Croton heliotropiifolius* extracts and essential oil obtained in 100 x g extract/ g vegetal dried material (%)

Croton heliotropiifolius	**Yield (%)**
Hexane	16.32
Ethyl acetate	6.4
Ethanol	2.03
Escentail oil	0.3

Source: own authorship

The antimicrobial activity against *Salmonella enterica* ATCC 14038 of fifty five extracts at 1mg/ml were tested, no one showed antimicrobial activity, Table 3.

Table 3: Antimicrobial activity of extracts from 17 plants using hexane, ethyl acetate and ethanol as solvents at a concentration of 1mg/mL

	Plant	Hexano	Acetato de etila	Etanol
1	*Artemisia absinthium Linné*	NA	NA	NA
2	*Calendula officinalis*	NA	NA	NA
3	*Cecropia Hololeuca Miquel*	NA	NA	NA
4	*Commiphora leptophloeos*	NA	NA	NA
5	*Costus spicatus Swartz*	NA	NA	NA
6	*Cuphea ingrate*	NA	NA	NA
7	*Jacarandá semiserrata Cham*	NA	NA	NA
9	*Laurus nobilis*	NA	NA	NA
8	*Miconia albicans*	NA	NA	NA
10	*Mikania hirsutíssima*	NA	NA	NA
11	*Momordica charantia Linné*	NA	NA	NA
12	*Pereskia aculeate*	NA	NA	NA
13	*Salvia officinalis*	NA	NA	NA
14	*Thuja Occidentalis Linné*	NA	NA	NA
15	*Tilia cordata*	NA	NA	NA
16	*Zea mays Linné*	NA	NA	NA
17	*Croton heliotropiifolius*	NA	NA	NA

* NA: no activity at 1mg/ml extract.

Source: own authorship

In order to determine the antimicrobial activity of plant fractions, five plants were chosen at random using hexane as solvent, as a result 22 fractions were tested, and however no one showed antimicrobial activity against *Salmonella enterica* ATCC 14028 at 1mg/ml, Table 4.

Table 4: Antimicrobial activity of hexane extracts of *Laurus nobilis*, *Thuja occidentalis*, *Costus spicatus*, *Savila occidentalis* and *Momordica charantia* against *Salmonella enterica* ATCC 14028

Plants	Number of fractions	Antimicrobial activity 1mg/ml
Laurus nobilis	F1, F2, F3 y F4	NA
Thuja occidentalis	F1, F2, F3 y F4	NA
Costus spicatus	F1, F2, F3, F4 y F5	NA
Savila occidentalis	F1, F2, F3 y F4	NA
Momordica charantia	F1, F2, F3 y F4	NA
TOTAL	22	NA

* NA: no activity at 1mg/ml extract.

Source: own authorship

Table 5. Antimicrobial activity of essential oils, secondary metabolites

No	Essential oil and secondary metabolites	Source	MIC [0.007-1 $mgml^{-1}$]
1	*Clove bud*	Sigma Aldrich	1 and 0.5 mg/ml
2	*Larus nobilis*	Oshadhi	NA
3	*Salvia officinalis*	Oshadhi	NA
4	*Rosmarinus officinalis*	Samia	NA
5	*Cymbopogon*	Samia	NA
6	*Oregano Selvagem*	Oshadhi	NA
7	*Croton heliotropiifolius*	This study	NA

8	Thymol	Sigma Aldrich	1 mg/ml
9	Carvacrol	Sigma Aldrich	NA

* NA: no activity at 1mg/ml extract.

Source: own authorship

Furthermore, the antimicrobial activity of the extracts was also evaluated against *Staphylococcus aureus, Escherichia coli* and *Candida albicans.*

4. Discussion

In this work we evaluated the antimicrobial activity from seventy three extracts against of *Salmonella* ATCC 14028, they did not showed activity at 1mg/ml. Solvent type and polarity can affect the extract quality, quantity, extraction velocity, inhibitory compounds, toxicity, other biological activity, and biosafety (ELOFF, 1998; ZHANG *et al*., 2019). The total secondary metabolites and their antioxidant capacity greatly depend on the solvent and plant part used for extraction (RAFIŃSKA *et al*., 2019).

Different to our work, LONGARAY *et al*. (2007) reported the antimicrobial activity of *Savila officinalis* essential oil from 5 to 10 mg/ml against *Salmonella* Typhimurium IBSal-101. Other study evaluated the bacterial growth inhibition curves of ten chemotypes of *S. officinalis* subsp. essential oil against *Shigella sonnei* CECT 413, *Escherichia coli* CECT 45, *Salmonella enterica* subsp. enterica CECT 443 and *Listeria monocytogenes* CECT 911. A total of 30 individual plants (3 per chemotype) were used in this assay. Concentrations between 625 and 40000 ppm of essential oil were tested.

Other study showed that methanol extract of *Thuja Occidentalis* contained the largest carotenoid showed the largest zone of inhibition in *Bacillus amyloliquifaciens* and *Salmonella* Typhi (POOJA *et al*., 2015). Besides, the methanol extract of *Momordica charantia* leaves against *Salmonella* Typhi in male albino rats (Sprague dawley) and the effects of treatment on liver function were evaluated as a result *Momordica* charantia leaf extract reported a potent antimicrobial against *S*. Typhi with hepatoameliorative potential (ADEYI *et al*., 2013).

In this work, *Croton heliotropiifolius* extracts and essential oil did not show antimicrobial activity against *Salmonella enterica* ATCC 14028. However, other study using leaves and stems to obtain essential oils from *Croton heliotropiifolius* showed antimicrobial activity against *Salmonella choleraesuis* and *Bacillus subtilis* above 500 mg/ml (ALENCAR *et al*., 2017), it could be related to the origin from the plant, and its chemical composition.According to PARK *et al.* (2019) *Clove bud* essential oil showed antimicrobial effect against Salmonella *Salmonella enterica* ATCC 14028.

The main oil constituents of *Clove bud* essential oil are eugenol (70–95 %), eugenol acetate (up to 20 %) and β-caryophyllene (12–17 %). Eugenol inactivated *Salmonella* Typhi within 60 min exposure, their MIC (0.0125%) and MBC (0.025%) reduced the viability and resulted in complete inhibition of the organism. The antibacterial activity of eugenol against *Salmonella* Typhi is attributed to increase the permeability of the membrane (DEVI *et al*., 2010). According to GIOVAGNONI *et al.* (2020), the dual mechanism of action of thymol and carvacrol enhance the effects associated with a *S*. Typhimurium *in vitro*.

In silico molecular docking and *in vitro* antimicrobial efficacy of carvacrol and thymol against multi-drug-resistant *enteroaggregative Escherichia coli* and non-typhoidal *Salmonella* spp. Were evaluated by docking studies employing 3D model of dispersin and ompC motifs with the carvacrol and thymol ligands and exhibited good binding affinity, besides this ligands were found to be zero violators of Lipinski's rule of five and exhibited drug-likenes, Carvacrol MIC and MBC for Salmonella strains (*S*. Enteritidis and *S.* Typhimurium), were from 0.12 to 0.25 µL/mL and 0.5 µL/mL, respectively, thymol MIC and MBC for Salmonella strains (*S*. Enteritidis and *S.* Typhimurium), were from 0.06 to 0.25 µL/mL and 0.5 µL/mL, respectively (ABISHAD *et al*., 2021).

Another important pharmacological action of thymol and carvacrol are the antifungal, antibacterial and anti-viral activity. Thymol showed antimicrobial activity *in silico* and *in vitro* against SARS-CoV-2 (SEADAWY *et al*., 2021). Besides, other study has been found that some monoterpenes, terpenoid phenols and phenyl propanoids such as anethole, cinnamaldehyde, carvacrol, geraniol, cinnamyl acetate, L-4-terpineol, thymol and pulegone were effective antiviral agents that have potential to inhibit the viral spike protein (ZHANG *et al*., 2020; KULKARNI et al., 2020).

In addition, both the United States Environmental Protection Agency (EPA) in the USA (Available online: https://www.epa.gov/ pesticide-registration/list-n-disinfectants-use-against-sars-cov-2-covid-19) and the Government of Canada (Available online: https://www.canada.ca/en/health-canada/services/drugs-healthproducts/disinfectants/covid-19/list.html) have placed on the list of disinfectants with evidence for use against COVID-19 preparations with thymol asthe active ingredient. These products are designed to disinfect external hard surfaces and hands in healthcare, institutional, or residential applications.

5. Conclusion

From all the extract evaluated in this study, only *Clove bud* oil and thymol decreased *Salmonella* Typhimurium 14028, both of them showed are widely described in the literature. The antifungal, antibacterial, anti-viral (especially anti Sars-Cov2) activity and synergism with bacteriophages to control *Salmonella* Typhimurium in chicken of this molecule increase the potential to develop innovative thymol-based products.

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Capítulo 4

Genomic analysis of *Salmonella enterica* serovar Enteritidis SE3 using a hybrid genome sequence assembly approach

Genomic analysis of *Salmonella enterica* serovar Enteritidis SE3 using a hybrid genome sequence assembly approach

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Abstract

Background: In Brazil, *Salmonella enterica* serovar Enteritidis is a significant health threat. The genome of *Salmonella* Enteritidis strain SE3 is largely uncharacterized, including mechanisms of drug resistance and virulence. The whole-genome sequence of *Salmonella* SE3 was obtained by hybrid sequencing assembly to improve mobile genetic element identification, and permit pangenome and phylogenetic strain analysis.

Results: *Salmonella* SE3 was isolated from soil at the Subaé River in Santo Amaro, Brazil, a region contaminated with heavy metals and organic waste. *De novo* hybrid sequencing assembly of *Salmonella* SE3 from Illumina HiSeq and ONT MinION whole genome sequencing yielded 10 contigs and showed 99.98% of identity with *Salmonella enterica* subsp. *enterica* serovar Enteritidis OLF-SE2-98984-6. Twelve *Salmonella* pathogenic islands, multiple virulence genes, multiple antimicrobial gene resistance genes, seven defense systems, seven prophages and a heavy metal resistance gene (*arsC*) were identified. Pangenome analysis of the *S. enterica* clade, including SE3, revealed an open pangenome, with a core genome of 2,137 genes. The accessory genome comprised 3,390 shell genes and 69,352 cloud genes.

Conclusions: Our study showed the effectiveness of a hybrid sequence assembly approach for environmental *Salmonella* genome analysis using HiSeq and MinION data. The hybrid genome assembly enabled identification of virulence and resistance genes, mobile genetic elements and pangenome analysis.

Keywords: Whole genome sequence, *Salmonella* SE3, hybrid assembly, and mobile elements.

1. Introduction

Salmonellosis, one of the primary causes of foodborne infections resulting from gram-negative enteropathogenic bacteria *Salmonella spp*, is a global threat to human health (Hernandez-Reyes, et al., 2013). Typhoidal *Salmonella* causes enteric fever in

humans, whereas non-typhoidal *Salmonella* (NTS) results in acute/chronic gastroenteritis. Annually, it is estimated that NTS is responsible for ~93.8 million infections and ~155,000 deaths (Majowicz et al., 2010).

NTS infections cause diarrhoea and a non-specific febrile illness that is clinically indistinguishable from other febrile illnesses (GBDN-TSID Collaborators, 2019). *Salmonella enterica* subspecies *enterica* has more than 2600 serovars according to unique somatic (O) and flagellar (H) antigenic formulae (Das, *et al*., 2018; Saleh *et al*., 2019). *Salmonella enterica* Typhimurium and *Salmonella enterica* Enteritidis are the main pathogens responsible for causing gastroenteritis in humans (Rabsch *et al*., 2002; Carden *et al*., 2015).

To prevent the occurrence of the main *Salmonella* serovars worldwide, several prevention and control measures are adopted in producing farms and food processing industries. In Brazil, *Salmonella* infection of flocks and transmission to poultry-derived food is a major transmission route for the pathogen. *Salmonella* is routinely managed on farms by poultry vaccination and laboratory testing (Available online: https://www.gov.br/agricultura/pt-br/assuntos/sanidade-animal-e-vegetal/saude-animal/programas-desaude-animal/pnsa/2003_78.INconsolidada.pdf). However, in recent decades several poultry diseases and foodborne *Salmonella* outbreaks have been reported in Brazil (Kipper *et al*., 2022).

Whole-genome sequencing (WGS) is useful in foodborne outbreak investigations and pathogen surveillance (Allard *et al*., 2016). Illumina short-read sequencing technology has proven to be robust for characterizing pathogens of clinical care (Gilchrist *et al*., 2015), but it is unable to resolve repetitive and GC-rich regions, thus producing unresolvable regions in the underlying genome assembly that are fragmented into independent contigs (Utturkar *et al*., 2014). These regions affect the ability to obtain the complete whole-genome structure, which is crucial to determine if some genes are co-regulated or co-transmissible and if they are located on the chromosome or plasmids (Ashton *et al*., 2015). Furthermore, the bias to identify key virulence genes during an outbreak investigation can also have negative impacts on public health assessment.

Nanopore sequencing technology generates long reads to facilitate the completion of bacterial genome assemblies but lacks sequencing depth in some repetitive regions (Madoui *et al*., 2015). However, Nanopore sequencing's long reads can span the wide repetitive regions and resolve GC-rich regions, making it useful for resolving full-length genome sequences (Chen *et al.*, 2020). However, Nanopore sequencing technology exhibits lower read accuracy which may produce systematic errors, as a result, it has only usually been applied as a complement to short-read sequencing (Rang *et al.*, 2018). Since the release of the MinION platform by Oxford Nanopore Technologies, nanopore chemistry, basecalling, and bioinformatic tools have been steadily evolving, in order to use raw Nanopore long reads independently to get more accurate bacterial genomes independent of other sequencing technologies (Jain *et al*., 2016).

The combination of both short reads for base-calling accuracy and long reads for structural integrity has recently been developed as a hybrid assembly approach to close whole-genome assemblies, such as those found in the Unicycler and SPAdes pipelines (Antipov *et al.*, 2016; Wick *et al*., 2017). Unicycler was specifically developed for hybrid assembly of bacterial genomes (Wick *et al*., 2017). Unicycler generates a short-read assembly graph and then uses long-reads to build bridges to resolve all repeats in the genome, performs multiple rounds of short-read polishing and finally, it produces a complete genome assembly (Chen *et al.*, 2020).

WGS is useful to identify mobile elements such as antimicrobial resistance genes (ARGs), prophages, defense systems, genomic islands, pathogenic islands, and virulence genes among others. In this study, a hybrid genome assembly using both MinION and HiSeq sequencing data was used to improve the assembly parameters and gene completeness, the identification of mobile elements, whole-genome phylogeny and pan-genome in a *Salmonella* var. Enteritidis SE3 isolated from the Subaé River, a polluted river including organic waste and heavy metals, soil in Santo Amaro, Brazil.

The goal of this study was to compare *Salmonella* SE3 genome assembly generated using no hybrid assembly from HiSeq and MinION data and hybrid assembly (HiSeq + MinION). And use the assembly with the best quality and completeness to improve the inferring of phylogenetic relationships, and pangenome and to determine the presence and type of mobile genetic elements.

2. Materials and methods

Environmental soil samples were obtained from the Subaé river basin in Santo Amaro neighbourhood, Salvador de Bahia, Brazil. Approximately 100 g of soil sample was collected from river soil (12°31'46.77"S 38°44'1.24"W). The sample was transported in a refrigerated box (4–8°C) to the laboratory where the analyses were done immediately.

