
**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS-
MICROBIOLOGIA APLICADA**

AVALIAÇÃO DE NOVOS SANITIZANTES DE AÇÃO ANTIMICROBIANA

CAROLINA ROSAI MENDES

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CAROLINA ROSAI MENDES

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas-Microbiologia Aplicada.

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Dedico aos que estão profundamente em meu coração e me fizeram ser quem sou.

Ao meu Pai, Mãe e Esposo.

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*“Você não pode mudar o vento,
mas pode ajustar as velas do barco
para chegar onde quer.”*

Confúcio (Período das Primaveras e Outonos)

RESUMO

Alimentos frescos são frequentemente vetores para surtos de doenças como intoxicação alimentar com vômitos, diarreias e desidratação. Essa categoria de alimentos não usa processamento convencional de esterilização. Portanto, a sanitização desses alimentos são constantemente uma preocupação de saúde pública. Atualmente, novas categorias de sanitizantes são aplicadas em alimentos de consumo humano, cujos compostos são moléculas alternativas ao cloro. Esses sanitizantes livre de cloro foram estabelecidos após a organização mundial da saúde (OMS) divulgar os efeitos negativos da molécula de cloro no organismo, como problemas endócrinos. As nanopartículas de óxido de zinco (NPsZnO) é um sanitizante versátil com aplicações promissoras na categoria de sanitizantes alternativos. As NPsZnO é um antimicrobiano ativo contra um amplo espectro de microrganismos patogênicos. Outra categoria é a sanitização eletrolítica, cuja produção de cloro residual depende exclusivamente do tipo de sais adicionado à solução para produção de uma água eletrolítica com atividade bactericida. Se os parâmetros do processo forem adequadamente controlados, o tratamento elimina as principais bactérias patogênicas. Compostos sanitizantes extraídos de extratos naturais são estudados em destaque para os óleos essenciais. Essa categoria, apresenta atividade bactericida, fungicida; Além de alguns óleos essenciais apresentarem ação antioxidante na superfície do alimento, sendo vantajoso para o desenvolvimento de compostos de ação antimicrobiana para uso como sanitizante. Portanto, nesse estudo foram avaliados diferentes sanitizantes alternativos com potencial antimicrobiano contra cepas patogênicas de interesse.

Palavras-chave: Livre de cloro; Antimicrobiano; Patogênico.

ABSTRACT

Fresh foods are often vectors for disease outbreaks such as food poisoning with vomiting, diarrhea, and dehydration. This category of foods does not use conventional sterilization processing. Therefore, the sanitization of these foods is a constant public health concern. Currently, new categories of sanitizers are applied to foods for human consumption, whose compounds are alternative molecules to chlorine. These chlorine-free sanitizers were established after the World Health Organization (WHO) disclosed the negative effects of the chlorine molecule on the body, such as endocrine problems. Zinc oxide nanoparticles (ZnO NPs) are a versatile sanitizer with promising applications in the category of alternative sanitizers. ZnO NPs is an antimicrobial active against a broad spectrum of pathogenic microorganisms. Another category is electrolytic sanitization, whose production of residual chlorine depends exclusively on the type of salts added to the solution to produce electrolytic water with bactericidal activity. If the process parameters are adequately controlled, the treatment eliminates the main pathogenic bacteria. Sanitizing compounds extracted from natural extracts are studied, with emphasis on essential oils. This category presents bactericidal and fungicidal activity; in addition, some essential oils present antioxidant action on the surface of the food, which is advantageous for the development of compounds with antimicrobial action for use as sanitizers. Therefore, this study evaluated different alternative sanitizers with antimicrobial potential against pathogenic strains.

Keywords: Chlorine free; Antimicrobial; Pathogenic

SUMÁRIO

INTRODUÇÃO JUSTIFICADA	p.10-20
CAPÍTULO 1: Água eletrolítica como sanitizante alternativo ao cloro livre	p.21-30
CAPÍTULO 2: Óleo essencial de neem como fungicida	p.31-41
CAPÍTULO 3: Nanopartícula metálica com atividade antimicrobiana	p.42-52
CAPÍTULO 4: Síntese e avaliação da ação antimicrobiana das MPsQTO	p.53-68
BIBLIOGRAFIA	p.69-75

INTRODUÇÃO JUSTIFICADA

A busca por alimentos frescos, minimamente processados, livres de produtos químicos tem influenciado na disponibilidade de mercado e na regulamentação do uso de vários produtos agrícolas fitossanitários. Produtos frescos e minimamente processados possuem um maior valor agregado quando comparados aos produtos processados e industrializados (Cossu et al. 2017). Apesar disso, o perfil do consumidor avalia as vantagens de escolher essa categoria de produtos.

O crescente interesse por alimentos naturalmente ricos em proteínas, minerais, vitaminas e cuja produção agrária valoriza práticas orgânicas ou de livre adição de químicos, levou a formação de um nicho de mercado saudável e consumidores conscientes.

Os alimentos frescos e minimamente processados têm grande aceitação no mercado internacional, entretanto anualmente cresce o mercado de consumidores interno. São produtos frescos, embalados e de consumo imediato que priorizam as características nutricionais, sensoriais, qualidade e segurança alimentar (Zamuner et al., 2020). Portanto, o produto deve ser limpo, higienizado, desprovido de partes com danos mecânicos ou biológicos e embalado de modo, a facilitar seu consumo e transporte.

Outra vantagem para essa categoria de alimentos é a redução de desperdícios, visando um maior aproveitamento do alimento (Aayush et al., 2022). Entretanto, os alimentos frescos e minimamente processados apresentam maiores desafios de conservação devido aos riscos de contaminação por microrganismos patogênicos ou que podem reduzir a qualidade do produto e principalmente o tempo de prateleira (Raffo & Paoletti., 2022).

Para garantir uma produção de frutos frescos seguros para o consumidor, o pós colheita segue para packing-house, uma instalação mecanizada que prevê a redução do contato humano direto com o alimento e realiza os processos de sanitização e embalagem em atmosfera modificada (Dilarri et al. 2021). Vários países incluindo o Brasil, utilizam produtos à base de cloro na forma de hipoclorito de sódio (NaOCl) em processos de sanitização de alimentos.

No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) junto ao Ministério da Agricultura, Pecuária e Abastecimento (MAPA) estabelecem legislações para o uso de substâncias biocidas em alimentos destinados ao consumo humano ou animal. De acordo com o Código da Regulação Federal nº 21 da Administração de Alimentos e Medicamentos dos Estados Unidos (FDA) Art. 49 adotado no Brasil pela ANVISA e compartilhado pelo MAPA, a concentração permitida do cloro em solução para uso em alimentos deve estar entre 50 ppm e 200 ppm (MAPA, 2018). Entretanto, a Organização Mundial da Saúde (OMS) divulgou dados sobre o efeito cumulativo do cloro livre, sendo responsável por problemas endócrinos como insuficiência renal, deficiência na absorção de iodo e aterosclerose (OMS, 2017).

O uso do cloro livre, quando em contato com a matéria orgânica leva à formação de compostos halógenos com potencial carcinogênico como os trihalometanos e os ácidos haloacéticos (EFSA 2015; Gil et al., 2019). Portanto, o Conselho da União Europeia (UE) adotou o novo regulamento (UE) 2018/605 (Regulamentação da Comissão Delegada da União Européia, 2018) com a categoria “Desreguladores Endócrinos” (DEs) em produtos sanitizantes para uso em alimentos destinados ao consumo humano. Essa categoria lista a proibição do uso de substâncias como o cloro livre produzido a partir do hipoclorito de sódio e eletrólise do cloreto de sódio, que são

nocivos à saúde em longo prazo (European Commission, 2018).

O regulamento permite que a UE proíba o uso do cloro livre em contato com alimentos e a entrada de produtos de destino alimentar no mercado interno com base na identificação do perigo à saúde, em vez de uma avaliação de risco considerando sua exposição.

No Brasil, a nova regulamentação da UE tem influenciado diretamente na escolha dos produtos sanitizantes usados em processos de higienização em frutos frescos e minimamente processados destinados exclusivamente à exportação para UE. Apesar disso, a ANVISA não apresentou qualquer retificação nas diretrizes de lei para o cloro livre destinados ao uso de produtos alimentares do mercado interno. Mesmo assim, os produtores junto as instalações de packing-house tiveram que se adequar as novas leis da UE para continuar com as exportações.

Em 2018 o Brasil ocupou a 23ª posição no ranking dos exportadores de frutos frescos, segundo a Associação Brasileira dos Produtores Exportadores de Frutas e Derivados (Abrafutas, 2018). No mesmo ano, as exportações de frutos frescos e minimamente processados tiveram aumento de 9% que ultrapassaram os US\$ 150 milhões na balança comercial do agronegócio (Abrafrutas, 2018). Embora ainda ocupe a mesma posição no primeiro trimestre de 2020, o país é o terceiro maior produtor de frutos do mundo, com 2,5 milhões de hectares cultivados. No entanto, apenas 3% da produção nacional de frutos frescos foram exportados no ano de 2019 (Secretaria de Agricultura e Abastecimento, 2019).

Esse setor está em ascensão entre acordos comerciais com o Mercosul e a Associação Europeia de Livre-Comércio (EFTA), formado por Suíça, Noruega, Islândia e Liechtenstein, a qual beneficiam a balança comercial do agronegócio brasileiro

(Mercosul-EFTA, 2019). O acordo garante benefícios fiscais imediatos como tarifa zero, os quais, ficam totalmente livres de sobre taxa de importação ou dependendo do produto recebem os benefícios com período de carência que podem variar entre quatro e dez anos.

O ano de 2019 marcou a fruticultura brasileira que alcançou a marca de 21% de aumento em volume exportado, cerca de 980 milhões de toneladas de frutos frescos comparados a 848 milhões em 2018, a qual gerou uma expectativa para alcançar a marca de US\$ 1 bilhão no futuro (Abrafrutas, 2020). Isso representa um aumento de 16% em valor na balança comercial do agronegócio cerca de US\$ 173,19 milhões em comparação a US\$ 158,86 milhões no mesmo período do ano de 2018 (Abrafrutas, 2019).

No entanto, a crise decorrente do vírus da Covid-19 tornou o ano de 2020 com baixa na balança comercial brasileira de exportação de frutos frescos.

A interrupção das atividades de importação no mercado interno da China durante o período de quarentena afetou diretamente os acordos comerciais no mesmo ano. Apesar da China nunca ter sido um grande comprador de frutos frescos brasileiro dentro do quadro de mercado internacional, gerou uma preocupação voltada aos países exportadores concorrentes que abasteciam anteriormente o mercado chinês. E novos acordos comerciais são considerados concorrentes diretos do Brasil nas exportações para novos destinos como a UE. Esse quadro reforça a necessidade de estabelecermos uma posição de maior confiança no mercado de exportadores para UE.

A meta é exportar produtos com a mesma qualidade e seguros a saúde, dentro das novas normativas vigentes da EU que impedem o uso de cloro livre como sanitizante de frutas frescas e minimamente processadas.

O MAPA incentiva a participação do Brasil nas exportações mundiais no setor

de alimentos. Entretanto, ocorre uma disparidade entre os regulamentos para o uso de sanitizantes e biocidas aprovados no Brasil e a UE, e ajustar essas diferenças são a maior preocupação.

O uso de produtos sanitizantes não aceitos pela UE pode gerar uma instabilidade no mercado de exportação, o que reflete na economia do segmento de fruticultura no Brasil.

O processo de sanitização do pós colheita é considerado a principal etapa para reduzir o número de micro-organismos a nível de segurança alimentar (Cossu, 2017).

Os substituintes alternativos ao cloro livre são peróxido de hidrogênio, ácido peroxiacético, ozono, extratos de origem orgânica e ultrassom para serem usados como sanitizantes em packing-house (Caccalano et al., 2021).

A substituição, também significa aumento no valor da produção que deve ser repassado no produto final para o consumidor. Portanto, é de extrema prioridade encontrar novos compostos com ação antimicrobiana que sejam aceitos pela UE de baixo custo e seguros à saúde do consumidor para garantir a contínua ascensão do mercado de exportação.

Alternativas ao uso de sanitizantes a base de cloro livre

A busca por produtos naturais que possam substituir produtos químicos como uma fonte alternativa verde tem sido adotada na UE, a ideia vai ao encontro com as práticas ambientais e renováveis. É um nicho de novas pesquisas para criação de produtos e aplicações.

Ácidos gálicos (galatos) sintetizados de matrizes orgânicas naturais têm sido estudados e aplicados como agentes inibidores de micro-organismos (Kubo et al., 2022).

Esse composto possui a vantagem de inibir bactérias Gram-positivas e Gram-negativas, podendo ser utilizados como um agente efetivo no combate de bactérias patogênicas humanas formadoras de biofilmes, como relatado por Kang et al. (2018).

Esses compostos apresentam também atividade anticarcinogênica, tendo potencial de uso para combate de células tumorais (Locatelli et al., 2023). No entanto, o uso dos galatos em produtos sanitizantes ainda não é aplicável pois a sua produção tem elevado custo e necessita de um composto ancorador estável.

Existe um interesse crescente nas propriedades antimicrobianas de óleos essenciais como alternativas aos produtos químicos biocidas para uso em packing-house. Óleos essenciais são descritos como líquidos hidrofóbicos de cadeia aromática que podem ser obtidos de praticamente de partes de vegetais como flores, raízes, folhas, frutos (Lira et al., 2020)

As substâncias que compõem o óleo essencial são consideradas, em geral, como GRAS (*Generally Recognized As Safe*, ou Geralmente Reconhecido como Seguro) pelo órgão de regulação americano (*Food and Drug Administration – FDA*), por não apresentarem efeitos de tolerância quando usados como aditivos alimentares. Estas características, associadas ao amplo espectro de atividades, como anti-inflamatória, antioxidante, antifúngica e antibacteriana, colocam os óleos essenciais como candidatos viáveis a serem explorados no desenvolvimento de compostos de ação antimicrobiana para uso em sanitizantes alimentares.

Alguns óleos essenciais já são utilizados na agricultura orgânica e outros descritos na literatura devido à ação bactericida, como o óleo de *Lippia gracilis*, *Unonopsis guatteroides*, *Origanum vulgare*, *Thymus vulgaris* (Aziz & Almasi, 2018; Santos et al., 2019; Yoshida et al., 2019; Hać-Szymańczuk et al., 2019).

Recentemente Zayed et al. (2019) descreveu as propriedades antioxidantes do óleo essencial de *Pimpinella anisum* (anis), o que aumentou o interesse para novas pesquisas e aplicações. Além disso, foi descrito a atividade antimicrobiana devido à presença do composto fenólico trans-anetol encontrado na proporção de 80% da composição do óleo (Kasouda et al., 2019).

O trans-anetol é um composto aromático de origem vegetal formado por trans-1-metoxi-4-(prop-1-enil)benzeno, também encontrado em menor concentração nos óleos de *Foeniculum vulgare* (Funcho), *Illicium veru* (anis estrelado), *Cuminum cyminum* (cominho) e *Salvia officinalis* (sálvia) (Anastasopoulou et al., 2019).

A capacidade antimicrobiana dos óleos essenciais depende principalmente da composição molecular do óleo, já que cada composto apresenta um mecanismo de ação específico.

Avaliar o tipo de molécula que compõe o óleo permite compreender melhor os mecanismos de ação antimicrobiana e a região de atuação na célula alvo.

Alguns compostos presentes nos óleos essenciais podem interagir na permeabilidade da bicamada lipídica, desse modo o composto leva ao desequilíbrio e lise celular; outros compostos podem atuar negativamente no sistema enzimático, o que desencadearia uma série de alterações na síntese de produtos considerados essenciais para a sobrevivência da célula ou inativação do material genético (Oliveira et al., 2011).

Compreender os mecanismos de ação das drogas antimicrobianas é parte essencial para desenvolver novas pesquisas com aplicabilidade, visando à segurança da saúde humana e ambiental. Apesar da capacidade antimicrobiana de vários óleos essenciais, sua aplicação pode ser restrita, devido à composição apolar do óleo que impede sua solubilidade em sistemas aquosos e à degradação e evaporação de alguns

compostos. Portanto, a encapsulação do óleo essencial em matriz biopolimérica é uma alternativa para aumentar a sua solubilidade, impedir a evaporação, reduzir a fotodegradação e oxidação do composto.

Matrizes biopoliméricas de fontes renováveis como a quitosana têm chamado muita atenção para o uso em sistemas de dispersão de medicamentos e encapsulação de compostos orgânicos na forma de hidrogéis, micro ou nanomateriais (Boardman et al., 2017). A quitosana é um biopolímero formado a partir da desacetilação da quitina o segundo polímero mais abundante na natureza, depois da celulose (Kou, 2020). É um produto natural, de baixo custo, renovável e biodegradável.

A molécula de quitosana é um copolímero composto por N-acetil-2-amino-2-desoxiD-glucopirranose e 2-amino-2-desoxi-D-glucopirranose, onde os dois tipos de unidades de repetição estão ligadas por (1→4)-β-glicosídica, estas unidades estão distribuídas em diferentes graus, dependendo do grau das porções acetiladas (Fleet, 1985). Assim como a maioria dos polissacarídeos e proteínas, a quitosana é constituída de cadeias poliméricas individuais que são mantidas juntas por ligações dipolo-dipolo e forças de van der Waals.

A quitosana possui cargas positivas na estrutura da cadeia do biopolímero principalmente pelos grupamentos amina NH_2 que estão presentes em grande quantidade e são facilmente protonados a NH_3^+ . Este biopolímero possui uma estrutura molecular quimicamente similar à da quitina, diferenciando-se somente nos grupos funcionais. A principal diferença entre eles é a presença de grupos amino na estrutura da quitosana. Suas propriedades são desejáveis devido à estrutura do polissacarídeo que permite biocompatibilidade, biodegradabilidade e não toxicidade (Bhattacharjee et al., 2020).

A encapsulação do óleo essencial pode ser realizada por meio da reticulação

intermolecular entre o grupo amino protonado e o agente reticulante fosfato aniônico do tripolifosfato (Samrot et al., 2020). Esse método cria redes de ligações cruzadas na matriz do biopolímero e aprisiona o composto orgânico de interesse.

O emprego de micropartículas de quitosana é reportado na literatura devido ao potencial uso para aplicações farmacêuticas como o encapsulamento de medicamentos descrito por Divya & Jisha (2018). Podendo ser aplicado em outras áreas como em sanitizantes para o controle microbiológico em frutos frescos, o qual ainda é um desafio, pois o produto deve eliminar os principais microrganismos patogênicos de origem alimentar sem ser prejudicial à saúde humana.

As nanopartículas são candidatas versáteis para combater microorganismos patogênicos devido às suas propriedades físico-químicas únicas e à capacidade de interagir com células microbianas na nano escala (Hetta et al., 2023).

A categoria de nanopartículas metálicas possui propriedade antimicrobiana através do mecanismo de ação que rompe a membrana celular bacteriana ou pela interferência nos processos celulares (Mamun et al., 2021).

As nanopartículas de prata são capazes de romper matrizes de biofilme, inibir a adesão bacteriana e induzir a morte celular microbiana (Bruna et al., 2021).

As nanopartículas de cobre tem efeito antimicrobiano ao liberar íons de cobre que agem no rompimento da membrana celular bacteriana (Ma et al., 2022).

As nanopartículas de óxido de zinco possuem propriedades antimicrobianas fotoativadas com produção de espécies de oxigênio reativo e íons de zinco. Essa capacidade os torna eficazes na interrupção de formação de biofilmes (Puspasari et al., 2022).

