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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

MICAELA GUIDOTTI TAKEUCHI

*Microevolução da transferência horizontal de genes: perspectivas ampliadas pela
intervenção de nanocompostos*

UBERLÂNDIA

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MICAELA GUIDOTTI TAKEUCHI

**MICROEVOLUÇÃO DA TRANSFERÊNCIA HORIZONTAL DE GENES:
PERSPECTIVAS AMPLIADAS PELA INTERVENÇÃO DE NANOCOMPOSTOS**

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Veterinárias, da Faculdade de Medicina Veterinária, da Universidade Federal de Uberlândia, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias.

Área de concentração: Saúde Animal
Orientadora: Prof.^a Dra. Daise Aparecida Rossi
Coorientador: Prof.^a Dra. Roberta Torres de Melo

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MICAELA GUIDOTTI TAKEUCHI

**MICROEVOLUÇÃO DA TRANSFERÊNCIA HORIZONTAL DE GENES:
PERSPECTIVAS AMPLIADAS PELA INTERVENÇÃO DE NANOCOMPOSTOS**

Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal de Uberlândia, como parte dos requisitos para a obtenção do título de Doutor em Ciências Veterinárias

Área de concentração: Saúde Animal

Uberlândia, 19 de julho de 2024

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À minha filha Maísa, por ser meu sol e minha certeza de que os sonhos são possíveis de serem realizados.

Dedico

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“O sagrado é a função de dar sentido”.

Mircea Eliade

DADOS CURRICULARES DA AUTORA

MICAELA GUIDOTTI TAKEUCHI - Nascida em Araras, Estado de São Paulo, em 08 de dezembro de 1982, filha de Hamilton Antônio Guidotti e Maria Alice Pedro Bom Guidotti. Médica Veterinária, graduada em junho de 2009 pela Universidade Federal de Lavras - UFLA. Em 2011, iniciou o mestrado na Universidade Federal de Goiás (UFG), no Programa de Pós-graduação em Sanidade Animal, Higiene e Tecnologia de Alimentos (UFG), concluído em junho de 2013. Atuou como médica veterinária na indústria de produtos cárneos e como docente entre 2011 e 2017 nos cursos de biotecnologia e medicina veterinária. Em 2020, iniciou o doutorado no programa de pós-graduação em Ciências veterinárias da Universidade Federal de Uberlândia (UFU) e a pós-graduação *lato sensu* na Universidade Federal de Alfenas (UNIFAL). Tem experiência nas seguintes áreas: métodos diagnósticos, microbiologia, patógenos, biologia molecular, saúde pública e segurança alimentar.

RESUMO

A resistência antimicrobiana (RAM) é um fenômeno natural, mas pode ser maximizada pela transferência horizontal de genes (THG) em ambientes que impõem pressão de seleção. O primeiro capítulo da tese aborda as condições gerais sobre a THG por meio da conjugação bacteriana, resistência antimicrobiana, utilização de inibidores do processo de conjugação e conceitos gerais importantes sobre nanocarreadores lipídicos (NLC). O segundo capítulo objetivou avaliar inibidores da conjugação (COINs) nanoencapsulado e não encapsulados de cobre na conjugação bacteriana e determinar a influência de coprodutos da indústria de produtos de origem animal na frequência de conjugação (FC). A conjugação bacteriana foi investigada em meio líquido e a sequência do genoma completo (WGS) da bactéria doadora (*Salmonella* Heidelberg doadora dos genes *bla*_{TEM-1}) e receptora (*Escherichia coli* J53 Az^R), bem como os transconjugantes após conjugação determinou a interferência dos coprodutos de origem animal *chicken juice* (CJ) e soro de leite (SL) na THG, e o uso de NCL em associação com complexos de cobre (NCLDRI12) e complexo de cobre não encapsulado (DRI12) como mitigadores desse processo. A sequência do genoma completo (WGS), permitiu determinar inibição da passagem de plasmídeos com genes de resistência a antibióticos, bem como uso da sintenia para visualização da troca ou deleção de blocos de genes. A presença dos coprodutos estimulou o aumento da FC, que porém foi reduzida na presença de DRI12. Entretanto, na presença de SL, este composto permitiu a passagem de todos os plasmídeos carreadores de genes de resistência a antibióticos. Na análise de similaridade, interferiu em trocas e deleções das regiões de colinearidade. Em comparação ao DRI12, NLCDRI12 não reduziu o número de transconjugantes, mas impediu a passagem dos plasmídeos IncC e IncX1_1. O terceiro capítulo objetivou avaliar o efeito dos coprodutos SL e CJ e NLC de sálvia e olíbano na FC. A cepa de SH foi caracterizada fenotipicamente como multirresistente e por PCR convencional para detecção do gene *bla*_{TEM}. Uma concentração de 0,39 mg/mL foi utilizada para os inibidores NLC de sálvia e olíbano na mitigação da THG. Os testes demonstraram que o soro de leite se mostrou como o principal potencializador na THG (aumento de três ordens de magnitude), mas as NCL de óleo essencial de sálvia e olíbano foram capazes de reduzir a FC em mais de 70 e 11 ordens de magnitude, respectivamente, sendo promissoras no controle da resistência antimicrobiana em ambientes industriais cujo desafio de controle é maior.

Palavras-chave: conjugação bacteriana; nanopartículas; resistência antimicrobiana; sequenciamento genômico completo.

ABSTRACT

Antimicrobial resistance (AMR) is a natural phenomenon, but it can be maximized by horizontal gene transfer (THG) in environments that impose selection pressure. The first chapter of the thesis covers general conditions on THG through bacterial conjugation, antimicrobial resistance, the use of inhibitors of the conjugation process and important general concepts on lipid nanocarriers (NLC). The second chapter aimed to evaluate nano encapsulated and non-encapsulated copper conjugation inhibitors (COINs) on bacterial conjugation and to determine the influence of co-products from the animal products industry on conjugation frequency (CF). Bacterial conjugation was investigated in liquid media and the whole genome sequence (WGS) of the donor bacterium (*Salmonella* Heidelberg donor of the blaTEM-1 genes) and recipient bacterium (*Escherichia coli* J53 AzR), as well as the transconjugants after conjugation determined the interference of animal by-products chicken juice (CJ) and whey (SL) in THG, and the use of NCL in association with copper complexes (NCLDRI12) and unencapsulated copper complex (DRI12) as mitigators of this process. Whole genome sequencing (WGS) made it possible to determine the inhibition of the passage of plasmids with antibiotic resistance genes, as well as the use of synteny to visualize the exchange or deletion of gene blocks. The presence of coproducts stimulated an increase in CF, but this was reduced in the presence of DRI12. However, in the presence of SL, this compound allowed all the plasmids carrying antibiotic resistance genes to pass through. In the similarity analysis, it interfered with exchanges and deletions of collinearity regions. Compared to DRI12, NLCDRI12 did not reduce the number of transconjugants, but it did prevent the passage of the IncC and IncX1_1 plasmid. The third chapter aimed to evaluate the effect of the coproducts SL and CJ and NLC from sage and olibanum on CF. The SH strain was characterized phenotypically as multidrug-resistant and by conventional PCR to detect the blaTEM gene. A concentration of 0.39 mg/mL was used for the NLC inhibitors of sage and olibanum in the mitigation of THG. The tests showed that whey proved to be the main potentiator in THG (an increase of three orders of magnitude), but the sage and frankincense essential oil NLCs were able to reduce CF by more than 70 and 11 orders of magnitude, respectively, making them promising for controlling antimicrobial resistance in industrial environments where the control challenge is greater.

Keywords: bacterial conjugation; nanoparticles; antimicrobial resistance; whole genome sequencing.

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LISTA DE ABREVIATURAS E SIGLAS

ARGS - Genes de resistência aos antibióticos (ARGs)

CDC - Centers for Disease Control and Prevention

CLSI - Clinical Laboratory Standards Institute

CIM - Concentração Inibitória Mínima

DNA - Ácido Desoxirribonucleico

DRI12: composto de cobre não encapsulado

DTA - Doenças Transmitidas por Alimentos

Escherichia coli J53 Az^R - *Escherichia coli* resistente a azida sódica.

EFSA - *European Food Safety Authority*

FAO - Organização das Nações Unidas para Alimentação e a Agricultura

ESBL - Beta-lactamase de espectro estendido

NLCDRI12 - Nanocarreador lipídico

Nm – Nanômetro

NP - Nanopartículas

OMSA - Organização Mundial de Saúde Animal

OMS - Organização Mundial da Saúde

PB - Pares de Base

PBP's -Proteínas ligadoras de penicilina

PCR - *Polymerase Chain Reaction*

PNUMA - Programa das Nações Unidas para o Meio Ambiente

TEM - nome do primeiro paciente (Temoniera) de quem a enzima foi isolada.

THG - Transferência Horizontal de Genes

WHO - *World Health Organization*

µg - Micrograma

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CAPÍTULO 1

CONSIDERAÇÕES GERAIS

A tese intitulada “Microevolução da transferência horizontal de genes: perspectivas ampliadas pela intervenção de nanocompostos” foi supervisionada pela Professora Dra. Daise Aparecida Rossi, que orientou todo o estudo. Os experimentos foram realizados na Universidade Federal de Uberlândia, no Laboratório de Biotecnologia Animal Aplicada (LABIO) e no Laboratório de Epidemiologia Molecular Experimental (LEPMOL).

A estrutura do documento seguiu as orientações delineadas pelo Programa de Pós-Graduação em Medicina Veterinária da Faculdade de Medicina Veterinária - UFU (PPGCV), com as seguintes seções:

- Capítulo 1- Fundamentação teórica sobre resistência antimicrobiana e transferência horizontal de genes. Ênfase maior foi dada para o processo de conjugação bacteriana e genes codificam a produção das enzimas β -lactamases. Além disso, este capítulo descreve as características de patógenos multirresistentes, potenciais inibidores da transferência horizontal de genes e a descrição de técnicas de sequenciamento genômico.
- Capítulo 2 - "*Nanopore genome sequence reveals the impact of metal compounds on horizontal gene transfer in mimetic food environments*". Artigo a ser submetido ao periódico "*Scientific Reports*" com fator de impacto 4,6. Os critérios para submissão estão disponíveis em: Guia para autores – Scientific Reports - ISSN 2045-2322 (online) | *Nature Portfolio*.
- Capítulo 3 - "*Interference with Bacterial Conjugation and Natural Alternatives to Antibiotics: Bridging a Gap*". Artigo publicado no periódico "*Antibiotics*" com fator de impacto 4.8.

1 INTRODUÇÃO

No âmbito da saúde global, a resistência antimicrobiana (RAM) tomou proporções de um desafio complexo e crescente. Abordar as estratégias colaborativas entre países e encontrar medidas que possibilitem alcançar resultados promissores são atualmente os pilares que permitirão conter esse problema de escala mundial (Tang; Millar; Moore, 2023; WHO, 2017).

A RAM tende a continuar nas discussões políticas entre países nas próximas décadas e recentemente a Organização Mundial da Saúde (OMS) anunciou avanços de monitoramento desse problema em escala global por meio do lançamento da Plataforma 2.0 do Sistema Global de Vigilância de Resistência e Uso de Antimicrobianos (GLASS). Essa plataforma foi atualizada no ano de 2023 e marca a vigilância em tempo real para melhor compreensão da extensão e distribuição da resistência antimicrobiana, bem como a rápida disseminação de dados críticos e procedimentos coordenados frente a ameaças emergentes (Sirijatuphat *et al.*, 2020; Tornimbene *et al.*, 2022).

As previsões são alarmantes caso esse problema não seja mitigado e as projeções preveem 10 milhões de mortes anualmente até o ano 2050 (Kumar; Arnipalli; Ziouzenkova, 2020; O'Neill, 2016). A utilização indevida e em excesso de antimicrobianos na produção animal e na saúde humana, acelerou o processo de desenvolvimento das superbactérias, que afetam não apenas a saúde humana e animal, mas também levam ao acúmulo maciço destes medicamentos no ambiente (Polianciuc *et al.*, 2020).

Diante deste cenário, estratégias para o enfrentamento dessa crise sanitária mundial têm sido avaliadas com abordagens multifacetadas que incluem: a utilização responsável de antimicrobianos, vigilância e rastreamento contínuo dos micro-organismos e moléculas críticas (Castro-Vargas *et al.*, 2020), abordagem *One Health* e desenvolvimento de novas moléculas que contribuam para controlar os principais patógenos (Hegemann *et al.*, 2023).

A Autoridade Europeia para a Segurança dos Alimentos (EFSA) demonstrou por meio de ferramentas interativas de visualização de dados, que os dados ainda são críticos e persistentes em *Salmonella* resistentes (índices > 20 a 50%) a ampicilina, a tetraciclina e a sulfametoxazol isoladas de humanos, bovinos e suínos (EFSA, 2024). Tendência semelhante são observados em *Escherichia coli* isoladas de animais produtores de alimentos e produtos alimentícios derivados. Em particular para este micro-organismo, o monitoramento é crítico, pois fornece informações cruciais sobre reservatórios de genes e disseminação de elementos genéticos móveis, como os plasmídeos. Também é crucial o acompanhamento dos resultados das medidas mitigadoras implantadas (EFSA, 2023).

O processo de conjugação bacteriana permite moldar a diversidade gênica entre espécies (Moura de Sousa; Lourenço; Gordo, 2023) e para confrontar essa crise silenciosa, novas moléculas alternativas aos antimicrobianos tradicionais têm sido testadas como medida para retardar a transferência de genes de resistência a antibióticos. A utilização de óleos essenciais e íons metálicos em associação ou não com nanopartículas têm sido avaliados, a fim de evitar a transferência horizontal de genes (THG).

Neste enfoque, propomos inovações tecnológicas e ações específicas, como a utilização da nanotecnologia, que oferece maior ação contra os mecanismos de troca gênica aliada à maior biodisponibilidade de biocompostos e maior estabilidade, sem afetar a biocompatibilidade (Ribeiro *et al.*, 2019). A utilização de carreadores lipídicos nanoestruturados (CLN) associados a óleos essenciais já demonstrou efeitos significativos em *Campylobacter jejuni* (Ribeiro *et al.*, 2021). Por isso, o conhecimento das taxas de conjugação bacteriana em condições que mimetizam a indústria de alimentos associada à aplicação de NLC no controle microbiológico e de processos de recombinação pode representar uma abordagem eficaz também para *Salmonella* e *E. coli* (EC).

2 OBJETIVO

Avaliar os efeitos de nanocompostos lipídicos estruturados (NLC) associados a dois óleos essenciais (sálvia e olíbano), NLC associada a cobre (NLCDRI12), além de cobre não encapsulado (DRI-12) na inibição bacteriana e na prevenção da THG de resistência a beta-lactâmicos entre cepas de *S. Heidelberg* e *E. coli* J53 Az^R, bem como avaliar os efeitos destes complexos de cobre na frequência de conjugação e transferência de genes por meio da técnica de sequenciamento do genoma completo (WGS). Este estudo visa compreender a eficácia desses nanocompostos na mitigação da disseminação de resistência a antimicrobianos em bactérias patogênicas, abordando tanto a ação direta dos compostos quanto a interrupção dos mecanismos de conjugação bacteriana em ambientes simulados da indústria alimentícia (presença de *chicken juice* e soro de leite).

3 REVISÃO DE LITERATURA

3.1 Resistência antimicrobiana

A resistência aos antimicrobianos é um problema global de saúde pública, com impacto na saúde humana, animal e ambiental (Iramiot *et al.*, 2020a). O tema é tratado na interface mundial e justifica-se por englobar a abordagem de saúde única, associada a diversos relatos de que o aumento do uso de antibióticos impulsiona a microevolução da resistência antimicrobiana e consequentemente, confere mecanismos de resistência semelhantes aos antibióticos comumente usados em humanos (Rabello *et al.*, 2020). No ano de 2019, esse problema entrou como uma das dez principais ameaças à saúde segundo a Organização Mundial de Saúde (WHO, 2019).

A THG é um fator primordial para a disseminação rápida de genes, mas a dinâmica total que amplifica as taxas de transferências da resistência aos antimicrobianos é ainda mal elucidada (Castañeda-Barba; Top; Stalder, 2024; Lermينياux; Cameron, 2019). Tornou-se um consenso geral de que o uso excessivo de antimicrobianos, bem como a utilização de dosagens incorretas ou combinações inadequadas, impulsionou os níveis de resistência bacterianos (Roberts; Zembower, 2021). Um estudo sistemático que contemplou 15 anos de avaliação sobre o consumo de antimicrobianos e envolveu 76 países determinou a regulação de medicamentos em três níveis para as bases indicadas (Liang *et al.*, 2021) como terapias de primeira e segunda linha (denominada de Access) e medicamentos de terceira linha (vigilância). Diferentemente do esperado, os antibióticos classificados como de vigilância (utilizados para indicações específicas e com restrição devido ao potencial de resistência mais alto) tiveram um aumento expressivo (90,9%) em razão do aumento da prevalência de micro-organismos multirresistentes (MDR) (Klein *et al.*, 2021).

Este cenário agravou-se após a era da síndrome respiratória aguda grave Corona Vírus 2 (SARS-CoV-2), e estudos robustos de revisões sistemáticas indicaram que estes índices foram alavancados também para *E. coli* (estimados em 87,5%) e o antibiótico ampicilina (Sulayyim *et al.*, 2022). Em maior grau de severidade, foram evidenciados que apenas 8% dos pacientes necessitavam de antibioticoterapia durante a pandemia de COVID-19, mas que entre 66% a 95% dos casos foram administradas classes de antimicrobianos de amplo espectro, mesmo na ausência de infecção bacteriana (Garcia-Vidal *et al.*, 2021; Goncalves Mendes Neto *et al.*, 2021; Zhou, F. *et al.*, 2020).

Um relatório do Centro de Controle e Prevenção de Doenças americano (CDC) no ano de 2021 demonstrou índices elevados de doenças transmitidas pelos alimentos (DTA's)

causados por *Salmonella* em períodos discriminados entre 2004–2008 e 2015–2016, estimadas em 159.000 e 222.000 infecções respectivamente (Medalla *et al.*, 2021). *Salmonella enterica* subsp. *enterica* serovar Heidelberg (SH) ocupou o quarto lugar na frequência de ocorrência em produtos de origem animal nos EUA, e no Brasil ocupa o segundo lugar segundo os dados do anuário dos programas de controle de alimentos de origem animal do DIPOA (MAPA - Ministério Da Agricultura Pecuária e Abastecimento, 2023). A capacidade de sobrevivência e propagação no ambiente representam importantes desafios para o controle deste sorovar nas fazendas avícolas e métodos tradicionais de manejo de resíduos demonstram-se não eficazes para interromper o ciclo de contaminação (Voss-Rech *et al.*, 2019). A adaptação genética às condições ambientais (Kaldhone; Foley; Ricke, 2017) e a multirresistência de cepas de SH associadas ao hospedeiro pode ser uma das razões para esta predominância (Vilela *et al.*, 2023). Além disso, estimativas temporais preveem valores futuros de aumento da resistência para drogas prioritárias da terapêutica antimicrobiana de 17% a 31% até 2030 para 52 países, incluindo pontos críticos discriminados no Brasil (Cravo Oliveira Hashiguchi *et al.*, 2019).

Frente ao cenário mundial da RAM, o Brasil desenvolveu um programa em 2018 denominado Plano de Ação Nacional para Prevenção e Controle da Resistência Antimicrobiana no Brasil (PAN-BR) seguindo objetivos instituídos por organizações internacionais, como a Organização Mundial da Saúde. O programa PAN-BR visa aplicar estratégias de prevenção, vigilância, pesquisa e monitoramento de infecções causadas por bactérias resistentes aos antimicrobianos (Corrêa *et al.*, 2022). O Brasil também atendeu os objetivos da Aliança Quadripartite (OMS/FAO/OMSA/PNUMA) para o enfrentamento da resistência antimicrobiana, e atualmente o Ministério da Agricultura e Pecuária (MAPA) trabalha no intuito de harmonizar as políticas públicas nacionais às exigências internacionais para garantir a proteção no âmbito da Saúde Única na segunda etapa do programa denominado PAN-BR AGRO (Pecuária, 2023).

Mundialmente, a União Europeia (UE) implementou uma nova legislação destinada a direcionar esforços para combater a resistência microbiana na saúde animal, limitando a utilização destas medicações em animais. A legislação, introduzida há três anos, prevê medidas para promover a utilização responsável e sensata de antimicrobianos em animais e o uso consciente na população humana. De acordo com a Comissão Europeia para a Saúde e Segurança Alimentar, até 2030, a meta é reduzir em 20% o consumo humano total de antimicrobianos e em 50% a utilização de antimicrobianos nos animais de criação e na aquicultura da UE (Directorate-General for Health and Food Safety, 2023). Entretanto, mesmo com estas ações globais o uso de antimicrobianos em escala registrou um aumento mundial de

46% no período compreendido entre os anos 2000 e 2018, resultando em um incremento de 9,8 para 14,3 doses diárias definidas (DDD) (Qi *et al.*, 2023)

Diante da complexidade da resistência antimicrobiana, que abrange tanto a saúde humana, animal quanto ambiental, torna-se imperativo um enfoque multidisciplinar e integrado para enfrentar esse desafio. A intersecção entre estes três pilares, abordada em uma visão de saúde única, evidencia a necessidade de medidas coordenadas e abrangentes para mitigar os impactos da ocorrência em bactérias patogênicas (Zinsstag *et al.*, 2023).

3.2 Transferência horizontal de genes

A identificação de fatores impulsionadores, como o aumento e a disseminação de genes de resistência por meio de elementos genéticos móveis, destaca a urgência de estratégias preventivas e de vigilância. Os esforços científicos na busca por inibidores da THG representam passos significativos para mitigar os problemas ocasionados. No entanto, o aumento persistente no consumo global de antimicrobianos sublinha a necessidade contínua de aprimorar e implementar medidas eficazes. Uma abordagem abrangente, que englobe a saúde única e a segurança alimentar, é essencial para enfrentar o desafio da resistência antimicrobiana e garantir um futuro sustentável para a saúde pública global (Jaffee, 2018).

Neste ponto, métodos e compostos não antibióticos que visam a redução na propagação de genes de resistência tem sido proposto como medida para reduzir sua incidência. Já foram descritos o uso de biochar, derivado da palha de arroz e amendoim (Fang *et al.*, 2022), ácido desidrocrepnínico (DHCA), derivado de plantas tropicais, inibidores eficazes de plasmídeos IncF e IncW (Fernandez-Lopez *et al.*, 2005) e o isotiocianato de benzila derivado de um processo hidrolítico natural em vegetais da espécie Brassica (Kwapong; Stapleton; Gibbons, 2019).

Em relação a esta estratégia específica, os inibidores da conjugação (COINs) desempenham um papel preventivo sem exercer uma ação terapêutica direta. Sua eficácia prática depende da administração em ambientes propícios à conjugação do plasmídeo. Para aplicação prática, a toxicidade das COINs deve ser avaliada, preferencialmente eliminada por completo, ao passo que sua estabilidade química precisa ser preservada (Getino *et al.*, 2016).

Trabalhos utilizando COINs enfatizam a importância da avaliação das características específicas de cada ambiente avaliado. Por exemplo, a adição de ácido graxo sintético ácido 2-hexadecínico (2-HDA) como suplementação alimentar na produção de peixes reduziu a frequência de conjugação de plasmídeos em até 10 vezes em ecossistemas aquáticos

controlados. Da mesma forma, em ensaios de transferência de plasmídeo realizados no intestino de camundongos, a adição de 2-HDA reduziu a eficiência da conjugação em 50 vezes (Getino *et al.*, 2015; Liu *et al.*, 2023; Palencia-Gándara *et al.*, 2021).

A realização eficaz da transferência plasmidial por conjugação em ambientes heterogêneos representa um desafio significativo. Embora várias propriedades abióticas tenham sido analisadas de forma isolada, o impacto desses fatores moduladores em conjunto ainda não foi completamente compreendido. É relevante o conhecimento de que diferentes fatores como a temperatura, pH, matéria orgânica, e disponibilidade de nutrientes desempenham papéis cruciais na determinação da ocorrência de conjugação e na eficiência de transferência (GARBUSU, 2017).

A presença de matéria orgânica em superfícies e ambientes representa uma fonte significativa de nutrientes que podem afetar as taxas de conjugação bacteriana (Shafieifini *et al.*, 2022). A presença de matéria orgânica na cadeia de processamento de alimentos proporciona substratos para as bactérias, facilitando sua colonização (CARVALHO *et al.*, 2019). Além do papel direto como nutriente, estudos demonstraram que determinados coprodutos como o exsudato de frango ou *chicken juice* (CJ) promovem a formação de biofilmes fortes (Melo *et al.*, 2017b) e a presença do soro de leite incrementa a multiplicação microbiana em sistemas operacionais determinados por bateladas (Selover; Waite-Cusic, 2021). Anteriormente, trabalhos demonstraram que esses coprodutos contêm proteínas, carboidratos e lipídeos que são essenciais para o desenvolvimento microbiano (Guidotti-Takeuchi *et al.*, 2023), de maneira que ampliam e favorecem a sobrevivência e a persistência na cadeia produtiva (Melo *et al.*, 2017b), determinando dessa forma, condições essenciais para a THG.

3.3 Cepas multirresistentes (MDR)

A resistência antimicrobiana (RAM) é um fenômeno que naturalmente ocorre nas populações microbianas. Investigações revelaram que micro-organismos bacterianos congelados, provavelmente há 13.000 anos em cavernas de gelo na Romênia, foram reativados e isolados e constatou-se que já apresentavam resistência a todas as classes de antimicrobianos testadas (Paun *et al.*, 2021). Anteriormente ao marco da descoberta da penicilina por Alexander Fleming em 1928 (Fleming, 1980), o controle de infecções bacterianas já era documentado no Egito, Grécia e China (Sengupta; Chattopadhyay; Grossart, 2013). Ao longo do tempo (Figura 1), foram identificadas novas descobertas de substâncias químicas com ação na parede e membrana da célula bacteriana (Silver, 2011), interferência na síntese de ácidos graxos, síntese

de proteínas e metabolismo energético e com efeitos adversos para o crescimento microbiano: 1930 – sulfonamidas; 1940 – aminoglicosídeos, tetraciclina, bacitracina, nitrofuranos, polimixina, fenicóis e cefalosporinas; em 1950 – macrolídeos, novobiocina, rifamicinas; 1953, eritromicina; 1960 – metilclina, trimetoprima, quinolonas, lincosamidas, fosfomicina; 1967 – gentamicina; 1970- carbapenêmicos, monobactâmicos, oxazolidinonas; 1972-vancomicina; 1985 – Imipenem e ceftazidime; 1986 – levofloxacina; 2000- linezolida; 2003- daptomicina; 2010 – ceftaroline (May, 2023; Ventola, 2015), e mais recentemente, em 2022, a clovibactina- (Shukla *et al.*, 2023).