2.1 *Salmonella* isolation

Salmonella was isolated according to the US Food and Drug Administration Bacteriological Analytical Manual (BAM/FDA, 2006). Briefly, 10 g or 10 mL of samples of each sample were pre-enriched in 100 mL lactose broth (supplier), at 37°C for 24 h, 0.1 mL of pre-enriched culture was transferred to 10 mL enriched in Tetrathionate (TT) broth (supplier) and incubated at 41°C for 24 h. Broth cultures from the selective enrichment broth were plated on Xylose-Lysine-Desoxycholate (XLD) agar (supplier), Bismuth sulfite agar (supplier) and Salmonella Shigella (SS) agar (supplier). Colonies characteristic of *Salmonella* having a slightly transparent zone of reddish color and a black center for XLD, gray or brown-black colonies with or without metallic sheen for Bismuth Sulfite Agar, and beige colonies with black centers for SS agar were identified and picked. Then, the isolates were tested biochemically using the Triple Sugar Iron (TSI) test. *Salmonella* strains were confirmed when they showed good to excellent growth, pink colonies with black centers were detected, and the agar was red (Asai, *et al.,* 2002).

2.2 DNA isolation

For bacteria, a single colony was enriched in 5 ml Luria Bertani (LB) broth, and 15 mL of enrichment broth was transferred to a centrifuge tube and centrifuged at 4000 rpm for 10 min. DNA from *Salmonella* strains were extracted and purified using the E.Z.N.A. Bacterial DNA Mini Kit (Omega Biotek, CA, USA) following the instructions provided by the manufacturer. For phages, a crude lysate was centrifuged the lysate as described. DNA isolation from phages was carried out using the E.Z.N.A. Viral DNA Mini Isolation Kit (Omega Biotek, CA, USA) following the instructions provided by the manufacturer. The quality and concentration of the bacteria and phage DNA was evaluated by Qubit Fluorometric Quantification (ThermoFisher Scientific, Waltham,

MA, US) and gel electrophoresis (1% of agarose gel, 80 V for 45 min in 1x TAE Buffer).

2.3 16S gene amplification

PCR amplification was performed using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA), 16S gene Amplification PCR for the amplification of the 16S rRNA gene was carried out using universal primers 27F (5′-AGAGTTTGATCATGGCTCAG-3′) as forward and 1492R (5′-GGTTACCTTGTTACGACTT-3′) as a reverse primer (Lane, 1991; Turner *et al*., 1999; Senthilraj, 2016). Approximately 10-100 ng of template was added to a reaction mix containing 10 µL Master Mix 2x (Qiagen), 1 µL primer 27 F (10 uM), 1 µL primer 1492R (10 uM), and 1 µL reverse primer (10 uM). PCR was performed with the following cycling conditions: initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 1 min, annealing from 50°C to 60°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 7 min. PCR products were visualized using Gelred (Biotium) on a 2% agarose gel which had been run at 80 V for 30 min. The separated PCR products were visualized under UV light and photographed.

2.4 16S rRNA gene sequencing and phylogenetic analysis

The amplified 16S PCR products were purified and sequenced at MACROGEN (Seoul, Korea) using the ABI 3100 sequencer with Big Dye Terminator Kit v. 3.1. The same 16S primer sequences used for PCR were used for sequencing. The sequences were assembled and trimmed using Geneious Prime and submitted to the Greengenes database (https://rnacentral.org/expert-database/greengenes). The sequences of this study and sequences de reference were aligned with Clustal W, and the evolutionary history was inferred using the Neighbor Joining Method (Nei & Kumar, 2000). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985). There were a total of 1552 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

2.5 Whole Genome Sequencing (WGS) by MinION and Illumina

Nanopore WGS sequencing was carried out at the Molecular and Computational Biology of Fungi Laboratory, Federal University of Minas Gerais (UFMG). The DNA library was prepared with a rapid sequencing kit (SQKRAD004, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. Libraries were sequenced with qualified FLO-MIN106 flow cells (R9.4.1, active pores number > 800) for 2 h on a GridION (Oxford Nanopore Technologies, Oxford, UK).

The quality of the sequencing was verified through the FastQC v0.11.9 program (https://github.com/s-andrews/FastQC). The Porechop v0.2.4 program (Wick et al., 2017) was used for the detection and elimination of the adapters, as well as for the demultiplexing of the Nanopore reads. Possible sequencing errors were treated with the Canu v2.1.1 monitor correction module (Koren *et al*., 2017). The de novo assembly based on Bruijn graphs of corrected sequences was carried out through the Flye v2.8.3 (Kolmogorov *et al*., 2019). The contigs obtained using de novo assembly were subjected to a polishing (correction of raw contigs) with the Racon v1.4.22 program (Vaser *et al*., 2017), which took the read mappings made with BWA v0 .7.17 (LI, 2013).

The Illumina sequencing library was prepared from genomic DNA [1 µg] using the NEBNext Fast DNA Fragmentation and Library Preparation Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's recommendations. The library quality was assessed using the Agilent 2100 Bioanalyzer equipment, and the paired-end DNA sequencing was carried out in the Illumina HiSeq 2500 platform. After sequencing, the raw read quality was assessed using the FastQC v0.11.5 software (https://github.com/s-andrews/FastQC, accessed on 15 January 2020).

2.6 Hybrid genome sequence assembly

MinION long-reads were assembled using the Racon pipeline with default parameters (Vaser *et al*., 2017) while Illumina short reads were assembled using the (i) SPAdes version: 3.15.3 (Bankevich *et al*., 2012), (ii) Unicycler (Wick *et al*., 2017) and (iii) Edena (Hernandez *et al*., 2008) software with default parameters. Hybrid assemblies using Illumina and MinION reads were performed using the software (i) MaSuRCA (Zimin *et al*., 2013), and (ii) Unicycler. Genome quality and completeness for each assembly were evaluated using QUAST v4.6.0 (Gurevich *et al*., 2013), and

BUSCO v4 (Benchmarking Universal Single-Copy Orthologs) (Simão *et al*., 2015). BUSCO analyses were performed using the database bacteria obd_10.

2.7 Serotype identification

The identification of the serotype was carried out from the de novo contigs, using the SeqSero2 v1.2.1 program (ZHANG *et al*., 2019).

2.8 Gene annotation

The annotation of genes for both the bacterial and plasmid genomes was performed through the predictor, based on hidden Markov models, Prokka v1.14.6 (SEEMANN, 2014).

2.9 Genome similarity assessment

Salmonella enterica genomes (16,638) were downloaded from the NCBI Genbank database on July 2022. Genomes with more than 500 contigs were removed, and contigs smaller than 500 bp were removed from the remaining genomes. Genome quality was evaluated with CheckM v.1.0.13 (Parks et al., 2015), using completeness and contamination score of >= 90% and <= 10%, respectively. Genome-distance estimation of genomes was performed with Mash v.2.2.1 (Ondov et al., 2016). Near-identical redundant genomes were removed using in-house scripts to cluster genomes assemblies sharing pairwise Mash distances less than 0.005 (~99,95% ANI identity) and cluster representatives were chosen based on assembly N50. Further, the genome dataset was taxonomically verified using the Genome Taxonomy Database (GTDB). To investigate the genomic relatedness of the *S. enterica* SE3 strain and Genbank genomes, a genome-distance tree was built using a combination-distance matrix of Mash and ANI values, computed with Mash v.2.2.1 and fastANI (Jain et al., 2018), respectively.

2.10 Pangenome analysis

The *S. enterica* pangenome analysis was performed with Roary v.3.6, using 90% identity threshold to determine gene clusters (Page et al., 2015). The Heaps law model was used to estimate the pangenome openness. Core genes (present in up to 95% of the genomes) were aligned with MAFFT v.7.394 (Katoh and Standley, 2013). SNPs were extracted from the core-genome alignment using SNP-sites v.2.3.3 (Page et al., 2016). The phylogenetic tree was constructed using IQ-TREE (Nguyen et al., 2014), with

ascertainment bias correction under the model GTR+ASC, and bootstrap support was performed using 1000 replicates. The resulting phylogenetic tree was visualized and rendered with iTOL v4 (Letunic and Bork, 2019).

2.11 Mobile genetic element identification and annotation

Genomic islands were identified using Island Viewer software (www.pathogenomics.sfu.ca/islandviewer/upload/) (Bertelli *et al*., 2017), virulomes were detected using VFanalyser/VFDB (www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) (Liu *et al*., 2022), resistomes were identified using ResFinder-4.1 (https://cge.cbs.dtu.dk//cgi-bin/webface.fcgi?jobid=61358037000023BC9E7A4C58) (Alcock *et al*., 2020), and CARD (https://card.mcmaster.ca/) (Jia et al., 2016), Prophages were identified using Phaster (phaster.ca) (Arndt *et al*., 2016), phage defence systems were detected using PADLOC (https://padloc.otago.ac.nz/padloc/) (Payne et al., 2022) and DefenseFinder (https://defense-finder.mdmparis-lab.com/) (Tesson et al., 2022). SPIFinder 2.0 was used to detect Pathogenic Islands (https://cge.cbs.dtu.dk/services/SPIFinder/) (Roer et al., 2016). BRIG was used to draw the chromosomal *Salmonella* genomes (http://brig.sourceforge.net/) (Alikhan et al., 2011).

3. Results

3.1 *Salmonella* isolation and characterization

Presumptive *Salmonella* were isolated from the soil of the Subaé River using *Salmonella* selective growth media. Isolates showed typical *Salmonella* characteristics: in XLD a slightly transparent zone of reddish color and a black center was noted for colonies, in Bismuth Sulfite Agar gray or brown-black colonies with or without metallic sheen were observed and in SS agar the colonies were beige with black centers. In biochemical tests, good growth was seen in TSI, acid and gas reaction at depth, alkaline surface (red), presence of H_2S, and 16S sequencing were performed to confirm isolate identification.

3.2 16S Analysis

The presumptive *Salmonella* isolates were confirmed by 16S PCR amplification (Dos Santos et al., 2019) and sequencing, followed by a sequence query of the Greengenes database. Analysis of the Query returned Coverage of 100% and an E value

of 0, with 99.91% identity to the same sequence, *Salmonella* serovar Enteritidis (ID: MT621365.1).

3.3 Whole genome sequencing of *Salmonella* isolate SE3

The *Salmonella* isolate, designated SE3, was analysed by whole genome sequencing. The number of reads from Illumina HiSeq sequencing was 15,997,283 and the number of reads from MinION sequencing was 13,326, after preprocessing. The MinION long reads had an average size of 5.1 kb, and the longest read was 28.841 kb..

3.4 Genome assembly

In this study, six whole genome sequence assembly strategies were tested. The HiSeq and MinION sequencing data were generated using the *Salmonella* SE3 isolate, one using no hybrid assembly of long reads (MinION), three using no hybrid assembly of short reads (Illumina HiSeq), two using hybrid assembly (Illumina HiSeq + MinION) (Table 2). For Illumina HiSeq assembly, Unicycler showed the best performance, with 31 contigs, total length of 4,683,367 bp, largest contigs of 1,262,086 bp and N50 of 478.501 bp.

Unicycler hybrid assembly showed the best performance for genome assembly overall (Table 1) (Figure 1). Genome hybrid assembly revealed 10 contigs, total length of 4,713,463 bp, largest contig of 519,108 bp and N50 of 2,750,500 bp.

For genome completeness, Unicycler HiSeq and Unicycler hybrid assembly showed the same result, 98.4 % of the orthologous genes (complete genes) searched, 99.4 % were single-copy genes, 1.6 % genes were not identified or missing, and there were no identified single and fragmented genes (Table 2).

Table 1. Summary statistics for the assembled genome of *Salmonella* SE3 using reads from Illumina HiSeq and Oxford Nanopore Technologies MinION.

Assembly method	**Racon**	**Unicycler**	**Edena**	**SPAdes**	**Unicycler**	MaSuRCA
Sequence data	**MinION**	**HiSeq**	**HiSeq**	**HiSeq**	**Hybrid**	**Hybrid**
Number of contigs	2	31	41	50	10	39
Number of contigs (≥ 0 bp)	2	65	54	111	18	42
Number of contigs (≥ 50 000 bp)	2	15	4,475,114	4,566,140	4	24
Largest contigs	4,671,311	1,262,086	488,615	1,276,166	2,750,500	519,108

Total length (≥50 000 bp)	4,730,597	4,683,367	4,701,851	4,805,245	4,713,463	4,585,719
GC (%)	52.18	52.14	52.15	51.85	52.16	52,15
N50	4,671,311	478,501	181,604	491,607	2,750,500	246,991
L50	1	3	8	3	1	7

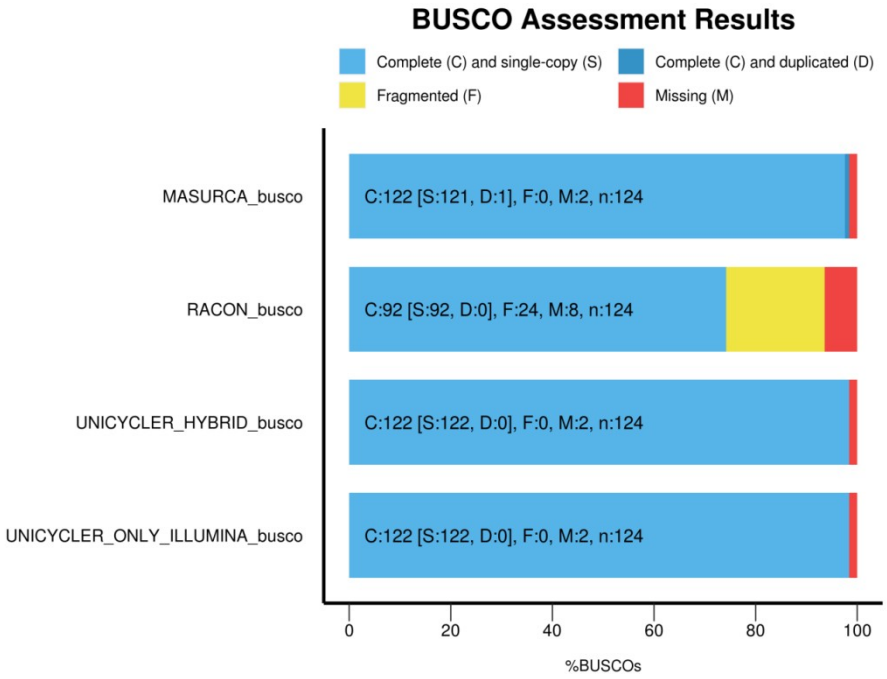

Figure 1. BUSCO completeness assessment of *Salmonella* SE3 genome

Table 2. Completeness assessment of *Salmonella* SE3 assemblies using BUSCO software.

Assembly method	Sequence data	Complete (%)	Single-Copy (%)	Duplicated (%)	Fragmented (%)	Missing (%)
Racon	**MinION**	74.2	74.2	0	19.4	6.4
Unicycler	**HiSeq**	98.4	98.4	0	0	1.64
Unicycler	**Hybrid**	98.4	98.4	0	0	1.64
MaSuRCA	**Hybrid**	98.4	97.6	0.8	0	1.64

3.5 Completeness of the genome annotation

Salmonella SE3 showed ~99,95% ANI identity **with** Salmonella enterica subsp. enterica serovar Enteritidis Durban. The genome of *Salmonella* SE3 was annotated using Prokka, Table 3 and supplementary material 1.