Outra categoria de sanitizante alternativo em destaque é a aplicabilidade da água

eletrolítica na contagem de coliformes fecais e outras cepas de interesse clínico (Zhao et al., 2021). A versatilidade das nanopartículas metálicas permitem a incorporação em vários materiais com a finalidade de agente antimicrobiano.

A água eletrolisada resulta da eletrólise de um sal em solução sob influência de corrente elétrica para a formação de novas moléculas com propriedades reativas e antimicrobianas. De acordo com Zhao et al. (2021) existem diferentes categorias de água eletrolisada como água eletrolisada neutra, água eletrolisada ácida e água eletrolisada alcalina. As diferentes águas eletrolisadas provem do tipo de sal utilizado durante os processos o que interfere no valor de pH da solução.

EUA e o Brasil utilizam a eletrólise da água com NaCl para geração de hipoclorito de sódio em processos industriais de sanitização (Ampaw et al., 2021). No entanto, o método pode ser aplicável com outros tipos de sais para que não haja a geração de molécula de cloro livre. Portanto, a utilização de sais como Na_2SO_4 , ZnSO_4 , e CaCl_2 podem apresentar um potencial uso na sanitização de frutas e legumes frescos sem a molécula de cloro livre produzida.

Os dispositivos de desinfecção eletroquímica podem ser divididos em duas categorias: Os eletrolisadores diretos, a qual interagem diretamente na água com a matéria orgânica; e os eletrolisadores para oxidantes mistos que necessitam de uma solução concentrada de sais para gerar “anólito”, uma mistura de espécies oxidantes fortes que podem incluir a produção de cloro livre, dióxido de cloro, peróxido de hidrogênio, ozônio e outros radicais de vida curta dependendo do sal adicionado durante os processos de eletrólise (Li et al., 2020).

Água eletrolisada ácida, também conhecida como água oxidante é gerada pela

eletrólise de uma solução diluída de cloreto de sódio (NaCl) em uma câmara com eletrodos anódico e cátodo separados por uma membrana, e essa água é obtida do lado do ânodo. Kim et al. (2020) relatou que seu produto tem forte efeito bactericida contra muitos patógenos de origem alimentar. No entanto, como ponto negativo essa eletrólise tem liberação de gás cloro (Kim et al., 2020).

A água eletrolisada neutra tem pH entre 5,5 a 6,5 e produz uma alta concentração de ácido hipocloroso (HClO) sendo considerado o íon hipoclorito (ClO⁻) ofator principal da atividade bactericida; Além de uma alternativa no processamento de sanitização de alimentos por não gerar cloro livre (Rahman et al., 2020).

A água eletrolisada alcalina é gerada por um cátodo e tem um valor de pH entre 9 e 11. Além disso, são utilizadas soluções de cloreto de magnésio (MgCl₂) ou cloreto de cálcio (CaCl₂) de acordo com Sun et al. (2022).

Nesse sentido, podemos avaliar que o controle microbiológico em frutos frescos ainda é um desafio, pois o produto deve eliminar os principais microrganismos patogênicos de origem alimentar, não ser prejudicial à saúde humana e estar dentro das normativas vigentes do destino de exportação. Portanto, nesse estudo foi aplicado diferentes sanitizantes para avaliar a atividade antimicrobiana contra cepas de interesse.

OBJETIVOS

Geral: Sintetizar diferentes compostos sanitizantes que apresentem atividade antimicrobiana contra uma seleção de cepas.

Específico: Avaliar a atividade antimicrobiana da água eletrolítica com sulfato

de sódio; óleo de *A. indica*; nanopartículas de óxido de zinco; óleo de *P. anisum* encapsulado em micropartícula de quitosana (MPsQTO).

CAPÍTULO 1

Água eletrolítica como sanitizante alternativo ao cloro livre

Nesse capítulo é discutido o uso da eletrólise em uma solução de sulfato de sódio (Na_2SO_4) em comparação com a solução de cloreto de sódio (NaCl) comumente aplicado como sanitizante de alimentos, mas recentemente restrita para sanitização de alimentos na UE. Como alternativa ao cloro livre o estudo busca avaliar a atividade antimicrobiana em cepas de *E. coli* com um novo tipo de água eletrolítica que não gera formação de cloro livre durante a eletrólise.

Os resultados são apresentados no artigo “Different electrolytic treatments for food sanitation and conservation simulating a wash process at the packinghouse” descrito por Mendes et al. (2023).



Different electrolytic treatments for food sanitation and conservation simulating a wash process at the packinghouse

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Abstract Microorganisms are predominantly responsible for food deterioration, necessitating the sanitization and removal of these entities from food surfaces. The packinghouse employs free chlorine in the sanitization process; however, free chlorine's propensity to react with organic matter, forming potentially toxic compounds, has led to its restriction or outright prohibition in several European countries. Therefore, this study aims to assess various washing methods, emulating packinghouse conditions, utilizing diverse forms of electrolyzed water to impede microbial proliferation and significantly enhance the food's shelf life. The subject

of investigation was cherry tomatoes. The findings revealed that electrolyzed water containing NaCl exhibited superior efficacy compared to electrolysis with Na₂SO₄. Both forms of electrolyzed water demonstrated noteworthy effectiveness in inhibiting microorganisms, resulting in a reduction of 2.0 Log CFU mL⁻¹ for bacteria and 1.5 Log CFU mL⁻¹ for fungi. The electrolyzed water also exhibited a comparable capability to free chlorine in removing fecal coliforms from the tomato surfaces. Notably, both electrolyzed water treatments extended the shelf life of cherry tomatoes by at least three days, accompanied by minimal or negligible residues of free chlorine. Consequently, the electrolyzed water formulations proposed in this study present themselves as promising alternatives to traditional packinghouse sanitizers.

Carolina Rosai Mendes and Guilherme Dilarri have contributed equally to this work (co-first authorship).

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Keywords Cherry tomatoes · Electrolyzed water · Free chlorine · Microorganism · Shelf life

Introduction

The demand from consumers for fresh, organic, minimally processed, and chemical-free foods is on the rise, impacting their availability and supply in grocery stores and markets. In North America and Europe, the search for and sale of fresh, organic, and chemical-free foods increased by 90% (Arbenz et al. 2015; Rahman et al. 2021). Furthermore, regulations governing the use of various agricultural plant protection products have become more stringent, aiming to ensure the safety of these fresh, minimally processed foods.

Fresh, minimally processed, and organic farming foods pose greater conservation challenges due to the risk of contamination by pathogenic microorganisms, which can compromise product quality and, notably, shelf life (Kim and Song 2017). Particularly, fruits may lose essential properties

and decay before reaching consumers, rendering even specific fruits unsuitable for sale (Dilarri et al. 2016). Moreover, bacteria classified as fecal coliforms, such as *Escherichia coli*, can lead to severe foodborne infections in humans, posing a significant food contamination concern (Martin et al. 2016).

To ensure consumer safety, the post-harvest process involves the packinghouse, where sanitization and controlled packaging procedures take place. The primary sanitizing agent used in most countries is chlorinated compounds, primarily sodium hypochlorite (NaOCl), often at a concentration of 200 ppm or higher (Weng et al. 2016; Dilarri et al. 2022). However, the continuous application of chlorine and chlorine-associated compounds raises concerns among public health organizations, leading to restrictions on its use or oversight of chlorine residue on food surfaces (EFSA 2015; Meireles et al. 2016).

The European Union has introduced a new category, 'Endocrine Disruptors' (EDs), for sanitizing products used in foods intended for human consumption. This category imposes limits on the maximum chlorate compounds allowed on fresh fruits (European Commission 2018; Dilarri et al. 2022). The focus on chlorine residue stems from the formation of byproducts (trihalomethanes and chloroform) that are known carcinogens and toxic compounds (São José and Vanetti 2012; Wang et al. 2013; EFSA 2015).

Electrolyzed water results from the electrolysis of a salt solution, with variations in time and electric current, yielding a water product with diverse properties and compounds. A broad spectrum of electrolyzed water exists, including neutral electrolyzed water, acid electrolyzed water, and alkaline electrolyzed water. Additionally, factors such as the type of salt utilized (NaCl, Na₂SO₄, ZnSO₄, and CaCl₂), in conjunction with the electrolysis equipment, electric current, and electrolysis time, can produce various types of electrolytic water characterized by distinct properties and by-products (Rahman et al. 2016; Ampia et al. 2021; Zhao et al. 2021).

The application of electrolyzed water has proliferated across diverse areas, finding utility in the clinical field of human health for the elimination of pathogenic microorganisms (Yan et al. 2021), as well as in the preservation and sanitation of foods (Rahman et al. 2016; Zhao et al. 2021). Electrolysis processes involving water are also deployed in environmental applications, such as the removal of dyes from the textile industry (Setodeh et al. 2021) and the degradation of organic matter present in sewage (Azizi et al. 2015).

The most widely used electrolyzed water involves NaCl, constituting the most extensively studied electrolysis process, given its low cost and high efficiency in microbial elimination. Conversely, electrolysis with Na₂SO₄ has not undergone extensive testing in the sanitization and preservation

of food. To date, no study has explored the use of Na₂SO₄ in food sanitation simulating a packinghouse scenario against fecal coliforms, such as *Escherichia coli*.

It is essential to highlight that, unlike electrolysis with NaCl, which generates free chlorine, electrolysis with Na₂SO₄ does not produce free chlorine. The antimicrobial components in electrolyzed water with Na₂SO₄ consist of reactive oxygen species (ROS), including peroxides, superoxide, hydroxyl radicals, singlet oxygen, and alpha-oxygen. Consequently, aside from its effectiveness in eliminating microorganisms, it leaves no residue in the food.

The objectives of this study were to evaluate electrolyzed water with both NaCl and Na₂SO₄ in the sanitization and preservation of food. Both electrolyzed waters were compared with the traditional NaOCl at 200 ppm used in conventional packinghouses. The electrolysis generated with both salts underwent characterization and was employed in immersion wash assays of cherry tomatoes, simulating a commercial packinghouse. The microorganisms on the food surface were analyzed both before and after the wash treatment. Additionally, *E. coli* was utilized as the fecal coliform contaminant evaluated in this study. Observations were made on the shelf life of cherry tomatoes after the sanitization treatment with the two electrolysis methods, and all data were subjected to statistical analysis to ascertain the efficacy and differences between the two proposed electrolyzed water solutions.

Materials and methods

Electrolysis

The electrolysis was conducted using a deionized water solution of Na₂SO₄ at 0.08 M or NaCl at 0.08 M. The process took place in a cell containing titanium (Ti) electrodes coated with positively and negatively charged TiO₂ and RuO₂, each with a geometric area of 40.6 cm² for the anode and cathode. The energy system was connected to an electric power source generated by the Dower model FCC-3005D. A magnetic stirrer was attached to the electrolytic system for agitation and homogenization of the electrolyzed water (Dilarri et al. 2016). The setup scheme of the electrolytic system is illustrated in Fig. S1 of the Supplementary Material. All electrolysis procedures were carried out for 3 min at 1 A and 8.7 V.

Conductivity, temperature, pH, and free chlorine levels of the produced electrolyzed water were monitored before and after electrolysis. Conductivity, temperature, and pH were measured using the Benchtop M1000 pH/mV/Conductivity/ISE/DO—Meters by LabForce (Muttentz, Switzerland). Free chlorine was quantified using the DPD colorimetric method with the Test Kit Model DL-DPDT by Del Lab (Araraquara,

Brazil), and the absorbance of the color solution was measured using the UV–Vis spectrophotometer Shimadzu—Model 2401-PC (Kyoto, Japan). The electrolysis processes were executed ten times, with all parameters analyzed and compared during their generation.

Fecal coliform evaluated

The fecal coliform employed in this study was the Gram-negative bacterium *E. coli* (ATCC 8739). *Escherichia coli* cells were cultured in a nutrient broth medium containing 5 g L⁻¹ of peptone and 3 g L⁻¹ of beef extract, supplemented with 15 g L⁻¹ of bacterial agar for solid medium. The cultivation was conducted at 35 °C for 12 h in a shaker operating at 200 rpm. All reagents utilized were sourced from Himedia Laboratories Ltd. (Mumbai, India).

Food selection and sanitization assays

The vegetable selected for this investigation was the cherry tomato (*Solanum lycopersicum* var. *cerasiforme*). All specimens were directly procured from the producer, bypassing any sanitization processes in a packinghouse. The cherry tomatoes were meticulously divided into three distinct groups, ensuring uniformity in size, weight, and the absence of punctures or deformities. Rigorous control measures were implemented to maintain the utmost consistency among the evaluated groups.

Sanitization assays were conducted with three independent replicates, each comprising three groups of 10 vegetables subjected to different treatments. This approach aimed to establish a robust dataset for subsequent statistical analyses. To mitigate the risk of immediate spoilage, the sanitization assays were performed promptly after the collection of tomatoes.

Prior to conducting the sanitization assays, wherein the contamination of tomatoes by *E. coli* cells was simulated to mimic fecal coliform contamination, a series of steps were executed. Initially, *E. coli* cells were cultured in a nutrient broth medium until reaching a concentration of 10⁸ CFU mL⁻¹. Subsequently, the medium underwent centrifugation at 6000 × g for 7 min, resulting in the discarding of the supernatant, and the cells were then resuspended in a saline solution (NaCl 0.96%). Following this, tomatoes were spray-inoculated with the *E. coli* suspension at a concentration of 10⁸ CFU mL⁻¹ until the run-off point, after which they were allowed to air-dry at room temperature for 4 h.

The sanitization tests encompassed the evaluation of four distinct treatments: Negative Control (NC) utilizing auto-claved tap water; Positive Control (PC) employing a NaClO solution (Chemical Abstract Service number 7681-52-9, purchased from Sigma-Aldrich, Taufkirchen, Germany) with a concentration of 200 ppm of free chlorine; Treatment 1

(T1) employing electrolyzed water with NaCl; and Treatment 2 (T2) utilizing electrolyzed water with Na₂SO₄. Sanitization assays involved immersion washing in 500 mL of the respective treatment for duration of 2 min. Subsequently, the tomatoes were rapidly dried using a non-heated airflow from a hair dryer for 30 s and then placed into sterile Becker flasks covered by a porous membrane.

The Becker flasks were maintained at room temperature (1 ± 26 °C), and individual analyses of vegetable treatments were conducted every 24 h. The assessments included checking for the state of deterioration based on parameters such as degree of deterioration, fungus coverage, color, and consistency. Values ranging from 0 to 4 were assigned, with 0 indicating no change, 1 assigned to changes covering up to 1/4 of the food, 2 to changes covering up to 1/2 of the food, 3 to changes covering up to 3/4 of the food, and 4 to changes affecting the entire food item. Additionally, the appearance of each type of vegetable was scrutinized, considering external color categorized as alive (A) for foods maintaining their appearance and dimmed (D) for those that lost their color.

Microbiological analysis

Microbiology analyses were conducted after the food sanitization assays. Sterile swabs were employed to sample the treated foods, and these samples were inoculated onto various culture media specified in the present study. Nutrient Agar (NA) was employed for bacterial growth, while Sabouraud agar, supplemented with 0.15% v/v chloramphenicol, was utilized for fungal growth. Subsequently, the plates were incubated for 24 h at 30 ± 1 °C for NA media and 72 h at 26 ± 1 °C for Sabouraud media. Following the incubation period, colony-forming units (CFU) of fungi and bacteria were enumerated for each treatment. Microbiology assays were conducted immediately after the sanitization process (time zero) and after twelve days.

A total of 9 plates with NA and 9 with Sabouraud were made and counted for each treatment evaluated (NC, PC, T1, and T2). Each treatment in the sanitization assays consisted of three equal and independent groups, resulting in the utilization of 3 plates of medium for each independent group and a total of 9 medium plates. Given the presence of three independent triplicates in the sanitization assays, a cumulative total of 27 medium plates of NA and Sabouraud medium were assessed for time zero and the final time, respectively.

To confirm and identify the presence of the fecal coliform used as contamination (*E. coli* cells), bacterial colonies exhibiting growth similar to that of *E. coli* on agar medium were subjected to diagnosis by PCR, following the protocol outlined by Tsen et al. (2002). This approach allows for the verification of the efficacy of treatments PC, T1, and T2 in eliminating all *E. coli* cells. The same was done for the NC,

seeking to validate the results with the rescue of *E. coli* cells from tomatoes surface.

Statistical analysis

All experimental assays were conducted with three independent groups, each comprising 10 vegetables per group. All assays were executed in triplicate and subjected to standard deviation analysis (SD) (Eq. 1) to assess experimental errors. All datasets underwent non-parametric statistical analysis using the Kruskal–Wallis (Dunn) test, with three degrees of freedom, performed using BioEstat 5.30 software.

$$SD = \sqrt{\frac{1}{N-1} \sum_{i=1}^N \left(\frac{Qie - Qic}{Qie} \right)^2} \quad (1)$$

where Qie and Qic ($\mu\text{g mg}^{-1}$) are experimental and calculated data, and N is the number of measurements made.

Results and discussion

The results of temperature, conductivity, and pH revealed clear distinctions between the two types of electrolyzed water produced in this study and tap water (Table 1).

Tap water exhibited consistent properties in terms of conductivity, pH, and temperature, despite the presence of free chlorine, which remained at very low levels. The Brazilian water treatment includes a chlorination stage, yet the concentrations are regulated to ensure that the level of free chlorine in the water does not exceed 2 ppm for consumers. In contrast, electrolyzed water with NaCl demonstrated significantly higher levels of free chlorine, potentially exerting antimicrobial effects at this concentration. Additionally, it displayed an alkaline pH and greater conductivity compared to tap water. These characteristics of electrolyzed water with NaCl arise from the generation of superoxide radicals and other antimicrobial agents such as Cl_2 , ClO^- , NaClO , HO_2 , and HOCl (Rahman et al. 2016). Although the levels of free chlorine generated by NaCl electrolysis raise health concerns (EFSA 2015; Meireles et al. 2016), registering 67.15 ppm, this concentration is comparatively low when juxtaposed with traditional food sanitization in packinghouses using free chlorine at 200 ppm (Weng et al.

2016; Dilarri et al. 2022). Therefore, the electrolyzed water generated and applied in this study is unlikely to result in high concentrations of chlorine residue on the food surface post-application.

NaOCl is known to be corrosive, posing risks to individuals involved in packinghouse or sanitization processes, warranting caution in its use (São José and Vanetti 2012; Dilarri et al. 2022). However, at the levels of NaOCl produced in this work, it was deemed safe for use with low corrosive potential. The NaCl electrolysis proves highly effective in sanitizing food and is authorized and employed in various countries worldwide (Gil et al. 2015; Rahman et al. 2016). Notably, due to the NaCl concentration and electrolysis time, the generated electrolyzed water with NaCl predominantly contains a free chlorine amount below 100 ppm and maintains a pH value close to neutrality. This aligns with recommendations from the US Environmental Protection Agency and the United States Department of Agriculture (Hricova et al. 2008). Therefore, it can be affirmed that the NaCl electrolyzed water produced in this study is less aggressive compared to other studied and applied food sanitizers (Gil et al. 2015; Rahman et al. 2016; Ampia et al. 2021; Dilarri et al. 2021, 2022).

Electrolysis using Na_2SO_4 avoids the generation of free chlorine, and additionally, it imparts an acidic pH to the solution owing to the formation of free H_2 and H^+ in the electrolyzed water. Na_2SO_4 electrolysis produces reactive oxygen species (ROS), including peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen, all serving as potent antimicrobial agents that induce damage to proteins, nucleic acids, and cytoplasmic membranes (Li et al. 2010; Dharmaraja 2017; Yan et al. 2021). Crucially, the ROS compounds do not foster resistance or mutations when applied against microorganisms, presenting an additional advantage of Na_2SO_4 electrolyzed water (Li et al. 2010; Dharmaraja 2017).