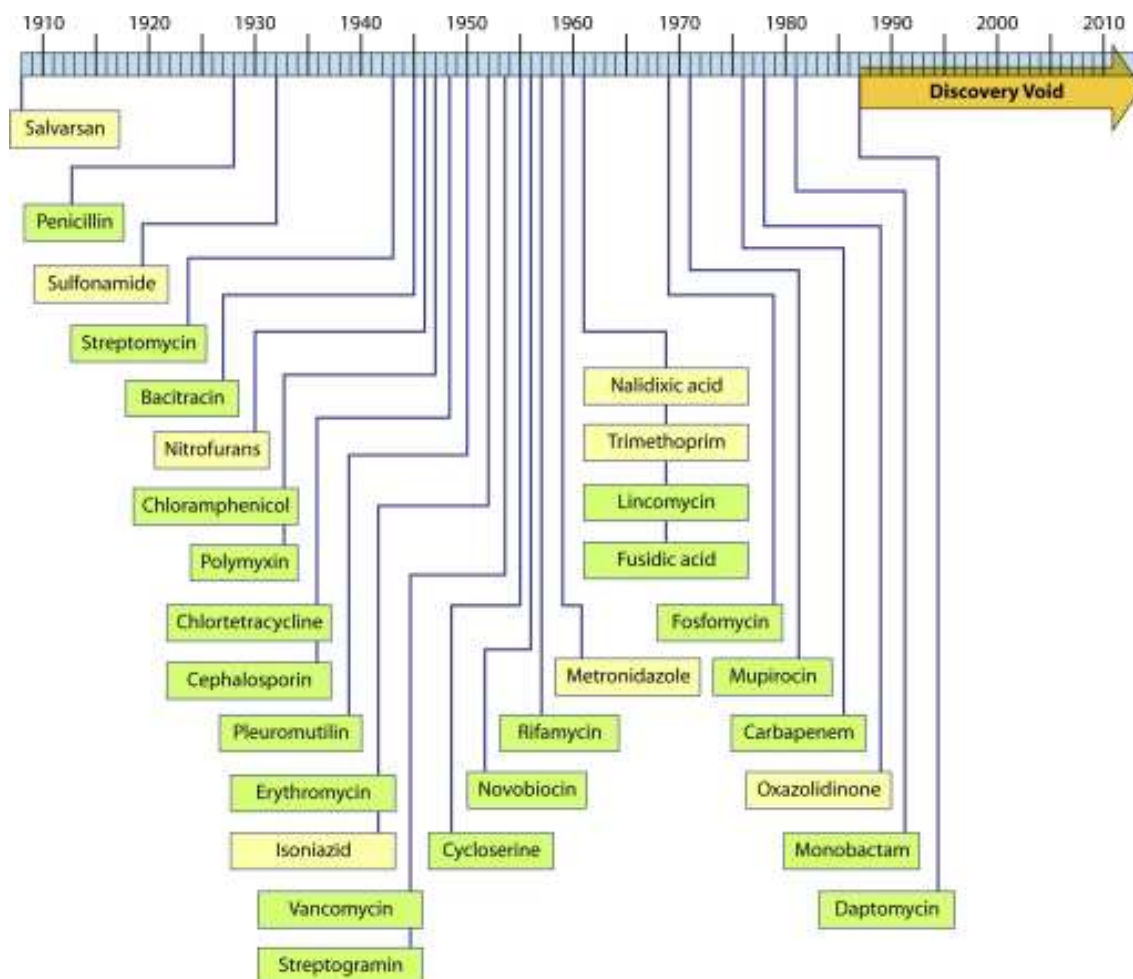
Entretanto, as rotas biossintéticas utilizadas pelas bactérias para evadir destes antimicrobianos evoluíram constantemente, mesmo antes da intervenção humana e da utilização clínica dos antibióticos na terapêutica de doenças. No ambiente natural, concentrações subinibitórias podem interferir negativamente na produção de fatores de virulência nos microrganismos, incluindo o desenvolvimento de resistência, bem como o estabelecimento das relações parasita-hospedeiro (Sengupta; Chattopadhyay; Grossart, 2013). Com base em dados de vigilância, a aquisição de resistência a importantes antibióticos (ARGs) por patógenos em ambientes naturais deve se tornar cada vez mais severa, com expansão crescente entre os países (Cravo Oliveira Hashiguchi *et al.*, 2019).

A RAM em bactérias patogênicas, como *Salmonella* e *E. coli* traz grandes desafios, principalmente devido a facilidade de disseminação destes patógenos (Iramiot *et al.*, 2020b) e sua presença no elo final da cadeia de alimentos (Heredia; García, 2018) como produtos lácteos (Hassani *et al.*, 2022) e cárneos (Cap *et al.*, 2020). Além disso, *Salmonella* e *E. coli* continuam sendo patógenos particularmente importantes causadores de DTAs no Brasil e no mundo (Foudraïne *et al.*, 2021), em particular, *Salmonella enterica* sorovar Heidelberg é considerado ambiental, principalmente por ser frequentemente isolado na produção de frangos de corte (Sielski Galvão Soares *et al.*, 2023). Desde meados de 2013, *Salmonella* Heidelberg passou a integrar o grupo dos dez sorovares com maior incidência no Brasil (Santos *et al.*, 2022) e nos Estados Unidos esse sorovar é responsável por altas taxas de infecções invasivas em humanos (ETTER *et al.*, 2019). As fontes alimentares correspondem por 65% da incidência, compreendidas por amostras ambientais, animais, matérias-primas e seres humanos (Santos *et al.*, 2023).

Em relação a *E. coli*, as enzimas de resistência mais estudadas são as β -lactamases TEM, responsáveis por 90% de resistência nessa espécie e que interferem na atividade dos β -lactâmicos porque o decompõem antes de atacar um alvo específico (Hussain *et al.*, 2021). Evidências mais importantes residem no fato de que mutações de nucleotídeo único em genes

que produzem β -lactamases TEM-1 levam à resistência às cefamicinas e aos carbapenêmicos (Ahmed *et al.*, 2014).

Figura 1 - Cronologia da descoberta de agentes antimicrobianos (Silver, 2011).



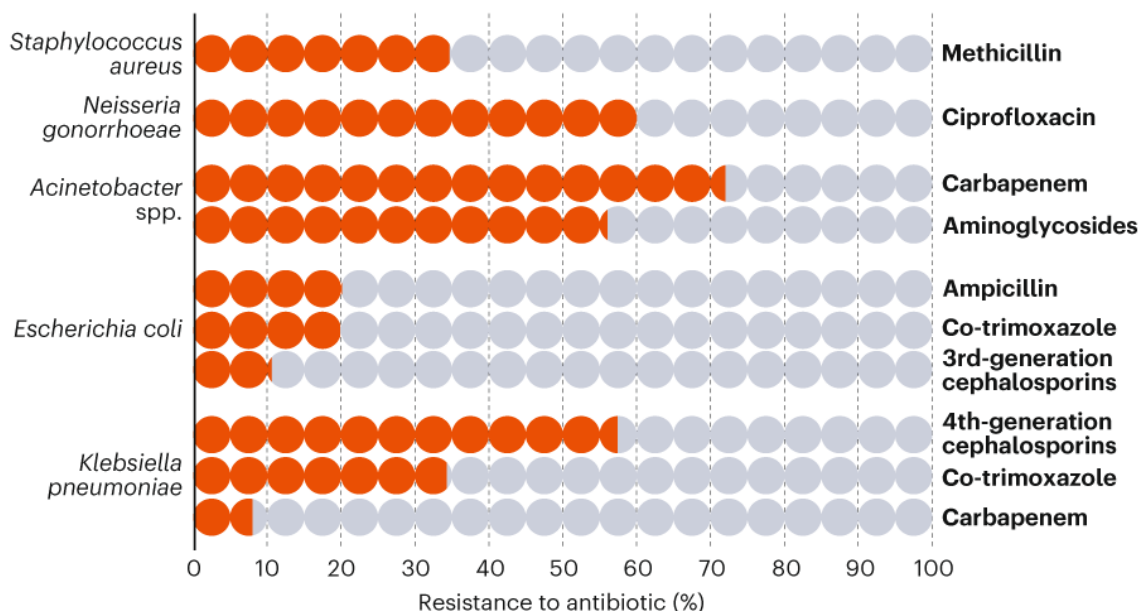
Fonte: Silver (2011)

Muitos estudos relataram a presença de patógenos classificados como MDR para classes de antimicrobianos de destaque como β -lactâmicos, aminoglicosídeos, tetraciclina e sulfonamidas (Figura 2) (Assoumy *et al.*, 2021).

A utilização de antibióticos está presente na rotina da medicina humana e veterinária, mas adjuvantes não antibióticos utilizados na agricultura disponíveis no ambiente também podem induzir uma co-resistência. Esta é a razão da presença de enzimas antigas como as β -lactamases de fontes ambientais serem como mecanismo de proteção aos β -lactâmicos naturalmente produzidos (Bush, 2018). A multirresistência em *E. coli* tornou-se uma questão relevante, amplamente observada em humanos e animais em todo o mundo. Esta espécie

bacteriana tem uma grande capacidade de acumular genes de resistência, principalmente por meio da THG (Poirel *et al.*, 2018).

Figura 2 - Resistência a diferentes classes de antimicrobianos em *Escherichia coli* e outros patógenos.



Fonte: Organização Mundial de Saúde (2022). Disponível em: https://www.nature.com/immersive/d41591-023-00043-5/index.html?utm_source=linkedin&utm_medium=social&utm_content=organic&utm_campaign=CONR_JRNL_S_AWA1_GL_SCON_SMEDA_NATUREPORTFOLIO

A enzima TEM-1 foi primeiramente identificada na Grécia no ano de 1963, em *E. coli* (DATTA; KONTOMICHALOU, 1965). Embora ocorram naturalmente em algumas espécies de bactérias, as β -lactamases foram mobilizadas em plasmídeos e se espalharam em resposta ao uso excessivo de antibióticos β -lactâmicos (Castanheira; Simner; Bradford, 2021). Em bactérias Gram-negativas, enzimas de amplo espectro como TEM-1 surgiram após a introdução de cefalosporinas de primeira e segunda geração. Posteriormente, foram introduzidos antimicrobianos β -lactâmicos de espectro expandido, refratários à hidrólise por essas enzimas. É preocupante que o gene *bla*_{TEM-1}, que codifica a β -lactamase TEM-1, precise apenas de alguns polimorfismos de nucleotídeo único (SNPs) específicos para evoluir para um gene que codifica uma β -lactamase de espectro estendido (ESBL), que pode degradar todas as moléculas de terceira geração, incluindo ceftriaxona (Huang; Palzkill, 1997; Ohnishi *et al.*, 2010). A situação é ainda mais complicada pelo fato de que os ARGs podem ser facilmente transferidos entre espécies bacterianas com a ajuda de elementos genéticos móveis como plasmídeos, integrons,

sequências de inserção (IS) e transposons (Singh; Singhal; Viridi, 2018). Vinte e oito grupos de incompatibilidade de plasmídeos foram descritos na família Enterobacteriaceae. As famílias de plasmídeos IncA/C2_1, IncX1_1 e Inc1_1_Alpha têm características específicas e estão associadas a diferentes patógenos (McMillan; Jackson; Frye, 2020).

Anteriormente denominados IncA/C2_1 e hoje referenciado como IncC é o grupo plasmidial mais estudado em *Salmonella* (Ambrose; Harmer; Hall, 2018). Geralmente são plasmídeos grandes com mais de 100kb e possuem alta capacidade de conjugação (Humbert *et al.*, 2019), permitindo a transferência entre diferentes espécies bacterianas. Frequentemente carregam múltiplos genes de resistência a antibióticos como β -lactâmicos, aminoglicosídeos, cefalosporinas, sulfonamidas e tetraciclinas (Cao *et al.*, 2018; Rozwandowicz *et al.*, 2018a). Esses plasmídeos são comuns em cepas de *Salmonella* (Iduu *et al.*, 2024), em cepas comensais e patogênicas de *E. coli* (Pauly *et al.*, 2021) e em cepas de *Klebsiella pneumoniae* em surtos nosocomiais (Yao *et al.*, 2021).

Em comparação, IncX1_1 são menores (30-50kb) e com espectro estreito de hospedeiros quando comparados aos plasmídeos IncC, mas possuem também alta eficiência conjugativa. Possuem elementos de inserção IS e transposons que facilitam a movimentação de genes de resistência (Zalewska, 2024). Já foram isolados em cepas de *Salmonella* (Hull *et al.*, 2022) na produção de suínos, constituindo-se também um alerta quanto ao elo final do processamento e abate de animais (Wu *et al.*, 2021).

IncII_1_Alpha possui tamanho variável entre 80-100kb e estão comumente associados à resistência e disseminação de genes de resistência a β -lactâmicos (Carattoli *et al.*, 2021) em isolados de *Salmonella* (Iduu *et al.*, 2024) e em aves de produção (Folster *et al.*, 2012). Possui alta capacidade conjugativa e frequentemente carregam genes de resistência a diferentes classes de antimicrobianos como aminoglicosídeos, tetraciclinas e trimetropim-sulfametoxazol (McMillan; Jackson; Frye, 2020).

3.4 Conjugação bacteriana

A conjugação bacteriana foi descrita pela primeira vez no ano de 1946 como mecanismo de reprodução sexual por Joshua Lederberg e Edward L Tatum (Lederberg; Tatum, 1953), e é responsável pela transmissão de moléculas de DNA plasmidial (Smillie *et al.*, 2010). Este mecanismo é presumivelmente o principal motor de troca gênica em comunidades bacterianas, e os elementos de conjugação, tais como plasmídeos conjugados, muitas vezes abrigam múltiplos genes de resistência a antimicrobianos (Wozniak; Waldor, 2010). Dentre os três

mecanismos de transferência horizontal de genes, a conjugação é universalmente conservada entre bactérias e ocorre em uma ampla variedade de ambientes, no qual impulsiona a microevolução e adaptação de cepas bacterianas à resistência aos antimicrobianos (Shen; Tang; Liu, 2022).

A transferência de DNA conjugativo requer mecanismos específicos e sincrônicos para realizar o transporte do DNA (Carranza *et al.*, 2021a) e a formação do par de acasalamento entre micro-organismos (Zechner; Moncalián; De La Cruz, 2017). A estabilização dos pares de acasalamento é dependente de adesinas ou *pili* especializados presentes na superfície das bactérias e são particularmente importantes em ambientes líquidos (caldo), nos quais a motilidade das bactérias e os fatores ambientais podem interferir na estabilidade do par de acasalamento (Neil; Allard; Rodrigue, 2021a). O processo de conjugação envolve três processos consecutivos distintos que podem ser didaticamente subdivididos em: processamento do DNA (relaxossoma), recrutamento e transporte (sistema de secreção tipo IV – T4SS) e transferência do substrato pelo pilus. O processamento e o recrutamento do DNA ocorrem no citoplasma e é necessário um aparelho de secreção transmembrana para o transporte do DNA processado de uma célula bacteriana para outra (Ilangovan; Connery; Waksman, 2015).

Este fenômeno, dependente de contato já é amplamente caracterizado e inicia-se no citoplasma da bactéria doadora com a montagem do relaxossomo em um local específico do plasmídeo, denominado de *OriT* ou origem de transferência. O relaxossomo necessita de proteínas acessórias denominadas TraY e TraM, necessárias para o reconhecimento eficiente da região *oriT* (De La Cruz *et al.*, 2010) e da relaxase TraI, uma proteína chave cujo papel é reconhecer a sequência de DNA no plasmídeo na região *oriT* do plasmídeo (Schildbach; Robinson; Sauer, 1998).

Para a etapa de recrutamento e transporte, a relaxase determina uma reação de clivagem no sítio *nic* e reage ao 5'-fosfato gerado, com posterior ligação ao sistema T4SS e pela proteína TraD (Lang; Zechner, 2012). Após a reação de clivagem, a síntese de DNA do doador começa a partir da extremidade 3' da fita clivada, de modo que a cópia de DNA de fita simples (ssDNA), que é transferida para a célula receptora, é substituída pela nova fita de DNA sintetizada (Carranza *et al.*, 2021a). O T4SS possui genes com função de formar os pares de acasalamento (*mpf*) que permite o contato próximo entre micro-organismo doador e receptor (Christie, 2016). Desempenham um papel essencial na biogênese do pilus (Waksman, 2019) e são únicos na mobilização do material genético para a bactéria receptora (Virolle *et al.*, 2020).

Por fim, o processamento do plasmídeo na célula transconjugante é um processo relativamente rápido, pois a entrada do plasmídeo ssDNA (fita simples) e sua conversão em

dsDNA (fita dupla) é concluída em cerca de quatro minutos em média. Mais importante ainda, o evento de conversão de ssDNA em dsDNA é o evento crucial que determina o programa de expressão do gene do plasmídeo (Couturier *et al.*, 2023). Os genes que codificam a maquinaria de conjugação podem ser transportados em plasmídeos ou replicons cromossômicos, mas as células receptoras não são estáticas nesse processo, podendo excluir ou modular a transferência (Raleigh; Low, 2013).

3.5 Mecanismo de resistência aos beta-lactâmicos

Os antimicrobianos β -lactâmicos são uma das classes de drogas mais utilizadas em escala mundial e atingem quase dois terços de todas as prescrições realizadas (Barreiro *et al.*, 2023; Bush; Bradford, 2016). São caracterizados por possuírem um anel β -lactâmico reativo, essencial para sua atividade bactericida (Pucci, 2012)

Bactérias possuem uma parede celular composta de um componente essencial, o peptideoglicano, que nas Gram negativas fica localizado abaixo da membrana externa bacteriana. A presença de enzimas bacterianas conhecidas como proteínas ligadoras de penicilina ou PBP's participam da biossíntese do peptideoglicano na parede celular bacteriana por meio da polimerização da cadeia de glicano (transglicosilação) e reticulação de cadeias peptídicas (transpeptidação) e possuem a função de auxiliar na síntese da parede celular durante o crescimento e a divisão, garantindo a sua integridade (Sharifzadeh *et al.*, 2020).

Cada tipo de bactéria possui uma coleção específica de proteínas ligadoras de penicilina (PBP's), variando de três a oito dessas enzimas por espécie (Georgopapadakou; Liu, 1980). A denominação das PBP's advém da afinidade em se ligar ao antibiótico penicilina, e portanto, são alvos dos antibióticos β -lactâmicos que agem impedindo a formação do peptideoglicano por meio do bloqueio da atividade de transpeptidação, ocasionando prejuízo na formação da parede celular, aumento do estresse osmótico e autólise (Mora-Ochomogo; Lohans, 2021).

O emprego terapêutico de β -lactâmicos enfrenta desafios decorrentes da emergência de bactérias resistentes a esses agentes e da rápida propagação dos mecanismos de resistência associados. Destaca-se a relevância clínica das β -lactamases, enzimas responsáveis pela degradação dos antibióticos β -lactâmicos, as quais representam o principal mecanismo de resistência observado em bactérias Gram-negativas (Castanheira; Simner; Bradford, 2021). Mais de 2.800 variantes únicas de beta-lactamases são atualmente identificadas, sendo o aumento nesse número atribuído, em parte, à crescente pressão exercida pelo uso difundido de

antimicrobianos beta-lactâmicos e à manipulação constante desses fármacos na tentativa de superar a produção bacteriana de beta-lactamases (Bush, 2018).

A presença de patógenos carreando genes de resistência a β -lactâmicos (β -RG's) não se limita apenas a ambientes hospitalares (Zhou, Y. *et al.*, 2020), sendo também observada em bactérias isoladas na população em geral, em alimentos e na exploração pecuária (Qi *et al.*, 2023). Determinadas variações particulares de β -lactamases, como as formas enzimáticas conhecidas como TEM, SHV e OXA, que são codificadas por plasmídeos, exibem uma disseminação ampla (Mendes *et al.*, 2019). A aquisição ou mutação das proteínas ligadoras de penicilina (PBP's) representa o principal mecanismo de resistência aos β -lactâmicos em bactérias Gram-positivas (Fisher; Mobashery, 2016). Entre os patógenos Gram-negativos, os mecanismos de resistência mais prejudiciais concentram-se na ação de β -lactamases. Essas enzimas podem ser codificadas cromossomicamente e produzidas em níveis elevados, ou transferidas por meio de elementos móveis (Bush; Bradford, 2016).

As bactérias Gram-negativas fazem uso de quatro mecanismos principais mais frequentemente utilizados para se protegerem do efeito letal dos antibióticos β -lactâmicos, que incluem a alteração no sítio ativo das PBP's, limitação da absorção pela modificação das proteínas porinas que obstruem a passagem dos β -lactâmicos para a membrana interna, produção de β -lactamases e o sistema de bomba de efluxo (C Reygaert, 2018).

As bactérias patogênicas frequentemente adquirem resistência aos medicamentos antibacterianos por meio da incorporação de genes que codificam a produção de β -lactamases, que quebram as ligações β -lactâmicas presente nos antibióticos β -lactâmicos, como penicilinas e cefalosporinas (Mohammad Shahid, Anuradha Singh, 2022). Essa ligação é crucial para a atividade dos antibióticos, pois imita a ligação peptídica que conecta a D-alanina terminal ao monômero de peptideoglicano. Essas enzimas frequentemente acumulam mutações nos genes da penicilinase e são frequentemente codificadas em plasmídeos auto transmissíveis, o que facilita sua rápida disseminação. (Akhtar; Fatima; Khan, 2022).

Existem dois sistemas de classificação amplamente reconhecidos para beta-lactamases: a classificação estrutural de Ambler (Grupos AD) e a classificação funcional de Bush-Jacoby-Medeiros (Grupos 1-4). As enzimas dos grupos A C e D de Ambler são serino-hidrolases atuantes no sítio ativo, enquanto as do grupo B são metalo-beta-lactamases, exigindo a presença de íons zinco como cofator para atividade hidrolítica (Shahid; Singh; Sami, 2022). Os genes de resistência aos beta-lactâmicos reconhecidos em plasmídeos e com importância para a saúde pública incluem: *bla*_{TEM} (Darphorn *et al.*, 2021), *bla*_{SHV} (Liakopoulos; Mevius; Ceccarelli, 2016) e *bla*_{OXA} (Ibrahim *et al.*, 2021; Vale *et al.*, 2021), que conferem resistência a

aminopenicilinas e cefalosporinas de espectro estreito. Organismos que carregam genes *bla_{CMY-2}*, conhecidos como produtores de AmpC mobilizados, podem inibir cefalosporinas de espectro estendido (3^a geração) e cefamicinas (2^a geração) (Ogunrinu *et al.*, 2020).

3.6 Nanopartículas

As abordagens de inibição das transferências de plasmídeos fornecem uma estratégia única para minimizar a resistência aos antimicrobianos em uma variedade de ambientes naturais, sendo a inibição do processo de conjugação um meio eficaz para remover plasmídeos de resistência de uma população bacteriana ao longo do tempo (Cabezón; De La Cruz; Arechaga, 2017; Graf *et al.*, 2019; Palencia-Gándara *et al.*, 2021; Xiao *et al.*, 2023). Novas estratégias surgiram ao longo do tempo para reduzir a ocorrência de plasmídeos que carregam RAM. Como alternativa promissora e indicativa da sua viabilidade, produtos comerciais alimentares já se encontram disponíveis para o mercado consumidor e incluem nanopartículas com função de aditivos associados a ácidos graxos ω -3 presentes em derivados lácteos, como em iogurtes (Ghorbanzade *et al.*, 2017) e em indústria de bebidas (Ilyasoglu; El, 2014).

A diversidade de aplicações de nanopartículas em matrizes alimentares para garantir a qualidade de alimentos para humanos e animais também está vinculada em oferecer vantagens como o carregamento de vitaminas e aumento da biodisponibilidade por meio do mecanismo de proteção contra degradação no trato gastrointestinal - TGI (Dasgupta; Ranjan, 2018). Além disso, essas associações podem ser multifuncionais e uma variedade de incorporações alimentícias como a Aquanova® e a *Lypo-Spheric Vitamin*, Livon Laboratories, Inc. Company Profile, Henderson, NV (EUA) incluíram nanopartículas para facilitar o descongelamento de produtos congelados por micro-ondas (Martin Möller *et al.*, 2009) em produtos patenteados pela Nestlé®, redução de gordura de 16% a 1% na produção de sorvetes (Unilever®, Londres, Reino Unido) (Shobhana Gupta, 2017a), incorporação de compostos antimicrobianos (Shobhana Gupta, 2017b) em embalagens, e na descontaminação de equipamento em procedimentos industriais (Silva; Cerqueira; Vicente, 2012).

Desta maneira, mais do que indicar a miniaturização de partículas, a formulação de compostos bioativos nanoparticulados abriu uma nova janela para lidar com sistemas biomiméticos que utilizam menores dosagens, incrementam a eficácia contra os micro-organismos ou permitem o aumento da bioatividade (Ribeiro *et al.*, 2021), devido a maior superfície de contato que pode interagir contra bactérias planctônicas resistentes a antibióticos e promover eficiência na inibição da transferência de plasmídeos (Li; Zhang, 2023).