Table 3. *Salmonella* SE3 showing annotated genome using Prokka tool kit

Annotated genome	Features
rRNA	20
tRNA	87

Repeat region	2
CDS	4403
mRNA	1

3.6 Genomic relatedness of *Salmonella* SE3

We downloaded all available *S. enterica* genomes in the Genbank database (n=16,638, July 2022), out of which 37 were removed after CheckM quality filtering. The set of *S. enterica* genomes were further filtered to remove highly fragmented and near-identical redundant genomes (see method for details), comprising a dataset with 1,598 genomes. Through genomic identity analysis performed with a combined matrix of all Mash and fastANI pairwise distances between genomes, we identified 159 genomes with incorrect taxonomic assignment. The distance tree built with the combined matrix showed that the *Salmonella* SE3 genome was located within the properly classified cluster of *S. enterica* genomes (Figure 2A). The *S. enterica* dataset comprised 1,439 *S. enterica* genomes sharing Mash distance values up to 0.03 (~97% ANI identity) (Figure 2B).

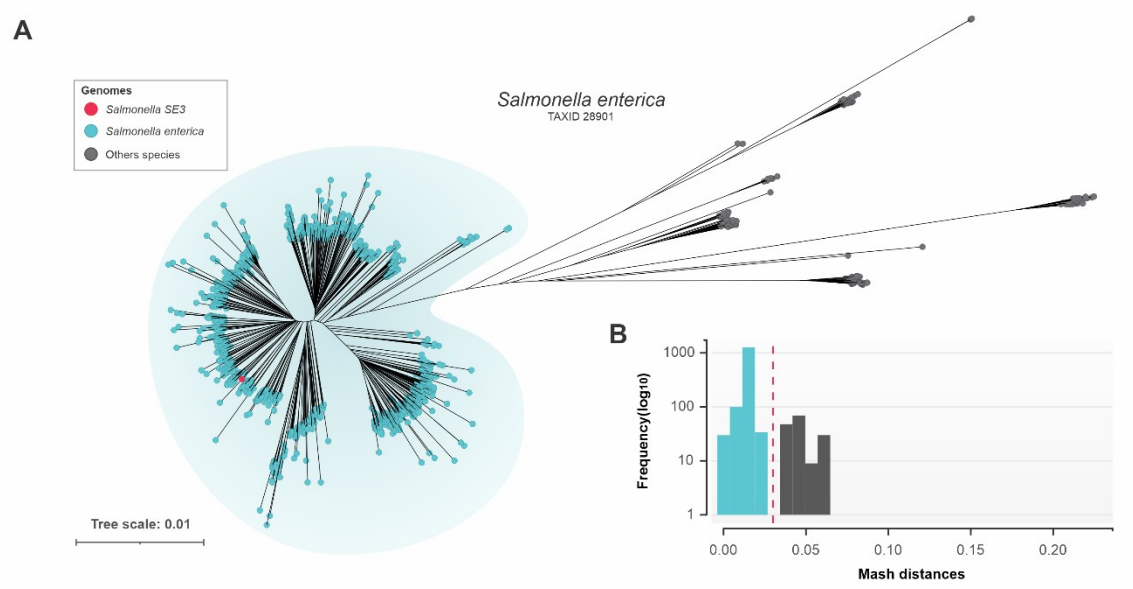

Figure 2. Genome similarity of *Salmonella* SE3. (A) Distance tree of *Salmonella enterica*, built using a combined matrix of all Mash and fastANI pairwise distances of *Salmonella* SE3 and 1,598 publicly genomes. Genomes classified by GTBD as *S. enterica* are shaded in blue. (B) Mash-distance values of *Salmonella* SE3 were calculated with 1,598 *Salmonella* genomes. The maximum Mash-distance threshold (0.03) used to select genomes is represented by a dotted line.

3.7 Pangenome analysis

The pangenome of 1,439 *S. enterica* genomes is composed of 74,995 gene clusters, including a core genome (present in at least 95% of the genomes) of 2,137 genes. The accessory genome comprises 3,390 shell genes (present from 15% to 95% of the genomes) and 69,352 cloud genes (present in up to 15% of the genomes) (Figure 3B). The Heaps law estimate supports an open pangenome (alpha= 0.52) for *S. enterica*. (Figure 3A), indicating a high genetic diversity, and the capacity of this sympatric species to rapidly acquire exogenous DNA. We also performed a maximum-likelihood phylogenetic reconstruction using 292,004 SNPs extracted from core genes. This analysis revealed that *Salmonella* SE3 belongs to a monophyletic clade containing 23 *S. enterica* strains of serovar Enteritidis (Figure 3C).

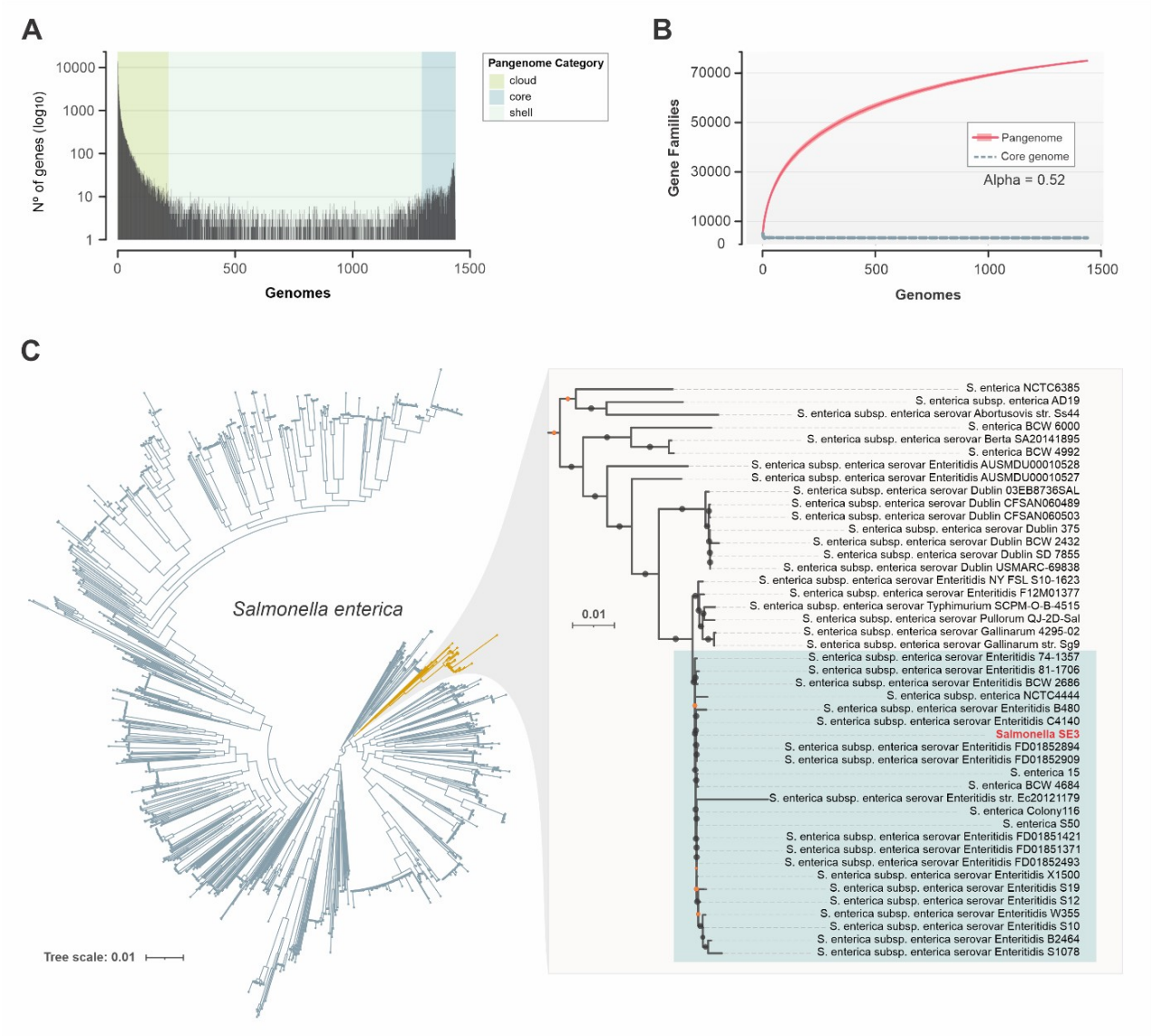

Figure 3. Pangenome of *Salmonella enterica* and phylogeny of *Salmonella* SE3. (A). Gene frequency of *S. enterica* pangenome. (B) Number of gene families in the *S. enterica* pangenome. The cumulative curve (in red) and an alpha value of the Heaps law less than one (0.52) supports an open pangenome. (C) core-genome SNP tree of *Salmonella enterica* highlighting the phylogenetic group contained the *Salmonella* SE3 genome. The monophyletic clade containing the serovar Enteritidis of *S. enterica* is shaded in cool grey. Bootstrap values below and above 70% are represented by orange and dark-grey dots, respectively.

3.8 Genome features

a) Resistome identification

Several resistance mechanisms were identified in *Salmonella* SE3 using the CARD database, resistance to aminoglycosides (alleles of *AAC(6')-Iy, kdpE, baeR*), fluoroquinolones (alleles *of MdtK, emrB, emrR, sdiA, Escherichia coli acrA, acrB, rsmA, adeF*), macrolides (alleles of *Klebsiella pneumoniae* KpnE, *K. pneumoniae* KpnF, H-NS, CRP), monobactam (*golS*), nitroimidazole (*msbA*), tetracycline (*E. coli* mdfA), cephalosporin (*Haemophilus influenzae* PBP3 conferring resistance to beta-lactam antibiotics, *E. coli* EF-Tu mutants conferring resistance to Pulvomycin, *E. coli uhpT* with mutation conferring resistance to Fosfomycin, *E. coli glpT* with mutation conferring resistance to Fosfomycin), Figure 4.

According to their mechanism of resistance, the genes were classified as antibiotic efflux (*golS, baeR, MdtK, K. pneumoniae* KpnE, *K. pneumoniae KpnF, H-NS, sdiA, mbsA, E. coli mdfA, kdpE, E. coli acrA, acrB, adeF, CRP, rsmA, emrB, emrR* and *marA*), antibiotic inactivation (*AAC(6')-ly*), antibiotic target alteration (*vanG, bacA, H. influenzae* PBP3 conferring resistance to beta-lactam antibiotics, *E. coli uhpT* with mutation conferring resistance to Fosfomycin, *E. coli EF-Tu* mutants conferring resistance to Pulvomycin, *E. coli glpT* with mutation conferring resistance to Fosfomycin, *E. coli* EF-Tu mutants conferring resistance to Pulvomycin, *pmrF*, *E. coli* acrAB-tolC with marR mutations conferring resistance to ciprofloxacin and tetracycline, *E. coli soxR* with mutation conferring antibiotic resistance and *E. coli soxS* with mutation conferring antibiotic resistance).

Resfinder identified resistance against aminoglycosides: tobramycin (*aac(6')-Iaa* (*aac(6')-Iaa*_NC_003197) and amikacin (*aac(6')-Iaa* (*aac(6')-Iaa*_NC_003197).

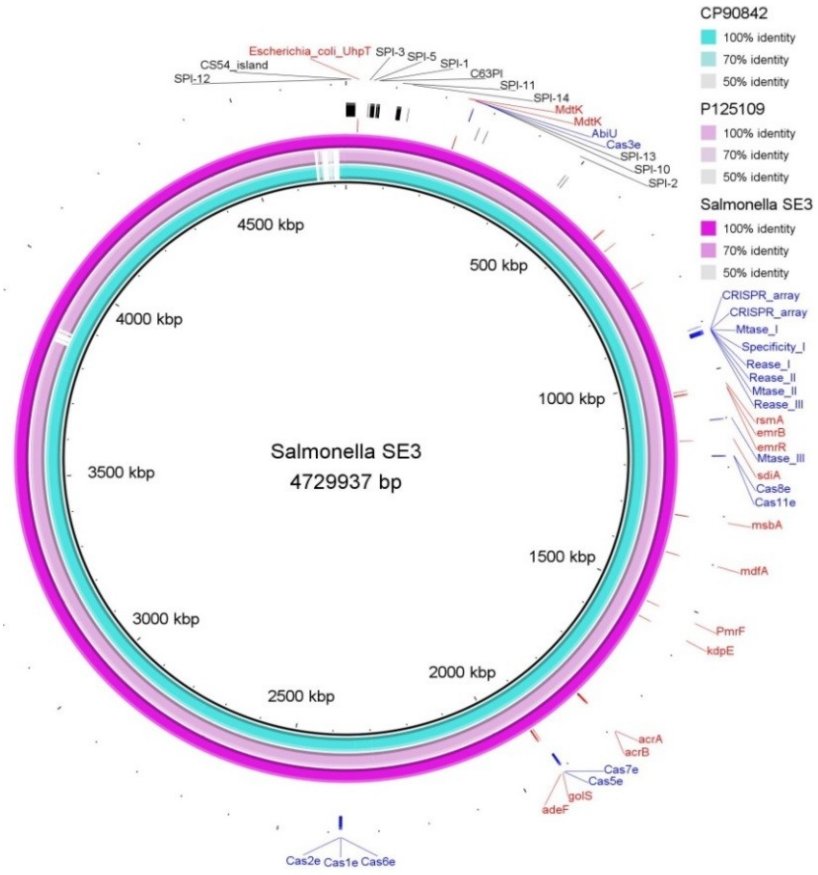

Figure 4. Salmonella SE3 antimicrobial resistance genes (red color), Salmonella Pathogenic Island (SP) (black color) and defense system (blue color) with two genomes of reference of *Salmonella* serovar Enteritidis (P125109 and CP9084.2).

b) Virome, genomic island and pathogenic island identification

In total, 144 potential virulence genes were identified in *Salmonella* SE3 using VFanalyser/VFDB, some of the most important identified were *invA*, *sipA*, *sipB*, *sipC*, *fepA*, *sopA*, *sopB*, *sopD*, *sopE2*, *pefA*, *pefB*, *pefC*,*pefD* and *ssaB*.

Genomic islands were detected using Island Viewer which uses three prediction methods: Integrated, IslandPath-DIMOB and SIGI-HMM. Twelve pathogenic islands were detected (Figure 2 and Table 4), and included virulence genes, secretion proteins, resistance genes, bacteriophage sequence regions, transposases and integrases. The gene arsC, encoding Arsenate reductase was identified in a genomic island. The *mdtK* gene (encoding multidrug resistance protein MdtK) was also identified in the resistome analysis. Virulence genes identified using Island Viewer was very similar to those identified using VFanalyser/VFDB, (Figure 5).