While the conductivity of both types of electrolyzed water did not exhibit significant differences, they consistently maintained higher levels than tap water due to the ions formed. This elevated conductivity may also contribute to an inhibitory effect against certain microorganisms.

The cherry tomatoes exhibited signs of decay after 8 days, particularly following exposure to tap water washing (Table 2). Since these vegetables had not undergone any packinghouse processing or sanitization, their accelerated

Table 1 Physicochemical values of the two kinds of electrolyzed water produced in the present study and the tap water

	Free chlorine (ppm)	Conductivity (mS cm^{-1})	pH	Temperature ($^{\circ}\text{C}$)
Tap water	1.4 ± 0.3	0.8106 ± 0.18	6.02 ± 0.06	23 ± 1.00
Electrolyzed water (NaCl)	67.15 ± 2.1	73.04 ± 0.48	8.02 ± 0.90	33 ± 0.80
Electrolyzed water (Na_2SO_4)	0	69.88 ± 1.59	4.80 ± 0.66	31 ± 0.78

Table 2 Classification of the vegetables after the sanitization assay

Days	Degree of deterioration				Fungus coverage				Consistency				Color				Kruskal–Wallis (Dunn)			
	NC	PC	T1	T2	NC	PC	T1	T2	NC	PC	T1	T2	NC	PC	T1	T2	NC	PC	T1	T2
1	1	1	1	1	1	1	1	1	1	1	1	1	A	A	A	A	n.s	n.s	n.s	n.s
2	1	1	1	1	1	1	1	1	1	1	1	1	A	A	A	A	n.s	n.s	n.s	n.s
3	1	1	1	1	1	1	1	1	1	1	1	1	A	A	A	A	n.s	n.s	n.s	n.s
4	2	1	1	1	1	1	1	1	2	1	1	1	A	A	A	A	n.s	n.s	n.s	n.s
5	2	1	1	1	1	1	1	1	2	1	1	1	A	A	A	A	n.s	n.s	n.s	n.s
6	3	2	1	2	1	1	1	1	3	1	1	2	D	A	A	A	n.s	<i>p</i> > .05	<i>p</i> > .05	<i>p</i> > .05
7	3	2	1	2	2	1	1	1	3	1	1	2	D	A	A	A	n.s	<i>p</i> > .05	<i>p</i> > .05	<i>p</i> > .05
8	4	3	2	3	4	2	2	2	4	2	2	3	D	A	A	A	n.s	<i>p</i> > .05	<i>p</i> > .05	<i>p</i> > .05
9	4	3	3	3	4	3	3	3	4	3	3	3	D	A	A	A	n.s	<i>p</i> > .05	<i>p</i> > .05	<i>p</i> > 0.5
10	4	3	2	3	4	3	3	3	4	3	3	3	D	A	A	D	n.s	<i>p</i> > .05	<i>p</i> > .05	n.s
11	4	4	3	4	4	4	3	4	4	4	3	4	D	D	D	D	n.s	n.s	n.s	n.s
12	4	4	4	4	4	4	4	4	4	4	4	4	D	D	D	D	n.s	n.s	n.s	n.s

n.s. Not significant

decay is expected. Despite rotting within a week at room temperature post-tap water washing, it is likely that the Negative Control (NC) treatment (2 min of immersion in tap water) merely removed surface dirt and debris without imparting any sanitizing activity. Notably, during the initial five days, the NC showed no significant difference compared to PC, T1, and T2, with distinctions becoming apparent only on the sixth day (Table 2). Remarkably, by the conclusion of the 12-day assay, the characteristics of the NC once again aligned with those of T1 and T2 (Table 2). This outcome underscores the pivotal role of washing processes in packinghouses, significantly extending the shelf life of foods (Kaewklin et al. 2018; Belgacem et al. 2021).

After six days of exposure to T1 and T2, cherry tomatoes exhibited favorable characteristics when compared with the Negative Control (NC) and Positive Control (PC), being statistically different from the NC. However, a distinction emerged between T1 and T2, with T2 manifesting signs of deterioration, loss of color, and a decrease in food consistency from day eight, indicative of a shorter shelf life compared to T1 and PC. By the tenth day, cherry tomatoes from T2 had already decayed, displaying all characteristics identical to those of the NC on the eighth day. While T2 extended the shelf life of cherry tomatoes by three days (a commendable outcome) it remained inferior to T1 and PC. Conversely, T1 increased the shelf life of cherry tomatoes by four days, proving more effective than T2 and statistically equivalent to PC. The color and food consistency of cherry tomatoes, following sanitization with T1 and PC, began to decline after the ninth day, attesting to their efficiency in sanitization and shelf life extension when compared with the NC.

The T1 contained antimicrobial compounds such as ClO^- , HO_2 , Cl_2 , HOCl , NaClO , and superoxides, all proven

to be efficient in inhibiting microorganisms and eliminating them without inducing resistance or mutation (Xuan and Ling 2019; Kim et al. 2019; Ampia et al. 2021). Notably, ClO^- , HO_2 , HOCl , and free chlorine act by damaging cytoplasmic membrane structures, ultimately leading to cell death (Dilarri et al. 2021). Consequently, for an organism to develop resistance to these compounds, mutations in multiple genes related to the structure of the cytoplasmic membrane would be necessary, rendering the emergence of mutants resistant to halogen compounds highly improbable. While the specific quantity of electrolysis byproducts was not identified in this study, the quantification of free chlorine, along with other parameters such as temperature, pH, and conductivity, affirms the generation of antimicrobial derivatives during the electrolysis of T1 and T2.

T2 demonstrated lower efficacy when compared to T1. Reactive Oxygen Species (ROS) are the primary agents in electrolyzed water with Na_2SO_4 against deteriorating microorganisms (Li et al. 2010). These agents are volatile, losing their effectiveness rapidly after electrolysis. Although the washing experiment was conducted immediately after electrolysis to minimize the loss of antimicrobial agents formed in the water, some may have still volatilized during the immersion washing process, diminishing the impact of T2 compared to T1. It is essential to recognize the capacity of certain microorganisms to resist ROS (Dharmaraja 2017; Ren et al. 2021), thus reducing the sanitizing effect of T2 and potentially resulting in a higher number of microorganisms on the food surface even after treatment. However, T2 demonstrated a significant extension in the shelf life of cherry tomatoes compared to the NC, establishing itself as a viable alternative for the sanitization process in packinghouses to enhance the

shelf life of vegetables. Another advantage of T2 lies in its non-generation of chlorine residues or derivatives on the food surface.

Microbiological assays revealed a significant difference between the electrolytic treatments and the NC, confirming that washing with the two types of electrolyzed water led to a substantial reduction in the number of bacterial colonies on the food surface. Both T1 and T2 were statistically equivalent to the PC, representing a reduction of 2 Log CFU mL⁻¹ in bacterial numbers in both electrolytic treatments when compared to the NC (Fig. 1). However, by the final time point (12 days), the number of bacterial colonies in each treatment had returned to the levels observed in the NC (Fig. 1). Surprisingly, even the traditional sanitization with free chlorine at 200 ppm exhibited statistical equivalence to the NC after twelve days, implying that it provides a similar level of protection and microorganism elimination as T1 and T2.

The fungi isolation results paralleled those of bacterial isolation, demonstrating a reduction of 1.5 Log CFU mL⁻¹. Although this reduction was less pronounced compared to bacteria, it still exerted a significant effect on removing fungi from the surface of cherry tomatoes (Fig. 2). Both electrolytic treatments were statistically equivalent at both time zero and after twelve days (Fig. 2). Additionally, there were consistently more yeast colonies observed than molds or mycelium fungi on all treatment plates, which constitutes an intriguing result.

The results of microorganism isolation from the sanitization assays during the final storage period indicated a persistent presence of the microbial population even after the treatments. This suggests that both the electrolytic treatments and the PC (which are commonly recommended sanitation agents in packinghouses) predominantly inhibited microorganisms rather than eliminating them entirely from the surface of cherry tomatoes. Notably, electrolytic

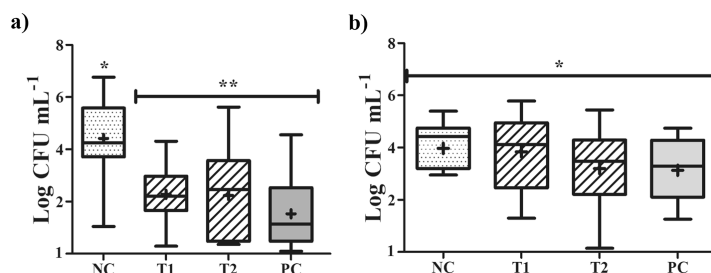


Fig. 1 Microbiological assays depicting bacteria colony numbers following washing treatments: **a** Bacteria isolation after the wash (time zero), and **b** Bacteria isolation at the final assay time (12 days). NC represents washing with tap water, PC denotes washing with a free chlorine solution at 200 ppm, T1 corresponds to washing with electrolyzed water with NaCl, and T2 signifies treatment with electro-

lyzed water with Na₂SO₄. Boxes illustrate the data distribution per treatment, with lines above and below representing the minimum and maximum values in the datasets; plus signs indicate the averages calculated for each distribution. Asterisks denote statistical differences among treatments, as determined by Kruskal–Wallis (Dunn) analysis

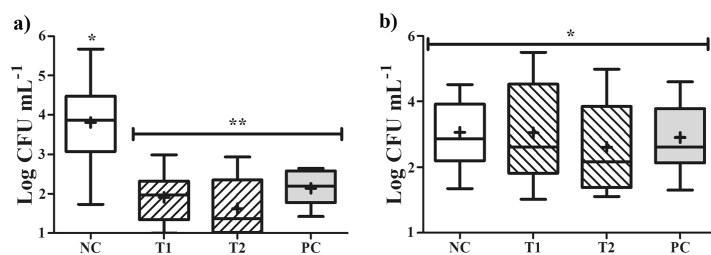


Fig. 2 Microbiological assays displaying fungi colony numbers following washing treatments: **a** Fungi isolation after the wash (time zero), and **b** Fungi isolation at the final assay time (12 days). NC stands for washing with tap water, PC refers to washing with a free chlorine solution at 200 ppm, T1 corresponds to washing with electrolyzed water with NaCl, and T2 signifies treatment with electro-

lyzed water with Na₂SO₄. Boxes illustrate the data distribution per treatment, with lines above and below representing the minimum and maximum values in the datasets; plus signs indicate the averages calculated for each distribution. Asterisks denote statistical differences among treatments, as determined by Kruskal–Wallis (Dunn) analysis

water and NaOCl at 200 ppm exhibit limited effectiveness in eliminating bacterial biofilms (Dilarri et al. 2016; Zhao et al. 2021; Yan et al. 2021), potentially contributing to the resurgence of microbial growth towards the end of the food storage period. Bacterial and fungal spores may also persist on the food surface, leading to renewed microbial growth once the antimicrobial compounds have completely volatilized. It is well-known that electrolyzed water is not effective in eliminating microbial spores (Pintaric et al. 2015).

Moreover, even at time zero (immediately after sanitizing), isolated microorganisms were detected on the surface of tomatoes washed with T1, T2, and PC (Fig. S2 of the Supplementary Material). Since the tomatoes were not kept in a sterile environment and were exposed to external air, other microorganisms or microbial spores might have come into contact with them, simulating real-world market storage conditions. It's noteworthy that the byproducts generated by the electrolysis of Na_2SO_4 exhibit a fast volatilization effect, resulting in a less sustained residual effect compared to electrolysis with NaCl or NaOCl at 200 ppm. Electrolysis with NaCl produces volatile compounds, but free chlorine persists for a longer duration on the food surface compared to the products from Na_2SO_4 electrolysis (SO_4^{2-} and H^+) (Li et al. 2010; Pintaric et al. 2015; Rahman et al. 2016). This is supported by the observed increase in the shelf life of cherry tomatoes by one additional day in T1 and PC compared to T2. These findings underscore that electrolytic treatments and NaOCl do not exhibit prolonged residual effects on microbial inhibition, and microorganisms reemerge once their biocidal agents have volatilized from the food surface.

The elimination of fecal coliform cells, represented by *E. coli* in the present study, was confirmed by PC, T1, and T2, with a reduction of more than 2 logs of *E. coli* cells rescued (Fig. 3). This result is particularly noteworthy given that the contamination with *E. coli* involved a saline solution with 10^8 CFU mL^{-1} , an excessively high number for real-life contamination scenarios. Nonetheless, T1, T2, and PC significantly reduced the bacterial load compared to NC (3.34 log of CFU mL^{-1} of *E. coli* cells), which only involved a wash with sterile tap water (Fig. 3).

Escherichia coli, as a key fecal coliform and a widely used bacterial species for analyzing food contamination (Martin et al. 2016; Kim and Song 2017; Kim et al. 2019), underscores the food safety aspect of using electrolytic water of NaCl and Na_2SO_4 for food sanitization, completely eliminating this fecal coliform from the food surface. Although this study did not analyze other clinical pathogens such as *Salmonella enterica*, *Salmonella typhi*, *Staphylococcus aureus*, and *Enterococcus faecalis*, Kim et al. (2019) demonstrated success in eliminating a range of pathogens with electrolyzed water. Similarly, Gil et al. (2015) investigated the application of electrolytic water in the fresh-cut industry and concluded that it is as effective as NaOCl in food

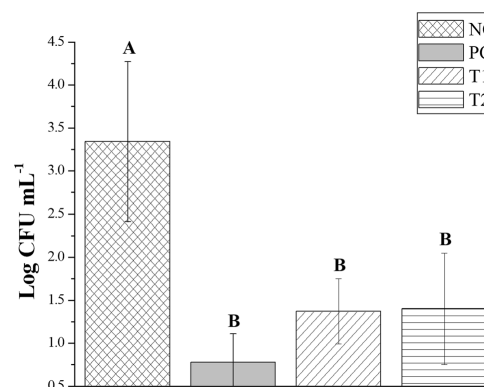


Fig. 3 *Escherichia coli* cells rescued from the tomato surface after the sanitization wash of each treatment at time zero. Bars represent averages of isolated cells. Data showing the same capital letters are not significantly different from each other based on Kruskal–Wallis Dunn analysis

disinfection, offering an eco-friendly and non-hazardous technology.

The present work revealed that many microorganisms associated with food rot regrew, indicating the persistence of microbiota in the food even after the sanitization treatments. This phenomenon was also observed in the PC during the final storage time, emphasizing that the shelf life and fecal coliform removal evaluated by electrolytic water were comparable to NaOCl. Consequently, based on the findings of this study and other investigations (Li et al. 2010; Rahman et al. 2016; Gil et al. 2015; Kim et al. 2019), it is reasonable to consider that foods sanitized with electrolytic water are as safe for consumption as those sanitized by the traditional NaOCl used in the food industry.

In summary, the electrolysis with Na_2SO_4 and NaCl has demonstrated efficacy as a viable sanitizer, effectively reducing a significant quantity of microorganisms and fecal coliform on the food surface. Moreover, it extends the shelf life by an additional three days without leaving any residual free chlorine, a notable advantage considering the increasing demand for food sanitizers devoid of chlorine residue (EFSA 2015).

It is crucial to recognize that while NaOCl at 200 ppm remains the most widely employed sanitizer in industrial settings due to its lower operational costs compared to electrolytic water, the constraints imposed by EU regulations on endocrine disruptors (EDs) will likely curtail its application. This limitation justifies the exploration of alternatives to NaOCl in packinghouses. Notably, water electrolysis stands out as a technology easily transferrable to large-scale industries (Chatenet et al. 2022). Many industries are already

adapting their sanitization processes to comply with stringent regulations regarding chlorine residues. Consequently, the study's findings emphasize the comparative aspects of applying electrolytic water in contrast to NaOCl, revealing minimal differences that could position electrolytic water as a viable alternative in packinghouses.

Conclusions

The effectiveness of both electrolytic waters in inhibiting microorganisms on the food surface, leading to a four-day extension in the shelf life of cherry tomatoes, has been established. The inhibitory effects of the evaluated electrolyzed waters were transient, with a limited residual impact on the food surface post-application. It is noteworthy that a residual microbiota persisted on the food surface even after the sanitization process, indicating that the effect of the electrolytic water did not eliminate all microorganisms, ultimately resulting in the rotting of cherry tomatoes after 12 days. Interestingly, similar results were observed in the case of the free chlorine solution at 200 ppm.

Both electrolyzed waters also demonstrated significant reductions in the tested fecal coliforms, with results statistically equivalent to those achieved with the free chlorine solution at 200 ppm. Consequently, electrolytic water derived from NaCl and Na₂SO₄ exhibited comparable efficacy to traditional free chlorine sanitation, suggesting it as a viable alternative to NaClO at 200 ppm for disinfecting fresh produce in packinghouses.

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Data availability The data that support the findings of this study are available from the corresponding authors upon request.

Declarations

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

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CAPÍTULO 2

Óleo essencial de neem como fungicida

A agência coordenada pela Organização das Nações Unidas (ONU) *Food and Agriculture Organization* (FAO), avaliou que 35% da produção de alimento global é perdida devido a contaminação por bactérias, fungos e pragas (Savary et al., 2019).

Nesse capítulo é avaliado a sanitização de citrus usando óleo essencial de neem contra o fitopatógeno *Phyllosticta citricarpa* causador da pinta-preta uma doença que reduz a produção, gera degradação e manchas na superfície do fruto (Sanches et al., 2019).

O citros tem elevada importância comercial, quando a qualidade do fruto decai reduz seu valor de mercado, a qual interfere na balança comercial do país. Segundo levantamento feito pelo Fundo da Defesa da Citricultura (FUNDECITRUS) a safra da laranja de 2021/2022 se encerrou com aproximadamente 264 milhões de caixa apenas no cinturão citrícola, região que compreende o estado de São Paulo e o Triângulo Sudoeste Mineiro, cuja apresenta os maiores números de produção de citros do país (FUNDECITRUS, 2022).

Uma alternativa fitossanitária que contribuem com a redução do patógeno é avaliado no artigo “Neem Essential Oil as an Antifungal Agent against *Phyllosticta citricarpa*” descrito por Schuch & Mendes et al. (2024).

Research Article

Neem Essential Oil as an Antifungal Agent against *Phyllosticta citricarpa*

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The fungus *Phyllosticta citricarpa* is a quarantine phytopathogen responsible for causing citrus black spot (CBS) disease. To export fruits to CBS-free countries, they must undergo a sanitation process to ensure disease control. In this study, neem essential oil (NEO) was tested against *P. citricarpa* for the first time as an alternative sanitizer. *In vitro* experiments were conducted to determine the inhibition concentration of NEO for *P. citricarpa*, and the mode of action of the essential oil was evaluated. *In vivo* assays were performed to simulate the sanitization process used in packinghouses. NEO was characterized by GC-MS/MS. The results revealed that NEO at 100 $\mu\text{L}\cdot\text{mL}^{-1}$ exhibited a similar inhibitory effect as copper oxychloride, suppressing $89.68 \pm 1.14\%$ of fungal mycelium growth. Fluorescence microscopy experiments demonstrated that NEO functions by disrupting the cytoplasmic membrane of fungal hyphae, leading to their death within 30 minutes of contact with NEO. GC-MS/MS characterization revealed a high presence of phenolic compounds, which serve as the primary antifungal agents responsible for the action against fungal hyphae. *In vivo* assays showed that NEO at 100 $\mu\text{L}\cdot\text{mL}^{-1}$ also reduced microorganisms (CFU mL^{-1}) by $93.00 \pm 3.88\%$ compared to the negative control. Overall, the results demonstrate that NEO can effectively serve as an alternative sanitizer against *P. citricarpa* in citrus packinghouses. Our findings allow future studies to explore the use of NEO for sanitizing other fruits and combating different phytopathogens to broaden its potential application in fruit sanitation for export.