Particularmente, a segunda geração de nanopartículas lipídicas, denominadas de nanocarreadores lipídicos são considerados seguros e promissores. São sistemas de multipartículas nanométricas com tamanho entre 50-500nm formados por uma matriz lipídica de lipídios sólidos e líquidos em temperatura ambiente que em conjunto são estabilizadas por um surfactante (Puri *et al.*, 2009). Em condições industriais, possuem a vantagem de serem incorporadas em alimentos e bebidas demonstrando vantagens contra espécies microbianas (Müller *et al.*, 2016). Devido a natureza desorganizada do seu núcleo (Figura 3), diferentes compostos bioativos podem ser carregados e vantagens como maiores padrões de estabilidade de armazenamento e toxicidade minimizada demonstram que estes sistemas podem ser transportadores ideais em rotas difíceis de alcançar (Khan; Sharma; Jain, 2023). Trabalhos tem demonstrado que os mecanismos de ação destes nanocarreadores contra espécies bacterianas estão centrados no tamanho das partículas e no seu formato, juntamente com suas cargas superficiais, culminando na morte de micro-organismos (Dyett *et al.*, 2021).

A entrega é direcionada e a interação com a bactéria pode ser diferente devido a diferenças na formulação. NLCs catiônicos são atraídos eletrostaticamente para a membrana e em contato com bactérias Gram-negativas, aumentam as taxas de liberação de compostos a fim de atingir o alvo específico e ocasionar reações enzimáticas que conduzem a ruptura da estrutura celular (Verma *et al.*, 2008). Para NLCs aniônicos, as ligações induzirão interações (Siek; Kandere-Grzybowska; Grzybowski, 2020) e posterior liberação do composto ao alvo.

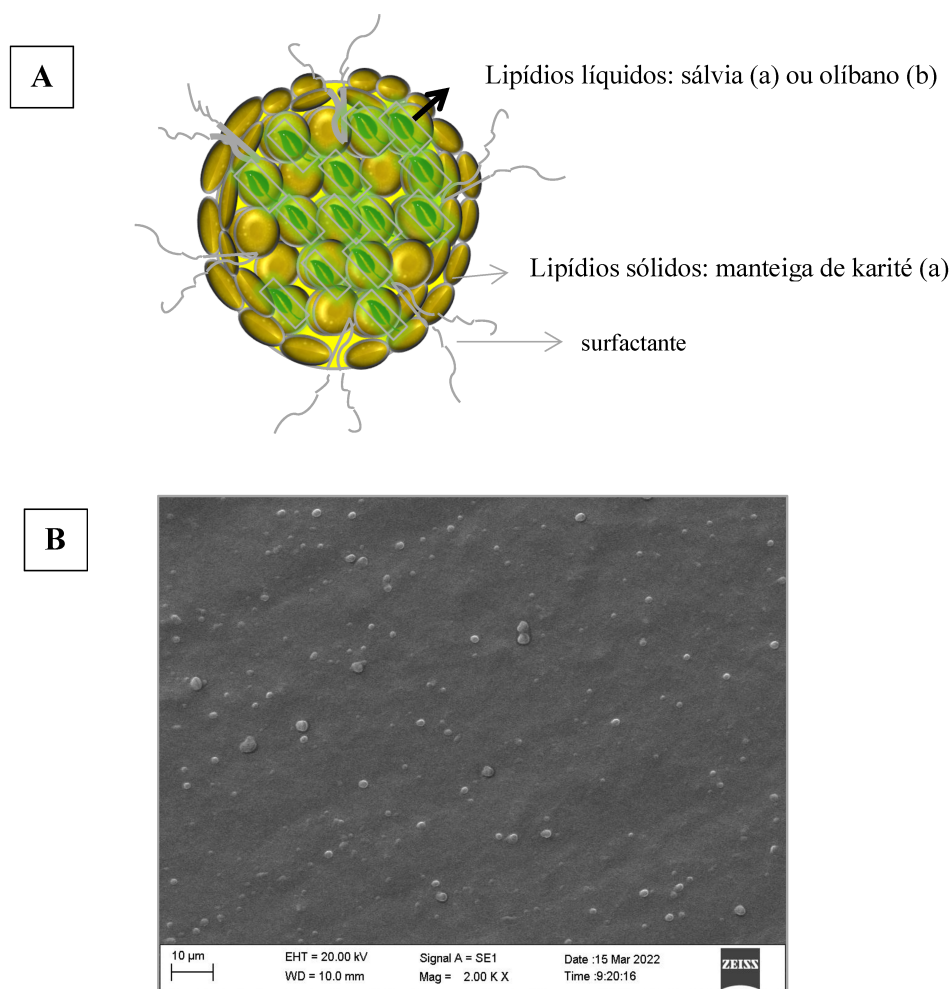
Além disso, especificamente para inibir os processos de THG, os efeitos físicos promovidos pelas nanopartículas de formato esférico apresentam maiores vantagens porque maximizam a interatividade com a membrana bacteriana, resultando em processos de tensão e estiramento (Linklater *et al.*, 2020) no centro primário da montagem do pili na membrana celular (Carranza *et al.*, 2021a). Dessa forma, demonstra-se que avanços na entrega de NLCs com efeitos bactericidas são dependentes não somente do tamanho e distribuição de nanopartículas adequadas (Keivani Nahr *et al.*, 2018a), mas também da sua estrutura, fatores primordiais que influenciarão os resultados (Haider *et al.*, 2020), pois determinam as taxas de liberação de compostos e a forma de interação com o micro-organismo (Azhar Shekoufeh Bahari; Hamishehkar, 2016).

Enfatiza-se o uso de NLCs como uma alternativa promissora ao analisar os aspectos de controle microbiológico e compatibilidade com alimentos. Pode ser preparado com diferentes misturas entre lipídios líquidos e sólidos, planejados molecularmente de acordo com a bactéria alvo, sendo resistente a variações de pH, formas de conservação dos alimentos e armazenado em temperatura ambiente, destacando aspectos desejáveis para a produção de um composto

eficaz que possa ser produzido em larga escala, para uso nas indústrias (Da Silva *et al.*, 2022; Nobari Azar *et al.*, 2020; Tang; Chen; Dong, 2023).

A tecnologia de nanopartículas em conjunto com compostos orgânicos ou inorgânicos é uma promissora alternativa para superar a resistência aos antimicrobianos em bactérias patogênicas em alimentos (Sultan *et al.*, 2018).

Figura 3 – Ultraestrutura (A) de nanocarreadores lipídicos (NLC) e microscopia de varredura de NLC (B) composto por lipídeos sólidos (manteiga de karité ou ucuuba) e lipídios líquidos (óleos essenciais de sálvia e olíbano).



Fonte: As autoras adaptado de GUIDOTTI-TAKEUCHI et al., 2022)

3.7 Complexo de cobre e nanopartículas de cobre

Devido a maior solubilidade em água e baixa toxicidade, os íons de cobre (II) podem agir em baixas concentrações como agente bacteriostático ou fungicida (Garg; Sharma, 2022).

Estas propriedades já eram utilizadas na civilização egípcia em tratamento de feridas (Dollwet, H.H., & Sorenson, 1985) e para esterilizar recipientes de água (Sudha *et al.*, 2009) até a descoberta dos antimicrobianos no início do século XX (Sportelli; Picca; Cioffi, 2014).

Suas propriedades antimicrobianas são amplamente documentadas (Salah; Parkin; Allan, 2021; Sun *et al.*, 2016) na prevenção e inibição de infecções (Wang, P. *et al.*, 2021), em propriedades bactericidas em superfícies (Faúndez *et al.*, 2004; Smith *et al.*, 2022), bem como em instalações hospitalares (Montero *et al.*, 2019) e no combate de patógenos importantes (Krasnovskaya *et al.*, 2020; Monteiro *et al.*, 2022a; Ude *et al.*, 2019; Zalevskaya; Gur'eva, 2021). Por ser um oligoelemento essencial e necessário para a atividade de diferentes enzimas do metabolismo energético em humanos e animais, respiração e síntese de DNA na célula (Iakovidis; Delimaris; Piperakis, 2011), a utilização destes compostos no desenvolvimento de novas moléculas torna-se atraente pelas suas potencialidades já evidenciadas (Paixão *et al.*, 2017).

Embora ainda ocorram incertezas quanto aos valores de referência dos limites de ingestão do metal por humanos (Bost *et al.*, 2016), índices de ingestão adequados de cobre para adultos correspondem a faixas entre 1,3-1,6 mg/dia para mulheres e homens, respectivamente, 0,7mg/dia para crianças menores de três anos e 0,4mg/dia para crianças menores de um ano (Scientific Opinion on Dietary Reference Values for copper, 2015).

Para os micro-organismos, o cobre é caracterizado como um micronutriente essencial e participa de funções biológicas, como cofator para cuproenzimas (Argüello; Raimunda; Padilla-Benavides, 2013). Particularmente em bactérias Gram-negativas, os mecanismos fisiológicos requerem processos para controle rígido da homeostase (El Zowalaty *et al.*, 2013), já que em excesso, as respostas antimicrobianas deste metal são desencadeadas de maneira deletéria, com danos nas funções proteicas e produção de espécies reativas de oxigênio (Dupont; Grass; Rensing, 2011).

Apesar dos mecanismos antibacterianos ainda não estarem completamente elucidados (Jin; Ren; Yang, 2016), duas vias principais da citotoxicidade são explicadas pelas reações de Fenton e de Haber-Weiss, $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{HO} + \text{HO}^\cdot + \text{Cu}^{2+}$; $\text{O}_2^- + \text{Cu}^{2+} \rightarrow \text{Cu}^{2+} + \text{O}_2$, respectivamente, por meio do qual os íons de cobre podem facilmente se inter converter entre os dois estados de oxidação com a produção simultânea de espécies reativas de oxigênio (Giannousi; Pantazaki; Dendrinou-Samara, 2017).

A capacidade de interferir no crescimento de micro-organismos tornou este metal de transição atraente para ser explorado no domínio da nanotecnologia, devido as propriedades

intrínsecas das nanopartículas que amplificam o potencial de ação do metal (Giannousi; Pantazaki; Dendrinou-Samara, 2017).

Na indústria de alimentos, estudos promissores já indicavam que superfícies de cobre em áreas para operações de processamento de alimentos possuíam efeito inibidor para importantes patógenos, incluindo *Campylobacter jejuni* e *Salmonella* entérica, entretanto, após manipulação de derivados cárneos, as concentrações do cobre aumentavam nos produtos manipulados (Faúndez *et al.*, 2004). Nestes derivados cárneos, como produtos de salsicharia e presunto, análises dos níveis de cobre revelaram que os níveis desse metal podem estar presentes em excesso (Sobhanardakani, 2018).

Em contrapartida, fatores que permitem tolerância ao cobre podem ser universais e presentes em organismos-chave da família Enterobacteriaceae como *Salmonella* e *EC* (Giachino; Waldron, 2020). Para contornar esse problema, diversas nanopartículas inorgânicas, incluindo as de cobre com atividades antimicrobianas específicas, possuem como vantagem a propriedade de distinguir o sistema celular bacteriano do sistema das células eucarióticas dos mamíferos por meio do sistema de transporte específico de procariotos e metaloproteínas (Modi *et al.*, 2023).

A resistência ao calor, estabilidade e baixo custo estão entre as principais vantagens da aplicabilidade de nanopartículas de cobre (Cu-Nps) (Sánchez-López *et al.*, 2020). A expressiva atividade antimicrobiana de Cu-NPs foi atribuída a maior transferência de elétrons entre micro-organismos e nanopartículas, resultado do contato direto e recepção de elétrons pelos nanocompostos, desencadeando a inibição do crescimento e ruptura da membrana bacteriana (Sánchez-López *et al.*, 2020).

Além disso, em células de *E. coli*, as Cu-NPs ocasionam múltiplos efeitos tóxicos incluindo degradação de DNA plasmidial, geração de espécies reativas de oxigênio (ROS), oxidação de proteínas e peroxidação lipídica (Chatterjee; Chakraborty; Basu, 2014).

3.8 Sequenciamento genômico

Após a elucidação da estrutura do DNA na década de 50 a partir dos trabalhos desenvolvidos por Rosalind Franklin e Maurice Wilkins (Zallen, 2003), e posteriormente por Watson e Crick, foi desvendada a estrutura de dupla hélice composta por diferentes bases nitrogenadas (Randall; Wilkins, 2023). Desde então, os progressos neste domínio da ciência foram constantes e permitiram identificar a ordem exata da sequência do DNA ou RNA, o que revolucionou o conhecimento sobre os micro-organismos (Thaventhiran *et al.*, 2020).

Abordagens diversas e avanços nas metodologias desenvolvidas foram implementadas desde 1975 com a finalidade de revelar a sequência desses aminoácidos (Sanger; Coulson, 1975). O sequenciamento envolve um conjunto de métodos para determinar a informação biológica disposta na ordem dos nucleotídeos em uma molécula de DNA, e dessa forma, entender a informação que essa molécula de DNA carrega ((ALEX; BRUNDHA; PRATHAP, 2020).

O sequenciamento de primeira geração, denominado de sequenciamento Sanger foi a técnica utilizada por mais de 40 anos, e nesta abordagem, o DNA amplificado ou molde é emparelhado com um *primer* oligonucleotídico, e posteriormente sofre extensão pela enzima DNA polimerase, que incorpora seletivamente uma mistura dos quatro desoxinucleotídeos trifosfatos (dNTPs: dATP, dGTP, dCTP, dTTP) ou trifosfatos de didesoxinucleotídeos de terminação de cadeia (ddNTPs: ddATP, ddGTP, ddCTP, ddTTP), que são modificados. A reação de alongamento pode ser interrompida à medida que os ddNTPs são incorporados, gerando fragmentos de DNA distinguíveis que podem variar de 800–1.000 pb (Sanger; Coulson, 1975; Sanger; Nicklen; Coulson, 1977). Para permitir o rastreamento desse processo, os nucleotídeos são demarcados radiotivamente com fósforo 32 e as sequências são separadas por eletroforese. A detecção é realizada por radiografia que permite a detecção dos nucleotídeos modificados e determina então, a sequência complementar de interesse.

O método de Sanger, passou por diversas modificações, e hoje, os marcadores radioativos não são mais utilizados e nucleotídeos modificados em uma única reação são marcados por fluorometria independente para cada um deles e a detecção é aprimorada por meio da introdução da eletroforese baseada em capilar, permitindo a dinamicidade das reações e verificação do resultado final em um eletroferograma (Heather; Chain, 2016). Estas foram as bases para o desenvolvimento de sequenciadores automatizados da primeira e segunda geração, também chamadas de *short reads*, capazes de gerar fragmentos de até 100pb, o que poderia ocasionar *gaps* na montagem do genoma.

O primeiro grande avanço do sequenciamento de primeira geração foi o Projeto do Genoma Humano (PGH). O genoma humano foi sequenciado paralelamente por dois grupos compostos por um consórcio público de cientistas ao redor do mundo (EUA, Reino Unido, Japão, França, Alemanha e China) que se uniram desde meados da década de 1990 para realizar essa ação com prazo até 2005 para finalização do projeto (International Human Genome Sequencing Consortium, 2004). Em 1988, a empresa *Celera Genomics* anunciou um rascunho do genoma humano com prazo final no ano de 2003. Devido a corrida científica ocasionada entre os dois principais grupos de pesquisa, a finalização do projeto foi realizada com dois anos

de antecedência pelo consórcio, com domínio público e a disponibilização gratuita das sequências (Barranco, 2021; Maxson Jones; Ankeny; Cook-Deegan, 2018). A tecnologia usada para o sequenciamento do genoma humano foi a denominada *shotgun*, que permitia a formação de fragmentos de DNA que posteriormente eram clonados, sequenciados e montados em longas sequências contíguas (*contig*). A sequência completa do DNA do genoma humano foi obtida após décadas de trabalho de um grande número de cientistas (Drmanac, 2023).

Menos de uma década depois, os instrumentos da "segunda geração" permitem que um único laboratório produza a mesma quantidade de dados em uma semana. A evolução ocorreu devido às limitações inerentes a abordagem desta primeira geração em relação aos custos, velocidade de rendimento e dificuldade de produção de maior quantidade de dados. Assim, foi desenvolvido um método de sequenciamento de segunda geração ou de nova geração (NGS) para satisfazer a grande busca de uma tecnologia mais barata e mais rápida (Mardis, 2013).

As abordagens de NGS, identificadas como plataformas Illumina ou Ion Torrent (ThermoFisher), geralmente iniciam-se com a preparação da biblioteca, amplificação em matriz, detecção de sequenciamento e análise e processamento de dados com bioinformática (Hu *et al.*, 2021). A NGS é fundamentalmente uma abordagem diferente para o sequenciamento que conduziu a diferentes descobertas inovadoras e trouxe uma nova complexidade na informação genômica, expandindo conhecimentos ilimitados relacionados com a diversidade de genomas, o transcriptoma e o epigenoma de qualquer espécie (Levy; Myers, 2016)

A evolução para a segunda geração iniciou-se em 2005 com o pirosequenciamento, que foi a primeira técnica que permitia o sequenciamento em ciclos, e cuja sequência é determinada pela detecção da liberação de pirofosfato quando nucleotídeos são adicionados ao modelo de DNA. Ao contrário da técnica de *shotgun*, nesta metodologia, a realização da clonagem do material não é necessária (Pervez *et al.*, 2022). Na reação devem estar presentes como componentes primordiais: o DNA *template*, o *primer* iniciador, a DNA polimerase, ATP sulfurilase, apirase, luciferase, luciferina e dNTPs modificados. A cada ciclo do sequenciamento, um tipo de nucleotídeo modificado é inserido na fita de DNA pela DNA polimerase, resultando na liberação de pirofosfato que na presença da ATP fosforilase produz ATP, que associado a luciferina e luciferase, emite luz que é captada pelo equipamento, gerando posteriormente um gráfico com picos. Terminado o processo, a apirase recicla os reagentes da reação e no próximo ciclo, outro tipo de nucleotídeo é adicionado, passível de ser incorporado pela DNA polimerase. Esta tecnologia foi muito utilizada em estudos com *snips*, que são polimorfismos de nucleotídeos único (variação de um único par de bases no genoma) (Ghemrawi *et al.*, 2023).

Por fim, os sequenciamentos de terceira geração, representam os avanços dos últimos anos no sequenciamento de DNA nos quais não são mais necessários a construção de bibliotecas a partir de fragmentos de DNA, pois os sequenciadores são capazes de sequenciar leituras longas quando comparadas com os métodos antecessores (Satam *et al.*, 2023). Os dados gerados são muito diferentes das gerações anteriores. Em 2011 foi desenvolvida pela *Pacific Bioscience*, uma técnica que utiliza a abordagem de molécula única em tempo real (SMRT), onde foi possível sequenciar a molécula de DNA em tempo real com nucleotídeos marcados com fluorescência. Este sequenciamento denominado PacBio SMRT Sequencing continua sendo utilizado, mas tem como desvantagem o custo elevado (Ardui *et al.*, 2018).

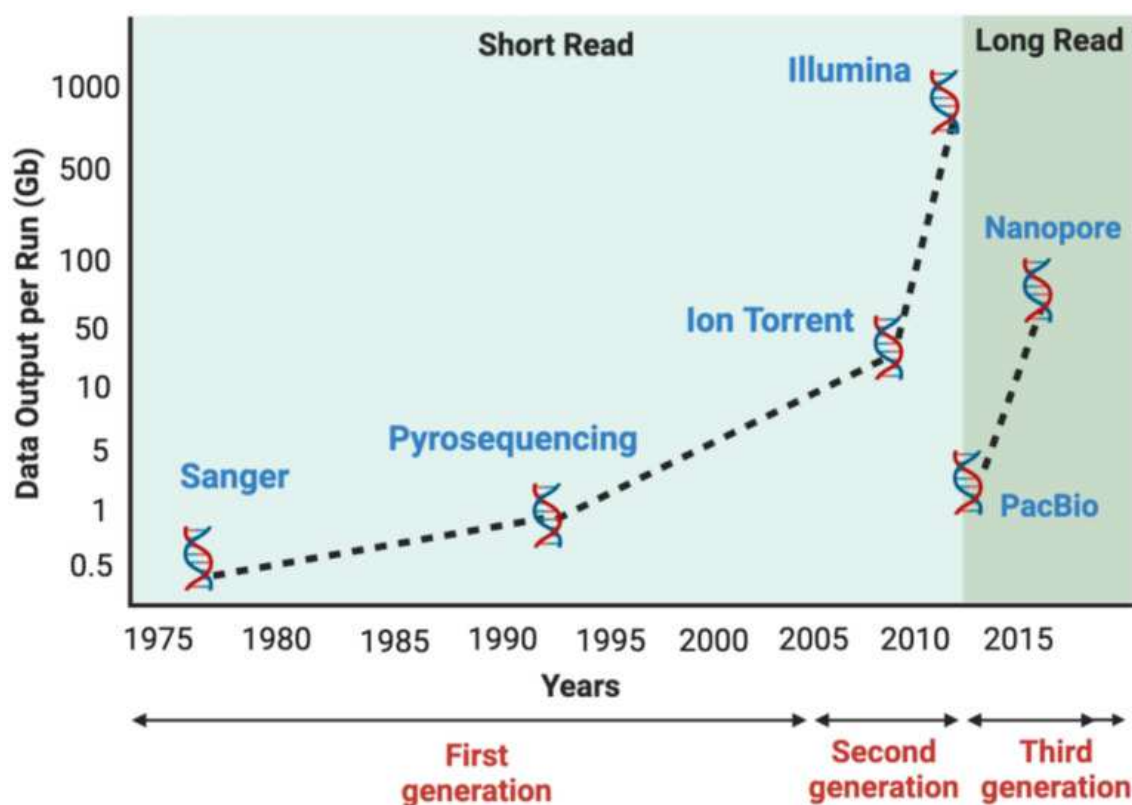
Dessa forma, em meados de 2014, a empresa *Oxford Nanopore Technologies* anunciou o desenvolvimento de outra técnica que sequenciava o genoma completo por meio da detecção da sequência de uma fita simples de DNA por sinais elétricos gerados pela passagem de cada fragmento por poros localizados em uma membrana condutora, gerando leituras superiores a 10kb (Amarasinghe *et al.*, 2020). Os instrumentos são agora cada vez mais automatizados e o processamento de maiores quantidades de amostras pode ser realizado. A vantagem destas tecnologias (MinION GridION e PromethION) é o custo reduzido (MinION tem valor aproximado de 1.000 dólares), a portabilidade e a análise em tempo real (MacKenzie; Argyropoulos, 2023).

Nestas últimas novas plataformas, a utilização de poros nanométricos dispostos em uma membrana é orientada por tensão e permitem a passagem de fitas simples de DNA por poros que funcionam como biossensores (Fu *et al.*, 2020). Os nanoporos podem ser proteínas biológicas ou sintéticas ou a combinação híbrida de ambos, que permitem a passagem de corrente elétrica (tensão positiva superior a 80 mV) de maneira constante entre o lado cis negativo da membrana e o lado trans positivo (Deamer; Akeson; Branton, 2016). No sistema, cada base nitrogenada ocasiona uma interrupção característica da corrente elétrica, que é captada por sensores e transformada em gráficos pelo *software* do próprio equipamento (Shi; Friedman; Baker, 2017). A discriminação e identificação das nucleobases individuais é relacionada com os sinais-ruídos ocasionadas pela passagem da base pelo nanopore. A grande vantagem dessa tecnologia é que consegue liberar leituras de até 23kb (Deschamps *et al.*, 2018), e dessa forma, revolucionou também as técnicas de bioinformática para realizar a análise e melhorar a precisão destes dados (Wang, Y. *et al.*, 2021). Os dados encontrados são depositados nos bancos de dados genômicos. Entre os bancos principais vinculados ao NCBI (*National Center for Biotechnology Information*): SRA (Sequence Read Archive Data), onde os dados

brutos podem ser disponibilizados, GEO DataSets (Gene Expression Omnibus, onde são depositados conjunto de dados de expressão gênica e GEO Profiles (Medicine, 2023).

Com a evolução da tecnologia (Figura 4), o sequenciamento por nanoporos tornou-se uma ferramenta importante e com facilidade de utilização, que permite múltiplas perspectivas sobre a propagação de micro-organismos, sua evolução e monitoramento ambiental de bactérias resistentes (Lobiuc *et al.*, 2023).

Figura 4 - Evolução dos métodos de sequenciamento genômico. Desenvolvimento e tecnologias de sequenciamento desenvolvidas.



Fonte: Satam *et al.*, 2023. Sanger: Método de Sanger, Pyrosequencing: pirosequenciamento, Ion Torrent: detecção e incorporação de nucleotídeos por mudanças no pH causadas pela liberação de íons de hidrogênio, Illumina: Usa nucleotídeos marcados com fluoróforos, Sequências longas: Nanopore: utiliza poros nanoscópicos através dos quais as moléculas de DNA são passadas, PacBio: Pacific Biosciences - utiliza a tecnologia SMRT (*Single Molecule Real-Time*), que sequencia DNA em tempo real através da observação da incorporação de nucleotídeos fluorescentes por uma DNA polimerase.

O sequenciamento de leitura longa (Oxford Nanopore) (Zhao *et al.*, 2023) vem sendo utilizado para desvendar a secreção de proteínas em conjugação bacteriana (Valenzuela-Gómez *et al.*, 2023), na translocação de genes *bla*_{CTX-M} presentes em elementos genéticos móveis (Bird

et al., 2022) e na avaliação de diversas β -lactamases em ambientes clínicos (Finton *et al.*, 2020). Importante destacar que em sua maioria, os estudos contemplam resultados com *EC* (Greig *et al.*, 2018).