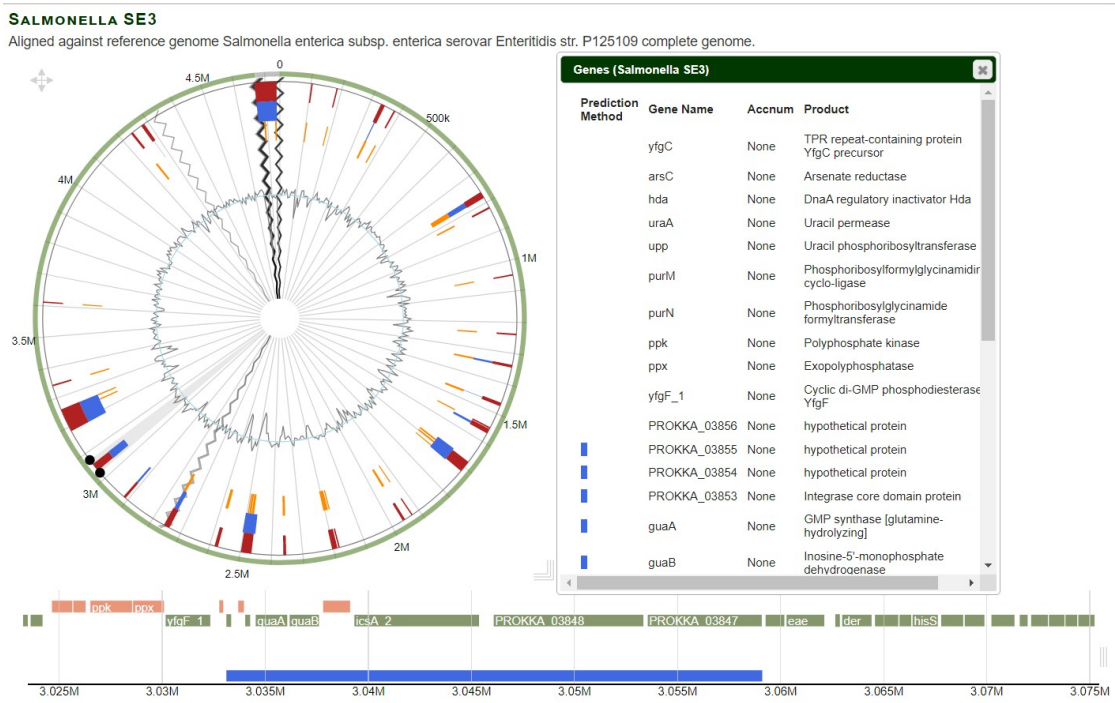

Figure 5. Distribution of SPIs (*Salmonella* Pathogenic Island) in *Salmonella* SE3 Genome Island. Three methods such as IslandPath-DIMOB, Integrated and SIGI-HMM were utilized for virulence/resistance gene annotations. The grey color circle represents GC content. Toxins (CcdB) and Antitoxin (CcdA) were also identified in SPI_1 and SPI_2. Hemolysin C (tlyC) was identified in SPI_10.

SPIFinder-2.0 was used to identify *Salmonella* pathogenic islands. The pathogenic island with the best match and high identity percentage was selected, Table 4. SPI-1, SPI-2, SPI-5, SPI-11, SPI-12, SPI-13, SP1-14, C63PI, CS54_island and not named were identified, among them Not named, SP1-1 and SPI-2 had 100% of Identity and a perfect match. SPI-11 had Gifsy-1_prophage in the insertion site, Table 5.

Table 4. Pathogenic islands identified in *Salmonella* SE3

No	SPI	Identity	Query/ Template length	Organism	Insertion site	Accession number
1	SPI-1	99.7	2705 / 2705	*Salmonella enterica* Typhimurium SL1344	fhlA/mutS	AF148689
2	SPI-2	100	642 / 642	*Salmonella enterica* Gallinarum SGC_2	tRNA-valV	AY956827
3	SPI-3	99.05	738 / 738	*Salmonella enterica* Typhimurium 14028s	tRNA_selC	AJ000509
4	SPI-5	99.11	9069 / 9069	*Salmonella* Typhimurium LT2	tRNA-serT	NC_003197

5	SPI-10	98.28	553 / 554	*Salmonella enterica* Gallinarium SGE_3	Not_published	AY956839
6	SPI-11	98.54	9085 / 15686	*Salmonella enterica* Choleraesuis SC_B67	Gifsy-1_prophage	NC_006905
7	SPI-12	97.14	5766 / 11075	*Salmonella enterica* Choleraesuis SC_B67	tRNA-pro	NC_006905
8	SPI-13	100	341 / 341	*Salmonella enterica* Gallinarum SGA_10	tRNA-pheV	AY956834
9	SPI-14	99.8	501 / 501	*Salmonella enterica* Gallinarum SGA_8	Not_published	AY956835
10	C63PI	99.12	4000 / 4000	*Salmonella enterica* Typhimurium SL1344	FhlA	AF128999
11	CS54_island	98.09	19669 / 25252	*Salmonella enterica* Typhimurium ATCC_14028	xseA-yfgK	AF140550
12	Not named	100	330/330	*Salmonella enterica* Enteritidis CMCC50041	--	JQ071613

3.9 Identification of antiviral defense systems

Several antiviral defense system virulence genes were identified using PADLOC and DefenseFinder tools (Table 6). Both tools identified several systems: Cas type IE, CBASS type I, CRISPR array, Restriction-modification (RM) RM type I, and RM type III. Similar antiviral systems and proteins were identified by PADLOC, with the exception of AbiU and RM type II (Table 5 and Figure 4).

Table 5. Antiviral defense systems of *Salmonella* SE3

Number	System	Subtype	Tool	Reference
1	AbiU	AbiU	PADLOC	Payne *et al*., 2022
2	Cas type IE	Cas3e	PADLOC	Payne *et al*., 2022
3	Cas type IE	Cas8e	PADLOC	Payne *et al*., 2022
4	Cas type IE	Cas11e	PADLOC	Payne *et al*., 2022
5	Cas type IE	Cas7e	PADLOC	Payne *et al*., 2022
6	Cas type IE	Cas5e	PADLOC	Payne *et al*., 2022
7	Cas type IE	Cas6e	PADLOC	Payne *et al*., 2022
8	Cas type IE	Cas1e	PADLOC	Payne *et al*., 2022
9	Cas type IE	Cas2e	PADLOC	Payne *et al*., 2022
10	CBASS_type_I	Cyclase	PADLOC	Payne *et al*., 2022
11	CBASS_type_I	Effector	PADLOC	Payne *et al*., 2022
12	CRISPR array	CRISPR array	PADLOC	Payne *et al*., 2022
13	CRISPR array	CRISPR array	PADLOC	Payne *et al*., 2022
14	RM type I	Mtase I	PADLOC	Payne *et al*., 2022
15	RM type I	Specificity I	PADLOC	Payne *et al*., 2022
16	RM type I	Rease I	PADLOC	Payne *et al*., 2022

17	RM type II	Rease II	PADLOC	Payne *et al*., 2022
18	RM type II	Mtase II	PADLOC	Payne *et al*., 2022
19	RM type III	Rease III	PADLOC	Payne *et al*., 2022
20	RM type III	Mtase III	PADLOC	Payne *et al*., 2022
21	Cas Class1 subtype I E1	Cas3 I 5	DefenseFinder	Tesson *et al*., 2022
22	Cas Class1 subtype I E1	Cas8e I E 1	DefenseFinder	Tesson *et al*., 2022
23	Cas Class1 subtype I E1	Cas2gr11 I E 2	DefenseFinder	Tesson *et al*., 2022
24	Cas Class1 subtype I E1	Cas7 I E 2	DefenseFinder	Tesson *et al*., 2022
25	Cas Class1 subtype I E1	Cas5 I E 3	DefenseFinder	Tesson *et al*., 2022
26	Cas Class1 subtype I E1	Cas6e I II II IV V VI 1	DefenseFinder	Tesson *et al*., 2022
27	Cas Class1 subtype I E1	Cas 1 I E 1	DefenseFinder	Tesson *et al*., 2022
28	Cas Class1 subtype I E1	Cas2 I E 2	DefenseFinder	Tesson *et al*., 2022
29	CBASS I 2	Cyclase SMODS	DefenseFinder	Tesson *et al*., 2022
30	CBASS I 2	2TM Gros	DefenseFinder	Tesson *et al*., 2022
31	RM Type III 2	Type III Reases	DefenseFinder	Tesson *et al*., 2022
32	RM Type III 2	Type III Mtases	DefenseFinder	Tesson *et al*., 2022
33	RM Type I 1	Type I S	DefenseFinder	Tesson *et al*., 2022
34	RM Type I 1	Type I Mtases	DefenseFinder	Tesson *et al*., 2022
35	RM Type I 1	Type I S	DefenseFinder	Tesson *et al*., 2022
36	RM Type I 1	Type I Reases	DefenseFinder	Tesson *et al*., 2022

* MTase=Methyltransferase I, Rease= restriction endonucleases,

3.10 Prophage identification

Of the prophages identified in *Salmonella* SE3 using PHASTER, two regions were intact, five regions were incomplete and 0 were questionable (Table 6). Proteins were identified in Gisfy and RE 2010 prophages including lysis, terminase, portal protein, protease, coat protein, tail shaft, attachment site, integrase, phage-like protein, transposase, fiber protein, plate protein, tRNA, hypothetical proteins and others.

Table 6. Details of prophage sequences annotated in *Salmonella* SE3 genome

Completeness	Score	Total Proteins	Region position	Most common Phage	No Accession	GC (%)
Incomplete	60	27	805989-831780	*Shigella* phage POCJ13	NC_025434	48.7
Intact	150	40	1041034-1072153	Phage Gifsy-2	NC_010393	47.22
Incomplete	50	13	1276587-1286489	Phage Gifsy-2	NC_010393	46.68
Incomplete	30	9	1698977-1705339	*Shigella* phage POCJ13	NC_025434	45.64
Intact	150	49	1081056-1124788	*Salmonella* phage RE-2010	NC_019488	51.18
Incomplete	20	8	1435195-1442595	*Escherichia* phage 500465-2	NC_049343	53.18
Incomplete	40	9	29216-37324	*Salmonella* phage RE-2010	NC_019488	52.44

4. Discussion

Salmonella SE3 was isolated from soil at the Subaé River in Santo Amaro, Brazil, a region contaminated with heavy metals and organic waste. The genome sequence of this isolate was determined using two different sequencing technologies and several different bioinformatics strategies. Both the DNA sequencing technology and assembly strategy affected the whole genome sequence produced.

In this study, three DNA sequence assembly strategies were tested on *Salmonella* SE3 data: hybrid (Hiseq + MinION) and non-hybrid (Hiseq and MinION). Hybrid assembly showed the lowest number of contigs followed by MinION assembly, Hybrid genome assembly resulted in a genome of 4.73 Mb, which was similar in size to that reported (4.68 Mb) for ***Salmonella enterica* subsp. *enterica* serovar Enteritidis str. P125109** NC_011294.1 **(GC: 52.17) (**Vaid et al., 2021). GC content of the assembled genome (52.16%) was more similar to ***Salmonella enterica* subsp. enterica serovar Enteritidis str. P125109** NC_011294.1 **(GC: 52.17) (**Vaid et al., 2021). On the other hand, according to Salmonella SE3 genome completeness (the process of determining the entirety, or nearly the entirety, of the DNA sequence of an organism's genome at a single time), no hybrid assembly (Illumina HiSeq) and hybrid assembly showed almost the same result.

HiSeq assemblies have been traditionally considered the “gold standard” because MinION sequencing could introduce high numbers of errors and consequently may interfere with high-quality genome annotations due to reduced accuracy in gene prediction, producing a large number of misannotated genes (González-Escalona et al., 2019; Taylor et al., 2019).

The *Salmonella* SE3 genome was located within the properly classified cluster of *S. enterica*. On the other hand, using genomic similarity analysis we identified 159 genomes with incorrect taxonomic classification, it is important to analyze these before developing a phylogenetic analysis because it could generate mistakes in the subsequent analysis. According to the pangenome analysis of *Salmonella* SE3, the core genome was composed for 2,137 genes and the accessory genome comprises: 3,390 shell genes and 69,352 cloud genes.

Besides, other study carried out a comparative genomic analysis of 44 genome sequences representing 17 serovars of *S. enterica*, pangenome estimated that the genus *Salmonella* displays an open pangenome as our results with Salmonella SE3, the structure comprising a reservoir of 10,775 gene families. Of these, 2847, 4657, and 3271 constitute the core gene families (CGFs), dispensable or accessory gene families (DGFs), and strain-specific gene families (SSGFs), respectively (Chand et al., 2020).

Salmonella SE3 showed an open pangenome. Park et al. (2019) constructed pangenomes of seven species in order to elucidate variations in the genetic contents of >27,000 genomes belonging to *Streptococcus pneumoniae*, *Staphylococcus aureus* subsp. *aureus*, *Salmonella enterica* subsp. enterica, *Escherichia coli* and *Shigella* spp., *Mycobacterium tuberculosis* complex, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Like our study, this work showed the pangenomes of *Salmonella enterica* subsp. enterica on the other six species has open as well as out study identified for Salmonella SE3.

There are some studies were the Pangenome of Salmonella (Park et al., 2019: Chand et al., 2020); were open like our study, It is important to note that , first the pangenome size is heavily influenced by the properties of the genomes used, this variation would likely result in inconsistencies. Second, the pangenomes will depend of the algorithm used. Lastly, by using a large number of different strains, relatively new genes were often included, which results in open pangenomes (Park et al. 2019).

The antimicrobial resistance gene profile of *Salmonella* SE3 genome identified genes potentially involved in resistance to aminoglycosides (*AAC(6')-Iy*, *kdpE*, *baeR*), fluoroquinolones (*mdtK*, *emrB*, *emrR*, *sdiA*, *acrA*, *acrB*, *rsmA*, *adeF*), macrolides (*kpnE*, *kpnF*, H-NS, CRP), monobactam (*golS*), nitroimidazole (*msbA*), tetracycline (*mdfA*), and cephalosporins (PBP3 mutants, EF-Tu mutants, *uhpT*, *glpT*). Other studies of *Salmonella* isolates from southern Brazil also reported the antibiotic resistance genes *mdf(A)* and *aac(6')-Iaa*, in *Salmonella* var *enteritidis*. However, differently to our study, other resistance genes were identified in *Salmonella* Enteritidis isolates, including *aac(3)-Iva*, *aph(3")-Ib*, *aph(4)-Ia*, *aph(6)-Id*, *tet(34)* and *tet(A)* (de Oliveira, et al., 2006; Vaz et al., 2010; Campioni et al., 2012; Achtman et al., 2012; Campioni et al., 2017; Mascitti et al., 2021). In the US, other antibiotic resistance mechanisms in *S. enterica* were described, such as resistance to aminoglycosides (*aadA*, *aadB*, *aacC*,

aphA, strAB), β-lactams (*blaCMY-2, PSE-1, TEM-1*), chloramphenicol (*cat1, cat2, cmlA, floR*), inhibitors of the folate pathway (*dfr, sul*), and tetracycline (*tetA, tetB, tetC, tetD, tetG,* and *tetR*) (Frye et al., 2013), none of these resistance genes was detected in our study.

In this work ten *Salmonella* pathogenic islands were identified in *Salmonella* SE3: SPI-1, SPI-2, SPI-5, SPI-11, SPI-12, SPI-13, SP1-14, C63PI, CS54_island and "not named". Whole genome analysis of a *S. enterica* serovar Typhimurium isolate (ms202) from a patient in India possessed six *Salmonella* pathogenicity islands: SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, and SPI-11 (Mohakud et al., 2022), but in our work, we did not identify SPI-4. The genes identified in SPI regions had homology to known transporters, drug targets, and antibiotic resistance genes and in a subset of genomic islands, genes that facilitate the horizontal transfer of genes encoding numerous resistance and virulence factors of regions belonging to type III secretion systems (T3SS). Vilela et al. (2022) analyzed the WGS of six *S.* Choleraesuis strains provided by the Brazilian *Salmonella* reference laboratory of the Oswaldo Cruz Foundation (FIOCRUZ-RJ), which receives *Salmonella* isolates from diverse isolation sources and regions of the country. Pathogenicity islands SPI-1, -2, -3, -4, -5, -9, -13, -14 and CS54 island were detected in five strains and SPI-11 in four strains. Similar to our study almost all these SPI with the exception of SPI 4 and SPI 11 were detected in our study. SPIs are common in *Salmonella* isolates and are mainly composed of genes associated with virulence functions. SPI-1 and SPI-2 are involved in the invasion of intestinal epithelial cells and survival and replication within phagocytic cells, respectively, through the formation of type 3 secretion systems. SPI-5 is related to fluid secretion and inflammatory response. Lastly, SPI-3, SPI-4, SPI-11, SPI-13, SPI-14, and CS54 are associated with *Salmonella* survival and adaptation to stresses within macrophages (Seribelli et al. 2021).