1. Introduction

The orange fresh fruit, a quintessential agricultural product, has annual export revenue of approximately 2 billion US dollars worldwide, rendering it a cornerstone commodity for major producer countries like the USA, Brazil, and China,

boasting remarkable profitability [1, 2]. Within the USA, fresh citrus fruits, including oranges, constitute one-fifth of all fresh fruit consumption, playing a pivotal role in the American diet [3]. Not only are citrus fruits esteemed for their flavor but they also harbor significant antioxidant properties, brimming with phytochemicals and vitamins

such as Vitamin C, which impart its many health benefits [4, 5]. However, this agricultural treasure is not without its challenges; natural phytopathogens inhabit citrus fruits, compromising their quality for consumption. Citrus is susceptible to quarantine diseases, imposing restrictions on their exportation and distribution to consumers. Among these quarantine diseases, citrus black spot (CBS) emerges as one of the most formidable adversaries, inflicting substantial losses upon producers [6, 7]. *Phyllosticta citricarpa*, the causative agent of CBS, ravages economically significant citrus species, precipitating fruit depreciation and orchard yield diminishment [8, 9]. The lifecycle of *P. citricarpa* within citrus orchards is complex, with contamination of leaves and fruits being commonplace [10, 11]. Notably, *P. citricarpa* spores can adhere to citrus fruit surfaces, with disease symptoms manifesting after approximately 40 days, although the majority tend to emerge closer between 110 to 200 days [11–13]. Symptoms may only become apparent as the fruit ripens or during postharvest stages, facilitating disease dissemination to new regions and countries. Therefore, it is imperative that countries grappling with CBS ensure the thorough eradication of *P. citricarpa* propagules, including mycelia and spores, from the fruit surfaces earmarked for exportation.

The CBS has entrenched itself in the USA and Brazil [1, 14], both major citrus producing countries. European Union classified the CBS as A1 quarantine phytopathogen (Phytosanitary Regulations 2019/2072), having a high control for the citrus fresh fruits exported to their countries [15, 16]. Therefore, to control the CBS and avoid its dissemination to the free disease areas, it is applied fungicides based on copper to protect the fruits during the period of susceptibility [17]. Copper oxychloride at 90 g·L⁻¹ of concentration is the mainly recommended copper-based formulation to control CBS [8, 18]. The Brazilian Ministry of Agriculture, Livestock, and Supply (MAPA) mandates thorough sanitation of citrus fruits at packinghouse facilities to ensure the eradication of any lingering phytopathogens on fruit surfaces [19]. Sanitizing agents utilized in this process must demonstrate efficacy against the targeted phytopathogen to guarantee the complete elimination of disease propagules [1, 19]. The current prevalent sanitation practice at Brazilian citrus packinghouses involves immersing fruits in a sodium hypochlorite solution at 200 ppm for a duration of 2 minutes [1]. So far, no study has been carried out to find a specific sanitizer for *P. citricarpa* that still has a residual effect and eliminates other microorganisms present on the citrus surface.

Essential oils have surfaced as a promising alternative amidst the emergence of novel organic sanitizers, particularly since their approval by the European Union Commission Regulation as suitable sanitizing agents for citrus fruits [1, 20, 21]. Essential oils are extracted from plant leaves, while vegetable oils are generally extracted from seeds. Despite originating from the same plant, essential oils may exhibit variations in composition owing to their extraction from different plant parts. A myriad of essential oils has been recognized for their potent antimicrobial properties on inhibiting microbial cells and biofilms [22]. Most

notably, *Azadirachta indica* essential oil, commonly referred to as neem essential oil (NEO), has demonstrated bactericidal efficacy against an array of clinical pathogens, in addition to serving as a natural mosquito repellent for textiles [23–25]. Neem leaf extract has also high efficacy in limiting the growth of fungi of the genus *Rhizopus* [26]. Furthermore, studies have showcased the antifungal activity of NEO against postharvest pathogens such as *Penicillium expansum*, *Monilinia fructicola*, and *Trichothecium roseum* [27]. NEO harbors a diverse array of therapeutic properties attributed to alkaloid, flavonoid, saponin, and steroid metabolites [28, 29]. Active compounds within NEO are categorized into two principal groups: isoprenoids and nonisoprenoids. Isoprenoids encompass triterpenoids and diterpenoids, including limonoids, gedunin, protomelinin, and azadirone. Nonisoprenoid compounds comprise sulfur, proteins, dihydrochalcone, carbohydrates, glycosides, and polyphenols [29]. Neem leaves additionally harbor nimbin, nimbandiol, 6-desacetylnimbin, nimbanene, nimbolide, n-hexacosanol, ascorbic acid, nimbiol amino acid, 17-hydroxyazadiradione, 7-desacetyl-7-benzoylgedunine, and 7-desacetyl-7-benzoylazadiradione [28]. Many of these organic chemical constituents within NEO exhibit potent bactericidal and fungicidal properties. Despite NEO's remarkable antimicrobial attributes, its efficacy against *P. citricarpa* has not been evaluated, nor has it been assessed as a sanitizer compound for citrus fruits.

Therefore, the aims of the present study were to evaluate the antifungal effect of NEO against *P. citricarpa* and its possible use as a sanitizer agent in citrus fruit packinghouses. This is a novel research study that has never been conducted before. *In vitro* assays were used to determine the inhibition concentration of NEO for *P. citricarpa*. The antifungal molecules present in NEO were identified through GC-MS/MS analysis, which allowed for a clear understanding of their mechanisms of action against the *P. citricarpa* hyphae. *In vivo* assays were carried out to simulate a commercial citrus packinghouse and compare the action of NEO with the copper oxychloride (the major agent in CBS control), bringing a novel organic sanitizing agent against CBS for the citrus fresh fruits market.

2. Materials and Methods

2.1. Essential Oil. The NEO sourced from Laszlo® (Sao Paulo, Brazil), licensed under N° 160 and a member of the aromatherapy trade council, was utilized. The NEO was cold pressed from *A. indica* leaves, exhibiting approximately 97% purity extracts.

2.2. Phytopathogen. This study used *P. citricarpa* (McAlpine) van der Aa (synonym: *Guignardia citricarpa* Kiely) (32758-ATCC), stored in silica spheres as outlined by Lange and Boyd [30]. This strain is from the collection of the Laboratory of Multidisciplinary Sciences, Sao Paulo State University (UNESP), Rio Claro campus, Brazil. Cultivation of the fungus occurred on both liquid and solid YPD medium (10 g·L⁻¹ of yeast extract, 20 g·L⁻¹ of bacteriological

peptone, 20 g·L⁻¹ of dextrose, and 15 g·L⁻¹ of agar for solid medium) over a 21-day period at 28 ± 1°C. All reagents for cellular growth were acquired from Himedia Laboratories Ltd. (Mumbai, India).

2.3. Antifungal Sensitivity Assays. The antifungal sensitivity assays to NEO were carried out by the growth of *P. citricarpa* in YPD agar plate, according to the methodology of CLSI AST guidelines [31]. A 10 mm radial fungal mycelium was placed at the center of an YPD agar medium plate. Firstly, the medium plates received different concentrations of the NEO before the *P. citricarpa* inoculation, where the essential oil concentrations in the medium were 40, 60, 80, and 100 µL·mL⁻¹. The NEO was diluted in 1% dimethylsulfoxide (DMSO) from Sigma-Aldrich (Darmstadt, Germany); therefore, for the negative control (NC), 100 µL of autoclaved deionized water with 1% DMSO was used. The positive control (PC) used in the experiments was a commercial copper formulation called Difere®, manufactured by Oxiquímica Agrociência Ltda. (Jaboticabal, Brazil). It contains 350 g·L⁻¹ of metallic copper and 588 g·L⁻¹ of copper oxychloride. This solution is commonly used as a fungicide in citrus farming. The concentration of Difere® used as PC in the present work was 226.44 g·L⁻¹ (90 g·L⁻¹ of copper oxychloride in the final solution). The area of fungal growth after 21 days at 28 ± 1°C was measured using ImageJ software (open source), and the graphs were plotted using Origin version 8 (OriginLab Corporation, USA). All antifungal sensitivity assays comprised three replicas and were conducted thrice. Data validation was performed using the standard deviation (SD)

$$SD = \sqrt{\frac{1}{N-1} \sum_{i=1}^N \left(\frac{Q_{ie} - Q_{ic}}{Q_{ie}} \right)^2}, \quad (1)$$

in which Q_{ie} and Q_{ic} are experimental and calculated data and N is the number of measurements carried out.

2.4. Fluorescence Microscopy Assays. The membrane integrity of *P. citricarpa* was evaluated using the Live/Dead-Cell Viability Kit from Thermo Fisher (Waltham, USA). Prior to assessment, fungal mycelium was cultured in YPD broth at 28 ± 1°C with agitation at 150 rpm for 10 days. Then, the mycelium was separated by centrifugation at 4000 × *g* Force and remained in contact for 30 min with NEO at the optimal concentration for inhibiting *P. citricarpa*, as determined in the antifungal sensitivity assays. SYTO-9 and propidium iodide (PI) dyes, at a concentration of 50 µM each, were employed to stain live (green) and dead (red) fungal hyphae, which were then observed under a fluorescence microscope. Fungal mycelium was immobilized on agarose-coated slides and examined using an Opticam O600R fluorescence microscope (Doral, USA), outfitted with a monochromatic Opticam 12.3 MP camera (Doral, USA). Image processing was conducted using OPTHD, Opticam software (Doral, USA). These

assays were performed in triplicate, with a minimum of 50 hyphae visualized per analysis.

2.5. Characterization of NEO by Gas Chromatography Tandem Mass Spectrometry (GC-MS/MS). GC-MS/MS analysis was employed to characterize the antifungal compounds present in NEO. The analysis was conducted using a Shimadzu GC-MS model QP-2010 Ultra gas chromatograph coupled with a flame ionization detector and mass spectrometer (GC-FID-MS) (Kyoto, Japan). A DB-5 fused silica column (300 mm × 0.25 mm × 1 µm df) was utilized, with helium gas (1 mL·min⁻¹) as the carrier at 89 kPa. NEO, extracted into ethyl acetate, was injected in a 10:1 split ratio mode. The initial injector temperature was maintained at 210°C for 2 minutes, followed by an increase to 280°C at a rate of 5°C/min. Electronic ionization at 70 eV and a scan rate of 0.5 scan/s were applied for sample analysis. MS1 level spectra processing parameters employing the Wavelet (ADAP) method were conducted according to Ni et al. [32]. All GC-MS data files (mzData) were processed using MZmine 2.53 software (open source), according to the parameters outlined by Elie et al. [33].

2.6. Sanitization of Fresh Fruits. The efficacy of NEO in eradicating *P. citricarpa* from citrus surfaces ensures the elimination of any *P. citricarpa* propagules, and thus sanitization assays mimicking packinghouse conditions were conducted. Fungal mycelium was cultured in YPD broth for 10 days at 28 ± 1°C under constant agitation of 150 rpm. Subsequently, the mycelium was separated via centrifugation at 4000 × *g* Force and resuspended in phosphate buffer (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄). For sanitization, fresh *Citrus sinensis* (L.) cv. Pera fruits were washed with neutral soap (Wash Fruit Aruá®, Matão, Brazil) at a concentration of 33.5% to remove debris, followed by drying. The experiment comprised four independent replicates, with each treatment group consisting of 15 oranges, following the methodology by Zamuner et al. [21] with modifications. Initially, the fruits were inoculated with *P. citricarpa* by spraying 200 mL of a saline solution (0.87% of NaCl) with 10⁶ spores per mL of the fungus. The fruits were air-dried at room temperature (23 ± 1°C) for 240 minutes and then washed with NEO diluted in 1% DMSO at the concentration determined in the antifungal sensitivity assays. Autoclaved deionized water with 1% DMSO served as the negative control (NC), while Difere® at 226.44 g·L⁻¹ (90 g·L⁻¹ of copper oxychloride) served as the positive control (PC). The washing step entailed submerging the fruits in each respective treatment solution for 2 minutes, followed by air-drying (without heat) for 30 seconds to remove excess solution, simulating the immersion bath and drying process in a citrus packinghouse [1]. At last, the fruits were incubated for 40 days at 23 ± 1°C in a sterile chamber to prevent contamination with other microorganisms. After the incubation period, the fruits from each treatment were individually washed in PBS, and 100 µL of the wash solution was spread on YPD agar plates and incubated for 21 days at 28 ± 1°C to facilitate *P. citricarpa*

growth and assess complete elimination from the fruit surface. Microbial counts were conducted, and the data were subjected to nonparametric statistical analysis using Kruskal–Wallis (Dunn) with three degrees of freedom. All sanitization assays were performed in triplicate with four independent replicates. Data validation was performed using standard deviation (equation (1)) to ascertain experimental errors. Graphs were generated using Origin 8 software (OriginLab Corporation, USA), while statistical analyses were conducted using BioEstat 5.0 (open source).

3. Results and Discussion

The antifungal sensitivity assays revealed promising results regarding the efficacy of NEO in inhibiting the growth of *P. citricarpa* (Figure 1). The fungal growth was notably affected by NEO, with the concentration of $100 \mu\text{L}\cdot\text{mL}^{-1}$ of the essential oil exhibiting a statistically equivalent inhibition to that of copper oxychloride (Figure 1). Furthermore, a concentration-dependent inhibition of *P. citricarpa* growth by NEO was observed, resulting in the formation of a dose-response curve. These findings underscore the specificity of NEO's activity, targeting the structural integrity of the fungal hyphae, rather than exerting a physicochemical influence akin to that of free chlorine, oxygen-containing reactive species (ROS), or pH [34]. The absence of a fixed dose-response inhibition, characterized by decay in inhibition relative to lower compound concentrations, is indicative of a physicochemical mode of action, as opposed to a targeted interaction with a specific cellular site inhibited by the antimicrobial agent. Khan et al. [35] similarly demonstrated via growth inhibition curves that neem oil operates via a specific mechanism against fungal hyphae, corroborating the outcomes of our study. Notably, Khan et al. [35] also reported favorable inhibition results using propyl disulfide from neem oil against *Lasiodiplodia theobromae* and *Neofusicoccum parvum*, with concentrations closely resembling those employed in our investigation.

Rodrigues et al. [36] similarly documented the inhibition of *Aspergillus carbonarius* using neem oil extracted from seeds, conducting experiments closely aligned with those in the present study. Rodrigues et al. [36] achieved approximately 95% inhibition of fungal growth with a concentration of $3 \mu\text{L}\cdot\text{mL}^{-1}$ of neem oil extracted from seeds. In comparison, our study employed $100 \mu\text{L}\cdot\text{mL}^{-1}$ to achieve a comparable inhibitory effect to that of copper oxychloride, resulting in $13.94 \pm 1.33 \text{ cm}^2$ of fungal growth area (equating to $89.68 \pm 1.14\%$ fungal growth inhibition). It is noteworthy that the comparatively lower results obtained in our study may stem from variances in neem oil composition and the species of fungus utilized, as opposed to those examined by Rodrigues et al. [36]. Furthermore, we attained a lower concentration of NEO relative to copper oxychloride at $90 \text{ g}\cdot\text{L}^{-1}$, while still achieving equivalent fungal inhibition. Additionally, Falsini et al. [37] demonstrated significant fungicidal efficacy with neem oil (extracted from both leaves and seeds) at a concentration of 1.5% when combined with a nanoformulation vehicle, thereby augmenting the inhibitory effect of neem oil. Despite this enhancement, the

concentration applied and the hyphae inhibition observed closely paralleled our findings, substantiating the effectiveness of pure NEO tested against *P. citricarpa* in our study. The utilization of pure essential oil presents advantages in terms of cost-effectiveness for application in packinghouses or orchards, albeit requiring higher concentrations for efficacy. This highlights the importance of further experimentation and suggests the potential viability of utilizing pure essential oils, offering cost benefits for farmers and industries alike.

To evaluate the action mechanisms of the NEO, fluorescence microscopy assays were carried out with the Live/Dead kit. In this method, the entry of propidium iodide into the cell, staining it red, indicates damage to the cytoplasmic membrane and subsequent cell death [38]. The results showed that the *P. citricarpa* hyphae were directly affected by the disruption of cytoplasmic membrane after the contact with the NEO (Figure 2). NEO exhibited rapid action within the initial 30 minutes of contact, with approximately 76% of *P. citricarpa* hyphae affected by the oil (depicted as red-stained hyphae) (Figure 2).

The hyphae of the fungal mycelium exhibited a red stain following exposure to $100 \mu\text{L}\cdot\text{mL}^{-1}$ of NEO, indicating the targeted effect of NEO on the cytoplasmic membrane of *P. citricarpa*. The control group demonstrated intact cell cytoplasmic membranes (depicted as green hyphae), with no discernible effect of the vehicle (1% DMSO). Silva et al. [39] previously correlated the action mechanisms of NEO against fungi such as *Aspergillus flavus* and *Penicillium citrinum* with cytoplasmic membrane disruption. The findings of our study align with those of Silva et al. [39], further substantiating the effect of NEO on the cytoplasmic membrane. Essential oils, in general, exert their effects on the cytoplasmic membranes of fungi, ultimately leading to cellular cytoplasm lysis [40, 41]. Similar actions have been observed in bacteria such as *Klebsiella pneumoniae* [42]. This study marks the first observation of this action mechanism of NEO in *P. citricarpa*, underscoring the novelty of our results. Identifying the mechanism of action of antimicrobial compounds is crucial for analyzing potential mechanisms of resistance in microorganisms. Consequently, understanding the mechanism of action ensures the safe use of essential oils against phytopathogenic microorganisms. Based on our results, it can be inferred that NEO does not select or induce resistance in *P. citricarpa*, as developing resistance against NEO would necessitate multiple genetic mutations in the hyphae of *P. citricarpa* to alter the composition and structure of the cytoplasmic membrane [43, 44].

The NEO characterization was done through GC-MS/MS. After identifying the different organic compounds found in NEO, it is possible to determine whether they have an active role in the antifungal action. Moreover, by performing GC-MS/MS analyses, the organic molecules can be identified, and it becomes possible to establish a correlation between the inhibitory mechanisms of action observed in fluorescence microscopy and the organic compounds present in the NEO. The mass spectral fragmentation patterns were compared with the National Institute of Standards and Technology Mass Spectral (NIST4-MS) database.