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2210 **CAPÍTULO 2**

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Nanopore reveals effects of metal compounds on horizontal gene transfer in simulated food environments

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Abstract: A aquisição de plasmídeos de resistência via recombinação gênica permanece pouco compreendida no ecossistema selvagem e na interface de *habitats* como na indústria alimentícia. Nossa abordagem envolveu a identificação de *hotspots* potencializadores da conjugação bacteriana entre *Salmonella* Heidelberg (SH) e *Escherichia coli* J53 Az^R (EC) e a análise de medidas que possam reduzir esse processo de microevolução. A proposta contemplou a análise do genoma completo de bactérias transconjugantes para verificar a interferência dos coprodutos de origem animal *chicken juice* (CJ) e soro de leite (SL), e em paralelo, o uso de complexo de cobre bruto (DRI12) e nanoestruturado em carreador lipídico (NLCDRI12) como mitigadores desse processo. A identificação do gene *bla*_{TEM} nos transconjugantes nos motivou à análise fenotípica de frequência de transconjugantes resistentes à ampicilina (TRA) em cada tratamento aliada a verificação genética sintênica. Detectamos um efeito potencializador do SL e inibidor de NLCDRI12 na transferência horizontal dos plasmídeos. Todavia a autenticidade sintênica de IncI1_1_Alpha na conjugação com NLCDRI12, garantiu uma elevada frequência de conjugação (5-6 logUFC/mL) em comparação aos tratamentos com DRI12 que interferiram na deleção ou trocas de regiões de similaridade de IncI1_1_Alpha promovendo contagens de transconjugantes mais baixas (1-2 logUFC/mL). Compostos de cobre e NLCDRI12 associada a cobre promoveram impactos positivos distintos no controle da transferência de plasmídeos e na frequência de linhagens transconjugantes resistentes a ampicilina em ambientes desafiadores com a presença de coprodutos da indústria de alimentos.

Keywords: Análise de sintenia, Cobre, Conjugação bacteriana, nanocarreadores, patógenos.

1. Introdução

Na era da exploração genômica, o advento do sequenciamento do genoma completo (WGS) marcou o início da compreensão da dinâmica das comunidades microbianas ¹. Dentro dos complexos mecanismos apresentados nessa temática, as investigações ocasionadas pelo impacto da transferência horizontal de genes (THG) e a combinação de inibidores contra a transferência de elementos genéticos móveis evidenciam uma estratégia nova, viável e promissora para minimizar os altos riscos relacionados a microevolução de micro-organismos quanto à resistência aos antimicrobianos em ambientes industriais ²⁻⁴.

A conjugação é um mecanismo dinâmico para a troca rápida de material genético que promove a adaptabilidade e a resiliência das populações microbianas, o que preocupa as autoridades sanitárias quanto a disseminação e descontrole da resistência aos antimicrobianos em escala mundial ⁵. Estudos demonstraram que múltiplos compostos são capazes de suprimir a conjugação bacteriana e estratégias alternativas para a inibição deste fenômeno têm adotado abordagens focadas em componentes cruciais da maquinaria de secreção ⁵⁻⁸. A capacidade de decifrar a interferência de compostos alternativos aos antimicrobianos utilizando como ferramenta a genômica na sua totalidade pelo WGS, não melhora somente a compreensão da evolução microbiana como também traduz conhecimentos sobre a complexa interação entre fatores ambientais e o processo de THG.

Os compostos metálicos foram reconhecidos pela sua capacidade de modular os processos biológicos, promovendo efeitos de co-seleção e facilidade na promoção da diversidade gênica ao longo do tempo ⁹. Entretanto, o entendimento de como compostos metálicos com diferentes estruturas químicas e conformações associados às mudanças ambientais ¹⁰ podem influenciar e regular a THG ainda é pouco explorada ¹¹. A interação entre compostos metálicos e o seu impacto na THG e saúde única continuam a ser um assunto a explorar, principalmente em micro-organismos resistentes aos antimicrobianos ¹². A ampla gama de informações fornecidas pelo sequenciamento completo do genoma (WGS) nos permite compreender de maneira abrangente como os nanocompostos e compostos metálicos afetam o processo de THG.

Nosso objetivo foi apresentar novas perspectivas sobre a relação entre compostos metálicos inovadores a base de cobre, na forma nanoencapsulado ou não encapsulado, no

processo de transferência horizontal de genes na presença de coprodutos (SL e CJ) mimetizadores das condições de indústria de alimentos de origem animal.

2. Material e Métodos

2.1 Desenho do Estudo

O estudo utilizou uma estirpe de *S. Heidelberg* (SH) como bactéria doadora e *Escherichia coli* J53 Az^R (EC), como receptora nos ensaios de conjugação bacteriana. SH foi fenotipicamente caracterizada como multirresistente a diferentes classes de antimicrobianos em estudo prévio, incluindo β -lactâmicos, sulfonamidas e tetraciclinas ¹³.

Ambas as estirpes foram submetidas a ensaios de conjugação sob condições distintas: sem tratamento, na presença de 5% de soro de leite (SL), na presença de 5% de *chicken juice* (CJ), com complexo de cobre DRI12 e nanocarreador lipídico associado ao cobre NLCDRI12. Os transconjugantes foram submetidos a análise de sequenciamento do genoma completo para identificação dos plasmídeos transferidos. A constatação plasmidial do gene *bla*_{TEM} instigou a análise fenotípica da frequência de *Escherichia coli* transconjugante (ECTs) resistentes a ampicilina nas distintas condições aliada à confirmação via PCR convencional e à comparação via análise sintênica plasmidial.

2.2 Coprodutos da indústria de alimentos

A inclusão de coprodutos de origem animal, CJ e SL a 5%, foi realizada para simular as condições encontradas na indústria de processamento de alimentos de origem animal ^{14,15}. Para obtenção do CJ, carcaças de frangos congeladas obtidas comercialmente foram descongeladas em temperatura entre 25-30°C. O exsudato obtido foi submetido a três ciclos de centrifugação a 14.000 g por 10 minutos a 4 °C, seguido pela filtração do sobrenadante através de um filtro com poros de 0,22 μ M (Kasvi®) para remoção de micro-organismos contaminantes ¹⁶. Da mesma forma, o SL proveniente de queijo Minas artesanal (leite não pasteurizado de vacas da raça Jersey produzido por coagulação enzimática) foi preparado e centrifugado como descrito anteriormente. Tanto o CJ quanto o SL foram armazenados a -20 °C e verificados quanto a esterilidade microbiana por meio da incubação a 37 °C/24h para utilização nos procedimentos de experimentação.

2.3 Inibidores da conjugação (COINs)

Compostos a base de cobre foram utilizados como potenciais inibidores da conjugação em duas formas distintas: DRI12 e NLCDRI12.

O complexo DRI12 trata-se do composto metálico de cobre (II) [Cu(4-FH)(phen)(ClO₄)₂] (4-FH = 4-fluorophenoxyacetic acid hydrazide and phen = 1,10-phenanthroline), avaliado anteriormente quanto as propriedades físico-químicas, citotoxicidade, seletividade em células cancerígenas e não mutagenicidade em testes com *Drosophila melanogaster*, bem como sua atividade antibacteriana ^{17,18}.

O complexo NLCDRI12 compreende uma nanopartícula associada ao DRI12 preparada a 200 µM e solubilizada na fase oleosa, baseada no método de emulsificação/ultrasonicação descrito por Javed et al (2022) ¹⁹. A fase lipídica foi composta por palmitato de cetila (lipídio sólido) e triglicerídeos dos ácidos cáprico e caprílico (lipídios líquidos) (15 %, m/v) e como surfactante polaxamer (3%, m/v). Estes lipídios foram selecionados por serem tradicionalmente empregados no preparo destas formulações e não apresentarem nenhuma atividade bioativa. Para a formulação, a fase aquosa composta por polaxamer (3% m/v) foi aquecida na mesma temperatura da fase lipídica. Para a formação da pré-emulsão, a fase aquosa foi gotejada à fase lipídica, sob agitação de 10000 rpm, por 2 min, em agitador Ultra-Turrax (Ultra-Turrax® T18). A microemulsão formada foi submetida à sonicação por 15 minutos. Entre a produção da microemulsão até a produção da nanoemulsão o sistema foi ultrassonicados por 25 minutos em amplitude de 65%. A nanoemulsão formada foi arrefecida em banho de gelo, até alcançar 25°C, a fim de solidificar as nanopartículas formadas.

2.4 Determinação da concentração alvo dos COINs

Os valores de Concentração Inibitória Mínima (CIM) e a concentração alvo para os testes de conjugação com NLCDRI12 e DRI12 foram determinados para cepas de *SH* e *EC* utilizando o método de microdiluição em caldo ²⁰. Cinco concentrações diferentes foram testadas: 12,5; 6,25; 3,125; 1,56 e 0,78 µg/mL para NLCDRI12 e 125; 62,5; 31,25; 15,62 e 7,81 µg/mL para DRI12. Resumidamente, as suspensões bacterianas foram preparadas com uma concentração equivalente a 0,5 na escala de McFarland em solução salina estéril a 0,85% e inoculadas em microplacas contendo caldo Müller-Hinton com os compostos em diferentes concentrações, seguidas de incubação a 37°C por 18-24 horas. Os controles incluíram meio de cultura sem bactérias e meio de cultura com bactérias sem os compostos. As leituras foram

realizadas visualmente para determinar o MIC, definido como a menor concentração sem turbidez.

Concentrações abaixo da CIM foram submetidas a contagem bacteriana para verificar a não interferência no crescimento bacteriano entre os grupos de controle e as formulações. Aliquotas de 10 µL de cada concentração foram inoculadas em ágar triptona de soja (TSA), e as unidades formadoras de colônias foram contadas após o crescimento, expressas em $\log_{10}$, e submetidas a análise estatística para garantir a igualdade de crescimento entre os grupos. Todos os experimentos foram conduzidos com três réplicas e três repetições.

2.5 Conjugação bacteriana associada a inibidores e coprodutos da indústria de alimentos

A transferência horizontal de genes foi avaliada por meio do processo de conjugação bacteriana em meio líquido conforme protocolo proposto por Guidotti-Takeuchi et al (2023)²¹. A formulação com nanopartículas indicou padrões de estabilidade quanto ao tamanho médio obtido de $239,8 \pm 3,2$ nm, índice de polidispersão (PDI) de $0,124 \pm 0,014$ e potencial zeta equivalente a $-31,3 \pm 1,4$ mV. As estirpes *SH* (doadora) e *EC* (receptora) foram individualmente reativadas em caldo infusão cérebro e coração (BHI, Sigma-Aldrich®) a 37°C por aproximadamente 6hs e depois transferidas para TSA (Biokar®) e incubadas *overnight* a 37°C. Cinco colônias de cada estirpe (doadora e receptora) foram selecionadas após o crescimento e inoculadas separadamente em tubos Falcon estéreis contendo 3 mL de caldo Luria Bertani (LB-Neogen®) e posteriormente, incubadas a 37°C em agitador de placas orbital com rotação de 60 rpm durante cerca de três horas. Após a incubação, a absorbância foi mensurada ($D.O._{600nm}$) em leitor de microplacas (Perlong DNM-9602) para confirmar a entrada na fase exponencial ($D.O.$ entre 0,4 e 0,5).

Os diferentes grupos foram submetidos aos ensaios de conjugação (3h a 37°C a 20 rpm). Foram preparados em oito tratamentos e um controle, realizados em três réplicas e três

repetições, seguindo a proporção de 1:4 da cepa receptora em relação à doadora, em um volume final de 1mL, conforme a seguinte descrição:

1. Conjugação controle: 1:4 da cepa receptora em relação à doadora.
2. Conjugação com coproduto: 1:4 da cepa receptora em relação à doadora e 5% de (i) SL ou (ii) CJ.
3. Conjugação com inibidor: 1:4 da cepa receptora em relação à doadora acrescido à concentração alvo de (iii) DRI12 ou (iv) NLCDRI12.
4. Conjugação com inibidor associado ao coproduto SL ou CJ (5%): 1:4 da cepa receptora em relação à doadora, adicionados a 5% de SL ou CJ e COIN DRI12 ou NLCDRI12 nas concentrações alvo: (v) SL + DRI12; (vi) SL + NLCDRI12; (vii) CJ + DRI12; e (viii) CJ + NLCDRI12.

2.6 Sequenciamento genômico e processamento de dados brutos

A cepa doadora SH, a receptora EC e os transconjugantes de EC foram sequenciados utilizando Rapid Sequencing gDNA - barcoding kit (SQK-RBK004) com o sequenciador MinION, Oxford Nanopore Technologies (ONT) por 72h. As colônias foram inoculadas em caldo BHI e incubadas a 37° C *overnight*, seguida de extração de DNA bacteriano com o kit Wizard Genomic ADN Purification (Promega). Adicionalmente, entre 5 e 10ng de DNA de cada amostra foi usada para preparar a biblioteca genômica. Os dados da leitura bruta foram pré processados através de uma análise de qualidade inicial para verificar a cobertura de leitura e o comprimento da sequência. As leituras com a maior integridade e qualidade foram selecionadas para a montagem do genoma. O Filtlong v.0.2.1 (WICK, 2017) foi usado para filtrar as leituras brutas pelo corte de qualidade do Phred Score ($Q > 20$). As estatísticas de resumo e qualidade foram processadas pelo Nanoplot v.1.41.0 (DE COSTER, 2018) e os adaptadores localizados nas extremidades das leituras filtradas foram removidos com o Porechop v.0.2.4 (WICK, 2017, 2021).

2.6 Análise de Bioinformática e montagem do genoma

Raven v.1.8.0²² foi usado para montar os genomas com metodologia de novo, que é uma metodologia que oferece forte poder estatístico. A qualidade da montagem do genoma foi verificada com Quast v.5.2.0²³. A anotação de genes via Prokka v.1.14.6²⁴ foi realizada com um corte de 98% de identidade de sequência e um valor de $p=0.05$. O Staramr v.0.9.1²⁵ foi

utilizado para alinhar os genomas montados com as bases de dados ResFinder, PlasmidFinder and PointFinder para a detecção de genes de resistência e plasmídeos bacterianos. A base de dados PLSDB (Plasmid Database)²⁶ foi utilizada para verificar os plasmídeos encontrados com 95% ou mais de identidade e um valor de $p = 0.05$.

2.7 Análise de sintenia

A avaliação de rearranjos e inversões genômicas entre os plasmídeos identificados e o plasmídeo de referência foi realizada com a técnica de sintenia. Os contigs identificados como plasmídeos contendo as mesmas Incs que o plasmídeo de referência com o gene TEM-1 foi submetido à análise utilizando o Mauve versão 20150226 build 10²⁷. Esta análise permitiu a comparação das organizações genômicas entre os plasmídeos identificados e o plasmídeo de referência. Todas as sequências começaram no gene *repA*.

2.8 Análise fenotípica de transconjugantes para genes *bla*_{TEM-1}

A aquisição plasmidial do gene *bla*_{TEM} nas *Escherichia coli* transconjugantes (ECT) incitou a análise fenotípica quantitativa de ECTs em todos os tratamentos. Para isso, cepas doadoras e a receptora preparadas na D.O._{600nm} entre 0,4 e 0,5 foram submetidas separadamente a diluições decimais seriadas (10^{-1} a 10^{-8}) em solução salina a 0,85% (Êxodo Científica®) e 100 µL de cada uma das diluições foram adicionadas à superfície de ágar MacConkey (Biokar®) suplementado com os antibióticos ampicilina (Vetnil®) na concentração de 90 µg/mL (para SH) e azida sódica (Sigma-Aldrich®) na concentração de 160 µg/mL (EC) para expressar sua resistência fenotípica. As placas foram incubadas a 37°C por 48hs e realizada contagem das unidades formadoras de colônias (UFC).

A frequência de conjugação (FC) relacionada a resistência a ampicilina com potencial associação à expressão do gene *bla*_{TEM-1} foi determinada calculando-se a razão entre o número de UFC transconjugantes (T) e o número de receptores (R) no início do processo de conjugação (FC: média T/média R)²⁸. Após a quantificação, cinco colônias transconjugantes foram selecionadas de cada ensaio de conjugação, e submetidas à extração de DNA bacteriano com kit de extração Wizard Genomic Purification (Promega) e confirmadas por PCR convencional quanto a presença do gene *bla*_{TEM-1}. As colônias foram inoculadas em caldo BHI e incubadas a 37°C *overnight* e posteriormente realizada a extração de DNA bacteriano com o kit Wizard Genomic ADN Purification (Promega). A PCR foi realizada em um volume final de 25 µL,

utilizando o kit master mix da Promega, sob as condições de amplificação e *primers* discriminados na Tabela 1.

Tabela 1. Reação em cadeia da polimerase para o gene *bla*_{TEM-1}

Gene	Sequência 5'→ 3'	Condições PCR (°C)	DNA (ng)	Reference
<i>bla</i> _{TEM}	CAGCGGTAAGATCCTTGAGA ACTCCCCGTCGTGTAGATAA	95°C – 5 min; 50°C – 45 sec; 72°C 90 sec; 72°C 10 min	10	²⁹

2.9 Análise estatística

Foram empregadas avaliações com estatística descritiva para a determinação do perfil de transferência plasmidial. Para comparação entre os tratamentos envolvendo compostos metálicos e coprodutos, a normalidade dos resultados foi verificada após a transformação dos dados para log₁₀ e foi realizado o teste ANOVA para comparar três ou mais variáveis. Todos os cálculos foram realizados utilizando o software GraphPad Prism 8.0.1, com um intervalo de confiança de 95%.

3.0 Resultados

3.1 Concentração alvo dos compostos de cobre

Ambos os compostos de cobre foram efetivos para o controle tanto de EC quanto de SH nas mesmas concentrações para ambos os micro-organismos, equivalentes a CIM de 12,5 e 125 µg/mL para NLCDRI12 e DRI12, respectivamente, o que deixa evidente o feito antimicrobiano de ambos. As demais concentrações avaliadas não alteraram o inóculo bacteriano de SH e EC ($p>0,05$), sendo consideradas ideais para os testes de inibição da conjugação bacteriana, por não influenciar no crescimento bacteriano de ambas as linhagens. Diante disso, definimos como

concentrações alvo de NLCDRI12 e DRI12 para os ensaios de conjugação os valores de 6,25 $\mu\text{g/mL}$ e 62,5 $\mu\text{g/mL}$, respectivamente (Figura 1).

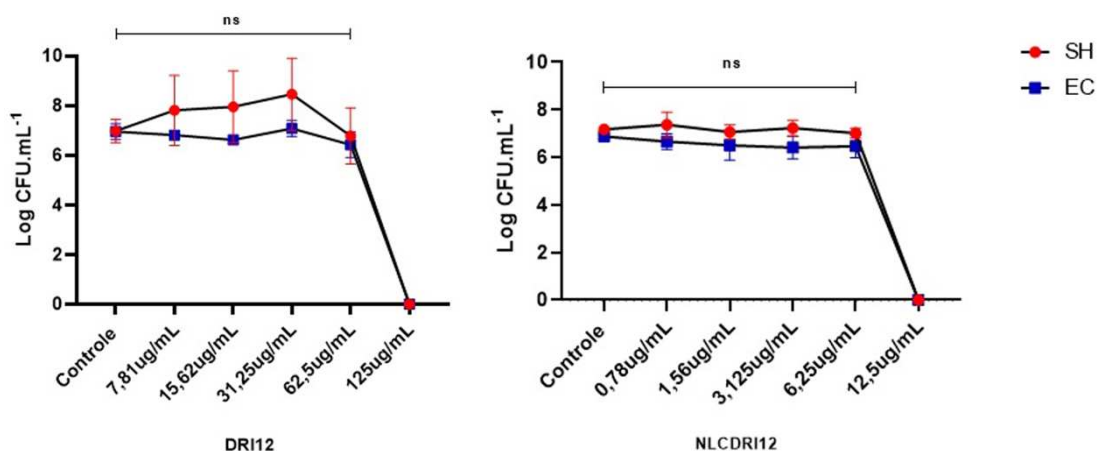


Figura 1. Valores médios e desvio padrão das contagens de SH e EC na presença de cinco diferentes concentrações e ausência (controle) dos compostos DRI12 e NLCDRI12. ns (não significativo): $p > 0,05$ usando teste *One way ANOVA*.

3.2 Sequenciamento genômico das cepas pré-tratamentos

A cepa receptora EC J53 Az^R, demonstrou-se susceptível e sem a presença de genes de resistência como esperado, com ausência de plasmídeos em seu genoma. Um total 28 genes de resistência foram revelados no cromossomo (Utg2004) de SH, incluindo genes inatos e adquiridos (TABELA SUPLEMENTAR). Quanto ao material genético móvel, observou-se a presença de três plasmídeos conjugativos IncC (anteriormente denominado IncA/C2_1) (Utg2000), IncX1_1 (Utg2002) e IncI1_1_Alpha (UTg2006), dos quais somente IncX1_1 não carregava genes de resistência.

Os genes de resistência *sul2*, *tetA*, *bla*_{TEM-1} e ANT (3') -IIa foram encontrados tanto em Utg2000 quanto em Utg2006, associados, respectivamente, à resistência a sulfonamidas, tetraciclina, ampicilina e estreptomicina.

3.3 Caracterização de Utg2006, Utg2000 e Utg2002

O desenho esquemático dos três plasmídeos, incluindo a disposição dos genes está descrito na Figura 2. Em Utg2006 foram identificados genes e proteínas responsáveis pela reparação, proteção e recombinação do DNA, que são cruciais para manter a estabilidade do plasmídeo na população bacteriana: (*umuC* e *umuD* -proteínas UmuC e UmuD, são componentes chaves do sistema SOS), proteína *klcA* (Proteína anti-restrição KlcA0, proteína associada a plasmídeos com função de suprimir a expressão de genes deletérios a célula

hospedeira), Noc (Nucleoid occlusion protein, com papel na divisão celular), *psiB* (Proteína PsiB, essencial para modular a resposta SOS), *rpnD* (nuclease promotor de recombinação RpnD, enzima importante para criar quebras controladas no DNA que permite a recombinação genética), *insK* (transposase, enzima que catalisa o movimento dos elementos de inserção para IS150).

Diretamente relacionados aos processos de conjugação foi encontrada a enzima *ais* (Lipopolysaccharide core heptose(II)-phosphate phosphatase), envolvida na modificação, manutenção da integridade e funcionalidade da membrana externa bacteriana, proteína *TcpT* (toxin coregulated pilus biosynthesis protein T), determinante na biossíntese do pili, proteína *RfaH* (proteína de antiterminação da transcrição) que afeta a expressão de operons envolvidos nos mecanismos de virulência e na resistência e *repA* (proteína de iniciação da replicação), essencial para a replicação do plasmídeo de maneira precisa e eficiente.

Para manutenção das funções plasmidiais foram encontradas a enzima *XerC* (Tyrosine recombinase *XerC*) envolvida na resolução de multímeros de plasmídeos em monômeros, que garante a adequada recepção da cópia de DNA e os genes *ANT (3")-IIa* (aminoglicosídeo nucleotidiltransferase) e *bla_{TEM1B}* (Beta-lactamase TEM) alvo desse estudo, determinante para a resistência aos antibióticos beta-lactâmicos, penicilinas e cefalosporinas de espectro restrito.

No plasmídeo *Utg2002* estavam presentes importantes proteínas que são componentes essenciais do sistema de secreção tipo IV (*VIRB1*, *VIRB4*, *VIR B9* e *VIRD4*), denominadas de sistemas minimizados da maquinaria de conjugação e que tem papel central na estabilização do T4SS e do recrutamento e acoplamento de substratos no canal de translocação e passagem de DNA. Além disso, este plasmídeo exibiu a presença do gene *RelE*, uma toxina que, por meio de um mecanismo ainda não elucidado, causa uma inibição significativa do crescimento celular e da formação de colônias, durante processos de escassez nutricional de aminoácidos.

Em *Utg2000* foi identificada a principal enzima envolvida no sistema microbiano de desintoxicação do mercúrio denominada mercúrio redutase (*MerA*), a qual desempenha a função de catalisar a conversão do Hg (II) inorgânico em Hg (0) gasoso. Além da *MerA*, proteínas de membrana como *MerP* e *MerT* realizam o transporte de Hg para o citoplasma, onde *MerA* o reduz para o estado volátil. A expressão global faz-se pela presença de operons *MerR* que são responsáveis pela repressão ou ativação de transcrição na ausência ou presença do metal pesado. A importância deste achado é enfatizada pelo fato de que os micro-organismos que apresentam resistência ao mercúrio tendem a ser selecionados e tornam-se mais abundantes em ambientes contaminados com este metal.

3.4 Transferência horizontal de genes e a interferência de coprodutos presentes em alimentos

Dos oito tratamento pré-definidos, apenas seis permitiram a análise robusta dos resultados de sequenciamento, excetuando as ECT tratadas somente com os coprodutos SL e CJ, assim como o controle. Observamos que independentemente do tratamento, a mobilidade do plasmídeo IncI1_1_Alpha (Utg2006) foi universal. Na presença de DRI12 e SL identificamos a mobilidade de todos os plasmídeos elencados na cepa doadora (Utg2000, Utg2002 e Utg2006). Nos demais tratamentos houve somente a transferência de Utg2006. Isso indica um efeito potencializador do SL e inibidor de NLCDRI12 na THG, uma vez que em todas as amostras tratadas com NLCDRI12 houve a passagem somente de Utg2006 nos transconjugantes. E, mesmo na presença de SL, o tratamento NLCDRI12 inibiu de forma mais eficiente a THG, superando os resultados com DRI (Figura 3).

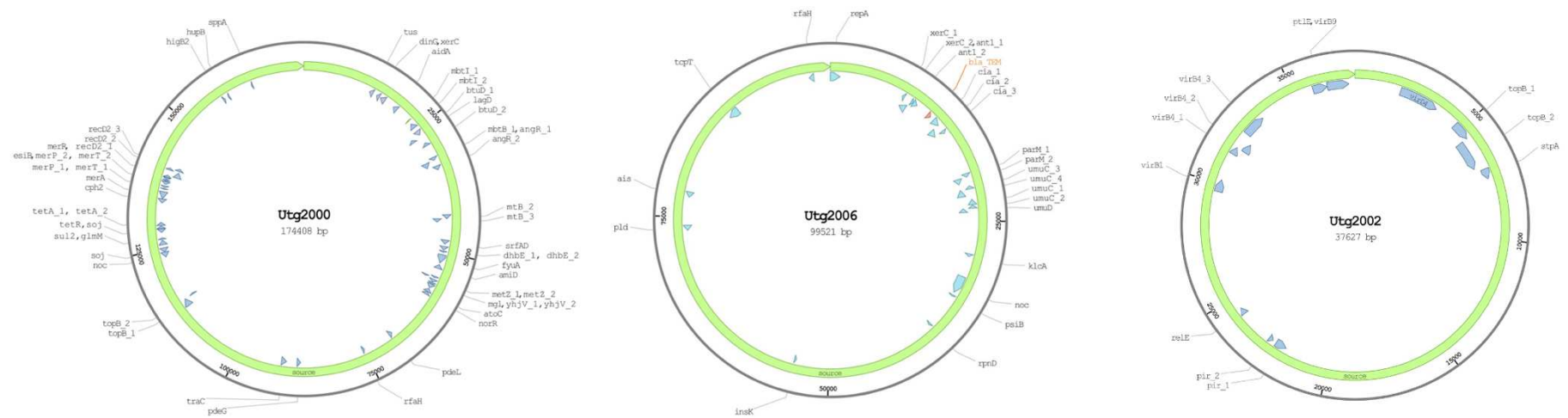


Figura 2. Mapas de plasmídeos presentes em SH: principais componentes proteicos e genes de resistência a antibióticos de Utg2006 (IncI1_1_Alpha), Utg 2002 (IncX1_1) e Utg 2000 (IncC).