In total, 144 potential virulence genes were identified using VFanalyser/VFDB, some of the most important identified were *invA, sipA, sipB, sipC, fepA, SopA, SopB, SopD, SopE2, pefA, pefB, pefC, pefD* and *ssaB* was detected in *Salmonella* SE3. Some of these virulence genes are also found in other serovars of *Salmonella.* Borah et al. (2022) investigated the prevalence, antimicrobial susceptibility, antimicrobial resistance and virulence genes of *Salmonella* isolates recovered from humans and different species of animals. Out of 1231 samples, 88 (7.15%) *Salmonella* isolates were obtained, among

which 21 (23.86%) belonged to *Salmonella enterica* subsp. *enterica* serovar Weltevreden, 22 (25%) to *S.* Enteritidis, 16 (18.2%) to *S.* Typhi and 14 (15.9%) to S. Newport; 7 (7.95%) isolates were untypable. Among the 88 isolates, some virulence genes as *invA*, *sipA*, *sipB* and *sipC* were detected irrespective of the serovar, whereas the *fepA* gene was present in 64.77% of the isolates belonging to serovars *S.* Enteritidis, *S.* Weltervreden, *S.* Typhi, *S.* Newport, *S.* Litchfield, *S.* Idikan and *S.* Typhimurium. Virulence genes were present in varying percentages among the *Salmonella* serovars studied as *sopB* (86.36%), *sopE2* (62.5%), *pefA* (79.54%) and *sefC* (51.14%). However, in our study, the gene *sefC* was not detected in *Salmonella* SE3.

The virulence genes identified are involved in several different processes, such as the *invA* gene usually codes for a protein in the inner bacterial membrane that is responsible for the invasion of intestinal cells of the host (Sharma et al. 2016; El-Sebay et al. 2017). The *fepA* gene encodes outer membrane receptor protein FepA, which participates in iron transport and plays a role in infection colonization in *Salmonella* (Zhang et al. 2020). T3SS-1 secretes proteins, termed effectors, across the inner and outer membranes of the bacterial cell. Some of the secreted effectors, including SipA (SspA), SipB (SspB), SipC (SspC) are encoded by genes located on SPI1 at centisome 63 of the bacterial chromosome. The remaining effectors, including SopA, SopB (SigD), SopD, SopE and SopE2 are encoded by genes that are scattered around the *S.* enterica serotype Typhimurium chromosome. Upon secretion from the bacterial cell, the SipB, SipC, and SipD proteins are thought to form a complex in the eukaryotic membrane that is required for translocation of the remaining effectors into the host cell cytoplasm (Raffatellu, 2005).The pefA gene which encoded the serotype associated plasmid (SAP) mediated fimbrial major subunit antigen of Salmonella enterica serotype Typhimurium (Woodward et al.1996). Plasmid-encoded fimbriae (Pef) expressed by Salmonella typhimurium mediate adhesion to mouse intestinal epithelium (Nicholson et al. 2000). The secretion system apparatus (SsaB) encoded by SPI-2 in *S. enterica* Typhimurium ms202as (Mohakud et al. 2022).

The gene *arsC,* encoding arsenate reductase, was found in the genome of *Salmonella* SE3. Arsenate reductase is essential for arsenate resistance and transforms arsenate into arsenite, which is extruded from the cell (Jackson and Dugas, 2003, Pei et al., 2021). This is of interest as *Salmonella* SE3 was isolated from the soil of Subaé River where heavy metal concentrations were above reference values (BRASIL, 2003;

de Andrade and Santos, 2013). In addition, mussels (*Mytella charruana*) gathered from the same region also contained lead, arsenic and cadmium in concentrations above reference values (BRASIL, 2003; de Andrade and Santos, 2013). Carvalho et al. (2018) also determined the quality of soils in 39 households from nearby Santo Amaro City, and the Residential Investigation Value (RIV) was exceeded by Lead (23.1% of the samples), Cadmium (7.7%), Nickel (2.6%), Zinc (25.6%), Arsenic (2.6%), and Antimony (7.7%). Li et al. (2021), studied examined the genes detected in the outbreak and non-outbreak *Salmonella* spp. by analyzing the data from the National Centre for Biotechnology Information (NCBI) Pathogen Detection Isolates Browser database. In this study also reported the *arsC* gene in *Salmonella* spp. besides others genes not identified in our study, as iron operon (e.g., genes *iroB* and *iroC*), the ars operon (e.g., genes *arsA, arsB, arsC,* and *arsR*), the pco operon (e.g., genes *pcoA, prcoB, pcoC, pcoD, pcoE,* and *pcoR*), and the sil operon (e.g., genes silA, silB, silC, silE, silF, silP, silR, and silS).

Several viral defence systems were detected in *Salmonella* SE3, including CRISPR-Cas type IE, CBASS type I, and RM type I and III systems. Similar antiviral systems and subtypes were identified by PADLOC and DefenseFinder tools, except for AbiU and RM type II only identified using PADLOC. So, Salmonella SE3 were in contact with several plasmids and phages, as a result it had to develop different defense systems. Besides, PADLOC identified the AbiU system, but DefenseFinder could not detect it.

Seven prophages were detected in the *Salmonella* SE3 genome, two were intact, and five were incomplete. By comparison, in *S. enterica* Typhimurium ms202 nine prophages were detected, two were intact, five were incomplete and two were questionable (Mohakud *et al*., 2022). Moreover, *Salmonella* SE3 had not only *Salmonella* prophage sequences (*Salmonella* phage RE-2010) but also prophages annotated as belonging to closely related genera *Shigella* (phage POCJ13) and

Escherichia (phage 500465-2), which may indicate horizontal gene transfer or polyvalent phages. A previous study has reported that phage populations in *S. enterica* contribute to horizontal gene transfer, including virulence and virulence-related genes within the subspecies (Hardt *et al*., 1998, Figueroa-Bossi *et al*., 2001, Switt *et al.,* 2015 and Worley *et al*., 2018). Further studies on *Salmonella* may uncover the receptor interaction mechanisms between phages and hosts that may lead to improving phage therapy as an option for the treatment or control of *Salmonella*.

5. Conclusions

Salmonellosis is a health care problem around the world, so genomic analysis of *Salmonella* isolates could be a key determinant for better control of salmonellosis. Our study showed the effectiveness of a hybrid sequence assembly approach for environmental *Salmonella* genome analysis using HiSeq and MinION data. *Salmonella* SE3 was determined to belong to a monophyletic clade containing 23 *S. enterica* strains of serovar Enteritidis. The hybrid genome assembly enabled mobile genetic elements, genomic islands, *Salmonella* Pathogenicity Islands, antiviral systems, antimicrobial resistance genes, virulence genes, and prophages to be identified in *Salmonella* SE3. Furthermore, a gene encoding heavy metal resistance, arsC, was detected. These data are important to inform the control of *Salmonella* and heavy metal pollution in the Santo Amaro region of Brazil.

6. References

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Capítulo 5

Isolation and genomic characterization of the polyvalent phage SF1 using MinION and Illumina platform

Isolation and genomic characterization of the polyvalent phage SF1 using MinION and Illumina platform

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Abstract

Background: Antimicrobial resistance is increasing despite new treatments being employed, so novel strategies are required to ensure that bacterial infections remain treatable. Bacteriophages (phages; bacteria viruses), have potential to be used as natural antimicrobial methods to control bacterial pathogens such as *Salmonella* spp.

Methods: In this study, a *Salmonella* bacteriophage, SF1, was isolated and characterized with respect to host range by plaque formation and morphology by transmission electron microscopy. Furthermore, the bacteriophage genome was sequenced by Oxford Nanopore Technologies (ONT) MinION and Illumina HiSeq platforms and assembled by Racon (MinION) and Unicycler (Illumina, Illumina+MinION). The genomes were annotated and analyzed, and their genomes compared with reference *Salmonella* bacteriophages CLC Genomics v. 9.5.3 (Illumina).

Results: Regarding to Quast statistics and genome annotation no hybrid assembly, MiION assembly showed the best results. Besides, no lysogenic cycle, antimicrobial resistance and virulence genes were identified in our work. SF1 bacteriophage showed activity against twenty seven strains: *Salmonella* var. Enteritidis, *Salmonella* var. Typhimurium, *Salmonella* var. Minnesota, *Shigella flexneri*, *Escherichia coli*, *Escherichia cloacae*, *Escherichia fergusonii*, *Citrobacter europeus*, *Citrobacter freundii*, *Corynebacterium pseudotuberculosis*, *Corynebacterium striatum*, *Glutamicibacter creatinolíticus*, *Klebsiella oxytoca*, *Listeria monocytogenes* and *Rodococos iaqui*. SF1 bacteriophage was effective against nine species, it is a polyvalent bacteriophage. Several proteins was identified in SF1 bacteriophage: Terminase, Major caps, receptor b, tail tube, DNA polymerase, DNA ligase, protein A1, putative proteins and several hypothetical proteins was detected.

Conclusions: MinION platform showed the best assembly for SF1 bacteriophage. The host range of SF1 bacteriophage was evaluated using sixty five strains, which showed

activity against twenty seven strains. SF1 bacteriophage showed to be a polyvalent bacteriophage. Two receptors were identified: receptor b and tail tube protein.

Key words: *Salmonella* spp., bacteriophages, whole genome sequence, polyvalent bacteriophage

1. Introduction

Diarrheal disease is the second leading cause of death in children under five years old, and was responsible for the deaths of 370,000 children in 2019 (https://www.who.int/health-topics/diarrhoea#tab=tab_1). *Salmonella enterica* is one of the main pathogens associated with food contamination; it is considered responsible for around 94 million cases of gastrointestinal illnesses and 155,000 annual deaths worldwide (Alby & Nachamkin, 2016; Eng et al., 2015; Mafi & Orenstein, 2020; Hernández-Díaz et al., 2022). In Brazil, during the period 2000-2011, *Salmonella* spp. were reported as the major cause of food-borne illness (42.27%), followed by *Staphylococcus aureus* (20.34%) and *Escherichia coli* (10.46%) (Tondo & Ritter, 2012).

The first surveillance data on resistance to antibiotics released by the WHO revealed high-levels of resistance in bacterial infections in high- and low-income countries (WHO, 2018). According to the Global Antimicrobial Surveillance System (GLASS, 2016-2017), there was a widespread occurrence of antibiotic resistance among 500,000 people tested for bacterial infection in 22 countries. The most commonly reported resistant bacteria were *E. coli, Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae,* followed by *Salmonella* spp. Antibiotic resistant infections are also associated with greater morbidity and mortality which increase healthcare costs. In low-income countries, affordability of second line drugs and reduced access to healthcare can restrict the use of newer broad-spectrum antibiotics, and as a result, increase of morbidity and mortality from antibiotic resistant infections in these countries (Bryce et al., 2016).

The increasing levels of antibiotic resistance in many bacterial infections have renewed interest in the exploitation of bacteriophages as therapeutic and biocontrol agents and in the study of the molecular mechanisms underpinning productive infection

(Jassim & Limoges, 2014; Mahony et al., 2011; De Smet et al., 2017; Tuner et al., 2021). Similarly, our understanding that prophages can influence the fitness, phenotype, and global metabolism of the host lysogen necessitates careful identification and genomic characterization of bacteriophages. In comparison to the Illumina sequencing platform, there are relatively few reports of bacteriophages sequenced solely by ONT or PacBio sequencing (Turner et al., 2021). We are not aware of any studies that have used hybrid genome assembly for *Salmonella* bacteriophages.

The host range is an important property to determine for bacteriophages, particularly if they are to be used for biocontrol. Some studies have reported polyvalent *Salmonella* bacteriophages, which were able to infect multiple genera. For example, WHR8 bacteriophage inefected *Escherichia coli* and *S. enterica* (Bielke *et al*., 2007) or Phage S144 infected *Enterobacter cloacae* and *Cronobacter sakazakii* (Gambino et al. 2020).

Considering the great potential of bacteriophages as antimicrobial agents in *Salmonella* biocontrol, this study was undertaken to isolate and characterize bacteriophages against a wide spectrum of *S. enterica* serovars isolated from the Subaé River in Santo Amaro, Salvador de Bahia-Brazil. Host range studies and whole genome sequencing was then used to characterize the bacteriophages identified.

2. Materials and methods

Environmental water samples were obtained from the Subaé river basin, Salvador de Bahia, Brazil. The basin has environmental impacts in its main watercourses arising from dumping of domestic, industrial effluents, agricultural and anthropological activities (Santana, 2013). Three samples of water (approximately 100 ml) were collected from the Subaé River, Santo Amaro city, Salvador de Bahia, Brazil (12°31'46.77"S 38°44'1.24"W). The samples were transported in a refrigerated box (4–8°C) to the laboratory where the analyses were done immediately.

2.1 Isolation and purification of *Salmonella* bacteriophages

In order to isolate DNA, 1 mL of water sample were added to 10 mL of bacteriophage buffer (10 mM Tris HCl (pH 7.5), 68.5 mM NaCl , 10 mM MgSO4 and 5mM $CaCl_2$), were homogenized and incubated for 10 min at room temperature, after that suspensions were filtered (0.22 µm membrane - Kasvi). For enrichment, 2.5 mL of

the filtered sample and 2.5 mL of log phase *Salmonella* Typhimurium ATCC 14028 and 10 mL of LB culture media (Kasvi) were mixed and incubated for 18 h at 37°C. Samples were centrifuged at 3500 rpm for 10 min and the supernatant filtered (0.22 µm membrane). 100 µL of filtrate, 100 µL of log phase *Salmonella* Typhimurium ATCC 14028 and 10 mM of $CaCl_2$ (Dinámica) were mixed and incubated for 10 min, and added to a LB soft-agar overlay plate, then incubated overnight at 37°C. A single clear plaque was selected and propagated on the host five times to ensure virulence and purity. 5 mL of bacteriophage buffer was added to each of 5 confluent lytic bacteriophage plates, centrifuged at 3500 rpm for 10 min and filtered (0.22 µm membrane). The filtered suspensions were ultra-centrifuged at 100,000 x *g* for 1.5 h and the supernatant discarded, 500 µL of SM buffer (50mM Tris–HCl (pH 7.5), 100mM NaCl, 8.1mM $MgSO_4$ and 0.01% (w/v) gelatin) was added to pellet and stored at 4°C (Raya & Piuri, 2017).

2.2 Preparation of high titer bacteriophage stocks

Purified bacteriophages were diluted serially in SM buffer (200 mM $NaCl_2$, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5 and 15% glycerol) to give a concentration that would provide confluent lysis of the host in a soft-agar overlay plate. Dilution plates with almost confluent lysis were chosen. To recover bacteriophages SM (5 mL) was added to each plate and left at room temperature for at least 1h, and the plates swirled regularly. The liquid was decanted, and contents were vortex-mixed before shaking for 30 min, and the overlay removed by centrifugation at 11 000 g for 10 min. 5.8% NaCl were added and incubated at 37 °C for 1h (Carey-Smith et al., 2006). Then, 10% PEG 6000 overnight at 4°C, the supernant removed by centrifugation at 15 000 g for 1 hour, the pellet was suspended in LB. The titer of the stock was determined by the overlay method.