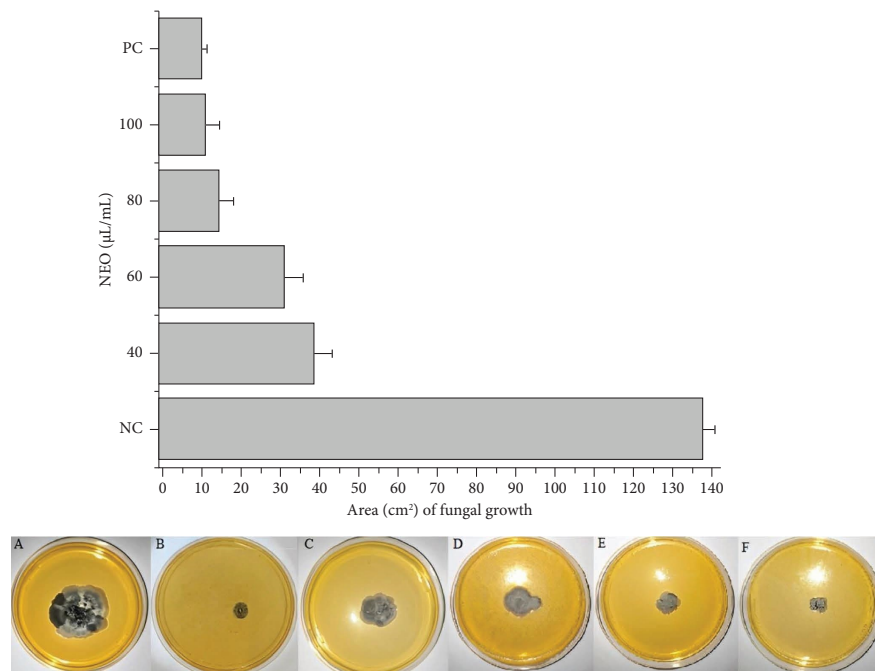


FIGURE 1: Comparative fungal growth of *P. citricarpa*, by area measured in the ImageJ software, after contact with different concentrations of NEO (40, 60, 80, and 100 $\mu\text{L}\cdot\text{mL}^{-1}$). PC was 90 $\text{g}\cdot\text{L}^{-1}$ of copper oxychloride, and NC was deionized water with 1% of DMSO. The photos of fungal growth of *P. citricarpa* on YPD agar plates are showed below of the graph, where A is NC; B is PC; C is NEO at 40 $\mu\text{L}\cdot\text{mL}^{-1}$ of concentration; D is NEO at 60 $\mu\text{L}\cdot\text{mL}^{-1}$ of concentration; E is NEO at 80 $\mu\text{L}\cdot\text{mL}^{-1}$ of concentration; F is NEO at 100 $\mu\text{L}\cdot\text{mL}^{-1}$ of concentration. Bars represent the average values of fungal growth area (cm^2); whiskers indicate the SD of the mean of three independent experiments used to verify experimental errors.

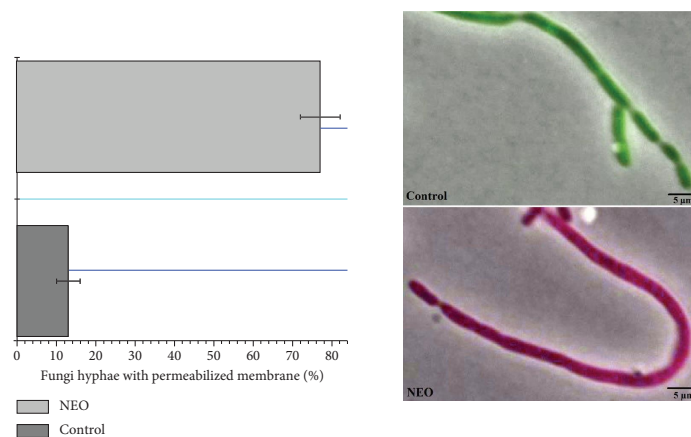


FIGURE 2: Percentage of *P. citricarpa* hyphae with the permeabilized cytoplasmic membrane. Hyphae with intact membranes are colored green, while the permeabilized membranes are colored red. The concentration of NEO was at 100 $\mu\text{L}\cdot\text{mL}^{-1}$, and the control was a sterilized saline solution (NaCl 0.87%) with 1% of DMSO. The pictures showed were at the overlay of Tx Red/eGFP and phase contrast images, with a magnification of $\times 100$. The experiment was performed thrice and at least 50 hyphae were evaluated per experiment ($n > 150$). Horizontal bars are the average percentage; whiskers are the average SD for verification of experimental errors.

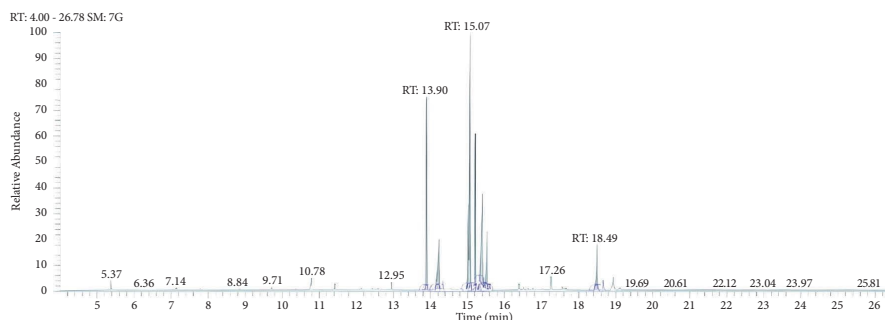


FIGURE 3: NEO spectral by GC-MS/MS.

The GC-MS/MS spectra displayed bands labeled according to retention time (RT) of different compounds identified by molecular weight (Figure 3). To identify the primary compounds, a table was compiled to provide further elucidation (Table 1).

Four of the primary molecules identified in NEO are represented in Figure 4, all of which have been associated with antifungal activity in previous studies [45–49]. Among the organic molecules observed in the GC-MS/MS analysis, particular emphasis is placed on 9-octadecenoic acid methyl ester, which emerges as the predominant compound in NEO. This compound has been documented as an effective agent against both fungi and bacteria in prior research [47, 50, 51]. On the other hand, 9-octadecenoic acid methyl ester is also a metabolite produced by several fungal species, and certain fungi have been reported to exhibit resistance to this compound [47, 52]. Moreover, the action mechanism of 9-octadecenoic acid methyl ester was not completely elucidated, and thus it is not possible to associate the 9-octadecenoic acid methyl ester with the *P. citricarpa* inhibition or the cytoplasmic membrane disruption.

Several phenolic molecules were identified in NEO through GC-MS/MS analysis, including syringol, 2,6-dimethoxyphenol, phenol, and 2,4-bis-1,1-dimethylethyl, which are present in significant quantities within the NEO composition (Figure 4). Compounds containing phenol as the active principle are widely recognized as biocides [49, 53]. Syringol, 2,6-dimethoxyphenol, phenol, and 2,4-bis-1,1-dimethylethyl have been previously associated with antimicrobial properties, particularly against fungi [45, 46, 54, 55]. Despite 9-octadecenoic acid methyl ester being the predominant chemical compound in NEO, it is plausible that phenol, 2,4-bis-1,1-dimethylethyl, syringol, and 2,6-dimethoxyphenol serve as the primary agents responsible for targeting the cell membrane of *P. citricarpa*. Both phenolic molecules are known to increase membrane permeability, leading to cytoplasm leakage and subsequent cell death in microorganisms [56, 57].

The 2,6-dimethyl-1,4-benzoquinone was also identified through GC-MS/MS analysis, marking the fourth molecule previously associated with antifungal properties found within NEO [48]. Quinones, including benzoquinones,

TABLE 1: Compounds identified using GC-MS/MS spectral.

Name of compound	Neem essential oil RT
9-Octadecenoic acid, methyl ester	15.07
6-Octadecanoic acid	15.41
Syringol, 2,6-dimethoxyphenol	13.90
Methyl stearate	15.21
L-ascorbic acid 2,6-dihexadecanoate	14.23
Oleic acid, (2,2-dimethyl-1,3-dioxolanyl) methyl ester	5.01
Phenol, 2,4-bis-1,1-dimethylethyl	18.49
2,6-Dimethyl-1,4-benzoquinone	10.79

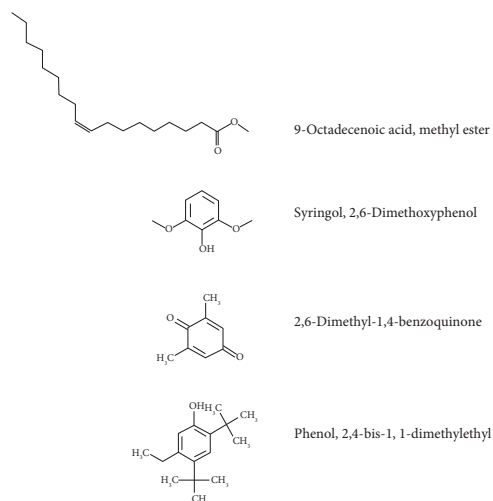


FIGURE 4: Molecular structure of compounds associated with the antifungal activity by NEO.

represent a class of aromatic compounds renowned for their broad-spectrum antimicrobial activity, with applications in the pharmacological field [49]. Benzoquinones are known to

inhibit DNA-gyrase, a mechanism of action (MOA) that has found utility in clinical drugs [58]. DNA-gyrase, a topoisomerase present in both prokaryotes and eukaryotes, emphasizes the broad antimicrobial interest in this class of molecules. However, due to the significant abundance of syringol, 2,6-dimethoxyphenol, phenol, and 2,4-bis-1,1-dimethylethyl compared to 2,6-dimethyl-1,4-benzoquinone, coupled with their mechanism of action involving cytoplasmic membrane permeabilization, it is reasonable to consider 2,6-dimethyl-1,4-benzoquinone as a secondary inhibitory agent. If the primary mode of action was through DNA division or enzymes such as DNA-gyrase, propidium iodide would be unable to penetrate the cytoplasm and alter the color of the hyphae in fluorescence microscopy assays. Thus, the fluorescence microscope, in conjunction with GC-MS/MS analysis, confirmed the cytoplasmic membrane disruption effect induced by NEO, with phenolic molecules identified as the primary agents responsible for inhibiting *P. citricarpa*. In summary, while 2,6-dimethyl-1,4-benzoquinone and 9-octadecenoic acid methyl ester exhibit certain inhibitory activity, they do not serve as the primary agents, instead possibly potentiating the action of phenolic compounds.

The CBS can appear even during the postharvest phase, facilitating the spread of the disease to new areas through transportation and storage. Fungal spores are disseminated through wind, with symptoms typically appearing approximately 40 days after infection. It is crucial that all propagules of *P. citricarpa* be eradicated from fruits or any materials exported to disease-free countries, as outlined by the European Food Safety Authority [59]. Consequently, sanitization assays were conducted, and recoveries of *P. citricarpa* spores on agar plates after 40 days were performed to assess the efficacy of NEO in removing any spores or mycelium from fruit surfaces. Figure 5 shows the microbial counts on agar plates following fruit washing with each treatment. Sanitization with NEO resulted in a significant reduction of $91.0 \pm 7.35\%$ in microorganisms ($\log_{10} \text{CFU mL}^{-1}$) compared to the negative control (NC) (Figure 5). This reduction closely paralleled that achieved with the copper-based fungicide (PC), with both treatments being statistically equivalent, thus underscoring the efficacy of NEO as an alternative for sanitizing citrus fruits in packinghouses. Notably, *P. citricarpa* was not isolated from the culture medium in any treatment except for the negative control, thus affirming the efficacy of NEO in completely eliminating any propagules of *P. citricarpa* from citrus fruit surfaces. Additionally, NEO demonstrated effectiveness in eliminating various microorganisms present on fruit surfaces, which persisted even after washing with neutral soap solution. This highlights the reduction of microorganisms on fruit surfaces and, in conjunction with the *in vitro* results (fluorescence microscopy and antifungal sensitivity assays), supports the assertion that NEO is effective in sanitizing citrus against *P. citricarpa* and other microorganisms.

It is noteworthy that, to date, no investigation has explored the use of NEO as a sanitizer for other fresh fruits. Silva et al. [39] utilized NEO against the fungi *Aspergillus flavus* and *Penicillium citrinum*, yielding favorable outcomes in

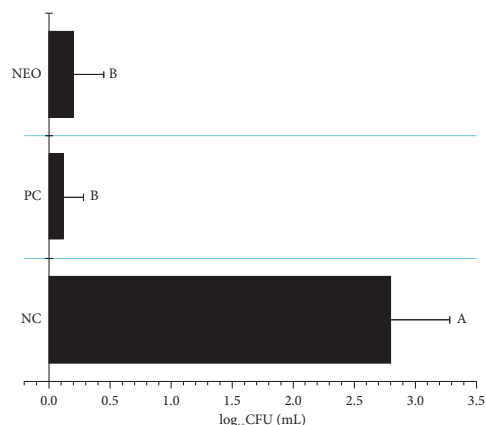


FIGURE 5: Colony forming units rescued from the surface of citrus fruits after the sanitization assays at the final time of 40 days. Bars represent the averages of rescued cells; whiskers indicate the SD of the means. Three independent experiments were performed. Data showing the same letters are not significantly different from each other based on the nonparametrical statistical analysis of Kruskal–Wallis (Dunn), with three degrees of freedom. Negative control (NC) were fruits washed using sterile deionized water with 1% of DMSO; positive control (PC) were fruits sanitized with copper oxychloride at $90 \text{ g} \cdot \text{L}^{-1}$ of concentration; NEO was applied at $100 \mu\text{L} \cdot \text{mL}^{-1}$ of concentration with 1% of DMSO as a vehicle. The $H = 56.5859$; $p < 0.05$ for NC to PC/NEO; the Z critical value was equal to 2.394 for all treatments; the Z-score value was 0.8957 for PC when compared with NEO, and the Z-score value was greater than 6.00 for NC when compared with PC or NEO.

protecting soybean seeds from these phytopathogens. Similarly, Khan et al. [35] demonstrated the efficacy of neem extract as an antifungal agent against mango rot phytopathogens *Lasiodiplodia theobromae* and *Neofusicoccum parvum*. These studies closely parallel our own in terms of results and application, as NEO has not previously been employed as a sanitizer against *P. citricarpa*. In addition to the novel findings presented herein, this study paves the way for further exploration of NEO's potential for sanitizing various types of fresh fruits and assessing its efficacy against other citrus phytopathogens. Many plant pathogens are categorized as quarantine organisms by the EU and other nations, and with escalating sanitization restrictions coupled with the quest for eco-friendly compounds, this study presents a viable alternative by proposing the application of essential oils such as neem for sanitizing exported fresh fruits.

4. Conclusion

The concentration of NEO required to achieve fungicidal action equal to copper oxychloride was found to be $100 \mu\text{L} \cdot \text{mL}^{-1}$, effectively inhibiting *P. citricarpa*. Fluorescence microscopy analysis demonstrated that NEO exerted a rapid effect, disrupting the cytoplasmic membrane of the fungus within 30 minutes of contact, thus representing the

primary mechanism of action. GC-MS/MS analysis corroborated these findings, identifying phenolic compounds (syringol, 2,6-dimethoxyphenol, phenol, and 2,4-bis-1,1-dimethyl ethyl) as the major antifungal agents in NEO. The utilization of NEO as a sanitizer for citrus fruits has proven highly effective, significantly reducing the number of microorganisms present on fruit surfaces after sanitization and effectively eliminating any remaining propagules of *P. citricarpa*.

In the future, further research can be conducted on other citrus fruits such as limes, lemons, tangerines, and grapefruit. Additionally, exploration of NEO's efficacy against other fruits and microorganisms could expand the availability of organic alternative sanitizers in the global fresh produce market.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

J.M. Schuch and C.R. Mendes conceptualized the study; performed data curation and formal analysis; investigated the study; carried out the experiments; proposed the methodology; validated the study; and visualized the study. C.R. Mendes and G.L. Cardoso carried out the sanitization of fresh fruit assays. C.A.V.L.R. Costamilan and P.R.M. Lopes performed statistical analysis and validated the results. R.N. Montagnolli, G.Dilarri, and E.D. Bidoia performed funding acquisition; were responsible for project administration; provided resources; supervised the study; and reviewed the original draft. Joyce Maria Schuch and Carolina Rosai Mendes contributed equally to this work and are co-first authors. All authors have reviewed this journal's licensing options, and all authors have the full power, authority, and capability (i) to agree to the terms of one of the licenses offered by this journal and (ii) to grant the rights set forth in such license for the publication of this submission. The authors also know the ethics statement from this journal and followed it.

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CAPÍTULO 3

Nanopartícula metálica com atividade antimicrobiana

Doenças disseminadas por contaminação alimentar representam desafios significativos para a saúde pública. Esse capítulo abrange as propriedades antimicrobianas das nanopartículas metálicas de óxido de zinco. A escala nano revoluciona a abordagem de inibir e controlar agentes infecciosos de origem alimentar.

As nanopartículas de óxido de zinco (NpsZnO) possui propriedades físico-químicas únicas e são candidatas promissoras para intervenções terapêuticas direcionadas. Ainda assim, seu potencial uso para aplicação em tratamentos antimicrobianos possui desafios em relação à biocompatibilidade, toxicidade e a necessidade de avaliar os efeitos de seleção microbiana após contato. Portanto, nesse capítulo foi avaliado os possíveis mecanismos de ação antimicrobiana para melhor compreensão e assim, prever possíveis seleções de linhagens de microrganismos resistentes em estudos futuro.

A atividade bactericida das NPsZnO contra uma seleção de patógenos clínicos foram determinadas, assim como os possíveis mecanismos de ação bactericida. Esses resultados são discutidos no artigo “Antibacterial action and target mechanisms of zinc oxide nanoparticles against bacterial pathogens” descrito por Mendes et al. (2022)



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Antibacterial action and target mechanisms of zinc oxide nanoparticles against bacterial pathogens

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Zinc oxide nanoparticles (ZnO NPs) are one of the most widely used nanoparticulate materials due to their antimicrobial properties, but their main mechanism of action (MOA) has not been fully elucidated. This study characterized ZnO NPs by using X-ray diffraction, FT-IR spectroscopy and scanning electron microscopy. Antimicrobial activity of ZnO NPs against the clinically relevant bacteria *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and the Gram-positive model *Bacillus subtilis* was evaluated by performing resazurin microtiter assay (REMA) after exposure to the ZnO NPs at concentrations ranging from 0.2 to 1.4 mM. Sensitivity was observed at 0.6 mM for the Gram-negative and 1.0 mM for the Gram-positive cells. Fluorescence microscopy was used to examine the interference of ZnO NPs on the membrane and the cell division apparatus of *B. subtilis* (*amy::pspac-ftsZ-gfpmut1*) expressing FtsZ-GFP. The results showed that ZnO NPs did not interfere with the assembly of the divisional Z-ring. However, 70% of the cells exhibited damage in the cytoplasmic membrane after 15 min of exposure to the ZnO NPs. Electrostatic forces, production of Zn²⁺ ions and the generation of reactive oxygen species were described as possible pathways of the bactericidal action of ZnO. Therefore, understanding the bactericidal MOA of ZnO NPs can potentially help in the construction of predictive models to fight bacterial resistance.

Nanoparticles (NPs) of metal oxide stand out in the field of antimicrobial compounds by their catalytic inhibition activity^{1,2}. However, their bactericidal mechanism of action (MOA) depends on several parameters, such as their morphology, composition and concentration^{3,4}. Zinc oxide (ZnO), magnesium oxide (MgO) and titanium dioxide (TiO₂) are substances recognized as safe when used as food additives or drug deliverers according to the Food and Drug Administration (FDA 2011) US Code of Federal Regulations (Title 21-CFR 182.8991)⁵. Zinc oxide nanoparticles (ZnO NPs) are the most promising inorganic materials that have bactericidal action and can be found in the composition of pharmaceutical drugs, sanitizers, cosmetics and food packaging processes⁶. However, the targets of ZnO NPs in bacteria of clinical importance are not fully understood. Zanet et al.⁷ carried out experiments using ZnO NPs against the model cell *Saccharomyces cerevisiae* in order to elucidate the main MOA, and concluded that the effects of ZnO NPs depend on their composition and dose.