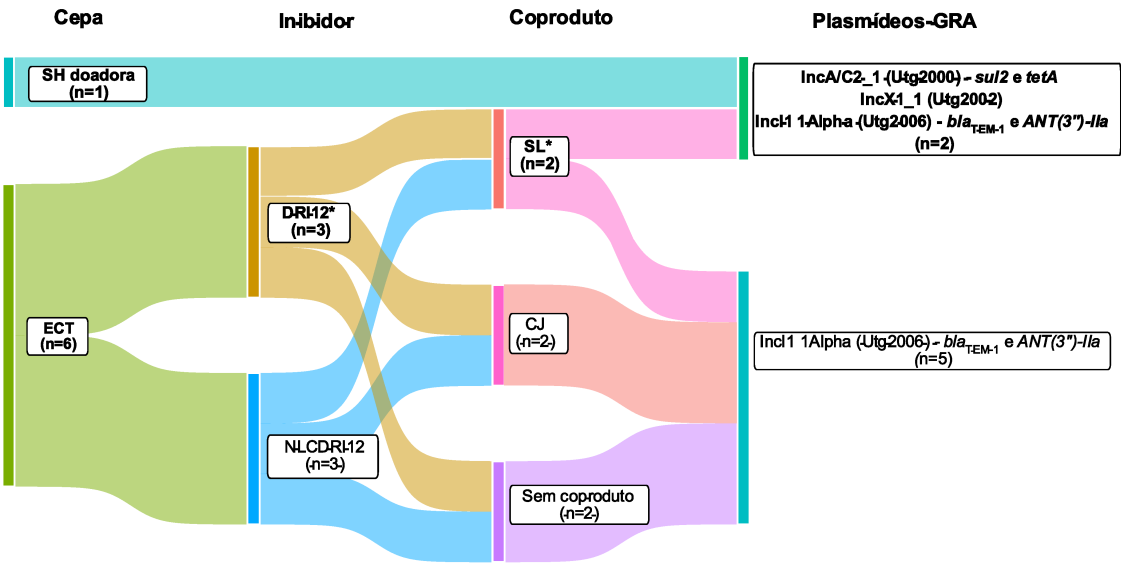


Figura 3. Transferência horizontal de genes e genes de resistência a antibióticos em transconjugantes de EC na presença e ausência de inibidores e de coprodutos da indústria de alimentos.

ECT: *Escherichia coli* J53 Az^R transconjugante, GRA: genes de resistência a antibiótico.

3.5 Classificação sintênica

A análise de sintenia foi aplicada para o plasmídeo IncI1_1_Alpha devido a sua passagem para todas *E. coli* transconjugantes (ECTs). O ensaio permitiu caracterizar a estrutura do plasmídeo em cada tratamento, considerando as diferentes regiões de similaridade. Uma linha de conexão foi desenhada por bloco colinear e as duas regiões contiguamente coloridas foram determinadas como blocos que podem ser comparados à sequência principal homóloga. Na Figura 4, a estrutura de rearranjo do plasmídeo indicou a presença de duas regiões de similaridade distintas, distinguíveis pelas cores diferenciadas em cada amostra, conforme

identificado em SH. As variações de sintenia de IncI1_1_Alpha classificou os plasmídeos de ECTs em três *clusters* principais (Figura 4 A, B e C).

O primeiro padrão (Figura 4A) compreende ao mesmo identificado na cepa doadora, e inclui os plasmídeos de ECTs submetidas ao tratamento com NLCDRI12 + SL e NLCDRI12 + CJ. Esse grupo contempla padrões de blocos conservados do plasmídeo com as mesmas regiões da cepa doadora (SH) evidenciadas pela mesma cor e disposição dos blocos de colinearidade, demonstrando forte sintenia com o plasmídeo de SH. O segundo grupo (Figura 4B) é evidenciado pelos plasmídeos de ECTs tratados com NLCDRI12, DRI12 e DRI12 + SL. Nesse padrão identificamos a reorganização/troca de posição das regiões de similaridade quando comparados a cepa doadora (SH).



Figura 4. Comparação de blocos colineares identificados no plasmídeo IncI1_1_Alpha e entre os transconjugantes de *E. coli* após eventos de transferência horizontal de genes. Blocos das mesmas cores representam correspondências de nucleotídeos homólogos. A: SH, NLCDRI12 SL e NLCDRI12 CJ. B: NLCDRI12 C, DRI12 C e DRI12 SL. C: DRI12 CJ.

O último modelo (Figura 4C) derivado do plasmídeo de ECT tratado com DRI12 + CJ revelou além da troca de posição das duas regiões de similaridade, deleção significativa de segmentos em um dos blocos, demonstrando baixa identidade aliada à elevada variação sintênica em relação ao plasmídeo de SH.

3.6 Avaliação fenotípica de resistência a ampicilina em *E. coli* transconjugantes (ECTs)

A partir das análises do sequenciamento do genoma de ECTs e verificação da transferência do plasmídeo Utg2006, e consequentemente do gene *bla*_{TEM-1}, determinamos fenotipicamente o número de ECTs em cada tratamento, aliada à resposta à informação sintênica identificada. Vale ressaltar que detectamos a presença de *bla*_{TEM-1} em todos os tratamentos e cepas submetidas à PCR.

As frequências de conjugação em todos os tratamentos com DRI12 (Figura 5) sofreram reduções significativas ($p=0,0001$) de 5,94 para 2,32 logUFC/mL na ausência de coprodutos (controle), 6,40 para 1,47 logUFC/mL na presença de SL e 6,52 para 2,04 UFC/mL na presença de CJ. Essa redução mantém a coerência com o rearranjo e deleção identificados na análise de sintenia dessas amostras.

Paradoxalmente, o uso de NLCDRI12 (concentração de 6,25µg/mL) não reduziu de maneira expressiva o número de transconjugantes (4,67 logUFC/mL somente NLCDRI12; 6,17 logUFC/mL associado ao SL e 5,01 logUFC/mL com CJ) ($p>0,05$) quando comparado ao DRI12. Todavia, tanto no tratamento NLCDRI12 + SL quanto NLCDRI12 + CJ obtivemos autenticidade sintênica ao plasmídeo de SH, coerente à expressão do elevado número de transconjugantes. A identificação do rearranjo sintênico no tratamento somente com NLCDRI12 justifica a menor contagem fenotípica entre esses três tratamentos, significativamente menor ($p<0,0001$) quando comparada ao NLCDRI12 + SL (diferença de 1,5 logUFC/mL).

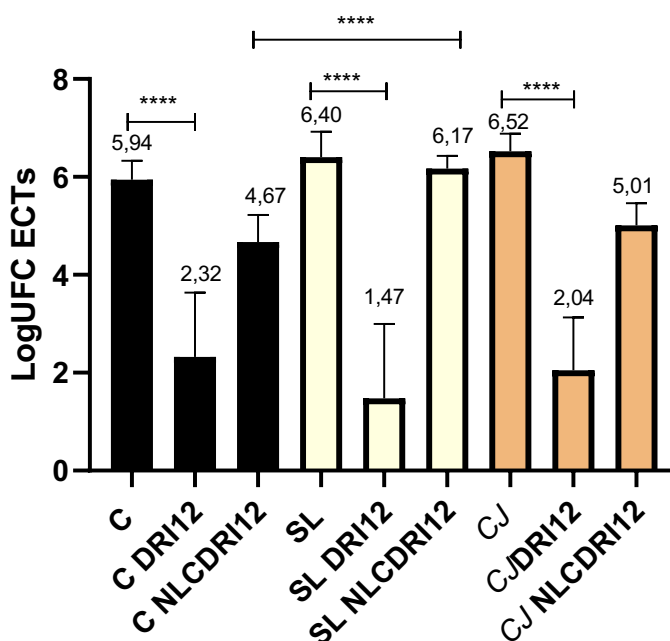


Figura 5. Avaliação fenotípica quantitativa de EC transconjugantes para resistência a ampicilina obtidas na presença e ausência de inibidores e coprodutos. C: controle. **** p < 0,0001, pelo teste *one way* ANOVA. ECTs: *Escherichia coli* transconjugantes.

3.7 Discussão

Para entender a dinâmica dos COINs, avaliamos neste estudo, um composto inovador derivado do cobre, denominado DRI12, sob duas formas de ação distintas (complexo de cobre e nanocarreador lipídico de cobre) como medida mitigadora da THG. Este composto apresentou efeito antimicrobiano (CIM) contra a cepa selvagem de SH e a cepa receptora na concentração de 125µg/mL e CIM dez vez menor para o mesmo composto nanoencapsulado. Particularmente, o DRI12 caracteriza-se pela ação do íon cobre (II) coordenado de modo bidentado pela hidrazida e fenantrolina. O perclorato, associado a esta estrutura é um contra íon e quando em solução se dissocia, de maneira que uma estrutura plana (hidrazida-cobre-fenantrolina) é formada, permitindo a sua intercalação com estrutura do DNA ³⁰.

Com uma ampla gama de utilização em diversas aplicações industriais ³¹ e agrícolas ³² tornou-se crucial entender o papel de cobre no controle microbiano ¹¹. Estudos científicos têm indicado a dualidade de ação ^{9,33} de diferentes compostos de cobre na co seleção de genes de resistência a antimicrobianos com metais ³⁴ e a indução da transferência de DNA plasmidial ³⁵. A sua aplicabilidade tem sido abordada também em formas promissoras como nanopartículas de cobre na produção de diferentes espécies como aves, pecuária e peixes ³⁶. Estudos prévios de DRI12 demonstram alta potência e ação sobre micro-organismos patogênicos, alta seletividade e baixa toxicidade, o que o torna um composto promissor para combater o câncer ¹⁸ e micro-organismos ³⁷. Aliado a isto, a encapsulação de DRI12 possibilitou a diminuição da concentração inibitória mínima e ofereceu ao complexo uma barreira física que prolonga a sua bioatividade, propiciando uma interação da nanopartícula/membrana bacteriana, aliado a vantagem que NLCDRI12 é um veículo passível de ser administrado em diferentes vias (*patente em progresso*).

Além disso, os complexos de cobre possuem eficácia em ambientes industriais e hospitalares ³⁸ por exibirem propriedades bactericidas ao serem incluídos em superfícies ³⁹, o que demonstra a diversidade de aplicação para o controle de patógenos, conforme identificamos também em nosso estudo. Entretanto, a ação do cobre na THG ainda é pouco explorada ¹¹, e em grande parte os estudos devem ser antecipados por relação de custo, caracterização e estudo

da molécula ¹⁸, avaliação de suas propriedades toxicológicas ⁴⁰, de estabilidade e interferência microbiológica ⁴¹.

Uma série de compostos tem sido avaliados como inibidores de conjugação e tem como alvo a inibição de plasmídeos ⁷. Essa abordagem amplifica-se como promissora pois um único COIN pode ser capaz de inibir mais de um grupo de incompatibilidade em ambientes de diferentes graus de seleção. Estimou-se que mais de 50% dos plasmídeos podem ser transferidos por conjugação ⁴² e somente na família *Enterobacteriaceae* já foram identificados 28 grupos de incompatibilidade. Entre esses, os plasmídeos das famílias IncC, IncX1_1 e IncI1_1_Alpha, encontrados na cepa *S. Heidelberg* utilizada nesse estudo como doadora, se destacam por estarem presentes em diferentes patógenos ⁴³. Os plasmídeos IncC, são comuns em cepas de *Salmonella* ⁴⁴ e cepas comensais e patogênicas de *E. coli* ⁴⁵, e podem carrear múltiplos genes de resistência a antibióticos, como β -lactâmicos, aminoglicosídeos, cefalosporinas, sulfonamidas e tetraciclina ⁴⁶. São geralmente grandes plasmídeos (> 100kb) que possuem alta capacidade de conjugação, permitindo a transferência entre diferentes espécies bacterianas ⁴⁷. Em comparação, os plasmídeos IncX1_1 são menores, com tamanhos entre 30-50kb, e têm um espectro de hospedeiros mais restrito em relação aos plasmídeos IncC, mas também apresentam alta eficiência de conjugação. Eles contêm elementos de inserção IS e transposons que facilitam a movimentação de genes de resistência ⁴⁸. Estes plasmídeos já foram isolados em cepas de *Salmonella* ⁴⁹ na produção de suínos, representando um alerta quanto ao elo final do processamento e abate destes animais ⁵⁰. Os plasmídeos IncI1_1_Alpha podem apresentar um tamanho intermediário entre 80-100kb e estão frequentemente associados à resistência e disseminação de genes de resistência a β -lactâmicos ⁵¹, aminoglicosídeos, tetraciclina e trimetopim-sulfametoxazol ⁴³, incluindo sistemas de produção avícolas ⁵².

A integração de conhecimentos sobre compostos de cobre, nanotecnologia e a compreensão global de mecanismos de THG utilizando-se de ferramentas modernas como WGS ⁵³ determina que a dinâmica das trocas gênicas está distante da neutralidade evolutiva e padrões distintos de repasse gênico estão associados a agentes extrínsecos não inerentes a maquinaria da conjugação bacteriana ⁵⁴. Ênfase maior tem sido dada a disseminação de genes de resistência ligadas ao aumento na administração e utilização de antibióticos ⁵⁵, superestimando os efeitos ocasionados por determinantes externos e subestimando as condições críticas de fatores não antibióticos, condições ambientais ou de outros compostos ⁵⁶. A interferência de níveis de nutrientes sobre o processo de conjugação tem sido relatada em diferentes ambientes ^{57,58} e estudos demonstram a interferência de fatores presentes em matrizes alimentares como um importante modulador da frequência de conjugação em produtos lácteos

e cárneos ⁵⁹⁻⁶¹. Particularmente, neste estudo, a presença de soro de leite associada ao DRI12 (Figura 3) permitiu a passagem dos três plasmídeos presentes na doadora. Os derivados lácteos tem sido relatados como potencializadores desse processo ⁶², com aumento de até dez vezes na transferência conjugativa ⁶³. A maioria dos estudos demonstraram que as FC são maiores na fase exponencial de crescimento da cultura ^{64,65} e transmissibilidade de genes de resistência maximizados em ambientes ricos em carboidratos e nutrientes disponíveis ⁴.

Neste estudo, a utilização de WGS propiciou aprofundar os conhecimentos da translocação de genes, demonstrando de maneira fiel os resultados presentes na célula. Os genes encontrados nos plasmídeos IncC (anteriormente denominado IncA/C2_1), *sul2* e *tetA* e IncI1_1_Alpha (ANT(3'')-IIa e *bla*_{TEM-1}) são notáveis por sua influência disseminada ⁶⁶ na família *Enterobacteriaceae* conferindo aos micro-organismos a capacidade de evadir-se da ação de agentes antimicrobianos ⁶⁷.

Baseados pela busca de inibidores potenciais frente a THG, as alterações ocasionadas pelo uso de NLCDRI12 versus DRI12 demonstraram dois mecanismos distintos de ação, mas igualmente importantes. A comparação entre a ação destes compostos possibilita não somente discriminar os grupos de incompatibilidade alvo, mas também podem fornecer informações sobre a epidemiologia ⁶⁸, a disseminação da resistência e a evolução do plasmídeo em determinada matriz. De forma específica, o grupo de incompatibilidade I1 (IncI1), carreador de *ant1* ANT (3'') -IIa e *bla*_{TEM-1B} neste trabalho, foi transferido para todas as ECTs, independentemente do tratamento utilizado. Este grupo chave de plasmídeos ⁶⁹ é bastante comum em *Salmonella* spp. de origem animal alimentar e de pacientes humanos ⁷⁰ e sua persistência é justificada devido aos baixos custos metabólicos conferidos às cepas receptoras e enfatizados nesse trabalho pela disseminação facilitada, mesmo na presença de inibidores.

A versatilidade na migração de IncI1_1_Alpha pode ser explicada pela codificação de dois pili sexuais, um rígido e espesso e outro fino e flexível, que garante a associação do par de acasalamento ⁷¹ e a estabilização para a transferência plasmidial em ambientes líquidos ⁷².

As células bacterianas dependem do controle de atividades e eventos celulares para manter a homeostasia e atividades fisiológicas normais e a presença de DRI12 determinou a alteração nesse metabolismo, expresso pela redução das taxas de frequência de conjugação de forma significativa juntamente com o rearranjo e deleção de regiões plasmidiais, como evidenciado pela técnica de sentença (Figura 4 B e C). Apesar disso, a associação com um ambiente rico em soro de leite e excesso de cobre, pode ter desencadeado respostas ao estresse ⁷³, o que permitiu a passagem dos três plasmídeos presentes na bactéria doadora. Em concordância, IncI1 e IncA são plasmídeos determinantes para a resistência a β -lactâmicos e

não há desvantagens para a célula bacteriana quanto ao custo de aptidão da aquisição de ambos os plasmídeos ^{72,74}.

A análise destas diferenças estruturais entre os micro-organismos é uma etapa crucial em diversos estudos de genômica comparativa, e a visualização de regiões genéticas intactas, interrompidas ou duplicadas ⁷⁵ possibilita aperfeiçoar o conhecimento sobre as funções desempenhadas pelos plasmídeos e que são importantes no estabelecimento clonal inicial e na manutenção endêmica na população hospedeira ⁷⁶.

O sinergismo presente em NLCDRI12 controlou a passagem plasmidial seletivamente, que pode ser justificada pela maior interação física da partícula com a membrana plasmática do patógeno, gerando ação sobre a montagem para iniciar a conjugação ⁷⁷. Entretanto, a contagens de ECTs em NLCDRI12 foi mantida, principalmente pela manutenção das regiões de colinearidade presentes na célula doadora (Figura 4 A), exceto pela redução no grupo controle de NLCDRI12 (4,67LogUFC/ml) que teve a FC reduzida em 1,5 logUFC/mL associada a trocas de regiões colineares quando comparado a SL NLCDRI12 (6,17LogUFC) (Figura 5).

As propriedades inerentes das NLC permitem a liberação controlada de cobre ⁷⁸, contribuem para a versatilidade de uso de compostos metálicos e são alternativas viáveis contra patógenos de importância em saúde pública ⁷⁹. Ademais, a capacidade de superar um ambiente com maior desafio de controle (presença de soro de leite) e o efeito prolongado de ação, podem ser explicadas pela natureza lipídica das NLCDRI12, resultando em liberação intermitente e interferência na THG ⁸⁰.

Apesar de não serem amplamente utilizados em estudos epidemiológicos, os rearranjos genéticos desempenham um papel crucial como força motriz na evolução dos plasmídeos. Recombinação, ganho e perda de fragmentos de DNA são processos-chave da evolução ⁸¹. A maior diversidade resultou do posicionamento, orientação e deleção de um bloco conservado avaliado pelo gráfico de sintenia. A partir de leituras longas do plasmídeo Utg2006 determinou que o rearranjo plasmidial ou o embaralhamento de segmentos não determinou alteração na montagem de *bla_{TEM-1}*, que foi confirmada pela sua identificação nos transconjugantes por PCR convencional e nem na manifestação fenotípica da resistência.

A presença de NLCDRI12 e DRI12 resultou em ilhas não sintênicas que podem resultar de uma combinação de rearranjos significativos, eventos de duplicação e aparecimento de genes específicos ⁸². O panorama regulatório é complexo e demonstra um alto grau de flexibilidade do genoma, com a identificação de regiões conservadas ao lado de *hotspots* de rearranjo. Em sua grande maioria, esses rearranjos são associados à recombinação de elementos de inserção (IS), demonstrando sua importância na plasticidade do genoma ⁸³. Particularmente no

plasmídeo Utg2006, IS150 estava presente, o que possibilita maior probabilidade de ser removida por seleção porque tendem a produzir mutações mais deletérias(CONSUEGRA et al., 2021). O mecanismo de amplificação do *bla*_{TEM} ainda não foi totalmente elucidado mas estudos recentes demonstram que este gene está localizado em um segmento de DNA contendo outros genes de resistência a antibióticos, como *aadA* (aminoglicosídeos), denominado módulo de resistência genômica ⁸⁵. Isso indica que a análise de sintenia pode ser uma técnica valiosa para acompanhar a disseminação do plasmídeo IncI1_1_Alpha e do gene *bla*_{TEM-1} e que pode contribuir para o estudo das cadeias de transmissão em diferentes dinâmicas ambientais.

De maneira específica, podemos verificar que NLCDRI12 diminuiu a diversidade de plasmídeos transferidos na avaliação por WGS quando comparado ao DRI12 entre as cepas bacterianas, manteve as mesmas contagens de ECTs mesmo na presença de fatores extrínsecos que maximizam o metabolismo microbiano e alterou em menor proporção os blocos de colinearidade.

A presença generalizada de compostos de cobre em ambientes como solos agrícolas, água, equipamentos médicos ⁸⁶ e sistemas de tratamento de efluentes industriais amplifica a importância desses achados. A exposição constante a esses compostos pode mitigar os fatores amplificadores para a seleção e propagação de cepas bacterianas resistentes, exceto quando estão associados a fatores moduladores como a presença de ambiente rico em nutrientes, como na indústria de lácteos.

Esses dados destacam a complexidade das interações multifatoriais entre agentes antimicrobianos, o ambiente e os mecanismos de resistência bacteriana, reforçando a necessidade de uma abordagem integrada para enfrentar o desafio global da resistência antimicrobiana. A compreensão dos mecanismos de THG associados a clareza da técnica de WGS em ambientes diversos, e não somente com a utilização de cepas puras é crucial para o desenvolvimento e formulação de estratégias de gestão e intervenção contra o desafio global da RAM.

3.8 Conclusão

Neste estudo direcionamos o uso de nanopartículas de cobre e complexo de cobre a dois patógenos específicos importantes causadores de doenças transmitidas por alimentos como medida de controle da transferência horizontal de genes por conjugação bacteriana. Avançamos na nossa compreensão da evolução microbiana como também abrimos caminho para potenciais aplicações em domínios que vão da microbiologia ambiental à biotecnologia. À medida que desvendamos a relação bioativo/micro-organismo em uma abordagem integrada a realidade

2814 industrial, reforçarmos a necessidade de obter respostas a partir da matriz primária onde
2815 ocorrem estes processos. O uso do sequenciamento do genoma completo permitiu desvendar os
2816 desafios da utilização de dois diferentes compostos e suas interações com bactéria, contribuindo
2817 para o panorama mais vasto da genômica microbiana e das suas aplicações. Por meio da
2818 investigação de domínios inexplorados da THG, destacamos a mitigação de NLCDRI12 na
2819 passagem seletiva de plasmídeos IncC e IncX1 e de DRI12 sobre a redução expressiva na THG
2820 e o impacto exercido pelo coproduto soro de leite em transferir genes de resistência a
2821 antibióticos, mesmo na presença de compostos metálicos.