2.3 Efficiency of plating

SF1 phage displayed the widest bactericide host range in the spot assays were selected for a more thorough assessment of productive infection as defined by the efficiency of plating (EOP). Each phage was tested three times for each of four different

dilutions against all the bacterial strains that it had been shown to be able to lyse in the spot assays. This was done under the same conditions as in the spot assays, i.e. using stationary phase bacteria. Besides, *Salmonella* ATCC 14028 and *Citrobacter freundii* strain I to be tested were grown overnight (18 hours) at 30°C and 100 µl of each of those cultures was used in double layer plaque assays together with 100 µl of diluted phage lysate. SF1 phage phage lysates was 10^{2}–10^{12} times dilutions from the phage stock. This means that EOP assay replicates for a particular phage were done in parallel on all bacterial strains tested, and also at concentrations comparable to what was used in the spot tests. The plates were incubated_overnight at 30°C and the number of plaque forming units (PFU) was counted for each combination. When the 10^{6} dilution did not result in any plaques, a lower dilution was tried afterwards to verify that the EOP was lower than 0.001. Finally, the EOP was calculated (average PFU on target bacteria/average PFU on host bacteria) along with the standard deviation for the three measurements.

The average EOP value for a particular phage bacterium combination was classified as "High production" when the ratio was 0.5 or more, i.e. when the productive infection on the target bacterium resulted in at least 50% of the PFU found for the primary host. An EOP of 0.1 or better, but below 0.5, was considered to be of "Medium production" efficiency, and between 0.001 and 0.1 as "Low production" efficiency. An EOP equal to or under 0.001 was classified as inefficient (Khan et al. 2012).

2.4 Bacteriophage host range determination

The host range of the isolated bacteriophages was determined by challenging them against sixty-five strains of bacteria (Table 6). Exponential growth phase suspensions of host strains were prepared, everyone was adjusted at DO=0.7 CFU/mL. Overlays were inoculated with 100 µL host and poured on a LB agar base plate previously marked in a grid to allow identification of each inoculum (Carey-Smith, 2006), after the solidification of soft agar, a 5 µl drop of bacteriophage (approximately 10^{9} PFU/ml) suspension was placed on double-layered agar LB plates containing each individual host strain as described by Raya & Piuri (2017).

2.5 DNA isolation

For DNA isolation, 2µl of 1 mg/mL DNAse (Sigma-Aldrich, St. Louis, USA) and 20 µl of 1 mg/mL RNAse (ThermoFisher Scientific, Waltham, USA) were added to 2ml of filtered culture supernatant for 30 min at 37 °C. Then, 40µl of 2 M $ZnCl_2$ were added, then the suspensions were incubated for 5 min at 37 °C, centrifuged at 12 000 *g* for 1 min. The supernatant was discarded, 1 ml of TES (0,1 M Tris-HCl pH 8; 0,1 M EDTA, SDS 0,3 %) was added to the pellet, incubated for 15 min at 60 °C. 40 µl of 20 mg/mL proteinase K (ThermoFisher Scientific, Waltham, USA) were added incubated for 90 min at 37 °C. Subsequently, 1.5ml of Ethanol and 6M Guanidine was added to 1ml of sample. Then, QIAamp MinElute Virus Kits (Qiagen, Hilden, Germany), columns was used for DNA isolation and purification.

2.6 Preparation of Libraries for Complete Genome Sequencing (WGS) with MinION

Nanopore sequencing was carried out at the Molecular and Computational Biology of Fungi Laboratory, Federal University of Minas Gerais (UFMG). The DNA library was prepared with a rapid sequencing kit (SQKRAD004, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. Libraries were sequenced with qualified FLO-MIN106 flow cells (R9.4.1, active pores number > 800) for 2 h on a GridION (Oxford Nanopore Technologies, Oxford, UK).

The quality of the sequencing was verified through the FastQC v0.11.9 program (https://github.com/s-andrews/FastQC). The Porechop v0.2.4 program (Wick et al., 2017) was used for the detection and elimination of the adapters, as well as for the demultiplexing of the Nanopore reads. Possible sequencing errors were treated with the Canu v2.1.1 monitor correction module (Koren et al., 2017). The de novo montage, based on de Bruijn graphs, of the corrected readings was carried out through the Flye v2.8.3 montage (Kolmogorov et al., 2019). However, the contigs obtained in the de novo montage were subjected to a polishing (correction of raw contigs) with the Racon v1.4.22 program (Vaser et al., 2017), which previously took the reads mappings made with BWA v0 .7.17 (Li, 2016).

2.7 Illumina sequencing

The sequencing library was prepared using 1 µg purified bacteriophage DNA with the NEBNext Fast DNA Fragmentation and Library Preparation Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's recommendations. The library quality was assessed using the Agilent 2100 Bioanalyzer equipment, and the paired-end DNA sequencing was carried out in the Illumina HiSeq 2500 platform. After sequencing, the raw reads quality was assessed using the FastQC v0.11.5 software (https://github.com/s-andrews/FastQC, accessed on 15 January 2020).

De Novo Genome Assembly and Assessment

The genome assembly was carried out using different approaches and software. MinION long-reads were assembled using Racon pipeline with default parameters (Vaser *et al*., 2017) while Illumina short reads were assembled using SPAdes version 3.15.3 (Shen, A., & Millard., 2021). Hybrid assemblies using Illumina and MinION reads were performed using the software Unicycler (Wick *et al*., 2017). Genome quality and completeness for each assembly were evaluated using QUAST v4.6.0 (Gurevich *et al*., 2013).

2.8 Genome annotation

The automated annotation of genes for both the bacterial and plasmid genomes was performed via Prokka v1.14.6 (Seemann, 2014). From these predictions, a manual selection was made for the genes of interest. Genomic comparison of the SF1 bacteriophage genome was carried out using the reference of *Salmonella* bacteriophage s131 (NC_048009.1) using Easyfig (Sullivan et al. 2011).

2.9 Genomic analysis

Completeness of bacteriophage genome sequences were tested using checkV (Nayfach, et al., 2021). Preliminary identification of closest relatives of SF1 bacteriophage was using PhageClouds (http://phagecompass.dk/). PhageLeads was used to predict therapeutic bacteriophage suitability (http://phagecompass.dk/). Abricate was used to identify antimicrobial resistance or virulence genes (Yukgehnaish et al., 2022).

2.10 Electron microscopy

To produce bacteriophages for electron microscopy, high titre stocks were prepared in LB media. Bacteriophage samples were placed on electron microscopy

(EM) grids and stained with 1% uranyl formate or 1% potassium phosphotungstate, and micrographs taken at a magnification of 135.000. Samples on EM grids were stained with 1% uranyl formate or 1% potassium phosphotungstate and micrographs taken at a magnification of 135,000 X at 120 kV using a Tecnai G2-12-FEI Spirit Biotwin EM instrument (Carey-Smith et al., 2006).

3. Results

3.1 Isolation of *Salmonella* bacteriophage SF1

Samples of water of Subaé River, Santo Amaro Brazil were tested for the presence of *Salmonella* bacteriophages. To attempt the isolation of these bacteriophages, cultures of *Salmonella* var. Typhimurium ATCC 14028 were inoculated into Subaé River water from the Santo Amaro region of Brazil and tested for the presence of plaques using the double-agar layer technique (see Section 5.3.1). After the subsequent isolation, purification of many bacteriophages, one bacteriophage, showed consistently clear plaques and was named SF1 (Figure 1). The titer of the bacteriophage stock solution was approximately 3.2 x 10^9 PFU/ml. The multiplicity of infection (MOI) was determined to be 60.

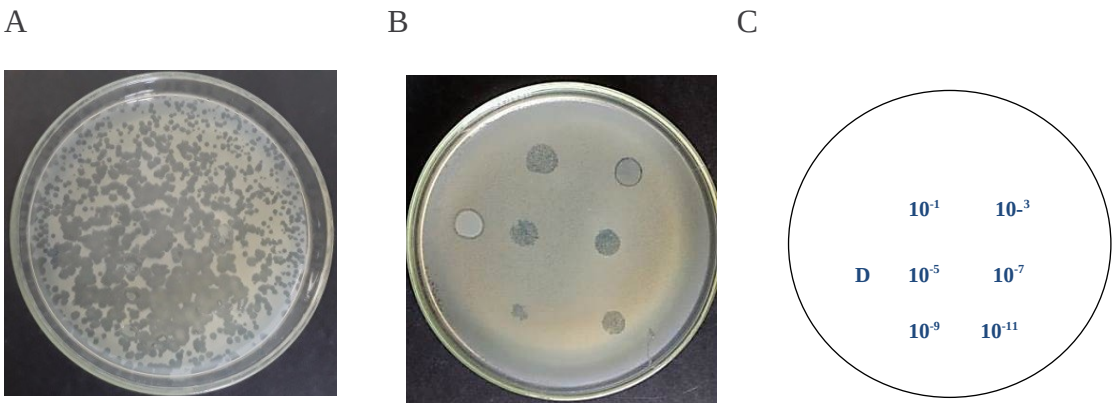

Figure 1. Plaques of Bacteriophage SF1 on the lawn of *Salmonella* Typhimurium ATCC 14028: A) Titer 10^{-4}. B) and C) Spot test using different titters: D (direct or 10^9), 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9}, and 10^{-11}.

3.2 Electron microscopy

Structural analysis of bacteriophage SF1 was performed using Transmission Electronic Microscopy (TEM). The dimensions of bacteriophage SF1 were: capsid length 159.38 nm, capsid width 155.15 nm and tail length 265.51 nm (Table 1 and Figure 2). The morphology of bacteriophage SF1 corresponded to the *Siphoviridae* family, *T5virus* genus (Gencay et al. 2019).

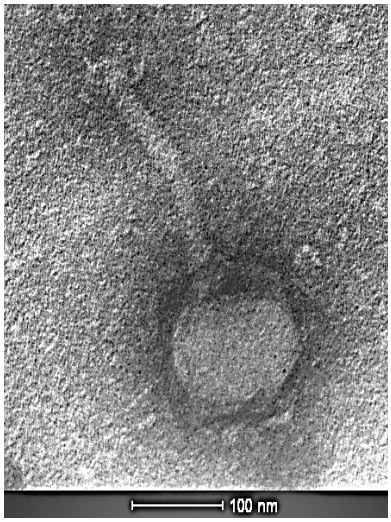

Figure 2. Electron micrograph of *Salmonella* bacteriophage SF1.

3.3 Whole genome sequencing of bacteriophage SF1

The Salmonella bacteriophage, designated bacteriophage SF1, was analyzed by whole genome sequencing. After preprocessing the number of reads using HiSeq was 40,000, and the number of reads MinION was 4,000. The mean coverage for HiSeq was 100 bp, and for MinION was 62 bp. Respect to the Illumina Miseq data the read number was 2,829,936 bp and for MinION was 22,541 bp.

3.4 Genome assembly

Three genome assembly methods were tested: non bybrid HiSeq Illumina (SPAdes assembly) and MinION sequencing and hybrid (Hiseq Illumina + MinION Unicycler assembly) Regarding to QUAST (tools for genome assemblies evaluation and comparison) analysis, MinION (Racon assembly) sequencing technology, Racon assembly showed the best parameters, 1 contigs, total length of 112 042 bp, largest contigs of 112 042 bp and N50 of 112 042 bp including the assembly of the reference (Salmonella bacteriophage s131) Table 1 and Figure 3.

According to the BLAST (Basic Local Alignment Search Tool analysis https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis, 92 % of query cover, 0 E-value was reported for SF1 bacteriophage using the three assemblies, 94.48% of identity using

MinION, 96.37% of identity using Hiseq assembly, 96.36% of identity using Hybrid assembly to the same bacteriophage, *Salmonella* bacteriophage S131 (NC_048009.1).

Table 1. Summary statistics for the assembled genome of SF1 using reads from Illumina HiSeq and Oxford Nanopore MinION.

Assembly method	Reference	Racon	Unicycler	Unicycler
Sequence data	**(NC_048009.1)**	**MinION**	**HiSeq**	**Hybrid**
Number of contigs	1	1	1	1
Number of contigs (≥ 0 bp)	1	1	112	1
Number of contigs (≥ 50 000 bp)	1	1	1	1
Largest contigs	110 091	112 042	110 012	110 012
Total length (≥50 000 bp)	110 091	112 042	110 012	110 012
GC (%)	39.22	39.15	39.17	39.17
N50	11091	112 042	110 012	110 012
L50	1	1	1	1

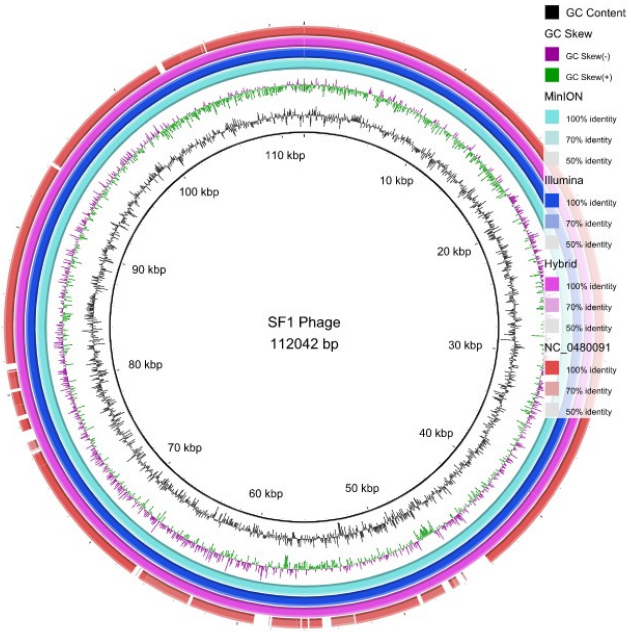

Figure 3. Comparison of genome assemblies for SF1

Comparing two non-hybrid (MinION/ Racon, and HiSeq/Unicycler) and one hybrid (MinION +HiSeq/Unicycler) assembly of SF1 bacteriophage with the reference genome *Salmonella* bacteriophage s131.

3.5 Genome annotation

Annotation of the three SF1 bacteriophage genome assemblies and the reference genome were undertaken using Prokka with the Caudovirales database (Table 2). No hybrid, MinION assembly showed the highest number of CoDing Sequence (CDS) (170) and the reference assembly showed the highest number of tRNA (23).

Table 2. Prokka data analyses using Caudovirales database

Values	Reference (NC_048009.1)	No hybrid MinION	No hybrid Hiseq	Hybrid (Hiseq + MinION)
CDS	155	170	161	156
tRNA	23	21	21	21

As a result, bacteriophage SF1 genome assembly using no Hybrid, MinION was chosen to compare the genome with the reference genome, Figure 3. Terminase, Major caps, receptor b, tail tube, DNA polymerase, DNA ligase, protein A1, putative proteins and several hypothetical proteins were detected Figure 4.