The synthesis of ZnO NPs can be achieved by chemical precipitation⁸, salt reduction⁹, sol-gel way based on an acetate precursor¹⁰, and sonochemical synthesis¹¹. However, different synthetic pathways yield ZnO particles with variable morphologies and sizes^{9,12,13}. Thus, their MOA, as well as their interaction with diverse cell structures, may vary significantly.

ZnO is a transition metal oxide and semiconductor with high binding energy which allows for a highly oxidative character¹⁴. This reaction leads to the formation of reactive oxygen species as the pathway of bactericidal action. In addition, another bactericidal MOA occurs through the release of zinc ions (Zn²⁺) that damage the

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cell membrane and may interrupt some metabolic pathways¹⁵. Thus, additional studies about the antibacterial MOA of ZnO NPs can relevantly contribute to the prediction of possible mechanisms of bacterial resistance and for the optimizing of the contact time and effective inhibition action.

Cell division is a critical process for microbial survival. Among the proteins involved in this process, FtsZ has a pivotal function in which it serves as a scaffold for the assembly of a multiprotein complex structure, the divisome, responsible for coordinating all the steps of cell division and cell wall remodeling¹⁶. The protein acts during cell division as an organizer of the cytoplasmic ring in bacteria and can be considered the main target of several bactericidal compounds¹⁷. Bactericidal agents act in diverse ways, such as inhibition of FtsZ in the cell division pathway, and can be identified by cytological profile of cells expressing FtsZ-GFP and observed by fluorescence microscopy¹⁸.

In this study, the model Gram-positive bacteria *Bacillus subtilis* (ATCC 19659) and a set of clinically relevant bacteria *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), and *Pseudomonas aeruginosa* (ATCC 27853) were used to determine the inhibitory concentration of ZnO NPs and to evaluate their effect on the bacteria cytological profile. The focus of the present study was to evaluate the action of ZnO NPs on cell morphology, chromosome organization, and protein production. Therefore, the cytoplasmic membrane and other proteins, such as FtsZ, which forms the scaffold for the divisome, were not included in the spectra of analysis. *Bacillus subtilis* FtsZ was used to evaluate any interference in the formation of the FtsZ ring.

Materials and methods

Synthesis of ZnO NPs. All reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany). ZnO NPs were synthesized by sonochemical-coprecipitation of 2 mM solution of zinc chloride followed by dripping it with ammonium hydroxide¹⁹. Next, the mixture was heated to 60 °C under continuous stirring until complete precipitation. The precipitate underwent ultrasonic bath sonicator (USC 1400) for 30 min to obtain NPs, followed by vacuum filtration using a 0.22 µm cellulose membrane, washing with deionised water and finally drying at 100 °C overnight.

Characterization of the synthesized nanomaterial. ZnO NPs were characterized using Fourier transform infrared spectrophotometer FT-IR (Shimadzu Model 8300), adjusted for scanning at 4000–400 cm⁻¹. For the analysis, a KBr pellet was made with the nanomaterial sample²⁰. Micrographs of ZnO NPs were taken by Scanning Electron Microscope (SEM)—JEOL JSM-IT100 operated at 30 kV coupled to a Bruker Quantax Energy Dispersive Detector (EDS), in order to study the morphological characteristics. The samples were coated with a gold layer by a metalization process before SEM readings. Finally, the crystalline structure of the ZnO NPs was characterized by X-ray diffraction powder (XRD, PHILIPS, X' pert-MPD system) using Cu Kα radiation (λ = 1.5418 Å). The X-ray wavelength was 0.15418 nm and the diffraction patterns were measured in the range of 2θ from 20° to 65°.

Bacterial strain and growth conditions. *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 19659), *Pseudomonas aeruginosa* (ATCC 27853) and the mutant *Bacillus subtilis* (*amy::pspac-ftsZ-gfpmut1*; a gift of Dr. F. Gueiros-Filho, IQ, USP, São Paulo, Brazil) expressing FtsZ-GFP were cultivated in nutrient broth medium (5 g L⁻¹ of peptone; 3 g L⁻¹ of beef extract; for solid medium was added 15 g L⁻¹ of bacterial agar) at 28 °C for 24 h in shaker at 200 rpm. All reagents for cellular growth were purchased from Himedia Laboratories Ltd. (Mumbai, India).

Antibacterial activity assay. The antibacterial activities expressed as Inhibitory Concentrations (ICs) of the ZnO NPs were determined by using the Resazurin Microtiter Assay (REMA)²¹. ZnO NPs at concentrations between 0.2 and 1.4 mM were placed in 96-well microplates. The strains were inoculated to independent trials at a final concentration of 10⁵ cells per 100 µL in each well and incubated for 12 h at 30 ± 1 °C. Nisin at 5 µg mL⁻¹ and Kanamycin at 20 µg mL⁻¹ from Sigma-Aldrich (Taufkirchen, Germany) were used as reference antibiotic (positive controls) against Gram-positive and Gram-negative bacteria, respectively. Nutrient broth medium was used as negative control. After the incubation period, the inhibition of cell growth was measured by the addition of 0.1 mg mL⁻¹ resazurin (Sigma-Aldrich; Taufkirchen, Germany) in each well. In live cells, resazurin is reduced to resorufin (a fluorescent compound) in the presence of NADH, which indicates cell activity¹⁸. The fluorescence intensity of resorufin was detected in a plate reader (Synergy H1N1—BioTek, Winooski, VT, USA) set to the excitation and emission wavelengths of 530 and 590 nm, respectively. The results of this assay were used to plot the correlation between ZnO NPs concentration and the inhibition of cell growth. Non-linear regression models were used to derive the IC₁₀₀ values for each bacterial strain (100% inhibitory concentration).

Effect of ZnO NPs on the membrane integrity. *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* at 10⁵ cells were exposed to ZnO NPs at concentrations equivalent to their IC₁₀₀ in 100 µL of media for 15 min. Next, 900 µL of phosphate buffer were added to stop the reactions. Next, cells were stained using 0.01 mg mL⁻¹ propidium iodide (PI) and 0.02 mg mL⁻¹ DAPI (4',6-diamidino-2-phenylindole). DAPI stains the nucleoid of every cell, whereas propidium iodide (PI) is a nucleic acid dye that penetrates only cells with damaged cytoplasmic membranes. Untreated cells were used as negative control, while positive control for damaged membranes was generated by heat-shock stress (Gram-negative) and Nisin treatment (Gram-positive). *B. subtilis* (*amy::pspac-ftsZ-gfpmut1*) expressing FtsZ-GFP was used to investigate the potential of the compound to interfere with the divisional septa. The bacterial cells were cultivated in the presence of 0.02 mM Isopropyl β-D-thiogalactopyranoside (IPTG) to induce the expression of FtsZ-GFP from the *pspac* promoter. Next, 100 µL of the cultures (adjusted to contain ~10⁵ cells) were exposed for 15 min to ZnO NPs at its respective IC₁₀₀. Cells were washed

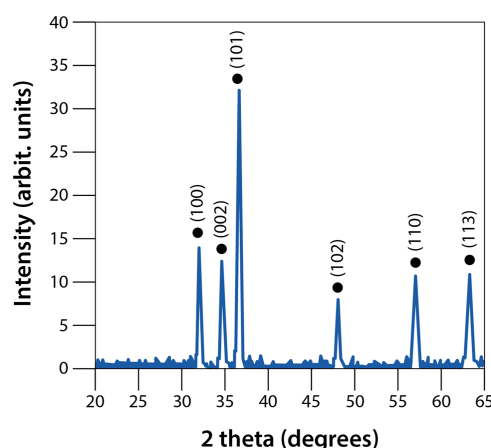


Figure 1. XRD power of ZnO NPs.

Lattice parameters <i>a</i> (Å)	Lattice parameters <i>c</i> (Å)	<i>c/a</i> ratio	Volume of unit cell (Å ³)	Average crystallite size (nm)	Microstrain ϵ ($\times 10^3$)
3.24	5.21	1.608	47.48	82.38	0.47

Table 1. Structural parameters of ZnO crystallite.

with water and resuspended in 100 μ L of 0.85% NaCl solution prior to microscope observation¹⁸. Cells were immobilized onto agarose-covered slides and visualized using an Olympus BX-61 (Tokyo, Japan), equipped with a monochromatic camera OrcaFlash 2.8 (Hamamatsu, Japan). Images were processed by the software CellSens version 11 (Olympus). One hundred cells were considered ($n = 100$) per treatment for quantifications.

Results and discussion

Characterization of ZnO NPs. The XRD peaks were consistent with ZnO crystallite. The analysis showed no extra peaks, which is due to the purity of the material applied during the synthesis of ZnO NPs. The positions of the diffraction peaks showed the same pattern found in the Joint Committee on Powder Diffraction Standards: 36–1451 database (JCPDS).

Figure 1 shows the diffraction peaks of ZnO NPs at (100), (002), (101), (102), (110), (103) which correspond respectively to the values in degrees (2 θ) at 31.34°, 34.50°, 36.32°, 47.60°, 56.68°, 62.94°. High diffraction peaks indicated the crystalline nature of the material²².

Table 1 shows the values of the structural parameters used to calculate the size of the ZnO crystallite by Eq. (1)²³. The high intensity peak at (101) was used to determine the lattice parameters.

$$D = \left[\frac{0.9\lambda}{\beta \cos \theta} \right] \times 100 \quad (1)$$

where D is the size of the ZnO crystallite; λ is the wavelength of Cu K α radiation at 1.5418 Å; θ is the Bragg diffraction angle, and β is the full width at half maximum intensity of the diffraction peak of the sample.

The crystallite size can be measured more accurately by high resolution X-ray diffraction (HRXRD) using the Bond method, which increases peak resolution to find the values of the Lattice parameters^{23,24}. In this study, we determined by XRD powder that most synthesized ZnO crystallites are around 80 nm in size. Similar results obtained through the synthesis of ZnO NPs by sonochemical-coprecipitation were shown by Khataee et al.²⁵.

The surface appearance and morphology of synthesized ZnO NPs were analyzed by SEM at 29.0 kx. Based on the images in Fig. 2, the ZnO NP showed complex bead and rod morphology. In addition, the ZnO NPs have an irregular size with formation of aggregated nanocrystallite.

FT-IR spectra are like molecular fingerprints that provide a valuable insight into chemical structures and their changes due to interactions with other molecules²⁶. FT-IR analyses detected the characteristic functional groups associated with the ZnO NPs (Fig. 3). The peak at 575 cm^{-1} corresponds to the stretching/vibration of the metal–oxygen bond in Zn–O. The peak at 3713 cm^{-1} corresponds to carbon residues identified during the sample measurement; and 1210 cm^{-1} belongs to the elongation of C–O. Hydrogen bonds are displayed at 1690

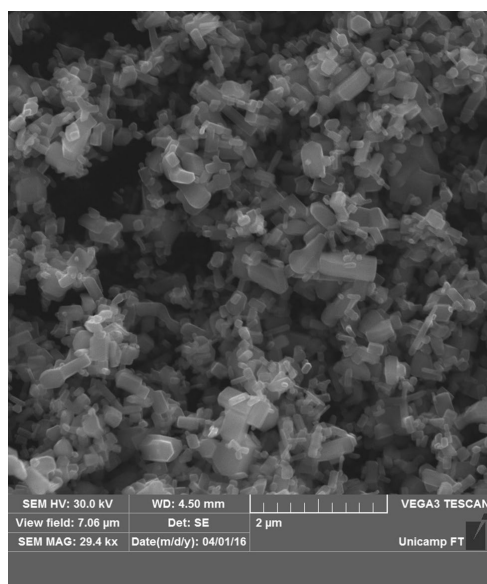


Figure 2. Surface morphology of ZnO NPs by SEM. 2 μm scale bar; 29.0 kx magnification.

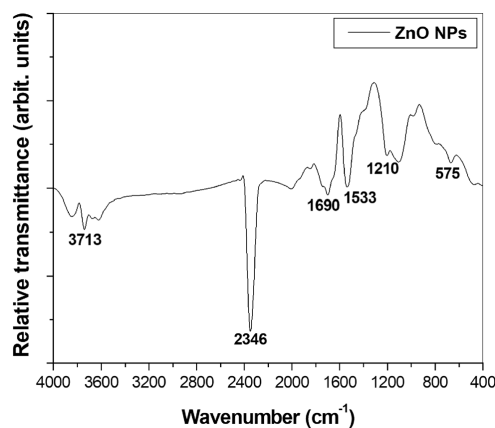


Figure 3. FT-IR spectrum of ZnO NPs.

and 2346 cm^{-1} , and they are ascribed to the stretching vibration of hydroxyl compounds. The hydroxyl group influences photocatalytic reactions in ZnO by generating superoxide radicals, which act as an antimicrobial²⁶.

Antimicrobial activity. The bactericidal activities of ZnO NPs against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis* were evaluated by monitoring cell respiration. Polynomial regression applied to the dose–response data was used to extrapolate the IC_{100} values, which were expressed as mM. The decrease in cell numbers observed after treatment is shown in by the plot containing the concentration of ZnO NPs versus the inhibition of cell growth (Fig. 4).

ZnO NPs inhibited growth of *E. coli* and *P. aeruginosa* with IC_{100} values of 0.6 mM for both strains. The IC_{100} values for *B. subtilis* and *S. aureus* were estimated at 0.8 and 1.0 mM, respectively. Gram-negative bacteria have a thin layer of peptidoglycan between two membranes, which is known to provide antimicrobial resistance²⁷. In

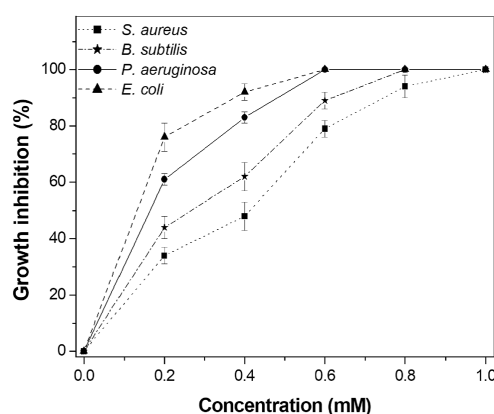


Figure 4. Antimicrobial activity of ZnO NPs determined by REMA.

addition, dissociated carboxyl groups present in the membranes generate negative charges on the cell surface. ZnO NPs, on the other hand, have a positive charge, with a zeta potential of +24 mV²⁸. As a result of electrostatic forces, damage to the cell membrane occurs due to electrostatic gradient differences across the negative membrane and the positive charges of the Zn²⁺ ions. Therefore, *E. coli* and *P. aeruginosa* died with the lowest concentration of ZnO NPs. Although the present study did not observe a large difference in the IC values for Gram-negative and Gram-positive, it is noteworthy that Gram-positive exhibited IC₁₀₀ values higher than Gram-negative. Similar inhibition in Gram-negative bacteria was previously reported by Yusof et al.²⁹, Saqib et al.³⁰ and Zubair and Akhtar³¹; however, with slight variations in the IC₁₀₀ values due to differences in the synthesis of the nanomaterial, which yields unique characteristics to each one of them. Overall, the results were close; however, our study not only investigated the percentage of inhibitory growth, but also the MOA target of ZnO NPs in clinical strains.

Effect of ZnO NPs on the bacteria cell. Bacterial cell division is a complex and dynamic process, which starts with the polymerization of the FtsZ protein in order to assemble the divisome, which will guide all the processes related to cell division and cell wall synthesis and remodelling³². FtsZ is the ancestral tubulin conserved in bacteria, which exerts its function dependent on the nucleotide guanosine triphosphate (GTP)^{33,34}. Some bactericidal compounds act by preventing the GTPase activity of FtsZ, which will inhibit cell division and lead to cell death¹⁷. In addition, blockage of the cell division process generally leads to cell filamentation, which can be easily accessed by fluorescence microscopy. Alternatively, by using mutant cells expressing labelled division proteins, e.g. FtsZ-GFP, one can follow the dynamics of division and study the effects compounds might have on the process.

B. subtilis expressing FtsZ-GFP was exposed to ZnO NPs at its IC₁₀₀ for 15 min, and afterwards observed under the microscope (Fig. 5). Note that even after ZnO NPs exposure, the cells still have intact bars perpendicular to the long axis of the rods, which is the normal profile for the Z-ring. This cytological profile was comparable to the control and did not show any disruption of the divisional ring.

The integrity of the membranes of *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* cells was investigated upon compound exposure using fluorescence microscopy. The results showed the disruption of cytoplasmic membranes in all strains after 15 min of exposure at IC₁₀₀ (Fig. 6). The filters Tx Red and DAPI Blue were applied together and used to visualize PI and DAPI. Cells with intact membranes are artificially stained in blue, while cells with damaged membranes are stained red³⁵. Thus, an increase in red-stained cells by PI is related to the increase in cell permeability due to damaged membranes.

In this study, all the bacterial species had their cytoplasmic membrane affected within the first 15 min of exposure to ZnO NPs. Treatment with the compound led to membrane damage in more than 70% of the cells. These results were expected since the bactericidal activity of ZnO NPs was already known, and its predominant MOA is associated with the cell membrane¹⁴.

ZnO is a transition metal oxide and semiconductor (which belongs to class II–VI) with wide band gap (3.3 eV), there is a general pattern expected. When the radiation has energy larger than the band gap of the ZnO, electron–hole pairs are formed. Electrons are promoted to the conduction band (CB). The hole generated in the valence band (VB) gets a strongly oxidizing character and oxidizing sites are created, which are capable of oxidizing water molecules or hydroxide anions and generate strong oxidizing species¹³. This reaction leads to

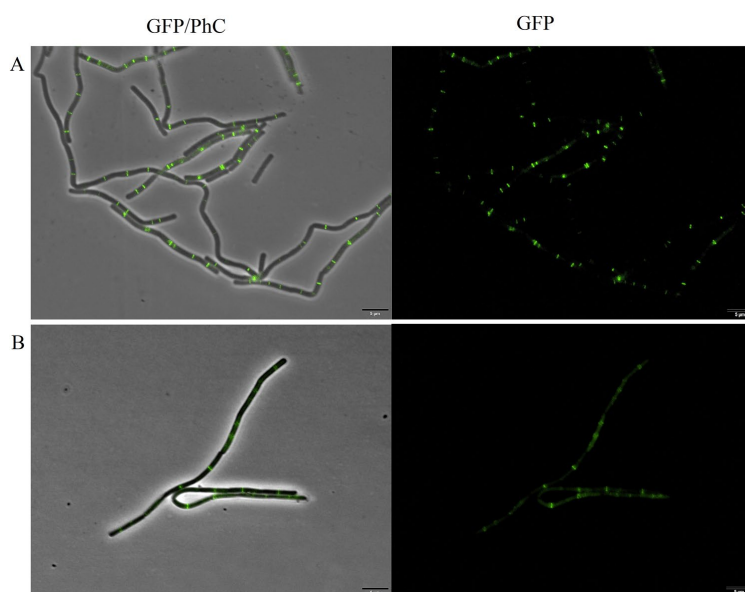


Figure 5. *B. subtilis* expressing FtsZ-GFP. (A) Control of cells grown in nutrient medium and diluted to 10^6 cells per mL^{-1} . (B) Cells after 15 min of exposure to ZnO NPs in the IC_{100} . GFP/PhC is the phase contrast images superimposed on the GFP fluorescence images. Scale bar $5\ \mu\text{m}$; $\times 100$ magnification.

the redox chain reaction with the generation of reactive oxygen species (ROS) formed by hydroxyl radical ($\text{OH}\cdot$), hydroperoxide radical ($\text{HO}_2\cdot$) and superoxide radical anion ($\text{O}_2^{\cdot-}$) as the pathways of bactericidal action³⁶.