2822 **Tabela Suplementar 1: Genes presentes no genoma (Utg2004).**

2823

CMY-59	CMY-59 is a beta-lactamase found in <i>Shigella</i> spp.	Cefamicina	
cpxA	CpxA is a membrane-localized sensor kinase that is activated by envelope stress. It starts a kinase cascade that activates CpxR which promotes efflux complex expression.	aminocoumarin; aminoglycoside	
FosA7	fosA7 is an enzyme that confers resistance to fosfomicin in <i>Escherichia coli</i> by breaking the epoxide ring of the molecule.	fosfomicin	
mdsC	MdsC is the outer membrane channel of the multidrug and metal efflux complex MdsABC.	carbapenem;cephalosporin;cephamycin;monobactam;penam;penem;phenicol	
mdsB	MdsB is the inner membrane transporter of the multidrug and metal efflux complex MdsABC. mdsB corresponds to 1 locus in <i>Pseudomonas aeruginosa</i> PAO1 (gene name: mexQ) and 2 loci in <i>Pseudomonas aeruginosa</i> LESB58.	carbapenem;cephalosporin;cephamycin;monobactam;penam;penem;phenicol	
mdsA	MdsA is the membrane fusion protein of the	carbapenem;cephalosporin;cephamycin;monobactam;penam;penem;phenicol	

	multidrug and metal efflux complex MdsABC.		
golS	GolS is a regulator activated by the presence of golD and promotes the expression of the MdsABC efflux pump.	carbapenem;cephalosporin;cephamycin;monobactam;penam;penem;phenicol	
<i>Escherichia coli</i> _ampH	AmpH is a class C ampC-like beta-lactamase and penicillin-binding protein identified in <i>Escherichia coli</i> .	cephalosporin;penam	
acrB	Protein subunit of AcrA-AcrB-TolC multidrug efflux complex. AcrB functions as a heterotrimer which forms the inner membrane component and is primarily responsible for substrate recognition and energy transduction by acting as a drug/proton antiporter.	cephalosporin;fluoroquinolone;glycylcycline;penam;phenicol;rifamycin;tetracycline;triclosan	
<i>Escherichia coli</i> _acrA	AcrA is a subunit of the AcrAB-TolC multidrug efflux system that in <i>E. coli</i> .	cephalosporin; fluoroquinolone; glycylcycline; penam; phenicol; rifamycin ;tetracycline ;triclosan	
kdpE	kdpE is a transcriptional activator that is part of the two-component system KdpD/KdpE that is studied for its regulatory role in potassium transport and has been	aminoglycoside	

	identified as an adaptive regulator involved in the virulence and intracellular survival of pathogenic bacteria. kdpE regulates a range of virulence loci through direct promoter binding.		
msbA	MsbA is a multidrug resistance transporter homolog from E. coli and belongs to a superfamily of transporters that contain an adenosine triphosphate (ATP) binding cassette (ABC) which is also called a nucleotide-binding domain (NBD). MsbA is a member of the MDR-ABC transporter group by sequence homology. MsbA transports lipid A a major component of the bacterial outer cell membrane and is the only bacterial ABC transporter that is essential for cell viability.	nitroimidazole	
marA	In the presence of antibiotic stress E. coli overexpresses the global activator protein MarA which besides inducing MDR efflux pump AcrAB also down- regulates synthesis of the porin OmpF.	carbapenem; cephalosporin; cephamycin; fluoroquinolone; glycylcycline; monobactam; penam; penem; phenicol; rifamycin, tetracycline, triclosan	

AAC(6')-Iy	AAC(6')-Iy is a chromosomal-encoded aminoglycoside acetyltransferase in <i>S. enteritidis</i> and <i>S. enterica</i> . Regulatory mutation required to increase expression of this chromosomally-encoded gene for resistance. In the specific system aminoglycoside resistance was due to a transcriptional fusion secondary to a chromosomal deletion in which the downstream aac(6')-Iy gene was placed under the control of the upstream nmpC promoter.	aminoglycoside	
H-NS	H-NS is a histone-like protein involved in global gene regulation in Gram-negative bacteria. It is a repressor of the membrane fusion protein genes <i>acrE</i> <i>mdtE</i> and <i>emrK</i> as well as nearby genes of many RND-type multidrug exporters.	cephalosporin;cephamycin;fluoroquinolone;macrolide;penam;tetracycline	
sdiA	SdiA is a cell division regulator that is also a positive regulator of <i>AcrAB</i> only when it's expressed from a plasmid. When the <i>sdiA</i> gene is on the chromosome it has no	cephalosporin;fluoroquinolone;glycylcycline;penam;phenicol;rifamycin;tetracycline;triclosan	

	effect on expression of acrAB		
mdtK	A multidrug and toxic compound extrusions (MATE) transporter conferring resistance to norfloxacin doxorubicin and acriflavine.	fluoroquinolone	
mdtB	MdtB is a transporter that forms a heteromultimer complex with MdtC to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex.	aminocoumarin	
mdtC	MdtC is a transporter that forms a heteromultimer complex with MdtB to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex. In the absence of MdtB MdtC can form a homomultimer complex that results in a functioning efflux complex with a narrower drug specificity. mdtC corresponds to 3 loci in Pseudomonas aeruginosa PAO1 (gene name: muxC/muxB) and 3 loci in Pseudomonas aeruginosa LESB58.	aminocoumarin	
baeR	BaeR is a response regulator that promotes the expression of MdtABC and AcrD efflux complexes.	aminocoumarin;aminoglycoside	

yojI	YojI mediates resistance to the peptide antibiotic microcin J25 when it is expressed from a multicopy vector. YojI is capable of pumping out microcin molecules. The outer membrane protein TolC in addition to YojI is required for export of microcin J25 out of the cell. Microcin J25 is thus the first known substrate for YojI.	peptide	
acrD	AcrD is an aminoglycoside efflux pump expressed in <i>E. coli</i> . Its expression can be induced by indole and is regulated by baeRS and cpxAR.	aminoglycoside	
emrR	EmrR is a negative regulator for the EmrAB-TolC multidrug efflux pump in <i>E. coli</i> . Mutations lead to EmrAB-TolC overexpression.	fluoroquinolone	
emrA	EmrA is a membrane fusion protein providing an efflux pathway with EmrB and TolC between the inner and outer membranes of <i>E. coli</i> a Gram-negative bacterium.	fluoroquinolone	
emrB	emrB is a translocase in the emrB -TolC efflux protein in <i>E. coli</i> . It recognizes substrates	fluoroquinolone	

	including carbonyl cyanide m-chlorophenylhydrazone (CCCP) nalidixic acid and thioactomycin.		
tolC	TolC is a protein subunit of many multidrug efflux complexes in Gram negative bacteria. It is an outer membrane efflux protein and is constitutively open. Regulation of efflux activity is often at its periplasmic entrance by other components of the efflux complex.	minocoumarin;aminoglycoside;carbapenem;cephalosporin;cephamycin; fluoroquinolone;glycylcycline;macrolide; penam; penem;peptide;phenicol; rifamycin; tetracycline; triclosan	
bacA	The bacA gene product (BacA) recycles undecaprenyl pyrophosphate during cell wall biosynthesis which confers resistance to bacitracin.	peptide	
CRP	CRP is a global regulator that represses MdtEF multidrug efflux pump expression.	fluoroquinolone;macrolide;penam	

Tabela Suplementar 2: Genes do Plasmídeo Utg2000

tus	DNA replication terminus site-binding protein
dinG	3'-5' exonuclease DinG
xerC	Tyrosine recombinase XerC
aidA	Quorum-quenching protein AidA

mbtI_1	Salicylate synthase
mbtI_2	Salicylate synthase
btuD_1	Vitamin B12 import ATP-binding protein BtuD
lagD	Lactococcin-G-processing and transport ATP-binding protein LagD
btuD_2	Vitamin B12 import ATP-binding protein BtuD
mbtB_1	Phenyloxazoline synthase MbtB
angR_1	Anguibactin system regulator
angR_2	Anguibactin system regulator
mbtB_2	Phenyloxazoline synthase MbtB
mbtB_3	Phenyloxazoline synthase MbtB
srfAD	Surfactin synthase thioesterase subunit
dhbE_1	2,3-dihydroxybenzoate-AMP ligase
dhbE_2	2,3-dihydroxybenzoate-AMP ligase
fyuA	Pesticin receptor
amiD	N-acetylmuramoyl-L-alanine amidase AmiD
metZ_1	O-succinylhomoserine sulphydrylase
metZ_2	O-succinylhomoserine sulphydrylase
mgl	L-methionine gamma-lyase
yhjV_1	Inner membrane transport protein YhjV
yhjV_2	Inner membrane transport protein YhjV
atoC	Regulatory protein AtoC
norR	Anaerobic nitric oxide reductase transcription regulator NorR
pdeL	Cyclic di-GMP phosphodiesterase PdeL
rfaH	Transcription antitermination protein RfaH
pdeG	putative cyclic di-GMP phosphodiesterase PdeG
traC	Protein TraC
topB_1	DNA topoisomerase 3
topB_2	DNA topoisomerase 3
noc	Nucleoid occlusion protein

soj	Sporulation initiation inhibitor protein Soj
glmM	Phosphoglucosamine mutase
tetR	Tetracycline repressor protein class A from transposon 1721
tetA_1	Tetracycline resistance protein, class C
tetA_2	Tetracycline resistance protein, class C
cph2	Phytochrome-like protein cph2
merA	Mercuric reductase
merP_1	Mercuric transport protein periplasmic component
merT_1	Mercuric transport protein MerT
esiB	Secretory immunoglobulin A-binding protein EsiB
merP_2	Mercuric transport protein periplasmic component
merT_2	Mercuric transport protein MerT
merR	Mercuric resistance operon regulatory protein
recD2_1	ATP-dependent RecD-like DNA helicase
recD2_2	ATP-dependent RecD-like DNA helicase
recD2_3	ATP-dependent RecD-like DNA helicase
higB2	Putative toxin HigB2
hupB	DNA-binding protein HU-beta
sppA	Putative signal peptide peptidase SppA

Tabela Suplementar 3: Genes do Plasmídeo Utg2002

virD4	Protein VirD4
topB_1	DNA topoisomerase 3
topB_2	DNA topoisomerase 3
stpA	DNA-binding protein StpA
pir_1	PI protein
pir_2	PI protein

relE	mRNA interferase toxin RelE
virB1	Type IV secretion system protein virB1
virB4_1	Type IV secretion system protein virB4
virB4_2	Type IV secretion system protein virB4
virB4_3	Type IV secretion system protein virB4
ptlE	Type IV secretion system protein PtlE
virB9	Type IV secretion system protein virB9

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Os autores declaram que não há conflitos de interesse

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Interference with Bacterial Conjugation and Natural Alternatives to Antibiotics: Bridging a Gap

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Abstract: Horizontal gene transfer (HGT) in food matrices has been investigated under conditions that favor gene exchange, although the major challenge lies in determining the specific conditions pertaining to the adapted microbial pairs in association with the food matrix. HGT is primarily responsible for enhancing the microbial repertoire for the evolution and spread of antimicrobial resistance and is a major target for the control of pathogens of public health concern in food ecosystems. In this study, we investigated *Salmonella* Heidelberg (SH) and *Escherichia coli* (EC) regarding gene exchange under conditions mimicking the industrial environment, with the coproducts whey (SL) and chicken juice (CJ). The *S. Heidelberg* strain was characterized in terms of antibiotic susceptibility standards and by PCR for the detection of the *bla*_{TEM} gene. A concentration of 0.39 mg/mL was determined to evaluate the anti-conjugation activity of nanostructured lipid nanocarriers (NLCs) of essential oils to mitigate β -lactam resistance gene transfer. The results showed that the addition of these coproducts promoted an increase of more than 3.5 (whey) and 2.5 (chicken juice) orders of magnitude in the conjugation process ($p < 0.01$), and NLCs of sage essential oil significantly reduced the conjugation frequency (CF) by 74.90, 90.6, and 124.4 times when compared to the transfers in the absence of coproducts and in the presence of SL and CJ, respectively. For NLCs from olibanum essential oil, the decrease was 4.46-fold for conjugations without inhibitors and 3.12- and 11.3-fold in the presence of SL and CJ. NLCs associated with sage and olibanum essential oils were effective in controlling the transfer of antibiotic resistance genes and are a promising alternative for use at industrial levels.

Keywords: bacterial conjugation; *bla*_{TEM}; chicken juice; nanostructured lipid carriers; whey

1. Introduction

Antimicrobial resistance (AMR) is one of the main global threats today, and recent reports estimate that it directly caused 1.27 million deaths in the year 2019 (Thompson, 2022). In Brazil, the National Health Surveillance Agency, through the Evaluation of National Indicators of Health Care-Related Infections (HAIs) and Microbial Resistance (MR), in the year 2021, estimated that *Escherichia coli* resistance to different classes of beta-lactams can reach rates of 47.5% to cephalosporins, 16.5% to carbapenems in hospital settings (Anvisa, 2022), and 74.28% and 62.85% to ampicillin and amoxicillin,

respectively, in samples from humans and animals (Santos *et al.*, 2020). Antimicrobial tolerance levels may increase through gene exchange between the two important pathogens *Salmonella* Heidelberg and *Escherichia coli* (Oladeinde *et al.*, 2019).

The presence of *bla*_{TEM} genes β -lactamases related to ampicillin resistance, has been reported in isolates of the family Enterobacteriaceae for more than 80.9% of the samples tested (Amador *et al.*, 2009) and in different environments (Bibbal *et al.*, 2007; Singh *et al.*, 2016; Sunde *et al.*, 2008). The *bla*_{TEM} gene is one of the most common variants of genes conferring beta-lactamase resistance to bacteria such as *E. coli* and *Klebsiella pneumoniae* as well as other Gram-negative bacteria (Tekiner; Özpınar, 2016). Its spread is facilitated by horizontal gene transfer (HGT), such as bacterial conjugation, a feature universally conserved among bacteria (Virolle *et al.*, 2020). Resistance mediated by the *bla*_{TEM} gene (Olesen; Hasman; Møller Aarestrup, 2004) stands out for its involvement in food matrices in the dissemination and microevolution of antibiotic-resistant bacteria (ANSHARIETA *et al.*, 2021; Samtiya *et al.*, 2022; Walsh *et al.*, 2007) and presents itself as a major challenge in the control of Gram-negative bacteria (Dewangan *et al.*, 2016; Effendi *et al.*, 2018).

In *S. Typhimurium*, ampicillin resistance rates exceed 75% (Liang *et al.*, 2019), and in response to this health challenge (Brazil, 2022), research is focused on finding actions that reinforce key interventions in combating antibiotic-resistant microorganisms and the mechanisms via which resistance genes are spread (Monteiro *et al.*, 2022b; Phelipe Augusto Borba Martins Peres; Roberta Torres de Melo; Paulo Marcel Armendaris; Fabiano Barreto; Tiago Follmann Perin; Ana Laura Grazziotin; Guilherme Paz Monteiro; Eliane Pereira Mendonça, 2022). In addition, monitoring AMR in *E. coli* is already a mandatory indicator to verify the occurrence of bacterial phenotypes resistant to clinically important antibiotics such as carbapenems, colistin, and beta-lactams in the environment (Anjum *et al.*, 2021) and in products of animal origin in Europe (Anjum *et al.*, 2021; Commission Implementing Decision 2013/652/EU, 2013).

Horizontal gene transfer is a major contributor to an increased diversity in the microbial genomic repertoire (Carranza *et al.*, 2021a), and conjugation is one of the most common mechanisms, particularly for the transfer of antibiotic resistance plasmids (Neil; Allard; Rodrigue, 2021b). This process is characterized by the dynamics of direct contact and assembly of the type IV secretion system (T4SS) of a donor bacterium and DNA transfer to a recipient bacterium (Alvarez-Martinez; Christie, 2009; Cascales; Christie, 2003). The overall rate of conjugation is determined not only by the type of plasmid that can be transferred but also by the cell physiology of the strains forming the conjugation pair, as well as the environmental conditions and the availability of energy (Lopatkin *et al.*, 2016), which directly influence the efficiency of conjugation and the subsequent growth of the transconjugants (Sheppard; Beddis; Barraclough, 2020).

Although extensively investigated for different factors, approaches to inhibit bacterial conjugation are still understudied. Thus, potential targets settle on the conjugative elements and the membrane assembly system of plasmid-encoded conjugation (Getino *et al.*, 2015). Thus, the approach that aims to associate anti-conjugative compounds with already demonstrated microbial properties to prevent the spread of resistance without acting as a growth inhibitor itself is of interest (Graf *et al.*, 2019).

Essential oils (EOs) are known for their antimicrobial properties that, in association with nanoparticles, have as advantages the overcoming of limitations of use in food, such as odor, besides increasing their efficacy and stability, promoting greater solubility and bioavailability. They are also natural and sustainable and can be

incorporated in the food industry. Nanoparticles can also improve the targeted delivery of EOs to specific tissues or cells (Guidotti-Takeuchi *et al.*, 2022). Promising work on the use of essential oil-loaded nanoparticles for the control of pathogenic microorganisms is already underway (Baldim *et al.*, 2022; Keivani Nahr *et al.*, 2018a), and researchers have already demonstrated that turmeric-loaded lipid nanocarriers (NLCs) can effectively control wild-type microorganisms such as *Pseudomonas aeruginosa* (Shajari *et al.*, 2020). However, little is known about the action of nanoparticles carrying bioactive compounds in gene transfer processes and the use of new molecules that can prevent or decrease bacterial conjugation. Understanding the dynamics of HGT allows explaining the complexity of some modulating factors of the transfer, and *in vitro* measurements of this process generate effective answers about the transfer rates among microorganisms.

2. Results

1.1. Physicochemical and morphological characteristics of lipid nanocarriers

The association of NLCs with different solid lipid matrices confer stability for the delivery of the EOs of olibanum (NpO) and sage (NpS). In a previous study, the nanocomposites were evaluated for particle size, polydispersity index, zeta potential, biocompatibility, and cytotoxicity to evaluate antimicrobial activity in *Campylobacter jejuni* (Ribeiro *et al.*, 2021). These characteristics were in line with the previous data and showed excellent results for size and polydispersity index. The zeta potential (mean: -36.6 to -31) indicated stability of the nanoparticles when evaluated by three subsequent measurements at 25°C for the NpO and NpS formulations (Table 1).

The average particle diameter ranged from 257.6 to 228.3 nm for sage and olibanum NLCs, respectively, with a polydispersity index equivalent to 0.15 and a zeta potential of approximately 30–35 mV. Although the size of the NLCs showed a slight increase in the presence of different EOs, the stability between the formulations remained within the parameters presented over time, indicating that the incorporation of the bioactive compounds does not significantly influence the size of the particles. The values obtained for the polydispersity index (PDI) were below 0.2 for all systems, without significant variations, demonstrating that the nanocomposites were in an acceptable state of distribution, with low variability and no aggregation. Furthermore, the image obtained with SEM, at the 10-micrometer scale, evidenced the morphology and distribution of the nanometric particles, characterizing a polydisperse system with particles of homogeneous spherical morphology, with the absence of agglomerations (Fig. 1).

Table 1. Physicochemical stability parameters of structured lipidic nanocarriers of essential oils.

Solid/Liquid Lipid	Size (nm)	PDI	Zeta Potential (mV)
Shea butter/sage EO	257.6 ± 3.00	0.147 ± 0.01	- 31.0 ± 0.1
Ucuuba butter/olibanum EO	228.3 ± 1.91	0.137 ± 0.05	- 36.6 ± 0.9

Solid lipids at a concentration of 100 mg/mL and liquid lipids at a concentration of 50 mg/mL. PDI: Polydispersity Index.

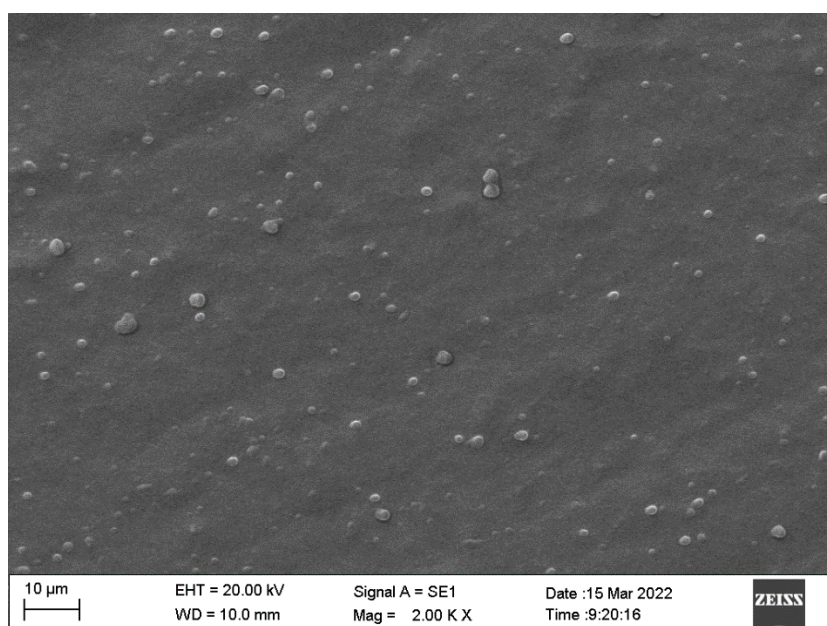


Figure 1. Scanning electron microscopy (SEM) image of the essential oil lipid nanocarrier formulation. Image taken at 20.000x magnification.

2.2. Minimum inhibitory concentration (MIC) of sage and olibanum lipidic nanocarriers.

The assay to evaluate the antimicrobial activity of the formulations with nanoparticles was confirmed by different *in vitro* experiments. The minimum bactericidal concentration (MBC) was 25 mg/mL for NpS and above 50 mg/mL for NpO, with an MIC of 1.8125 mg/mL for both carriers. From this, the optimum concentration of 0.390625 mg/mL (390.62 mg/L) was selected for NpS and NpO, considered ideal for the inhibition of bacterial conjugation but insufficient to kill the bacteria; it also did not result in a significant difference in the counts of *E. coli* and *S. Heidelberg* ($p > 0.05$) compared to the control (Table 2).

Table 2. Mean values and standard deviation of *Salmonella* Heidelberg and *Escherichia coli* counts in the presence and absence of inhibitors at a sage and olibanum NLC concentration of 0.390625 mg/mL.

	Control	NpS	* <i>p</i>	NpO	* <i>p</i>
<i>Escherichia coli</i> (log UFC)	6.88 ± 0.09	6.87 ± 0.17	>0.99	6.71 ± 0.25	0.95
<i>Salmonella</i> Heidelberg (log UFC)	7.17 ± 0.20	6.68 ± 0.42	0.13	6.72 ± 0.25	0.23

* *p*-value. NpS: nanoparticle with sage EO and NpO: nanoparticle with olibanum EO. NLC: nanostructured lipid carriers' formulations

The counts of donor, recipient, and transconjugant bacteria in the absence or presence of inhibitors did not significantly differ among the replicates ($p > 0.05$). Despite the evaluation of the effects of different factors on overall HGT rates, few studies have encompassed and weighed selective pressures within natural environments. At the macro-level, the frequency of conjugation (FCR) was 2.23%, whereas when supplementing the conjugation process with *CJ* (chicken carcass co-product) and SL (homemade Mina's cheese co-product, Federal University of Uberlândia Farm, MG/Brazil), the FCR was 6.22% and 8.16%, respectively.

2.3. Spectral evaluation and co-product supplementation

One of the characterization techniques used to evaluate the different constituents of SL and *CJ* is infrared vibrational spectroscopy, in which bands referring to the stretching of bonds present in the molecules (constituents) are observed. The region of

the vibrational spectra adopted was 4,000 to 650 cm^{-1} . The bands for SL, shown in Figure 2, were identified between 3,400 and 3,000 cm^{-1} , referring to the stretching of lipid bonds $\nu(\text{O-H})$ and $\nu(\text{CH}_2)$. The fat in the whey was confirmed due to the presence of the C-H bond stretching band (3,000–2,800 cm^{-1}) (Botelho *et al.*, 2015) from triglycerides (Upadhyay; Jaiswal; Jha, 2018).

In the wavenumber region between 1,700 and 1,000 cm^{-1} , there are vibrational modes of the binding bands related to fatty acids, proteins, and polysaccharides, and the intensity of the bands observed can be related to the concentration of proteins present in the samples (Andrade *et al.*, 2019). The most intense bands observed at 1,700 and 1,500 cm^{-1} refer to the bond stretches $\nu(\text{C=O})$, $\nu(\text{C-N})$ and $\delta(\text{N-H})$, $\nu(\text{C-N})$, respectively, from the amide functional groups (I and II) present in the peptide bonds (Andrade *et al.*, 2018). The band observed at 1,080 cm^{-1} can be attributed to the stretching of the O-H bond present in carbohydrates, such as lactose (El Darra *et al.*, 2017).

The bands referring to bond stretching ($\nu\text{N-H}$, $\nu\text{O-H}$, νCH_2 , νCH_3) were observed in a region of 3,500–3,300 cm^{-1} for Amide A and at 3,200–3,000 cm^{-1} for Amide B from protein constituents of the sample chicken juice. In the region of 1,800 to 1,600 cm^{-1} , the bands of the bonds ($\nu\text{C=O}$, $\delta\text{N-H}$, $\nu\text{C-H}$) were observed for Amide I, whereas those in the region of 1,570–1,470 cm^{-1} ($\delta\text{N-H}$, $\nu\text{C-H}$) were observed for Amide II and those at 1,350–1,250 cm^{-1} ($\nu\text{C-N}$, $\delta\text{N-H}$, $\nu\text{C=O}$, $\delta\text{O-C-N}$) for Amide III; these bands are characteristic of the protein content of the sample (Wang *et al.*, 2018). In the region between 1,220 and 900 cm^{-1} , we observed bands referring to the stretching of $\nu\text{C-O}$, $\nu\text{P-O}_2$ - bonds from the polysaccharides.

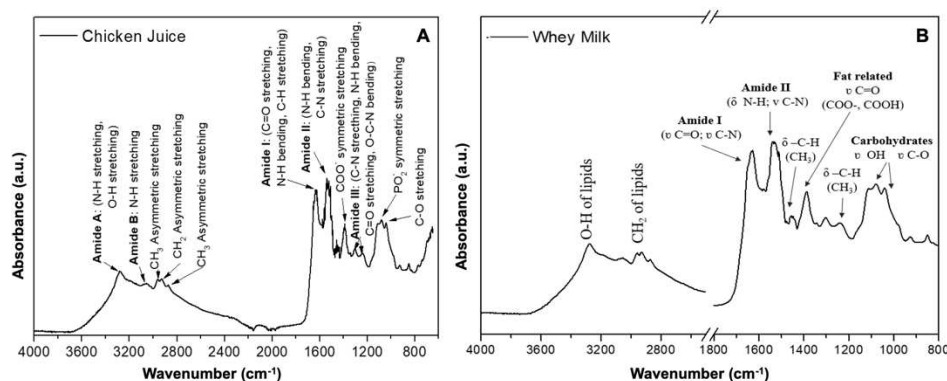


Figure 2. Comparison of FTIR spectra for chicken juice (A) and whey (B). a.u.: absorbance units.

2.4. Gene exchange and inhibitors of the conjugation process.

Via SEM, we visualized gene transfer based on the presence of sexual pili (Fig. 3). The samples presented a high cell density, enabling stabilization and contact between pairs. As shown in Figures 3A and 3B (arrows), the occurrence of sexual pili connections was homogeneous in all samples.

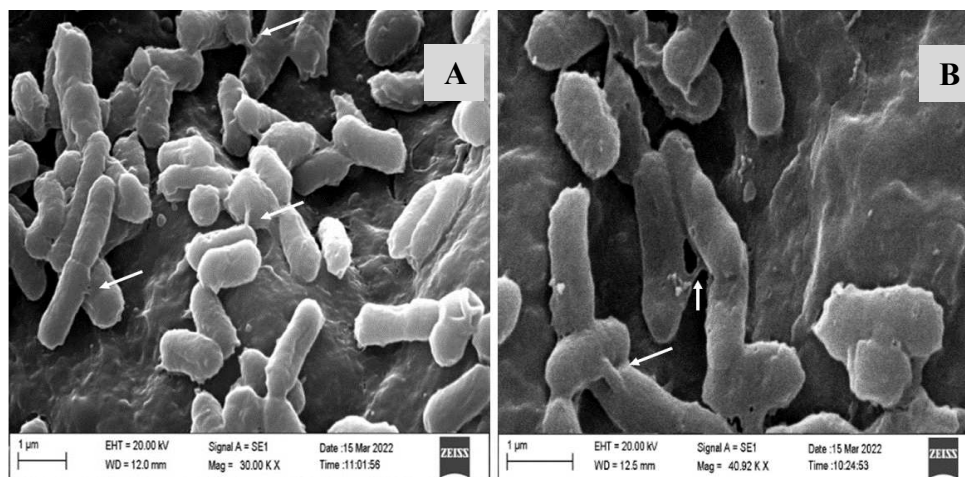


Figure 3. Scanning electron microscopy (SEM) image of bacterial conjugation between *Salmonella* Heidelberg and *Escherichia coli* (A and B), demonstrating multiple pili for horizontal gene transfer. Images taken at magnifications of 30.000x and 40.000x.