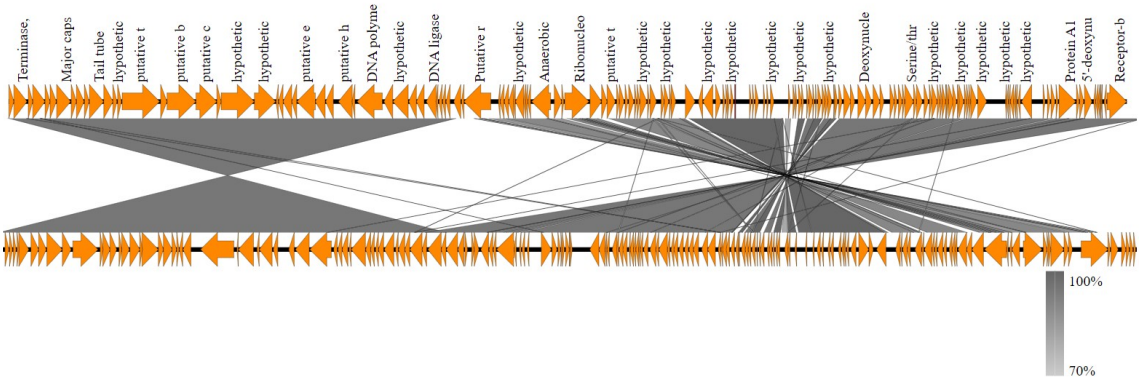

Figure 4. Genome comparison of bacteriophage SF1using Illumina assembly and Salmonella bacteriophage S131 (the reference genome, NC_048009.1).

3.6 Genomic analysis

a) In order to determine genome completeness, checkv software was carried out, the three assemblies showed high quality, lower completeness of 88.16% and upper completeness of 100%, no host contamination were detected, and 137 genes.

b) According genome sequencing using PhageClouds, Phage_NBSal003 from 130 genomes, top mach with bacteriophage_NBSal003, distance of 0.037562.

c) Regarding to PhageLeads analysis SF1 bacteriophage could not find any predicted temperate lifestyle genes. Besides, could not find any antimicrobial resistance or virulence in card.

Besides, the TEM classification marched with genomic classification, the bacteriophage SF1 as Salmonella phage S131 (NC_048009.1).

3.7 Host range activity

To identify the host range specificity of SF1 bacteriophage, sixty five bacterial strains were tested for infectivity using the spot test (Table 4). SF1 bacteriophage showed antimicrobial activity against twenty seven strains: *Salmonella* var. Enteritidis, *Salmonella* var. Typhimurium, *Salmonella* var. Minnesota, *Shigella flexneri*, *Escherichia coli*, *Escherichia cloacae*, *Escherichia fergusonii, Citrobacter europeus*, *Citrobacter freundii*, *Corynebacterium pseudotuberculosis*, *Corynebacterium striatum*, *Glutamicibacter creatinolíticus*, *Klebsiella oxytoca*, *Listeria monocytogenes* and *Rhodococcus equi,* Table 3. SF1 bacteriophage was effective against three serovar of *Salmonella:* var. Enteritidis, var. Typhimurium and var. Minnesota. It also was effective against nine genera, so it can be considered a polyvalent bacteriophage.

Table 3. Host range activity of Bacteriophage SF1 against several bacteria

No	Microorganismos	Antimicrobial activity
1	*Bacillus subtilis* strain I	-
2	*Citrobacter europeus*	+
3	*Citrobacter freundii* ATCC8040	+
4	*Citrobacter freundii* strain I	-
5	*Citrobacter freundii* strain II	+
6	*Citrobacter freundii* strain III	+
7	*Corynebacterium lactis* strain 2447	+
8	*Corynebacterium pseudotuberculosis* strain 1002	-
9	*Corynebacterium pseudotuberculosis* strain 258	-
10	*Corynebacterium pseudotuberculosis* strain 31	-
11	*Corynebacterium pseudotuberculosis* strain 316	-
12	*Corynebacterium pseudotuberculosis* strain BD57	-
13	*Corynebacterium pseudotuberculosis* strain Cp13	-
14	*Corynebacterium pseudotuberculosis* strain N1	+
15	*Corynebacterium pseudotuberculosis* strain T1	-
16	*Corynebacterium striatum* strain 1961	+
17	*Corynebacterium ulcerans* strain 809	-
18	*Dietiza* sp. Strain I	-
19	*Escherichia cloacae* ATCC 23355	+
20	*Escherichia coli*	-
21	*Escherichia coli* ATCC 2592	+
22	*Escherichia coli* strain EC2	+
23	*Escherichia coli* strain EC3	+

24	*Escherichia coli* strain EC7	-
25	*Escherichia coli* strain EC8	-
26	*Escherichia fergusonii*	+
27	*Glutamicibacter creatinolíticus* strain LGCM259 (NCBI)	+
28	*Klebsiella oxytoca* ATCC 49131	+
29	*Listeria monocytogenes* strain I	+
30	*Listeria scotia*	-
31	*Rhodococcus iaqui* strain 1	+
32	*Salmonella enterica* var Typhi strain Ia	+
33	*Salmonella enterica* var. Enteritidis ATCC 13076	+
34	*Salmonella enterica* var. Enteritidis strain SE3	+
35	*Salmonella enterica* var. Enteritidis strain SE4	+
36	*Salmonella enterica* var. Heidelberg strain SH1	-
37	*Salmonella enterica* var. Heidelberg strain SH10	-
38	*Salmonella enterica* var. Heidelberg strain SH2	-
39	*Salmonella enterica* var. Heidelberg strain SH3	-
40	*Salmonella enterica* var. Heidelberg strain SH4	-
41	*Salmonella enterica* var. Heidelberg strain SH5	-
42	*Salmonella enterica* var. Heidelberg strain SH6	-
43	*Salmonella enterica* var. Heidelberg strain SH7	-
44	*Salmonella enterica* var. Heidelberg strain SH8	-
45	*Salmonella enterica* var. Heidelberg strain SH9	-
46	*Salmonella enterica* var. Minnesota strain SM1	+
47	*Salmonella enterica* var. Minnesota strain SM10	+
48	*Salmonella enterica* var. Minnesota strain SM2	-
49	*Salmonella enterica* var. Minnesota strain SM3	-
50	*Salmonella enterica* var. Minnesota strain SM4	-
51	*Salmonella enterica* var. Minnesota strain SM5	-
52	*Salmonella enterica* var. Minnesota strain SM6	-
53	*Salmonella enterica* var. Minnesota strain SM7	-
54	*Salmonella enterica* var. Minnesota strain SM8	-
55	*Salmonella enterica* var. Minnesota strain SM9	-
56	*Salmonella enterica* var. Typhi strain I	-
57	*Salmonella enterica* var. Typhi strain II	-
58	*Salmonella enterica* var. Typhi strain III	-
59	*Salmonella enterica* var. Typhi strain IV	-
60	*Salmonella enterica* var. Typhimurium ATCC 14028	+
61	*Salmonella enterica* var. Typhimurium ATCC 14088	+
62	*Salmonella enterica* var. Typhimurium strain II	+
63	*Salmonella enterica* var. Typhimurium strain III	+
64	*Salmonella enterica* var. Typhimurium strain IV	-
65	*Shigella flexneri* Castellani and Chalmers 12022	+

4. Discussion

A *Salmonella* bacteriophage, SF1, was isolated from water of the Subá Riber in Santo Amaro, Brazil. The phage produced clear plaques on double-agar overlay plates which indicates that the phage is a virulent phage. Visualization of the structure by TEM indicated SF1 was a member of the *Siphoviridae* family, *T5virus* genus.

In this study no hybrid and hybrid assembly was used for Bacteriophage SF1 genome assembly, however according the Quast analysis the best assembly was MinION, Racon assembly showed the best parameters, it is related with the prokka annotation, where the highest number of CDS was detected in No hybrid, MinION assembly, as a result the quality of the MinION assembly was the best for Bacteriophage SF1 assembly.

Similar to our study, Llanos et al. (2019) identified the *Salmonella* bacteriophage S131, genome length was 119,416 bp, 170 genes, 18 tRNA and GC of 39.6, using MiSeq Illumina platform, (SPAdes assembler). In our study MinION assembly showed the best quality and quantitative parameters for assembly of SF1 bacteriophage.

Some estudies reported that using different technologies as Illumina, Oxford Nanopore Technology (ONT), or PacBio sequencing, as well as our study (Illumina and ONT), it is recommend aiming for between 25 and 100 × coverage. Hundred or thousand-fold over-coverage will generally not improve assembly, is unnecessarily expensive, and may result in assembly errors (Rihtman et al., 2016; Pightling et al., 2014; Desai et al., 2013; Wang,et al. 2013 and Turner et al., 2021).

In comparison to Illumina, there are relatively few reports of bacteriophages sequenced solely by ONT or PacBio sequencing. However both ONT and PacBio could be applied for the detection of modified nucleotides or for bacteriophages shown to be refractory to conventional sequencing approaches (Lu et al., 2014; Tuner et al., 2021). Our study showed that the best result between ONT, Illumina and hybrid sequencing was ONT for bacteriophage SF1. Although no major differences were detected between four assemblies and the reference *Salmonella bacteriophage* s131. However, it could be possible that the pipeline used for hybrid assembly in this study needs to be improved because we adapt the pipeline of hybrid assembly used for bacteria and not for bacteriophages.

Most of the protein coding sequences identified in the SF1 genome were hypothetical proteins. Terminase, Major capsid, receptor b, tail tube, DNA polymerase, DNA ligase, protein A1, putative proteins and several hypothetical proteins was detected. Like our study, ES18 terminase was identified in *Salmonella* bacteriophage ES18, this protein can move substantial distances along the DNA between recognition and cleavage of DNA destined to be packaged. Replicated bacteriophage, DNA is recognized at a pac site by the bacteriophage terminase, a cut is made in the DNA at or near that point, and a processive series of packaging events proceeds (Casjens et al. 2005). Rivera et al. (2022), described *Salmonella* bacteriophage STGO-35-1 and like our study receptor-binding proteins and tail tube protein (receptor) were identified, along with structural protein like major capsid, DNA polymerase. Sattar et al. (2022), reported a novel *Salmonella* bacteriophage (SSBI34) and evaluated its therapeutic potential. Similar to our study, it encodes a DNA ligase, DNA polymerase I and III, indicating SSBI34′s independence from host polymerases for DNA replication. Moreover, protein A1 was detected in our study, which is involved in the degradation of host DNA and the shutoff of host genes and bacteriophage pre-early genes (https://www.uniprot.org/uniprotkb/Q6QGT3/entry). According to the genomic analysis, SF1 bacteriophage did not have a lysogenic cycle, and antimicrobial resistance and virulence genes were not detected, so Bacteriophage SF1 has a great potential to be used in phage therapy, biological control and it could be used as immunogenic agent in vaccines.

Bacteriophage SF1 was effective against nine species in a spot test assay, suggesting it may be a polyvalent bacteriophage. Interestingly SF1 bacteriophage is active against some bacteria outside of the Enterobacteriaceae family. Although more studies are required to better understand phage host range.

Use of the term lysis from without (LO) has similarly drifted from its original meaning. Lysis from within (LI) is normal bacterial lysis induced intracellularly by phage proteins. Use of the term lysis from without (LO) has similarly drifted from its original meaning. Lysis from within (LI) is normal bacterial lysis induced intracellularly by phage proteins. Alternatively, Mg2+ addition to media (25mM) can reduce at least *E. coli* susceptibility to LOv, (Abedon, 2011). Besides, in this work we used $CaCl_2$ (10 mM) form *Salmonella* ATCC 14028 and *Citrobacter freundii* strain I susceptibility.

“Lysis from without is caused by adsorption of phage above a threshold value. The cell contents are liberated by a distension and destruction of the cell wall. The adsorbed phage is not retrieved upon lysis. No new phage is formed.” Nonetheless this description, LOv is not always easily induced upon phage adsorption unless phage densities are high, cells are inhibited in their gene expression, or cell envelope stability otherwise is low. In addition, not all phages may be inherently capable of inducing lysis from without. If there is reason to suspect that LOv might be interfering with phage therapy or other experiments then at least *in vitro* testing should be performed (Abedon, 2011) As a result it is important to carry out EOP assay of SF1 phages that have antimicrobial effect on the other bacterias (Table 4).

Mahmoud *et al* (2018) reported that *Salmonella* bacteriophages Salmacey1, Salmacey2, and Salmacey3 had lytic effect on four *Salmonella* serovars *S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Typhi. Like our study, all these Salmacey1, Salmacey2, and Salmacey3 also infected one strain of *Citrobacter freundii*. Moreover, the two bacteriophages (Salmacey1, Salmacey2) had a lytic effect on *Enterobacter cloacae* and Salmacey3 was able to infect *E. coli* suggesting that these bacteriophages are polyvalent. Other studies isolated and characterized *Salmonella* bacteriophages that could infect strains of *E. coli* and *S. enterica* serovar Choleraesuis (Bielke *et al*., 2007). Gambino et al. 2020, characterize the novel polyvalent bacteriophage S144, a member of the *Loughboroughvirus* genus, and showed plaques on specific serovars of *Salmonella enterica* subsp. *enterica* and on *Cronobacter sakazakii*. Similar to our study it is a polyvalent bacteriophage and it was not previously reported in the literature. The distinct profiles of *Salmonella* susceptibility may be explained by the non-specific binding receptors on the bacterial host or different resistant mechanisms during bacteriophage infection (Bielke *et al*., 2007).

Kim et al. (2021), characterized KFS-EC3, a polyvalent and lytic bacteriophage, which was isolated from slaughterhouse sewage, can efficiently infect *E. coli* O157:H7, *Salmonella* spp., and *Shigella sonnei*. Compared to our study, we identified the bacteriophage infectivity for *Salmonella* var. Enteritidis, *Salmonella* var. Typhimurium, *Salmonella* var. Minnesota, *Escherichia coli*, and *Shigella flexneri.* Problably, it is the first time that bacteriophage for *Corynebacterium pseudotuberculosis*, *Corynebacterium striatum*, and *Glutamicibacter creatinolíticus* has been described. It is likely that the receptors in receptor b and tail tube protein are responsible for the broad host specificity

of SF1 bacteriophage. The study of these receptors and specificity of hosts could be interesting for the knowledge of bacteriophage-host interaction for the ecology and evolution and for the application of bacteriophages in the pharmacy industry. Besides, the Phylogenic analysis of SF1 bacteriophage could be important for future studies.

5. Conclusion

In summary, this study we isolated a phage from was isolated from water of the Subá Riber in Santo Amaro, Brazil.The MinION platform was better for the assembly of SF1 bacteriophage than Illumina Hiseq and hybrid (MinION + Illumina Hiseq). No lysogeny genes, antimicrobial resistance and virulence genes were identified in the SF1 bacteriophage genome, which indicate that it is safety and have a therapeutic potential SF1 bacteriophage was a polyvalent bacteriophage active against *Salmonella, Escherichia, Corynebacterium, Klebsiella, Listeria and Rhodococcus species*. SF1 bacteriophage has the potential to be an alternative treatment to antibiotics for control of *Salmonella* and other species in healthcare and agriculture.

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3. DISCUSSÃO INTEGRADA

Neste trabalho fizemos uma revisão dos bacteriófagos como alternativa aos antibióticos na área clinica. A resistência antimicrobiana está aumentando apesar de novos tratamentos serem empregados. Com uma diminuição na taxa de descoberta de novos antibióticos, isso ameaça levar a humanidade de volta a uma “era pré-antibiótica” de atendimento clínico. Os bacteriófagos (fagos) são uma das alternativas mais promissoras aos antibióticos para uso clínico. Os níveis crescentes de resistência a antibióticos a muitas infecções nosocomiais renovaram o interesse na exploração de bacteriófagos como agentes terapêuticos e de biocontrole e no estudo dos mecanismos moleculares subjacentes à infecção produtiva (JASSIM & LIMOGES, 2014; MAHONY et al., 2011; DE SMET et al., 2017; TUNER et al., 2021).