Oxidative stress in the bacterial cell can be induced by ROS generation produced from ZnO NPs, which leads to the inhibition of protein synthesis and DNA replication¹⁴. In this situation, the ZnO conductivity increases, close to the “band gap” of the UV-spectrum characterized by high emission energy. The electronic excitation can destabilize the charges present in the cytoplasmic membrane resulting in their rupture. ZnO can also damage the cytoplasmic membrane by releasing Zn^{2+} ions from the dissolution of ZnO in aqueous solution. The Zn^{2+} ion acts as an inhibitor of the glycolytic enzyme through the thiol group oxidation due to specific affinity for the sulphur group³.

The MOA reported in this study are represented in schematic drawing shown in Fig. 7.

ZnO NPs can be attached to the surfaces of Gram-positive and Gram-negative bacteria through different pathways. The teichoic acid in the peptidoglycan layer and the lipoteichoic acid in the membrane are the source of negative charges in the cell surface. Positive charges from ZnO NPs are attracted to the cell surface by electrostatic interactions, and the difference in electrostatic gradient leads to damage in the cell surface^{37,38}. Teichoic and lipoteichoic acids act as a chelating agent on Zn^{2+} ions, which are then carried by passive diffusion across membrane proteins (Fig. 8). Moreover, the bactericidal action can occur by different mechanisms, such as adsorption in the bacterial surface, formation of different intermediates and electrostatic interactions.

The electrochemical gradient is generated by the movement of hydrogen ions across the cell membrane, which facilitates the diffusion of metallic ions³⁶. This mechanism is associated with the size of the material, whose small particles would have better electrostatic interactions. Thus, the ZnO target for inhibitory action is dependent on different factors such as concentration, size and time of interaction.

Zanet et al.⁷ showed that ZnO NPs affect the cell morphology and DNA. However, this can be a side effect, since the main target of ZnO NPs ends up being the first structure they have contact with and consequently act, such as the cytoplasmic membrane. Siddiqi et al.¹³ through SEM and TEM analysis concluded that ZnO NPs damage the cell membrane, and right after go to the cytoplasm, where they interact with other cell structures. Our results also showed damage to the cell. Therefore, it can be concluded that ZnO NPs are multi-target compounds and affect several structures of bacteria cells, but their main mechanism of action is in the cytoplasmic membrane, being other structure effects a consequence/secondary effect after the membrane rupture.

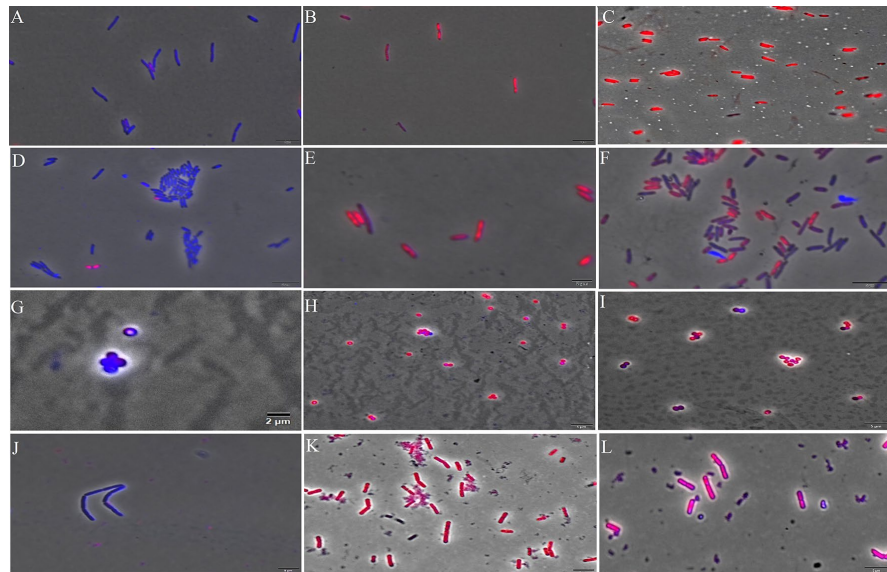


Figure 6. Fluorescence microscopy in cells stained with DAPI and PI after 15 min of exposure to ZnO NPs. Cells with intact membranes are artificially stained in blue, while cells with damaged membranes are stained in red. (A) *E. coli* (ATCC 8739) cells in nutrient broth medium (negative control); (B) *E. coli* (ATCC 8739) cells treated with heat-shock stress (positive control); (C) *E. coli* (ATCC 8739) cells treated with ZnO NPs at IC_{100} ; (D) *P. aeruginosa* (ATCC 27853) cells in nutrient broth medium (negative control); (E) *P. aeruginosa* (ATCC 27853) cells treated with heat-shock stress (positive control); (F) *P. aeruginosa* (ATCC 27853) cells treated with ZnO at IC_{100} ; (G) *S. aureus* (ATCC 6538) cells in nutrient broth medium (negative control); (H) *S. aureus* (ATCC 6538) cells treated with nisin at $5 \mu\text{g mL}^{-1}$ (positive control) (I) *S. aureus* (ATCC 6538) cells treated with ZnO at IC_{100} ; (J) *B. subtilis* (ATCC 19659) cells in nutrient broth medium (negative control); (K) *B. subtilis* (ATCC 19659) cells treated with nisin at $5 \mu\text{g mL}^{-1}$ (positive control); (L) *B. subtilis* (ATCC 19659) cells treated with ZnO at IC_{100} . Scale bar $2 \mu\text{m}$; $\times 100$ magnification.

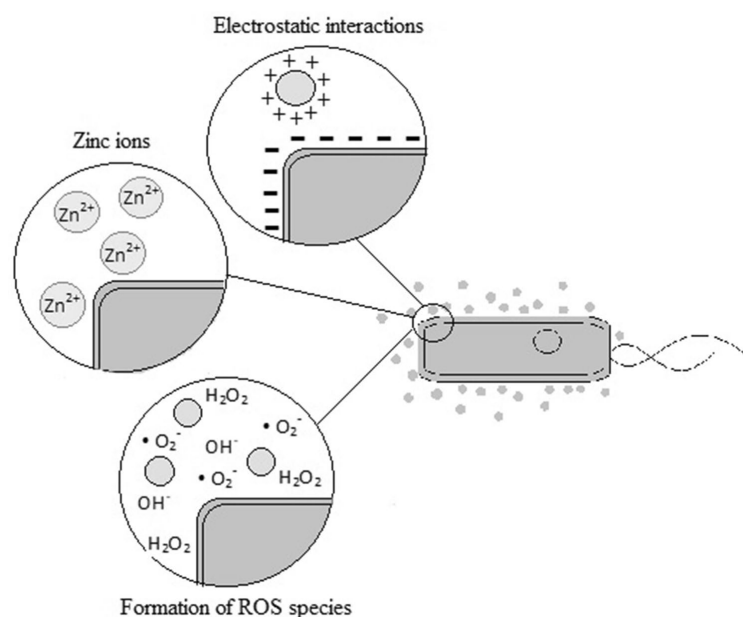


Figure 7. Model of the main bactericidal MOA of ZnO NPs which target the cytoplasmic membrane and cell wall.

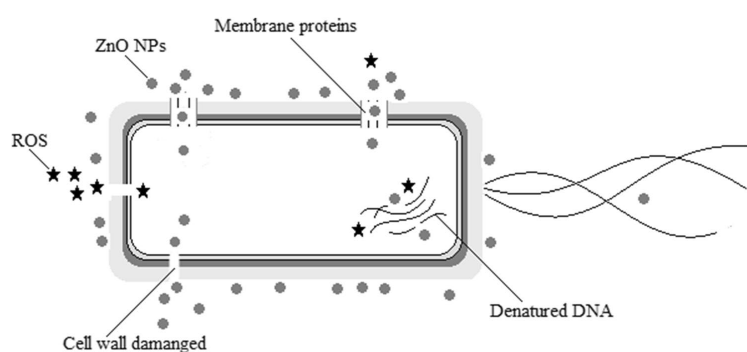


Figure 8. Cell model for the main mechanism of bactericidal action of ZnO NPs.

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C.R.M. and G.D.: conceptualization, methodology, fluorescence microscopy analyses, FT-IR assays, validation, formal analysis, writing original draft; C.F.F., V.M.R.S. and P.R.M.L.: methodology, formal analysis; E.D.B. and H.F.: conceptualization, methodology, funding acquisition, supervision, writing original draft; P.B.d.M.: SEM and X-ray assays, writing original draft; R.N.M.: conceptualization, visualization, writing/review and editing, supervision, funding acquisition, project administration.

Competing interests

The authors declare no competing interests.

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CAPÍTULO 4

Síntese e avaliação da ação antimicrobiana das MPsQTO

Nesse capítulo, foi realizado um estudo com o óleo essencial de *P. anisum* encapsulado na matriz biopolimérica de quitosana na escala micro.

A micropartícula de quitosana com óleo essencial (MPsQTO) foram sintetizadas para aumentar a superfície de contato do composto, além disso foi avaliado a ação antimicrobiana contra cepas clínicas. Ao final desse estudo, foi realizado um teste de toxicidade das MPsQTO usando uma célula modelo para demonstrativos de mutagênese de acordo com a EPA- Environmental Protection Agency (EPA, 1996). Desse modo, foi possível avaliar o potencial uso do composto como sanitizante.



Anise essential oil immobilized in chitosan microparticles: a novel bactericidal material for food protection

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Abstract

Foodborne infections in humans are one of the major concerns of the food industries, especially for minimally processed foods (MPF). Thereby, the packaging industry applies free chlorine in the sanitization process, ensuring the elimination of any fecal coliforms or pathogenic microorganisms. However, free chlorine's propensity to react with organic matter, forming toxic compounds such as trihalomethanes and haloacetic acid. Therefore, the present work aimed to synthesize a novel organic biomaterial as an alternative to free chlorine. Chitosan microparticles were produced, with *Pimpinella anisum* (anise) essential oil immobilized in the biopolymer matrix (MPsQTO). The characterization of this biomaterial was done through GC–MS/MS, FT-IR, and SEM. Antimicrobial assays proved that the MPsQTO presented antibacterial activity for *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* at 300 $\mu\text{L mL}^{-1}$ of concentration. The fluorescence microscope also showed the MPsQTO targets the cytoplasmatic membrane, which is responsible for cell death in the first minutes of contact. Studies with the mutant *B. subtilis* (*amy::pspac-ftsZ-gfpmut1*) and the *Saccharomyces cerevisiae* D7 also proved that the biomaterial did not affect the genetic material and did not have any mutagenic/carcinogenic effect on the cells. The sanitization assays with pumpkin MPF proved that the MPsQTO is more effective than free chlorine, increasing the shelf-life of the MPF. Consequently, the novel biomaterial proposed in this work is a promising alternative to traditional chemical sanitizers.

Keywords GC–MS/MS · Food safety · Pathogen microorganisms · Packinghouse · *Pimpinella anisum* · Sanitization

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Introduction

Minimally processed foods (MPF) are those that are sanitized and packaged for immediate consumption. In order to reduce waste and keep the food quality, MPF usually goes through sanitization processes, securing a long shelf life and ensuring safe consumption (Cossu et al. 2017). Therefore, the industry is highly concerned about the food security of MPF, inspecting for maintaining conservation and keeping it free of pathogenic microorganisms (Kim and Song 2017). Due to the cut processing, MPF is more susceptible to microbial infection (Liu et al. 2021). Among the main contaminating microorganisms, it is possible to highlight *Salmonella typhi*, as one of the main causes of food poisoning and intoxication in humans (Lee et al. 2015). Another bacterium that causes food contamination and intoxication in humans is *Pseudomonas aeruginosa*. This pathogenic bacterium is responsible for several virulence factors and is capable of forming biofilms, which can contaminate food and packaging industry equipments more easily (Abebe 2020; Li et al. 2023). Moreover, bacteria classified as fecal coliforms, such as *Escherichia coli*, can lead to severe foodborne infections in humans (Martin et al. 2016), which warns the importance of guaranteeing the total elimination of these microorganisms in MPF. The main agent used in the food industry as a sanitizer is sodium hypochlorite (NaOCl), being applied at a packing-house with a concentration of 200 ppm in an immersion bath for 2 min (Weng et al. 2016; Battagin et al. 2021; Dilarri et al. 2022). However, the World Health Organization (WHO) released data results about the cumulative effect of free chlorine in food, which is related to endocrine problems such as kidney failure, iodine absorption deficiency, and atherosclerosis disease (World Health Organization (WHO) 2017). Chlorine-based biocides, when in contact with organic matter, lead to the formation of halogen compounds such as trihalomethanes and haloacetic acid (Gil et al. 2019). Trihalomethanes and chloroform are formed after the chemical reaction of free chlorine with organic matter, having carcinogenic and mutagenic effects in the cell of the organism (Wang et al. 2013; EFSA 2015), becoming worrying compounds for public health. Therefore, the Council of the European Union (EU) adopted a new regulation in 2018 (2018/605 Regulation of the Delegated Commission of the European Union) with the category of “Endocrine Disruptors” (EDs), which prohibits and limits the concentration of free chlorine presents on the surface of the food after the packinghouse process (EFSA 2015; European Commission 2018; Dilarri et al. 2022). Nowadays, MPF is sanitized by other compounds beyond NaOCl, such as peracetic acid, essential oils (EO), and ozone (Rahman et al. 2016; Falcó

et al. 2019; Dilarri et al. 2022). Even so, microbiological control in MPF is still a challenge, as the industry must eliminate the main pathogenic microorganisms of the food without being harmful to human health, and preserving the characteristics of the MPF.

Due to their low cost, bactericidal action mechanisms, and being from a biological source, EO is growing in use as food sanitizers (Falcó et al. 2019; Zamuner et al. 2020; Battagin et al. 2021). Some EO are already used in organic agriculture due to their bactericidal action, such as the oil of *Lippia gracilis* (Santos et al. 2019), *Unonopsis guatterioides* (Yoshida et al. 2019), *Origanum vulgare* (Hać-Szymańczuk et al. 2019), and *Thymus vulgaris* (Aziz and Almasi 2018). Zayed et al. (2019) also described the antioxidant properties of *Pimpinella anisum* (anise) EO, which increased interest in further research and applications of this oil. In addition, Ksouda et al. (2019) described the antimicrobial activity of the EO of anise and associated it with the trans-anethole compound present in the oil. Additionally, the minimum inhibitory concentration (MIC) of *P. anisum* EO was calculated for major food-contaminating bacteria, such as *E. coli* (MIC of 2.5 µg mL⁻¹), *S. typhi* (MIC of 2.0 µg mL⁻¹), and *P. aeruginosa* (MIC of 3.0 µg mL⁻¹) (Abdel-Reheem and Oraby 2015). Consequently, due to its antioxidant and antimicrobial effects, with the MIC values for several species of microorganisms already defined, the use of *P. anisum* EO has become increasingly explored in the food and pharmaceutical industries (Sun et al. 2019). *P. anisum* is a grassy herb of the family Apiaceae, native to the eastern Mediterranean and Southwest Asia, being known and used in traditional Chinese medicine for its antimicrobial, anti-inflammatory, bronchodilator, expectorant, and insecticidal effects (Tavallali et al. 2017; Hashem et al. 2018; Sun et al. 2019). It is considered one of the most promising EO for technological applications and polymeric encapsulations (Albuquerque et al. 2022). However, to the present date, neither sanitizing tests with EO of *P. anisum* has been carried out with MPF.

Another point that limits the application of EO is the non-polar properties of the molecule, which impossibility, the solubility, and difficulty in the application of the oil. Therefore, the encapsulation of the EO in a polymeric matrix can be a way to increase the solubility and dispersion, reducing the photo-degradation and oxidation of the compound. Polymeric matrix also increases the residual effect of the antimicrobial compound, since it will remain on the applied surface for a longer period (Tamayo et al. 2016). Chitosan is a biopolymer composed of N-acetyl-2-amino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose linked by a (1 → 4)-β-glycosidic bond (Islam et al. 2017). This biopolymer is easily obtained from the deacetylation of chitin present in the crabs, lobsters, and langoustines shells; therefore, it is possible to extract it using fishery residues as its primary source, which makes it

a low-cost polymer (Dilarri et al. 2018; Mendes et al. 2020). Another advantage of chitosan is its ability to be complexed in different forms, being also possible to bring it to a micro-scale. These multi-complexation capacities open a great range for the use of this biopolymer in many different areas. The use of chitosan microparticles was described by Divya and Jisha (2018), which showed potential application for the dispersion of non-polar substances. Nevertheless, to the present moment, anise EO has never been immobilized in a chitosan matrix and applied against pathogenic microorganisms.

Pumpkins (*Cucurbita maxima*) belong to the Cucurbitaceae family and are extensively grown worldwide since many countries' diets include substantial amounts of this fruit (Pereira et al. 2020). Pumpkins are rich in phenolic compounds, such as flavonoids and phenolic acids, carotenoids, and vitamins (vitamin A, thiamine, riboflavin, vitamin B6, Vitamin C, vitamin E, and vitamin K) (Guz et al. 2018; Pereira et al. 2020). Pumpkin production has a great economic and social value in Brazil, with an estimated 27.08 kg per capita consumption, according to the Brazilian Institute of Geography and Statistics (Santos et al. 2016). Because it is a huge vegetable, it presents issues with marketing, storage, and handling, resulting in product loss (Soares et al. 2018). As a result, it is a vegetable that has the potential to grow in the market for minimally processed foods, increasing sales and value (Santos et al. 2016; Soares et al. 2018). Currently, it is possible to find several MPF of the many varieties of pumpkins offered in Brazil, and this trend is only going to grow. Due to these characteristics, the pumpkin MPF was selected as the target for the tests in the current study.

Therefore, this study aims to evaluate the EO of *P. anisum* encapsulated into the biopolymer matrix of chitosan in the micro-scale (MPsQTO) to increase the dispersion and contact surface of the EO. It was evaluated the performance of the bactericidal action of MPsQTO against *E. coli*, *S. typhi*, and *P. aeruginosa*, which are pathogenic microorganisms, and *Bacillus subtilis* that are the Gram-positive bacteria model for microbiological assays. In addition, ecotoxicological assays with the mutant *Saccharomyces cerevisiae* D7 were carried out to certify the toxicity of the MPsQTO for eukaryotic cells. In order to verify the effectiveness of the antimicrobial material produced in the present work, we conducted sanitization tests using MPF of pumpkin. The goal was to assess the shelf life and reduction of microorganisms in the MPF and check any changes in its initial characteristics after applying MPsQTO to the pumpkins.

Materials and methods

Microorganisms

E. coli (ATCC 8739), *S. typhi* (ATCC 19430), *B. subtilis* (ATCC 6633), and *P. aeruginosa* (ATCC 27853) are

bacterial strains associated with foodborne; therefore, they were selected as target microorganisms for this study. The mutant *Bacillus subtilis* (*amy::pspac-fitsZ-gfpmut1*) expressing FtsZ-GFP was used in the fluorescence microscope assays. All the bacteria used in the present work were cultivated in a nutrient broth medium (5 g L⁻¹ of bacteriological peptone; 3 g L⁻¹ of beef extract; for the solid medium was added 15 g L⁻¹ of bacterial agar) at 30 ± 1 °C for 24 h in a shaker at 200 rpm of agitation. The mutant yeast *S. cerevisiae* D7, a test organism for evaluation of the mutagenic/carcinogenic effect of the MPsQTO, was cultivated in YPD medium (15 g L⁻¹ of agar for solid medium; 20 g L⁻¹ of bacteriological peptone; 20 g L⁻¹ of dextrose; 10 g L⁻¹ of yeast extract) at 27 ± 1 °C, with agitation of 200 rpm for liquid growth. All reagents for cellular growth were purchased from Kasvi Ltda. (São José dos Pinhais, Brazil).