Conjugation in liquid medium was chosen for the tests, based on the conjugation rates obtained in a previous study (Rosanne Aparecida Capanema Ribeiro, Micaela Guidotti-Takeuchi, Roberta Torres de Melo, Carolyne Ferreira Dumont, Bárbara de Araújo Brum, Thais Jansen Martins, Wendell Guerra Luana Munique Sousa Ramos, 2023). We used the pair of microorganisms for which we determined an increase of more than 2.5 to 3.5 orders of magnitude in the percentage of frequency of conjugation in the presence of *CJ* and *SL*, when compared to the traditional process (without the presence of coproducts). The pre-established 3-hour period for evaluating the conjugation process represents the shift flow configurations present in animal product industries. The presence of the coproduct's whey and chicken exudate in this interval increased the gene transfer by 0.4 and 0.5 log CFU/mL ($p = 0.013$), respectively. The use of NpS had the greatest impact, with expressive inhibition results in all treatments, and reduced the conjugation efficiency by up to 124-fold when animal origin coproducts were added. The NpO showed more effective results in the traditional conjugation model (reduction of four orders of magnitude) and in the presence of *CJ* (reduction of 9 orders of magnitude). Moreover, it is important to note that *SL* promoted a higher increase in conjugation frequency when compared to *CJ*, resulting in a greater challenge for inhibition with NCL, which was reduced by three orders of magnitude (Fig. 4).

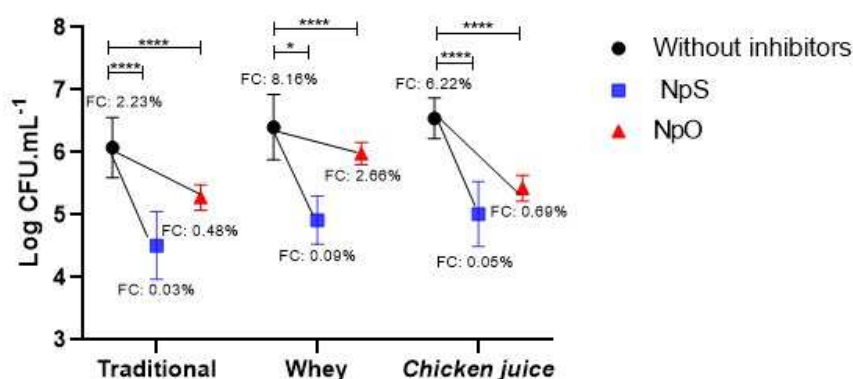


Figure 4. Comparison of conjugation frequencies obtained with and without 0.39 mg/mL of NLCs with essential oils of sage (NpS) and olibanum (NpO). WI: Without inhibitors; T: traditional; W: Whey; CJ: chicken juice. FCR: Receptor conjugation frequency in percent. *** $p < 0.0001$ and * $p < 0.01$, statistical difference by one-way ANOVA for the comparison with and without treatment with NLCs.

2.5. Molecular analyses

Molecular analyses by conventional PCR demonstrated the presence of the *bla*_{TEM} gene in all transconjugant colonies for the three compound treatments evaluated and their respective supplements. This result confirms the phenotypic characteristics presented in the transconjugant plates containing sodium azide (160 µg/mL) and ampicillin (90 µg/mL).

3. Discussion

Bacterial conjugation events occur in various environments and are ubiquitous (Coque, T.M., Cajal, R., Graham, D.W., Pruden, A., So, A.D., Topp, E., Grooters, 2023; Davison, 1999). They can also occur in bacterial communities present even in animal products, such as fresh milk (Tóth *et al.*, 2020) and chicken meat, and the genes can potentially be transferred during food consumption (Li *et al.*, 2022). Mimicking the food production chain in this study, with the use of *CJ* or *SL* coproducts, evidenced the construction of a model in this area, associated with environmental conditions that more accurately represent the extrinsic factors that can influence HGT, such as microbial mobility, fluid movements, and the microenvironment that can disturb the stabilization of the mating pair (Neil; Allard; Rodrigue, 2021a), depending on the junction between receiver and donor through the pilus (Low *et al.*, 2022).

The conjugation process, in the presence of organic matter coproducts (chicken juice or whey), demonstrated that the intrinsic factors present in the production chain, especially in production systems with continuous and automated batches, can promote the growth of certain unwanted bacterial subpopulations (Selover; Waite-Cusic, 2021). This makes their presence a primary factor in assessing HGT in these environments. The gradual process of DNA transfer in liquid medium and under agitation ensured efficient recombination rates when incubated for 3 hours, which is equivalent to approximately one-third of a work shift in the food industry. The frequency of conjugation is generally higher in environments under agitation compared to static environments, probably due to the increased number of collisions between cells (Headd; Bradford, 2018).

Under these conditions the gene exchange process of the *bla*_{TEM} gene was maximized by three and four orders of magnitude when incorporating coproducts of *CJ* and *SL*, respectively. Under optimal conditions, the culture of recipient cells can be converted into transconjugants within 30 min of the addition of donor cells at an optimal temperature of 37 °C, so that the mating pair requires only 5-7 min to allow DNA transfer (Funnell, Barbara E., 2004). Thus, active growth in a nutrient-rich environment and under optimal conditions would favor conjugation by sustaining the energy expenditure required for this process (De La Cruz *et al.*, 2010). Few studies have focused on the extrinsic factors that interfere with bacterial conjugation (BC) (Li *et al.*, 2022), i.e., the conditions that allow microbial fitness for plasmid transport costs (Lopatkin *et al.*, 2017). Environments with an optimal temperature (Mishra *et al.*, 2021) or that have nutrient availability (Alderliesten *et al.*, 2020) simulate natural conditions of conjugation rates under different experimental conditions (Shafieifini *et al.*, 2022); although these procedures are simplified, they are ideal for measuring specific rates and isolating modulating factors (Moralez *et al.*, 2021). There is strong evidence that bacterial growth increases the frequency of conjugation, and most research on conjugation is performed on growing bacterial populations or populations subjected to growth conditions (Headd; Bradford, 2018).

The composition of coproducts present in processed food can be evaluated by the FTIR spectroscopy technique, which has the advantages of demonstrating the structural differences in the molecules of different types of foods and determining the use and bioavailability of nutrients (Reynolds; Harmon; Cecava, 1994). In this sense, the use of whey as an inducer of recombinant protein production by *Escherichia coli* resulted in an

improvement in the fitness and efficiency in protein expression, without interfering with membrane fluidity and permeability (De Divitiis *et al.*, 2023). Thus, the use of lactose as a carbon source, present in SL, by *E. coli* maximized the metabolism and microbial machinery due to the greater availability of micronutrients. After using glucose and in the presence of lactose, *E. coli* grows faster compared to when it uses other carbon sources as intracellular lactose is degraded by the enzyme LacZ (encoding β -galactosidase) into galactose and glucose, of which the latter can directly enter the Embden-Meyerhof-Parnas pathway (Ammar; Wang; Rao, 2018). The interval between 1,450 and 600 cm^{-1} , present in *CJ*, is considered the fingerprint region, with characteristic bands coming from different biomolecules besides proteins, such as lipids, phospholipids, and nucleic acids (Candoğan; Altuntas; İğci, 2021). As for SL, enteric bacteria such as *E. coli* can use nitrogen-containing organic compounds as sole nitrogen sources (Van Heeswijk; Westerhoff; Boogerd, 2013). Recently, it has been observed that this microorganism can use DNA as an excellent source of carbon and nitrogen to grow rapidly (Huang *et al.*, 2022). These results show that in addition to isolating certain transfer pathways, the full biological context requires the integration of biophysical knowledge with environmental and growth conditions (Morales *et al.*, 2021).

In this context, we found that *CJ* and SL contained components of the amide grouping, with effects on biological activity (Ji *et al.*, 2020). These results may facilitate a more reliable characterization of the extrinsic factors that may interfere with and maximize gene exchange and can aid in discovering the contribution of these components individually or together to the ratio and frequency of conjugation in animal product environments.

In addition, previous studies have demonstrated the importance of dairy (Alexa (Oniciuc) *et al.*, 2020) and meat products (Monte *et al.*, 2019) as reservoirs of antibiotic resistance genes and in maintaining the transmission of antibiotic resistance, especially when the cold chain and proper heat treatment are not maintained (Li *et al.*, 2022; Liu, J. *et al.*, 2020). Environments with low nutrient levels (Shafieifini *et al.*, 2022), in the food industry, coproducts offer an optimal environment for multiplication and gene exchange. In a previous study, conjugation efficiency was maximized in environments of higher hydrodynamic stress, with consequent exposure to greater encounters between donor and recipient cells, favoring conjugation compared to static environments (Patkowski *et al.*, 2023). The establishment of contact between donor and recipient cells can be considered as limiting the rate of microbial conjugation as well as determining plasmid specificity (Virolle *et al.*, 2020).

Naturally, the environmental niches present in food processing, from primary production to the industry (Likotrafiti *et al.*, 2018), are critical for the persistence of resistance genes, representing an important link for transfer to the microbiota of the final consumer and the environment (Lambrecht *et al.*, 2019; Oniciuc *et al.*, 2019). Furthermore, plasmid transfer rates can be affected by the number of plasmids that the donor cell possesses since gram-negative bacterial cells can have between one and five simultaneous pili (Christie, 2016) and perform multiple plasmid transfer as a strategy to increase transfer and survival rates (Darphorn *et al.*, 2022). In this process, contact with the recipient cell favors the assembly of multiple T4SS systems in the donor cells, triggering the transfer of more than one copy of plasmid DNA to the recipient cell (Carranza *et al.*, 2021b). The type IV conjugative secretion system (T4SS) is a multi-subunit complex (García-Cazorla *et al.*, 2018) anchored to the membrane and responsible for elaborating a long extracellular filament - the conjugative pilus - that is essential for ADN transfer (Waksman, 2019).

As a measure of interfering with this process, determining the minimum inhibitory concentration of compounds that stop or decrease conjugative ADN transfer may be an effective option for controlling HGT (Bragagnolo *et al.*, 2020). Platforms using nanotechnology associated with biocompatible compounds, such as EOs, have gained

prominence for their versatility of use (Swain *et al.*, 2023). In the present study, the optimal dilution of these nanocompounds was determined before the conjugation experiments, with low concentrations for both NLCs, ensuring that most primary applications are safe (Al-Dahmash *et al.*, 2021; Bekhit, 2022; Mot *et al.*, 2022; Ribeiro *et al.*, 2021) and avoiding interference with bacterial growth and anticonjugation activity (Apiwatsiri *et al.*, 2021). This approach consists of associating EOs derived from medicinal plants and highlighting the action of promising new compounds that are bacterial conjugation inhibitors (COINS) *in vitro*. EOs are a rich source of bioactive compounds that can modify bacterial resistance (Oyedemi *et al.*, 2016), not only by hindering the growth of gram-negative bacteria but also by inhibiting transfer in a specific manner through interference with ADN replication (Cabezón; De La Cruz; Arechaga, 2017). In parallel, the adhesion interface and physicochemical effects play an important role in modulating the conjugative transfer (CT) process, irrespective of the microbial physiological response (Tang *et al.*, 2022).

Regarding the association of these EOs with nanoparticles to inhibit conjugation, the physical effects promoted by spherical shaped NpS present greater interactivity with the bacterial membrane, resulting in tension and stretching processes (Linklater *et al.*, 2020) in the plasma membrane, which is the primary assembly center for the initiation of the conjugation process (Carranza *et al.*, 2021c). Microspheres with adequate particle size and distribution are primary factors to optimize the results because they influence the release rate of the bioactive compound, interfering with the results obtained (Azhar Shekoufeh Bahari; Hamishehkar, 2016).

Some promising conjugation inhibitors (COINS) such as unsaturated fatty acids (2-hexadecanoic acid - 2HDA), seem to affect TrwD, belongs to the ATPase secretion superfamily, binding to bacterial membranes (García-Cazorla *et al.*, 2018), which hinders pilus biogenesis and ADN translocation (Atmakuri; Cascales; Christie, 2004). Indeed, the current mechanisms explaining the functionality of the structural components of type VI secretion do not exhibit specificity with target receptors on the cell surface, but this can be explained by a mechanical process (symbolically called "shot and bomb") in which perforation of the receptor cell by force or enzymatic activity of the pilus occurs (Virolle *et al.*, 2020). Unsaturated fatty acids can prevent plasmid transfer *in vitro* (Getino *et al.*, 2016), but to date, NLCs carrying bioactives have not been described as potential COINs. Using the delivery system (NLC), we observed an expressive reduction in conjugation frequency promoted by NpS, which caused HR to decrease by 74 orders of magnitude in the absence of animal industry coproducts. However, when the challenge was greater, in the presence of SL and CJ, this inhibition reached 90 and 124 orders of magnitude, respectively. In previous studies, CJ supplementation allowed the maintenance of viable microorganisms and maximized the results for the formation of strong biofilms, evidencing that this by-product facilitates the survival of bacterial cells in the production chain, increasing the challenges in food safety (Brown *et al.*, 2014; Melo *et al.*, 2017a).

It is important to stress that the NLC complex associated with sage and olibanum EOs interfered with the conjugation process, even at low concentrations. Therefore, modern experimental approaches, such as the use of bioactive compounds associated with NLCs (Bashiri *et al.*, 2020; Keivani Nahr *et al.*, 2018b; Sharma *et al.*, 2022), may favor a greater interaction with cells due to a higher surface-to-mass ratio (Huh; Kwon, 2011). Plants are a source of bioactive compounds, and a current approach is to isolate species that allow new insights into the inhibitory mechanisms of HGT at the molecular and cellular levels (Virolle *et al.*, 2020) as a measure to control antimicrobial resistance.

Robust screenings of bioactive molecules have already shown a 100-fold reduction in plasmid transfer frequencies (Cabezón; De La Cruz; Arechaga, 2017). Among these compounds, dehydrocrepnic acid (DHCA) (Fernandez-Lopez *et al.*, 2005) from tropical plant seeds (Gussoni *et al.*, 1994) and tanzawaic acids A and B inhibit the

conjugation of IncF and IncW plasmids and IncW and IncFII conjugative plasmids, respectively, without affecting cell growth (Cabezón; De La Cruz; Arechaga, 2017).

The essential oil of olibanum is derived from species of the genus *Boswellia* (Van Vuuren; Kamatou; Viljoen, 2010) and presents, as main markers, boswellic acid in commercially standardized extracts at concentrations of 37.5% to 65% . It also contains two promising agents, namely p 11-keto- β -boswellic acid (KBA) and 3- O-acetyl-11-keto- β -boswellic acid (AKBA,) which are present in all *Boswellia* species and are responsible for its pharmacological effects (Gerbeth *et al.*, 2011).

Similarly, *Salvia officinalis* L. (sage) of the Lamiaceae family is rich in phenolic compounds, mainly rosmarinic acid and yunnaneic acid (Afonso *et al.*, 2019), and used in medicines, cosmetics, and food flavoring (Lekbach *et al.*, 2019). In addition to the already known activities, it has recently been evaluated regarding the modulation of bacterial virulence and resistance, with promising results (Křížková *et al.*, 2023).

Studies investigating the long-term effects of these formulations on stability and toxicity are crucial for the validation of new systems (De M. Barbosa *et al.*, 2018). These formulations showed a greater versatility of action in different pathogen control processes and a lower toxicity in previous studies (Ribeiro *et al.*, 2021), which highlights their potential in preventing HGT in the food production chain. Thus, the use of biocompounds is focused on preventing the mobilization of plasmids in important environments from the food safety point of view.

In conclusion, two NLCs associated with sage and olibanum EOs were evaluated regarding the inhibition of the conjugation process. The animal production coproducts SL and *CJ* influenced HGT. As a mitigating measure, the use of NLCs associated with EOs of sage and olibanum promoted a significant reduction in FC.

3. Materials and Methods

3.1. Origin of strains

The study used a strain of *S. Heidelberg* that showed phenotypic resistance to five antimicrobials (ampicillin, cefoxitin, sulfamethoxazole, tetracycline, and clavulanic acid) which represent four important classes in human and veterinary medicine (Melo *et al.*, 2021). This strain was investigated for resistance genes by conventional PCR and contains the CMY-2, and *bla*_{TEM} genes and *luxS*, linked to the *quorum sensing* system. The *E. coli* strain J53AzR was used as the recipient strain (Matsumura; Peirano; Pitout, 2018).

3.2. Nanostructured lipidic carriers

The NLC formulations, composed of different matrices of solid lipids (shea butter and ucuuba) and liquid lipids (EOs of sage and olibanum) were previously formulated and tested on *Campylobacter jejuni* (Ribeiro *et al.*, 2021) at the Biotechnology Institute (Federal University of Uberlândia). Briefly, the solid and liquid lipids (EOs of salvia and olibanum at 50 mg/mL) were heated in a water bath (10°C above the melting point of the solid lipid). Subsequently, an aqueous solution of the biosurfactant Plantaren 1200® was heated to the same temperature as the lipid phase and added to the oil phase under high-speed (10,000 rpm) stirring for 2 min in an Ultra-Turrax mixer (IKA Werke Staufen®, Germany). Next, the systems were ultrasonicated for 12 min in a Vibracell high-end sonicator (Sonics & Mat. Inc®, Danbury, USA) operated at 500 W and 20 kHz, in alternating 30-s cycles (on/off). Sage (NpS) and olibanum (NpO) nanoparticle formulations were stored at 25°C. The physicochemical stability of the nanoparticles was previously validated (Ribeiro *et al.*, 2021) and re-evaluated in this work at three different time points to verify long-term stability.

3.3. Determination of the minimum inhibitory concentration (MIC)

The MIC values of essential oil-associated lipid carriers for *S. Heidelberg* and *Escherichia coli* J53AzR were determined using the broth microdilution method (EUCAST, 2022). Eight different concentrations were tested: 6.25, 3.125, 1.5625, 0.78125, 0.390624, 0.1953, 0.0976, and 0.0488 mg/mL. Briefly, bacterial suspensions were prepared at a concentration corresponding to 0.5 on the McFarland scale in sterile 0.85% saline solution and then inoculated into microplates containing Müller–Hinton broth with the two compounds alone at the different concentrations, followed by incubation at 37°C for 18–24 hours. For control, we used culture medium without the addition of bacteria and culture medium with the addition of bacteria and without the addition of the respective compounds. Readings were performed visually with the determination of the MIC, corresponding to the lowest concentration where no turbidity was observed. At concentrations immediately below the MIC, a bacterial count was performed to check if there was a difference between the counts of the control groups compared to the formulations. For the tests, a 10-μL aliquot of each product concentration was plated on soy tryptone agar (TSA), and after growth, the colony-forming units were counted and the results expressed in log₁₀, followed by statistical evaluation to ensure that there were no differences in growth between the groups. All assays were performed in three replicates and three repetitions.

3.4. Chicken juice and whey

The addition of animal by-products, namely chicken juice (CJ) and whey (SL), was performed to mimic food industry conditions (Brown *et al.*, 2014). The CJ was collected from a commercial chicken carcass frozen and thawed at 25–30°C. Subsequently, it was centrifuged three times at 14,000 rpm for 10 minutes at 4°C, followed by filtration of the supernatant through a 0.22-μm pore size filter (Kasvi®) to remove contaminants. Similarly, the whey was prepared and sterilized, collected from homemade Mina's cheese (Jersey breed animals) produced by enzymatic coagulation. Subsequently, 100 μL of the filtered by-product was inoculated into a TSA plate to verify the absence of microbial growth. Both CJ and SL were stored at -20°C.

3.5. Fourier-Transform Infrared (FTIR)

The composition of the coproducts was evaluated by FTIR. A diamond disk was used as the crystal material in the ATR unit as an internal reflection element. For collection, 20 μL of CJ and SL each was applied to a high-throughput system based on aluminum wafers and heated at 60°C on a hotplate for 3 minutes, followed by the application of pressure to provide contact with the crystal. Spectra were collected by scanning in the absorbance mode from 4,000 to 650 cm⁻¹, and 32 scans were performed per analysis at a resolution of 4 cm⁻¹. The infrared spectra were recorded on a portable ATR-FTIR spectrophotometer coupled to an attenuated total reflectance unit (Agilent Technologies, Agilent Cary 630, Santa Clara, CA, USA). After this evaluation, the spectral data were processed and analyzed using OriginPro 8.0 and MS-Excel.

3.6. Bacterial conjugation and inhibition of HGT by nanocarriers

The evaluation of the conjugation process followed the methodology proposed by (Rodriguez-Grande; Fernandez-Lopez, 2020)), with adaptations. Initially, the HS and CE strains were individually reactivated in brain- and heart-infused pre-enrichment broth (BHI, Sigma-Aldrich®) at 37°C for at least 6 hours and then repotted onto TSA(Biokar®), with overnight incubation at 37°C. After growth, approximately five colonies of each strain (donor and recipient) were selected and inoculated separately in sterile Falcon tubes containing 3 mL of Luria Bertani broth (LB-Neogen®) and incubated at 37°C in an orbital plate shaker with 60 rpm rotation for approximately three hours.

After incubation, absorbance was measured (O.D._{600nm}) in a microplate reader (Perlong DNM-9602) to check if the exponential phase was reached (O.D. between 0.4 and 0.5).

After absorbance determination, donor and recipient were individually subjected to serial decimal dilution (10^{-1} a 10^{-8}) in 0.85% saline (Êxodo Científica®). and 100 µL of each dilution was added to the surface of MacConkey agar (Biokar®) supplemented with the antibiotic's ampicillin (Vetnil®) at a concentration of 90 µg/mL (for *S. Heidelberg*) and sodium azide (Sigma-Aldrich®) at a concentration of 160 µg/mL (*E. coli* J53Az^R) to phenotypically express their resistance. The plates were incubated at 37°C for 48 hours, with the subsequent counting of colony-forming units (CFU). The groups submitted to the conjugation assays for 3 hours at 37°C at 20 rpm were prepared in the following proportions:

i) Traditional conjugation (no supplements): 180 µL of the donor strain (*S. Heidelberg*) and 720 µL of the recipient strain (*E. coli* J53Az^R) represented the co-culture in a 1:4 ratio, added to 100 µL of each NLC (salvia or olibanum).

ii) SL coproduct conjugation (5%): 170 µL of the donor strain (*S. Heidelberg*) and 680 µL of the recipient strain (*E. coli* J53 Az^R), preserving the 1:4 ratio, added to 50 µL of SL and 100 µL of each of the NLCs in separate reactions.

iii) *CJ* coproduct conjugation (5%): 170 µL of the donor strain (*S. Heidelberg*) and 680 µL of the recipient (*E. coli* J53 Az^R), preserving the ratio of 1:4, added to 50 µL of *CJ* and 100 µL of each of the NLCs in separate reactions.

Similarly, conjugation was performed in the absence of inhibitors, maintaining the ratio of donor and recipient (1:4):

i) Traditional conjugation (no supplements): 800 µL of the donor and 200 µL of the recipient.

ii) Conjugation with SL coproduct (5%): 190 µL of the donor strain, 760 µL of the recipient, and 50 µL of SL.

iii) *CJ* coproduct conjugation (5%): 190 µL of the donor strain, 760 µL of the recipient, and 50 µL of *CJ*. After recombination, the co-cultures were subjected to serial decimal dilution (10^{-1} to 10^{-8}) in 0.85% saline, followed by surface plating on MacConkey agar (Biokar®) supplemented with ampicillin (90 µg/mL) and sodium azide (160 µg/mL) for the determination of transconjugative colonies of resistant *E. coli* J53Az^R. All assays were performed in triplicate and in three repetitions.

3.7. Identification of recombinants

The transfer of resistance to β-lactams was investigated by phenotypic and genotypic methods, using conventional PCR to detect the *bla*_{TEM} gene (molecular weight 643 bp). Five transconjugating colonies were selected from each of the conjugates (traditional) and treatments (NpO and NpS), with and without supplements. The colonies were inoculated into BHI broth and incubated at 37°C overnight, followed by bacterial ADN extraction with the Wizard Genomic ADN Purification kit (Promega). The PCR was performed in a final volume of 25 µL, using the Promega master mix kit, under the amplification conditions and with the primers listed in Table 3. Amplification was performed in a thermal cycler (Eppendorf®) with 30 amplification cycles: denaturation at 95°C for 5 min, annealing at 50°C for 45 s, extension at 72°C for 90 s, and final extension at 72°C for 10 min. The ADN was amplified according to the standardization conditions described in Table 3, and the amplified products were visualized via 1.5% agarose gel electrophoresis using 0.5x TBE buffer (Invitrogen) and, as a molecular weight standard, 100-bp marker (Invitrogen).

Table 3. Primers and conditions used to identify the *bla*_{TEM} gene.

Gene	Sequence 5'→3'	PCR Conditions (°C)	ADN (ng) Reference
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	CAGCGGTAAGATCCTT	95°C – 5 min; 50°C		
	GAGA	– 45 sec; 72°C 90	10	(Ahmed <i>et al.</i> , 2007)
<i>bla_{TEM}</i>	ACTCCCCGTCGTGTAG	sec; 72°C 10 min		
	ATAA			

3.8. Gene recombination frequency

The conjugation frequency (CRF) was measured by calculating the ratio between the number of transconjugants (T) and the number of recipients (R) at the beginning of the conjugation process (CRF: mean T/mean R) (Suhartono; Savin, 2016). For standardization, transconjugants were counted on plates that contained between 25 and 250 colonies (Silva, 2017). Transconjugants were defined as the recipient cells (*E. coli* J53Az^R) that received the plasmid containing the ampicillin resistance gene from a donor cell (*S. Heidelberg*) at the end of the conjugation process, counted by the average value of repeats for each treatment. Calculations were performed separately for conjugations with and without the addition of SL and CJ and for the respective inhibitor treatment groups (NpO and NpS).