Embora mais de um século de terapia fágica principalmente ad-hoc tenha envolvido experimentação clínica substancial, a falta de padrões de orientação regulatória e execução eficaz de ensaios clínicos significa que a terapia para doenças bacterianas infecciosas ainda não foi amplamente adotada. No entanto, vários estudos de caso e ensaios clínicos recentes mostram-se promissores na abordagem dessas preocupações. Há um número crescente de ensaios clínicos de Fase I/II bem executados que descrevem a segurança e eficácia da terapia fágica. Há uma melhor compreensão da farmacologia, imunologia, segurança e potencial de resistência bacteriana. Tecnologias como engenharia genética, sequenciamento de genoma completo e metagenômica também fornecem novas ferramentas para aperfeiçoar estratégias terapêuticas de fagos. No entanto, ainda existem lacunas de dados sobre sua eficácia e falta de padronização e estruturas regulatórias adequadas que precisam ser resolvidas antes que a terapia fágica

possa ocupar seu lugar na medicina convencional. Dado o renovado interesse e ímpeto no campo da terapia fágica, há razões para estar otimista de que esses desafios podem ser superados nos próximos anos.

Nesse estudo, fizemos também à revisão sistemática e meta-análise para avaliar e melhorar a aplicação de tratamentos naturais contra patógenos de origem alimentar, como o biocontrole de fagos. A maioria dos depositantes de patentes para biocontrole em alimentos (73,18%) foram empresas privadas, e a minoria dos depositantes foram Universidades (26,82%). A Intralytix Inc. tem mais patentes do que qualquer outra empresa com 41% do total de patentes neste campo. Em contraste, as patentes para o biocontrole de fagos de patógenos de plantas foram registradas principalmente pela academia (56%), com uma minoria (37%) ligada à indústria (sem requerentes conjuntos) e 7% eram requerentes conjuntos (HOLTAPPELS et al., 2019).). A distribuição geográfica de ambos os documentos de patentes e artigos científicos sobre biocontrole de fagos em alimentos relatados neste trabalho foi principalmente (> 80%) na América do Norte e Europa, em comparação na Ásia e na América do Sul apenas com contribuições menores.

Ao examinar os métodos de aplicação de fagos nos artigos selecionados, coquetéis de fagos foram usados em 50% dos estudos de *Listeria* e 44,44% dos estudos de *Salmonella*. Para a maioria das aplicações, é provável que coquetéis de fagos sejam necessários para obter uma boa cobertura de todas as cepas, pois a maioria dos fagos é intrinsecamente estreita na faixa de hospedeiros (ROSS et al., 2016), no entanto, existem algumas exceções, como P100, que pode infectar ~ 95% de cepas de *L. monocytogenes* nos serovares 1/2 e 4 (GUENTHER et al., 2009).

A meta-análise de efeitos aleatórios foi realizada e revelou: (i) efeito antimicrobiano significativo dos fagos de *Listeria* em maçã, suco de maçã, pêra e suco de pêra (p-val = < 0,0001), (ii) efeito antimicrobiano significativo de fagos de *Salmonella* em ovos, maçã e frango pronto para cozinhar (p-val = 0,0001), (iii) nenhuma heterogeneidade (I2 = 0%, tau2 = 0) foi identificada na meta-análise, (iv) foi detectado viés de publicação em fagos de Listeria, mas não em fagos de Salmonella. (v) Os fagos ListShield e Felix01 apresentaram o melhor resultado para controle biológico de *Listeria* e *Salmonella*, respectivamente, (vi) concentração de fago e bactérias (p-val=1,05x105 e < 2x10-16), tempo (1,44x10-9) e o alimento (8,16x10-5) teve efeito

significativo no controle biológico de *Listeria*, (vii) a temperatura (p-val= 0,00825) e o tempo (p-val=0,00374) tiveram efeito significativo na atividade antimicrobiana dos fagos de Salmonella.

Alguns fagos são naturalmente resistentes a altas influências físico-químicas ambientais, como temperatura, pH, salinidade e desinfetantes, tornando-os potenciais agentes de biocontrole para uso no processamento de alimentos ou na fazenda para melhorar a segurança alimentar (BINETTI et al., 2002, TOMAT et al. , 2014).

A meta-análise mostrou que fagos específicos para patógenos de origem alimentar: *Salmonella* spp. e *L. monocytogenes* reduziram significativamente os patógenos nos alimentos, mas foi detectada alta heterogeneidade. Essa heterogeneidade pode ser explicada por análises de subgrupo de tipos individuais de alimentos em ambos os casos. SABITOVA et al., (2020) relataram que uma meta-análise combina idealmente os resultados de vários estudos que são altamente comparáveis em design, intervenção e população de pacientes. No entanto, na vida real, as metanálises frequentemente contêm vários estudos relativamente pequenos que diferem em muitos aspectos (SABITOVA et al., 2020), portanto, a análise de subgrupos é necessária.

Diversas alternativas naturais aos antibióticos têm sido desenvolvidas, uma delas são os metabólitos secundários de plantas. Neste estudo, a atividade antibacteriana de Dezessete plantas (*Artemisia absinthium Linné, Calendula officinalis, Cecropia Hololeuca Miquel, Commiphora leptophloeos, Costus spicatus Swartz, Cuphea ingrate, Jacarandá semiserrata Cham, Laurus nobilis, Miconia albicans, Mikania hirsutíssima, Momordica charantia Linné, Pereskia aculeata, Salvia officinalis, Thuja Occidentalis Linné, Tilia cordata, Zea mays Linné* e *Croton heliotropiifolius*) da região semiárida do nordeste do Brasil foram macerados empregando hexano, acetato de etila e etanol para produzir 51 extratos. Foram avaliados seis óleos essenciais produzidos comercialmente (*Larus nobilis, Salvia officinalis, Rosmarinus officinalis, Cymbopogon, Orégano Selvagem* e *Clove bud*) e os óleos essenciais de *C. heliotropiifolius* (obtidos por hidrodestilação), timol e carvacrol contra *Salmonella* 14028 usando abordagens *in vitro*.

A maioria dos extratos de produtos naturais testados neste estudo não apresentou atividade antimicrobiana significativa contra *Salmonella enterica* subsp. enterica ATCC 14028. No entanto, o óleo essencial de botão de cravo-da-índia e o timol mostraram atividade contra *Salmonella* ATCC 14028 na concentração de 1mg/ml. Segundo

GIOVAGNONI et al. (2020), o duplo mecanismo de ação do timol e carvacrol potencializa os efeitos associados a um *S.* Typhimurium in vitro. Do lado do hospedeiro, o timol e o carvacrol possuem propriedades anti-inflamatórias e antioxidantes que podem impedir a cascata de citocinas inflamatórias devido à infecção por *Salmonella*.

O óleo de botão de cravo é um líquido incolor ou amarelo, os principais constituintes do óleo são eugenol (70–95%), acetato de eugenol (até 20%) e β-cariofileno (12–17%). DEVI et al. (2010), reportaram que o eugenol inativou *Salmonella* Typhi em 60 min de exposição; seus MIC (0,0125%) e MBC (0,025%) reduziram a viabilidade e resultaram na inibição completa do organismo. A atividade antibacteriana do eugenol contra *Salmonella* Typhi é atribuída ao aumento da permeabilidade da membrana.

Neste trabalho, Salmonella SE3 também foi isolada do solo do Rio Subaé em Santo Amaro, Brasil, região contaminada com metais pesados e resíduos orgânicos. Doze ilhas patogênicas de Salmonella, múltiplos genes de virulência, múltiplos genes de resistência a antimicrobianos, sete sistemas de defesa, sete profagos e um gene de resistência a metais pesados (*arsC*) foram identificados. Além disso, determinou-se que o Pan-genoma é aberto, também foi identificado o core genoma (presente em pelo menos 95% dos genomas) de 2.137 genes e o genoma acessório com 3.390 genes shell (presentes de 15% a 95% dos genomas) e 69.352 genes cloud (presentes em até 15% dos genomas).

Sete profagos foram detectados no genoma de Salmonella SE3, dois estavam intactos e cinco incompletos. Em comparação, em *S.* enterica Typhimurium ms202 foram detectados nove profagos, dois estavam intactos, cinco incompletos e dois questionáveis (Mohakud et al., 2022). Além disso, Salmonella SE3 não tinha apenas sequências de profago de Salmonella (fago Salmonella RE-2010), mas também profagos anotados como pertencentes a gêneros intimamente relacionados *Shigella* (fago POCJ13) e *Escherichia* (fago 500465-2), o que pode indicar transferência gênica horizontal ou fagos polivalentes. Alguns estudos prévios reportaram que as populações de fagos em *S.* enterica contribuem para a transferência horizontal de genes, incluindo virulência e genes relacionados à virulência dentro da subespécie (HARDT et al., 1998, FIGUEROA-BOSSI et al., 2001, SWITT et al., 2015 e WORLEY et al., 2018). Mais

estudos sobre *Salmonella* podem revelar os mecanismos de interação do receptor entre fagos e hospedeiros que podem levar à melhoria da terapia fágica como uma opção para o tratamento ou controle de Salmonella.

Por outro lado, o fago SF1 foi isolado e caracterizado, incluindo a determinação da atividade antimicrobiana pela formação de placas e visualização da morfologia com microscopia eletrônica de transmissão. Além disso, o genoma do fago foi sequenciado pelo sequenciamento ONT MiION e Illumina Hiseq, três tipos de montagens foram avaliados, no híbrido (MinION e Hiseq) e híbrido (MinION + Hiseq), os genomas foram anotados e analisados, e seus genomas foram comparados com o fago de Salmonella de referência.

Em relação à estatística Quast e anotação do genoma, a montagem na plataforma MinION apresentou os melhores resultados. Além disso, não foram identificados genes de ciclo lisogênico, resistência antimicrobiana e virulência em nosso trabalho.

Também, o fago SF1 mostrou atividade antimicrobiana contra: *Salmonella* var. Enteritidis, *Salmonella* var. Typhimurium, *Salmonella* var. Minnesota, *Shigella flexneri*, *Escherichia coli*, *Escherichia cloacae, Escherichia fergusonii*, *Citrobacter europeus, Citrobacter freundii*, *Corynebacterium pseudotuberculosis, Corynebacterium striatum, Glutamicibacter creatinolíticus, Klebsiella oxytoca*, *Listeria monocytogenes* e *Rodococos iaqui*. O fago SF1 foi eficaz contra treze espécies bacterianas, é um fago polivalente. Várias proteínas foram identificadas no fago SF1: Terminase, Major caps, receptor b, tail tube, DNA polimerase, DNA ligase, proteína A1, proteínas putativas e várias proteínas hipotéticas foram detectadas.

É a primeira vez que a atividade antimicrobiana contra *Corynebacterium pseudotuberculosis*, *Corynebacterium striatum* e *Glutamicibacter creatinolíticus* por um bacteriófago é reportada. Além disso, é a primeira vez que o bacteriófago tem atividade contra treze espécies de bactérias. É possível que os receptores: receptor b, e a proteína do tubo da cauda estejam envolvidos na alta atividade antimicrobiana contra várias bactérias. O estudo destes receptores e especificidade dos hospedeiros pode ser interessante para o conhecimento da interação fago-hospedeiro tanto nos estudos de ecologia e evolução quanto na aplicação na indústria farmacêutica. Nesse estudo, não foi avaliado o analise filogenético do fago SF1, mas pode ser importante para estudos futuros.

4. CONCLUSÕES GERALES

A resistência antimicrobiana está aumentando globalmente, e novos tratamentos são urgentemente necessários para enfrentar esse desafio na assistência médica. Embora a terapia fágica para infecções bacterianas exista há mais de um século, a crise de resistência a antibióticos está fornecendo um impulso renovado para a terapia fágica cumprir sua promessa de longa data como tratamento clínico. Conforme descrito aqui, há um número crescente de ensaios clínicos de Fase I/II bem executados que descrevem a segurança e eficácia da terapia fágica. Há uma melhor compreensão da farmacologia, imunologia, segurança e potencial de resistência bacteriana. Tecnologias como engenharia genética, sequenciamento de genoma completo e metagenômica também fornecem novas ferramentas para aperfeiçoar estratégias terapêuticas de fagos. No entanto, ainda existem lacunas de dados sobre sua eficácia e falta de padronização e estruturas regulatórias adequadas que precisam ser resolvidas antes que a terapia fágica possa ocupar seu lugar na medicina convencional. Dado o renovado interesse e ímpeto no campo da terapia fágica, há razões para estar otimista de que esses desafios podem ser superados nos próximos anos.

Além disso, avaliamos a eficiência de fagos previamente patenteados como controle biológico de frutas, hortaliças e carnes. Nossas meta-análises revelaram que a concentração inicial de fagos e bactérias, tempo e alimento foram associados a um efeito antimicrobiano na *Listeria*. A temperatura e o tempo foram associados a um efeito antimicrobiano sobre *Salmonella*. Os fagos ListShield e Felix01 apresentaram o melhor resultado para controle biológico de *Listeria* e *Salmonella*, respectivamente. Uma abordagem sistemática como a que usamos aqui ajudará a informar futuras aplicações de fagos em patógenos bacterianos de origem alimentar e destaca a necessidade de melhorar a comparabilidade dos resultados para fornecer a melhor confiança nas conclusões de tais estudos.

Por outro lado, de todos os extratos avaliados neste estudo, apenas o óleo de broto de cravo e o timol diminuíram a *Salmonella* Typhimurium 14028, ambos se mostraram amplamente descritos na literatura. A atividade antifúngica, antibacteriana, antiviral (especialmente anti Sars-Covid2) e o sinergismo com bacteriófagos para o controle de Salmonella Typhimurium em frangos dessa molécula aumentam o potencial de desenvolvimento de produtos inovadores à base de timol.

A salmonelose é um problema de saúde no mundo inteiro, portanto, a análise genômica de isolados de *Salmonella* pode ser uma determinante chave para um melhor controle da salmonelose. Nosso estudo mostrou a eficácia de uma abordagem de montagem de sequência híbrida para análise do genoma de *Salmonella* de origem ambiental usando dados HiSeq e MinION. Além disso, Salmonella SE3 mostrou um Pan-genoma aberto.A montagem do genoma híbrido permitiu que elementos genéticos móveis, ilhas genômicas, ilhas de patogenicidade de *Salmonella*, sistemas antivirais, genes de resistência antimicrobiana, genes de virulência e profagos fossem identificados em Salmonella SE3. Além disso, um gene que codifica a resistência a metais pesados, *arsC*, foi detectado. Esses dados são importantes para informar o controle da poluição por Salmonella e metais pesados na região de Santo Amaro no Brasil.

Em resumo, neste estudo determinamos que a plataforma MinION deu o melhor resultado para a montagem do fago SF1 do que o montagem Illumina Hiseq e o híbrido (MinION + Illumina Hiseq). Além disso, não foram identificados genes de ciclo lisogênico, resistência antimicrobiana e virulência em nosso trabalho. O fago SF1 é um fago polivalente, o SF1 mostrou atividade contra: *Salmonella var. Enteritidis*, *Salmonella var. Typhimurium*, *Salmonella var. Minnesota*, *Shigella flexneri*, *Escherichia coli*, *Escherichia cloacae*, *Escherichia fergusonii*, *Citrobacter europeus*, *Citrobacter freundii*, *Corynebacterium pseudotuberculosis*, *Corynebacterium striatum*, *Glutamicibacter creatinolyticus*, *Klebsiella oxytoca*, *Listeria monocytogenes* e *Rodococos iaqui*. Dois receptores, receptor b e proteína do tubo da cauda foram identificados. O fago SF1 tem o potencial de ser uma alternativa ao antibiótico na terapia fágica e no controle biológico.

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