3.9. Scanning electron microscopy (SEM)

3.9.1. SEM - Conjugation

The ultrastructure of conjugation in all treatments was evaluated by scanning microscopy, using a modified method (Brown *et al.*, 2014), considering the growth conditions in Luria Bertani broth (LB-Neogen®) and conjugation at 37°C for 3 h/20 rpm. After conjugation, samples were fixed on 1-cm² (PU) slides (Habasit Cleandrive TM, Reinach, CH) and fixed with 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M PBS buffer (pH 7.4) at 4°C overnight. Subsequently, the fixative was removed, and the samples were washed with PBS buffer. The samples were post-fixed for 2 hours with 1% osmium tetroxide and washed three times with PBS buffer. The dehydrated series was applied with ethanol solutions (30, 40, 50, 60, 70, 80, and 90% and then three times at 100%) for 20 minutes for each step. The samples were dried in a CPD (critical drying point) system (Leica EM CPD300; Leica, Wien, Austria) using liquid carbon dioxide as a transition fluid, coated with a 20-nm gold layer (SCD 050, Baltec), and exposed on a Zeiss Supra 55 FEG SEM operating at 5 kV. Images were captured with standard ZEISS SmartSEM.

3.9.2. SEM - NLC

For SEM, the samples were adhered to the stub with a glass coverslip and subsequently sprayed with gold for 120 s at 30 kV. After this, the sage (NpS) and olibanum (NpO) nanoparticles were visualized in a JEOL field scanning electron microscope (model JSM 5800LV) operating at variable voltage from 0.3 to 30 kV with a tungsten filament, using the SemAfore 5.21 imaging system software at 40,000x and 30,000x magnification.

3.10. Statistical analysis

The results were subjected to percentage descriptive analysis to calculate the conjugation frequency. The transconjugant count and the gene transfer rate were log-transformed so that the normality of the residuals could be assessed. Analysis of variance, one way ANOVA, was performed to compare the assays of the replicates and possible differences in the results obtained. For the comparison of two variables, the t-test was used. All analyses were performed using the GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego CA, USA).

4. Conclusions

Two nanostructured lipid carriers associated with sage and olibanum essential oils were evaluated to act as inhibitors of conjugation. Coproducts of animal origin, namely whey and chicken juice potentiated horizontal gene transfer. As a mitigating measure, the use of nanocarrier associated with essential oils of sage and olibanum promoted a significant reduction in conjugation frequency. The inhibitory effect provided by sage nanoparticle and olibanum is a promising alternative to mitigate the microevolution of resistance gene transfer in the food industry and motivates us to conduct further studies with these molecules, with the aim to control microorganisms of public health importance.

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2375 You should submit any Supplementary Information together with the manuscript so that
2376 we can send it to referees during peer-review. This will be published online with accepted
2377 manuscripts.

2378 It's vital that you carefully check your Supplementary Information before submission as
2379 any modification after your paper is published will require a formal correction.

2380 Please avoid including any "data not shown" statements and instead make your data
2381 available via deposition in a public repository (see '[Availability of materials and data](#)' for
2382 more information).

2383 If any data that is necessary to evaluate the claims of your paper is not available via a
2384 public depository, make sure you provide it as Supplementary Information.

2385 We do not edit, typeset or proof Supplementary Information, so please present it clearly
2386 and succinctly at initial submission, making sure it conforms to the style and terminology
2387 of the rest of the paper.

2388 To avoid any delays to publication, please follow the guidelines below for creation,
2389 citation and submission of your Supplementary Information:

1. You can combine multiple pieces of Supplementary Information and supply them as a single composite file. If you wish to keep larger information (e.g. supplementary videos, spreadsheets [.csv or .xlsx] or data files) as another separate file you may do so.

2. Designate each item as Supplementary Table, Figure, Video, Audio, Note, Data, Discussion, Equations or Methods, as appropriate. Number Supplementary Tables and Figures as, for example, "Supplementary Table S1". This numbering should be separate from that used in tables and figures appearing in the main article. Supplementary Note or Methods should not be numbered; titles for these are optional.

3. Refer to each piece of supplementary material at the appropriate point(s) in the main article. Be sure to include the word "Supplementary" each time one is mentioned. Please do not refer to individual panels of supplementary figures.

2403 4. Use the following examples as a guide (note: abbreviate "Figure" as "Fig." when
 2404 in the middle of a sentence): "Table 1 provides a selected subset of the most active
 2405 compounds. The entire list of 96 compounds can be found as Supplementary
 2406 Table S1 online." "The biosynthetic pathway of L-ascorbic acid in animals
 2407 involves intermediates of the D-glucuronic acid pathway (see Supplementary Fig.
 2408 S2 online). Figure 2 shows..."

2409 5. Remember to include a brief title and legend (incorporated into the file to appear
 2410 near the image) as part of every figure submitted, and a title as part of every table.

2411 6. Keep file sizes as small as possible, with a maximum size of 50 MB, so that they
 2412 can be downloaded quickly.

2413 7. Supplementary video files should be provided in the standard video aspects: 4:3,
 2414 16:9, 21:9.

2415 If you have any further questions about the submission and preparation of Supplementary
 2416 Information, please email: scirep.admin@nature.com.

2417 ***Figure legends***

2418 Please begin your figure legends with a brief title sentence for the whole figure and
 2419 continue with a short description of what is shown in each panel. Use any symbols in
 2420 sequence and minimise the methodological details as much as possible. Keep each legend
 2421 total to no more than 350 words. Provide text for figure legends in numerical order after
 2422 the references.

2423 ***Tables***

2424 Please submit any tables in your main article document in an editable format (Word or
 2425 TeX/LaTeX, as appropriate), and not as images. Tables that include statistical analysis of
 2426 data should describe their standards of error analysis and ranges in a table legend.

2427 ***Equations***

2428 Include any equations and mathematical expressions in the main text of the paper. Identify
 2429 equations that are referred to in the text by parenthetical numbers, such as (1), and refer
 2430 to them in the manuscript as "equation (1)" etc.

2431 For submissions in a .doc or .docx format, please make sure that all equations are provided
 2432 in an editable Word format. You can produce these with the equation editor included in
 2433 Microsoft Word.

2434 ***General figure guidelines***

2435 You are responsible for obtaining permission to publish any figures or illustrations that
 2436 are protected by copyright, including figures published elsewhere and pictures taken by

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2438 without appropriate permission.

2439 You should state the source of any images used. If you or one of your co-authors has
2440 drawn the images, please mention this in your acknowledgements. For software, you
2441 should state the name, version number and URL.

2442 Number any figures separately with Arabic numerals in the order they occur in the text
2443 of the manuscript. Include error bars when appropriate. Include a description of the
2444 statistical treatment of error analysis in the figure legend.

2445 Please do not use schemes. You should submit sequences of chemical reactions or
2446 experimental procedures as figures, with appropriate captions. You may include in the
2447 manuscript a limited number of uncaptioned graphics depicting chemical structures - each
2448 labelled with their name, by a defined abbreviation, or by the bold Arabic numeral.

2449 Use a clear, sans-serif typeface (for example, Helvetica) for figure lettering. Use the same
2450 typeface in the same font size for all figures in your paper. For Greek letters, use a
2451 'symbols' font. Put all display items on a white background, and avoid excessive boxing,
2452 unnecessary colour, spurious decorative effects (such as three-dimensional 'skyscraper'
2453 histograms) and highly pixelated computer drawings. Never truncate the vertical axis of
2454 histograms to exaggerate small differences. Ensure any labelling is of sufficient size and
2455 contrast to be legible, even after appropriate reduction. The thinnest lines in the final
2456 figure should be no smaller than one point wide. You will be sent a proof that will include
2457 figures.

- 2458 • Figures divided into parts should be labelled with a lower-case, bold letter (**a**, **b**,
2459 **c** and so on) in the same type size as used elsewhere in the figure.
- 2460 • Lettering in figures should be in lower-case type, with only the first letter of each
2461 label capitalised.
- 2462 • Units should have a single space between the number and the unit, and follow SI
2463 nomenclature (for example, ms rather than msec) or the nomenclature common to
2464 a particular field.
- 2465 • Thousands should be separated by commas (1,000).
- 2466 • Unusual units or abbreviations should be spelled out in full or defined in the
2467 legend.
- 2468 • Scale bars should be used rather than magnification factors, with the length of the
2469 bar defined on the bar itself rather than in the legend.

2470 In legends, please use visual cues rather than verbal explanations such as "open red
2471 triangles". Avoid unnecessary figures: data presented in small tables or histograms, for
2472 instance, can generally be stated briefly in the text instead. Figures should not contain
2473 more than one panel unless the parts are logically connected; each panel of a multipart
2474 figure should be sized so that the whole figure can be reduced by the same amount and
2475 reproduced at the smallest size at which essential details are visible.

2476 ***Figures for peer review***

2477 At the initial submission stage, you may choose to upload separate figure files or to
2478 incorporate figures into the main article file, ensuring that any figures are of sufficient
2479 quality to be clearly legible.

2480 When submitting a revised manuscript, you must upload all figures as separate figure
2481 files, ensuring that the image quality and formatting conforms to the specifications below.

2482 ***Figures for publication***

2483 You must supply each complete figure as a separate file upload. Multi-part/panel figures
2484 must be prepared and arranged as a single image file (including all sub-parts; a, b, c, etc.).
2485 Please do not upload each panel individually.

2486 Please read the [digital images integrity and standards](#) section of our [Editorial and](#)
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2494 For optimal results, you should supply all line art, graphs, charts and schematics in vector
2495 format, such as EPS or AI. Please save or export it directly from the application in which
2496 it was made, making sure that data points and axis labels are clearly legible.

2497 ***2. Photographic and bitmap images***

2498 Please supply all photographic and bitmap images in a bitmap image format such as tiff,
2499 jpg, or psd. If saving tiff files, please ensure that the compression option is selected to
2500 avoid very large file sizes. Please do not supply Word or Powerpoint files with placed
2501 images. Images can be supplied as RGB or CMYK (note: we will not convert image
2502 colour modes).

2503 Figures that do not meet these standards will not reproduce well and may delay
2504 publication until we receive high-resolution images.

2505 ***3. Chemical structures***

2506 Please produce Chemical structures using ChemDraw or a similar program. All chemical
2507 compounds must be assigned a bold, Arabic numeral in the order in which the compounds
2508 are presented in the manuscript text. Structures should then be exported into a 300 dpi
2509 RGB tiff file before being submitted.

2510 4. Stereo images

2511 You should present stereo diagrams for divergent 'wall-eyed' viewing, with the two panels
2512 separated by 5.5 cm. In the final accepted version of the manuscript, you should submit
2513 the stereo images at their final page size.

2514 *Statistical guidelines*

2515 If your paper contains statistical testing, it should state the name of the statistical test, the
2516 n value for each statistical analysis, the comparisons of interest, a justification for the use
2517 of that test (including, for example, a discussion of the normality of the data when the test
2518 is appropriate only for normal data), the alpha level for all tests, whether the tests were
2519 one-tailed or two-tailed, and the actual P value for each test (not merely "significant" or
2520 "P < 0.05"). Please make it clear what statistical test was used to generate every P value.
2521 Use of the word "significant" should always be accompanied by a P value; otherwise, use
2522 "substantial," "considerable," etc.

2523 Data sets should be summarised with descriptive statistics, which should include the n
2524 value for each data set, a clearly labelled measure of centre (such as the mean or the
2525 median), and a clearly labelled measure of variability (such as standard deviation or
2526 range).

2527 Ranges are more appropriate than standard deviations or standard errors for small data
2528 sets. Graphs should include clearly labelled error bars. You must state whether a number
2529 that follows the $\pm$ sign is a standard error (s.e.m.) or a standard deviation (s.d.).

2530 You must justify the use of a particular test and explain whether the data conforms to the
2531 assumptions of the tests. Three errors are particularly common:

- 2532 • Multiple comparisons: when making multiple statistical comparisons on a single
2533 data set, you should explain how you adjusted the alpha level to avoid an inflated
2534 Type I error rate, or you should select statistical tests appropriate for multiple
2535 groups (such as ANOVA rather than a series of t-tests).
- 2536 • Normal distribution: many statistical tests require that the data be approximately
2537 normally distributed; when using these tests, you should explain how you tested
2538 your data for normality. If the data does not meet the assumptions of the test, you
2539 should use a non-parametric alternative instead.
- 2540 • Small sample size: when the sample size is small (less than about 10), you should
2541 use tests appropriate to small samples or justify the use of large-sample tests.

2542 *Chemical and biological nomenclature and abbreviations*

2543 You should identify molecular structures by bold, Arabic numerals assigned in order of
2544 presentation in the text. Once identified in the main text or a figure, you may refer to
2545 compounds by their name, by a defined abbreviation, or by the bold Arabic numeral (as
2546 long as the compound is referred to consistently as one of these three).

2547 When possible, you should refer to chemical compounds and biomolecules using
2548 systematic nomenclature, preferably using [IUPAC](#). You should use standard chemical

2549 and biological abbreviations. Make sure you define unconventional or specialist
2550 abbreviations at their first occurrence in the text.

2551 *Gene nomenclature*

2552 You should use approved nomenclature for gene symbols, and employ symbols rather
2553 than italicised full names (for example Ttn, not titin). Please consult the appropriate
2554 nomenclature databases for correct gene names and symbols. A useful resource is [Entrez](#)
2555 [Gene](#).

2556 You can get approved human gene symbols from HUGO Gene Nomenclature Committee
2557 (HGNC), e-mail: hgnc@genenames.org; see also www.genenames.org.

2558 You can get approved mouse symbols from The Jackson Laboratory, e-
2559 mail: nomen@informatics.jax.org; see also www.informatics.jax.org/mgihome/nomen.

2560 For proposed gene names that are not already approved, please submit the gene symbols
2561 to the appropriate nomenclature committees as soon as possible, as these must be
2562 deposited and approved before publication of an article.

2563 Avoid listing multiple names of genes (or proteins) separated by a slash, as in
2564 'Oct4/Pou5f1', as this is ambiguous (it could mean a ratio, a complex, alternative names
2565 or different subunits). Use one name throughout and include the other at first mention:
2566 'Oct4 (also known as Pou5f1)'.

2567 *Characterisation of chemical and biomolecular materials*

2568 *Scientific Reports* is committed to publishing technically sound research. Manuscripts
2569 submitted to the journal will be held to rigorous standards with respect to experimental
2570 methods and characterisation of new compounds.

2571 You must provide adequate data to support your assignment of identity and purity for
2572 each new compound described in your manuscript. You should provide a statement
2573 confirming the source, identity and purity of known compounds that are central to the
2574 scientific study, even if they are purchased or resynthesised using published methods.

2575 *1. Chemical identity*

2576 Chemical identity for organic and organometallic compounds should be established
2577 through spectroscopic analysis. Standard peak listings (see formatting guidelines below)
2578 for ¹H NMR and proton-decoupled ¹³C NMR should be provided for all new compounds.
2579 Other NMR data should be reported (³¹P NMR, ¹⁹F NMR, etc.) when appropriate. For
2580 new materials, you should also provide mass spectral data to support molecular weight
2581 identity. High-resolution mass spectral (HRMS) data is preferred. You may report UV or
2582 IR spectral data for the identification of characteristic functional groups, when
2583 appropriate. You should provide melting-point ranges for crystalline materials. You may
2584 report specific rotations for chiral compounds. You should provide references, rather than

2585 detailed procedures, for known compounds, unless their protocols represent a departure
2586 from or improvement on published methods.

2587 *2. Combinational compound libraries*

2588 When describing the preparation of combinatorial libraries, you should include standard
2589 characterisation data for a diverse panel of library components.

2590 *3. Biomolecular identity*

2591 For new biopolymeric materials (oligosaccharides, peptides, nucleic acids, etc.), direct
2592 structural analysis by NMR spectroscopic methods may not be possible. In these cases,
2593 you must provide evidence of identity based on sequence (when appropriate) and mass
2594 spectral characterisation.

2595 *4. Biological constructs*

2596 You should provide sequencing or functional data that validates the identity of their
2597 biological constructs (plasmids, fusion proteins, site-directed mutants, etc.) either in the
2598 manuscript text or the Methods section, as appropriate.

2599 *5. Sample purity*

2600 We request evidence of sample purity for each new compound. Methods for purity
2601 analysis depend on the compound class. For most organic and organometallic
2602 compounds, purity may be demonstrated by high-field ¹H NMR or ¹³C NMR data,
2603 although elemental analysis ($\pm 0.4\%$) is encouraged for small molecules. You may use
2604 quantitative analytical methods including chromatographic (GC, HPLC, etc.) or
2605 electrophoretic analyses to demonstrate purity for small molecules and polymeric
2606 materials.

2607 *6. Spectral data*

2608 Please provide detailed spectral data for new compounds in list form (see below) in the
2609 Methods section. Figures containing spectra generally will not be published as a
2610 manuscript figure unless the data are directly relevant to the central conclusions of the
2611 paper. You are encouraged to include high-quality images of spectral data for key
2612 compounds in the Supplementary Information. You should list specific NMR
2613 assignments after integration values only if they were unambiguously determined by
2614 multidimensional NMR or decoupling experiments. You should provide information
2615 about how assignments were made in a general Methods section.

2616 Example format for compound characterisation data. mp: 100-102 °C (lit.^{ref} 99-101 °C);
2617 TLC (CHCl₃:MeOH, 98:2 v/v): R_f = 0.23; [α]_D = -21.5 (0.1 M in n-hexane); ¹H NMR
2618 (400 MHz, CDCl₃): δ 9.30 (s, 1H), 7.55-7.41 (m, 6H), 5.61 (d, J = 5.5 Hz, 1H), 5.40 (d, J
2619 = 5.5 Hz, 1H), 4.93 (m, 1H), 4.20 (q, J = 8.5 Hz, 2H), 2.11 (s, 3H), 1.25 (t, J = 8.5 Hz,
2620 3H); ¹³C NMR (125 MHz, CDCl₃): δ 165.4, 165.0, 140.5, 138.7, 131.5, 129.2, 118.6,
2621 84.2, 75.8, 66.7, 37.9, 20.1; IR (Nujol): 1765 cm⁻¹; UV/Vis: λ_{max} 267 nm; HRMS (m/z):
2622 [M]⁺ calcd. for C₂₀H₁₅C₁₂NO₅, 420.0406; found, 420.0412; analysis (calcd., found for

2623 C₂₀H₁₅Cl₂NO₅: C (57.16, 57.22), H (3.60, 3.61), Cl (16.87, 16.88), N (3.33, 3.33), O
 2624 (19.04, 19.09).

2625 7. Crystallographic data for small molecules

2626 If your manuscript is reporting new three-dimensional structures of small molecules from
 2627 crystallographic analysis, you should include a .cif file and a structural figure with
 2628 probability ellipsoids for publication as Supplementary Information. These must have
 2629 been checked using the IUCR's [CheckCIF](#) routine, and you must include a PDF copy of
 2630 the output with the submission, together with a justification for any alerts reported. You
 2631 should submit crystallographic data for small molecules to the [Cambridge Structural](#)
 2632 [Database](#) and the deposition number referenced appropriately in the manuscript. Full
 2633 access must be provided on publication.

2634 8. Macromolecular structural data

2635 If your manuscript is reporting new structures, it should contain a table summarising
 2636 structural and refinement statistics. Templates are available for such tables
 2637 describing [NMR](#) and [X-ray crystallography](#) data. To facilitate assessment of the quality
 2638 of the structural data, you should submit with the manuscript a stereo image of a portion
 2639 of the electron density map (for crystallography papers) or of the superimposed lowest
 2640 energy structures (≥ 10 ; for NMR papers). If the reported structure represents a novel
 2641 overall fold, you should also provide a stereo image of the entire structure (as a backbone
 2642 trace).

2643 **Registered Reports**

2644 Registered Reports are original research articles which undergo peer-review prior to data
 2645 collection and analyses. This format is designed to minimize publication bias and research
 2646 bias in hypothesis-driven research, while also allowing the flexibility to conduct
 2647 exploratory (unregistered) analyses and report serendipitous findings. If you intend to
 2648 submit a Registered Report to *Scientific Reports*, please refer to detailed guidelines [here](#).
 2649

2650 **2 Normas do periódico Antibiotics - Capítulo 3**

2651

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- 2667 • Promoting Equity, Diversity and Inclusiveness within MDPI Journals
- 2668 • Resource Identification Initiative
- 2669
- 2670 Submission Checklist
- 2671 Please:
- 2672 1. Read the Aims & Scope to gain an overview and assess if your manuscript is suitable
- 2673 for this
- 2674 journal;
- 2675 2. Use the Microsoft Word template or LaTeX template to prepare your manuscript;
- 2676 3. Make sure that issues about publication ethics, research
- 2677 ethics, copyright, authorship, figure formats, data and references format have been
- 2678 appropriately considered;
- 2679 4. Ensure that all authors have approved the content of the submitted manuscript.
- 2680 5. Authors are encouraged to add a biography (optional) to the submission and post it
- 2681 to SciProfiles.
- 2682
- 2683 Manuscript Submission Overview
- 2684 Types of Publications
- 2685 Antibiotics has no restrictions on the maximum length of manuscripts, provided that the
- 2686 text is concise and comprehensive. Full experimental details must be provided so that the
- 2687 results can be reproduced. Antibiotics requires that authors publish all experimental
- 2688 controls and make full datasets available where possible (see the guidelines on
- 2689 Supplementary Materials and references to unpublished
- 2690 data).
- 2691 Manuscripts submitted to Antibiotics should neither be published previously nor be under
- 2692 consideration for publication in another journal. The main article types are as follows:
- 2693 • Article: These are original research manuscripts. The work should report scientifically
- 2694 sound experiments and provide a substantial amount of new information. The article

should include the most recent and relevant references in the field. The structure should include an Abstract, Keywords, Introduction, Materials and Methods, Results, Discussion, and Conclusions (optional) sections, with a suggested minimum word count of 4000 words.

- Review: Reviews offer a comprehensive analysis of the existing literature within a field of study, identifying current gaps or problems. They should be critical and constructive and provide recommendations for future research. No new, unpublished data should be presented. The structure can include an Abstract, Keywords, Introduction, Relevant Sections, Discussion, Conclusions, and Future Directions, with a suggested minimum word count of 4000 words.

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Manuscripts for Antibiotics should be submitted online at susy.mdpi.com. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website. Once you have registered, click here to go to the submission form for Antibiotics. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

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Authors are encouraged to use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office antibiotics@mdpi.com. Accepted file formats are:

- Microsoft Word: Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, we encourage you to use the Antibiotics Microsoft Word template file. Please insert your graphics (schemes, figures, etc.) in the main text after the paragraph of its first citation.
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When preparing manuscripts in LaTeX, we encourage you to use the Antibiotics LaTeX template files. You can now also use the online application writeLaTeX to submit articles directly to Antibiotics. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.

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Antibiotics now accepts free format submission:

- We do not have strict formatting requirements, but all manuscripts must contain the required sections: Author Information, Abstract, Keywords, Introduction, Materials & Methods, Results, Conclusions, Figures and Tables with Captions, Funding Information, Author Contributions, Conflict of Interest and other Ethics Statements. Check the Journal Instructions for Authors for more details.

- Your references may be in any style, provided that you use the consistent formatting throughout.

It is essential to include author(s) name(s), journal or book title, article or chapter title (where required), year of publication, volume and issue (where appropriate) and pagination. DOI numbers (Digital Object Identifier) are not mandatory but highly encouraged. The bibliography software package EndNote, Zotero, Mendeley, Reference Manager are recommended.

- When your manuscript reaches the revision stage, you will be requested to format the manuscript according to the journal guidelines.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work. It should explain why the manuscript fits the scope of the journal.

Any prior submissions of the manuscript to MDPI journals must be acknowledged. If this is the case, it is strongly recommended that the previous manuscript ID is provided in the submission system, which will ease your current submission process. The names of

proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

All cover letters are required to include the statements:

- We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal.
- All authors have approved the manuscript and agree with its submission to (journal name).

Author Biography

Authors are encouraged to add a biography (maximum 150 words) to the submission and post it to SciProfiles. This should be a single paragraph and should contain the following points:

1. Authors' full names followed by current positions;
2. Education background including institution information and year of graduation (type and level of degree received);
3. Work experience;
4. Current and previous research interests;
5. Memberships of professional societies and awards received.

Manuscript Preparation

General Considerations

- Research manuscripts should comprise:
 - o Front matter: Title, Author list, Affiliations, Abstract, Keywords.
 - o Research manuscript sections: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).
 - o Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.

Additional point:

Manuscripts describing the use of computational modelling and/or molecular docking programs to predict the structures or activity of new antibiotics will not be considered, unless they present additional supporting data, such as biological test results (using microorganisms and/or pure protein).

- Review manuscripts should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured

reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.

- Case reports should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.

- Graphical Abstract:

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, TIFF, or SVG. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.

- Acronyms/Abbreviations/Initialisms should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.

- SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.

- Accession numbers of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and Expression Data.

- Equations: If you are using Word, please use either the Microsoft Equation Editor or the Math Type add-on. Equations should be editable by the editorial office and not appear in a picture format.

• Research Data and supplementary materials: Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about Supplementary Materials and Data Deposit for additional guidelines.

• Preregistration: Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.

• Guidelines and standards: MDPI follows standards and guidelines for certain types of research. See https://www.mdpi.com/editorial_process for further information.

Front Matter

These sections should appear in all manuscript types • Title: The title of your manuscript should be concise, specific and relevant. It should identify if

the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used. Please do not include abbreviated or short forms of the title, such as a running title or head. These will be removed by our Editorial Office.

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3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: <https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use>

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