EO of *P. anisum*

P. anisum EO was obtained from Nikura® (London, UK), a member of the Aromatherapy Trade Council (ATC). The EO has 97% of purity and was extracted by cold pressing processes to reduce degradation/volatilization of the organic compounds, maintaining the major properties of the anise oil.

MPsQTO synthesis

The chitosan used in the present work was purchased from Sigma-Aldrich (Darmstadt, Germany), CAS 9012-76-4, being manufactured from shrimp shells. This chitosan has more than 75% degree of deacetylation, 0.38 g mL⁻¹ of density, 7.00 of pH, and 80 mesh of particle size.

The chitosan ionic gelling process was carried out through the method proposed by Hembram et al. (2016), where 1 g of powdered chitosan was dissolved in 35 mL of acetic acid solution at 5% of concentration (m v⁻¹). Next, the chitosan ionotropic gel was subjected to an ultrasonic bath—Elma X-tra LSM (Singen, Germany) for 20 min at 45 kHz, which allowed the polymer chain to break into a smaller size. Then, *P. anisum* EO was added reaching a final concentration of 5% in the total volume. The next step was the addition of tripolyphosphate (TPP) solution at 3% with 5.60 of pH 5.6 under constant stirring for 60 min at 21 °C. This step is fundamental for the chemical complexation of the oil in the biopolymer, and hence, crosslinking the MPsQTO. The separation of MPsQTO from liquid medium was performed by centrifugation at 6000 × g for 10 min.

Scanning electron microscopy (SEM)

The surface morphology, size, and shape of the MPsQTO produced in the present work were determined by

micrographs captured in SEM JEOL Model JSM-6360LV (Tokyo, Japan), equipped with backscattered secondary electron detectors coupled to an energy-dispersive analyzer (EDS) operating in high vacuum with a mechanical pump for the primary vacuum (10^{-3} Torr) and secondary vacuum (around 10^{-6} Torr), with an accelerating voltage of 15 kV.

For the preparation of the material, firstly, the MPsQTO was freeze-dried, and then the sample was metalized with 20-nm gold layer per sputtering deposition system. In that manner, the gold was deposited in all cavities and pores, overall covering the material independent of its irregularity. Afterward, the material was placed on carbon adhesive tape for SEM analysis. The images were obtained using an acceleration voltage of 5 kV and $\times 400$ magnification.

Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR characterizations were carried out with the FT-IR spectrophotometer Shimadzu Model IR Prestige 21 (Kyoto, Japan). The analysis processes were done through the pellet formation with 149 mg of KBr and 1 mg of the freeze-dried MPsQTO under 40 kN of compression. Next, the pellets were scanned at wavelengths between 4000 and 400 cm^{-1} , with bands reading at 4 cm^{-1} of resolution across 32 scanning procedures. For the identification of the peaks in the FT-IR spectrum, we used the ReactIR™ software for automated analysis. The software includes the latest library updated in 2023.

Gas chromatography–mass spectrometry (GC–MS/MS)

P. anisum EO was extracted in ethyl acetate and injected in the 1:30 split-ratio mode into the equipment GC–MS Model QP-2010 Shimadzu (Kyoto, Japan). Rtx-5MS capillary column ($60\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$) of fused silica and low-polarity phase was used with helium gas (1.5 mL min^{-1} of purge flow) at a pressure of 43.8 kPa and column flow of 0.84 mL min^{-1} . The analysis parameters were determined in this study using the electronic acceleration of 70 eV for ionization and injector temperature at $220\text{ }^{\circ}\text{C}$ for 2 min; next, it was increased to $290\text{ }^{\circ}\text{C}$ at a rate of $5\text{ }^{\circ}\text{C min}^{-1}$. All files from the GC–MS (mzData) were processed using NIST software.

Antibacterial activity assays

Firstly, the cell numbers per volume of each bacterial strain were determined by colony counting on plate, finding in this way, the values of CFU mL^{-1} of each bacterial strain before the initiation of the test. Subsequently, the respective cell suspensions were diluted at $100\times$ in a fresh medium to start the assays, reaching a final number of 10^5 CFU mL^{-1} of

each strain evaluated in the present work. Then, MPsQTO was added to each medium with the respective bacterial strain, varying the MPsQTO concentration in a range of 5 to $300\text{ }\mu\text{L mL}^{-1}$. For the positive control (PC), it was used Nisin at $5\text{ }\mu\text{g mL}^{-1}$ against Gram-positive bacteria strains, and Kanamycin at $20\text{ }\mu\text{g mL}^{-1}$ for the Gram-negative bacteria strains, being both antibiotics purchased from Sigma-Aldrich (Taufkirchen, Germany). The negative control (NC) was the broth medium without any compound. The total final volume of the Erlenmeyer flask containing the broth medium with bacteria and the MPsQTO was 20 mL. The flasks were incubated at $30 \pm 1\text{ }^{\circ}\text{C}$ for 4 h and 200 rpm of constant agitation. After incubation, samples of $100\text{ }\mu\text{L}$ of each flask were diluted in a solution of NaCl at 0.86%, and spread onto nutrient agar plates for CFU mL^{-1} counting. Each treatment was composed of three repetitions, and this experiment was done in three independent replicates. Data validation was performed using the standard deviation (SD), as shown in Eq. 1, and all the graphs were plotted using Origin version 8 (OriginLab Corporation, USA).

$$\text{SD} = \sqrt{\frac{1}{N-1} \sum_{i=1}^N \left(\frac{Q_{ie} - Q_{ic}}{Q_{ie}} \right)^2} \quad (1)$$

where N is the number of measurements carried out, and Q_{ie} and Q_{ic} are experimental and calculated data.

Fluorescence microscopy

E. coli, *S. typhi*, *B. subtilis*, and *P. aeruginosa* at 10^5 cells were exposed to MPsQTO for 15 min at the bactericidal concentration, determined previously in the antibacterial activity assays. After the exposure time, the cells were centrifuged at $6000 \times g$ for 7 min, the supernatant was discarded, and the cells were resuspended in 1 mL of saline solution (NaCl 0.86%). Next, the bacteria cells were stained with the Live/Dead BacLight kit (Thermo-Scientific L7012, Waltham, MA, USA), composed of the nucleic acid dye SYTO9 (that stains all cells, naturally passing through the cytoplasmic membrane), and the propidium iodide (PI) (stains only cells with damaged cytoplasmic membranes) (Savietto et al. 2018). The PC for the Gram-positive bacteria was the Nisin antibiotic, and for the Gram-negative bacteria, the damaged membranes were generated by heat-shock stress (Savietto et al. 2018). For NC, it was used cells without any treatment.

Analysis of the potential action of the MPsQTO in the interference/disruption of the divisional septa in bacteria was carried out through the fluorescence microscope assays with the *B. subtilis* (*amy::pspac-ftsZ-gfpmut1*). The mutant cells were cultivated in the presence of 0.02 mM Isopropyl β -D-thiogalactopyranoside (IPTG) to induce the expression of FtsZ-GFP from the *pspac* promoter. Subsequently, 1 mL of the culture with 10^5 CFU mL^{-1} was exposed to the

MPsQTO at its bactericidal concentration for 15 min. Later, the mutant cells were centrifuged at $6000 \times g$ for 7 min, the supernatant was discarded, and the cells were resuspended in 1 mL of 0.86% NaCl solution prior to microscope observation. The NC was the mutant cell without contact with any compound.

Cells were immobilized onto agarose-covered slides and observed using an Opticam Fluorescence Microscopy O600R (Doral, FL, USA), equipped with a monochromatic camera Opticam—12.3 MP (Doral, FL, USA). Images were processed through the software OPTHD—Opticam (Doral, FL, USA). One hundred cells were considered ($N=100$) per treatment for quantification and data analysis. All microscope assays were done in three independent replicates.

Mutagenic and carcinogenic assays with *S. cerevisiae* D7

For the mutagenic/carcinogenic assays with the mutant *S. cerevisiae* D7 (ATCC MYA4852TM) strain, it was used the standard protocol recommended by the US Environmental Protection Agency (EPA-USA 1996). Where, an aliquot of the cell stock was plated with YPD-agar, which should show 95% growth of white colonies (Zimmermann et al. 1975). Then, a white colony was selected to grow in YPD liquid medium for 18 h at 28 °C. Afterwards, the cells were centrifuged at $6000 \times g$ for 7 min, and subsequently, resuspended in $100 \times$ volume of 0.1 M phosphate buffer. Next, MPsQTSO at the bactericidal concentration was added to the buffer solution with the *S. cerevisiae* D7 cells. For NC, it was used sterile deionized water in the same volume proportion ($v v^{-1}$) of the treatment with the MPsQTSO. The PC, it was used ethyl methanesulfonate Sigma-Aldrich (Taufkirchen, Germany) at 1% of concentration. After 6 h of contact with each compound, an aliquot of each sample was inoculated by spread plate in YPD-agar and incubated at 27 ± 1 °C for 18 h. The change in the phenotypic expression of the yeast strain is related to the mutation expressed after contact with the evaluated compound. Experiments were performed with three independent triplicates with a minimum count of 100 cells per plate. The data was validated using the SD (Eq. 1) to identify experimental errors. Then, the data underwent non-parametric statistical analysis using Kruskal–Wallis (Dunn) with three degrees of freedom. Graphs were created using Origin 8 software (OriginLab Corporation, USA), and statistical analyses were carried out using BioEstat 5.0 (open source).

Sanitization assays

The MPF vegetable selected for this study was the pumpkin (*Cucurbita pepo* L.). All the pumpkins were directly acquired from the producer, being cut on the bench, and

without through any sanitation processes at packinghouse. The pumpkin pieces were meticulously divided into three distinct groups, ensuring uniformity in size and weight. The sanitization tests were conducted with three independent replicates, each comprising three groups of homogeneous pumpkin pieces. Right after the pumpkin cut and separation process, the sanitization assays were carried out with three treatments: NC with autoclaved tap water; PC with employing a NaClO solution (Chemical Abstract Service number 7681–52-9, purchased from Sigma-Aldrich, Taufkirchen, Germany) with a concentration of 200 ppm of free chlorine; and MPsQTSO at the bactericidal concentration (T1) determined previously in the antibacterial activity assays. The MPF wash in this study was done through by the spraying of each treatment solution until the run-off point in their respectively evaluated group. The pumpkins were kept under contact with the test compounds for 2 min. Next, pumpkins were quickly dried with the help of a hair dryer (non-heated airflow) for 30 s, and then placed into sterile containers covered by a porous membrane. The pumpkins were kept under constant temperature of 13 ± 1 °C, thus simulating the storage of the MPF vegetable. The pumpkins were individually analyzed every 24 h, checking for the state of deterioration, fungus coverage, bacteria biofilm, color, and consistency. The deterioration observations were started in the time zero (after the wash) to the final time (when all pumpkins of all treatments were rotten).

Results and discussion

MPsQTO characterization

Through FT-IR studies, it was possible to identify the main groups of chemical interaction between the biopolymer and the EO of *P. anisum*. The chemical bonds that drive the encapsulation processes of the EO are shown in the spectrum of biopolymer before and after the immobilization in the MPsQTO matrix (Fig. 1). The main point of the chemical ligation was identified in the peak at 1380 cm^{-1} , which disappeared after the immobilization of the oil in the biopolymer matrix. This is due to the chemical interaction between the carboxylic groups present in the biopolymer molecule with the oil. The 1646 cm^{-1} band is attributed to the elongation of the bond of the $-C$ and $-N$ molecules found in both materials due to the amino group present in the biopolymer and alaninol molecule in the EO. The absorbance of this region has an increased intensity, which is related to the increase in the concentration of the chemical groups after the addition of the EO. This result is confirmed by the deformation of the peak at 1427 cm^{-1} , which indicates an increase in the curvature of the spectrum by the addition of the amino group. The

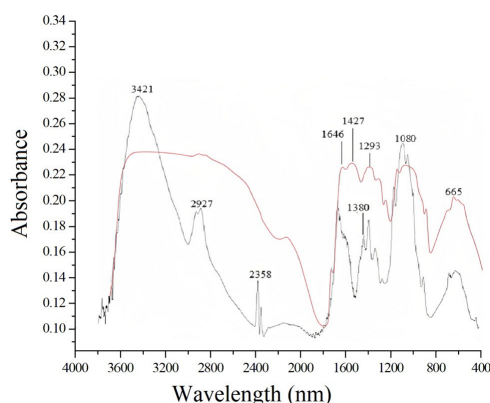


Fig. 1 FT-IR spectrum of the biopolymer before and after the immobilization of EO of *P. anisum*. The black line is the biopolymer chitosan, and the red line is the chitosan after the immobilization of the EO of anise in its polymeric matrix (MPsQTO)

peaks at 2927 and 2358 cm^{-1} show the asymmetric elongation of the $-\text{CH}_3$ and $-\text{CH}_2-$ groups, respectively. The peaks, between 3000 and 2850 cm^{-1} , are identified by the characteristics of the vibration of CO_2 and hydrogen molecules. The peak at 650 cm^{-1} shows the stretching of the aromatic ring present in both materials.

To determine the main *P. anisum* EO compounds, it was used the GC–MS/MS spectrometry with the NIST library for identification of the molecules. The anethole molecule was identified in higher concentration by the peak at 148 m z^{-1} ; alanol at 44 m z^{-1} ; benzaldehyde at 135 m z^{-1} ; α -terpinol at 1143 m z^{-1} ; and α -longipinene at 119 m z^{-1} (Fig. 2). Other compounds were associated in lower concentrations such as carvacrol, borneol, caryophyllene, and γ -terpinene. The highlight is the anethole molecule with ~89.0% of concentration in the analyzed *P. anisum* EO.

The morphological characterization and particle size of MPsQTO were evaluated by SEM (Fig. 3). The structural morphology of MPsQTO was irregular and had a great number of porous. Furthermore, the size of the particles was evaluated with a range of 540 to 700 μm . MPsQTO also showed cavities, which allow the EO to adhere in the biopolymer. The great number of porous and cavities also increase the contact surface of the MPsQTO with the microorganisms and the applied surfaces. The SEM images also showed that MPsQTO does not behave like a mycelium; on the contrary, the EO is available in all pores, which allows a high dispersion of the bactericidal compound. In addition, this conformation increases the solubility, improving the antimicrobial action in the sanitization process. The porosity of the material also allows for better anchoring interaction

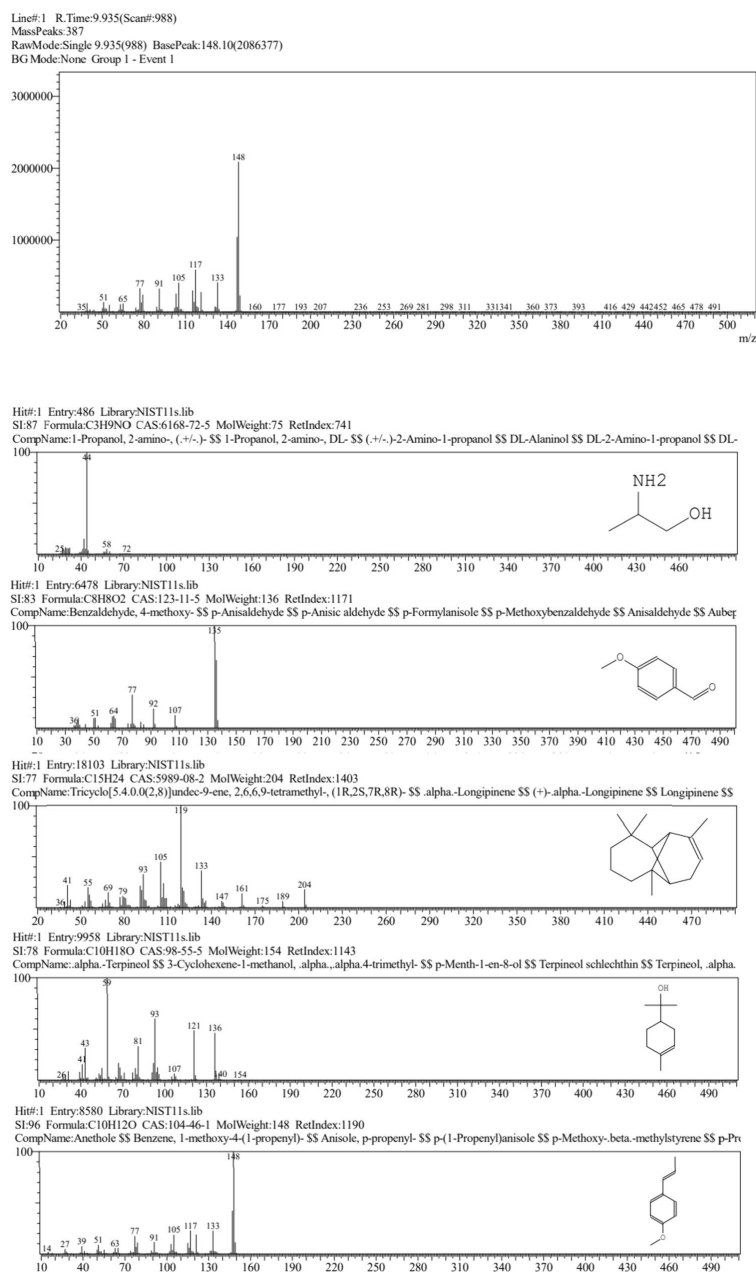
between the chemical bonding groups of the oil and the polymeric matrix.

Antimicrobial activity of MPsQTO

The bactericidal activities of MPsQTO showed different inhibition responses, and the bactericidal concentration also varied for each bacterial species evaluated. *S. typhi* was the more susceptible bacteria to the MPsQTO, where 20 $\mu\text{L mL}^{-1}$ of MPsQTO was bactericidal for the *S. typhi* (Fig. 4). *S. typhi* is the bacterium that presents the lowest MIC for the non-immobilized EO of *P. anisum*, with its MIC at 2 $\mu\text{g mL}^{-1}$ of concentration, as reported by Abdel-Reheem and Oraby (2015). This corroborated our results, as it also presented the lowest minimum bactericidal concentration for MPsQTO, when compared to the other bacterial strains evaluated in the present work. Another factor supporting the action of the immobilized oil is that MPsQTO exhibited bactericidal action at 20 $\mu\text{L mL}^{-1}$, which is proportional to the MIC of the pure *P. anisum* EO. As a result of immobilization, the concentration of MPsQTO must be raised to match the MIC value of pure *P. anisum* EO. This is because the contact surface and availability of the antimicrobial agent have decreased (Braun et al. 2024). On the other hand, immobilizing EO offers benefits such as increased solubility and dispersion. It also extends the antimicrobial residual effect on the food surface while limiting its impact on the food's characteristics and properties (Tamayo et al. 2016; Albuquerque et al. 2022; Braun et al. 2024).

The Gram-positive bacteria *B. subtilis*, was also shown to be susceptible to MPsQTO, since 60 $\mu\text{L mL}^{-1}$ of MPsQTO led all the *B. subtilis* cells to death (Fig. 4). Nevertheless, *E. coli* and *P. aeruginosa* were more resistant to MPsQTO. The MPsQTO was only bactericidal for *E. coli* and *P. aeruginosa* at concentrations of 300 and 200 $\mu\text{L mL}^{-1}$ (Fig. 4), respectively. In a direct comparison with *S. typhi* and *B. subtilis*, it can be stated that it was necessary to add ten times the concentration of MPsQTO to achieve the same antimicrobial effect with *E. coli* and *P. aeruginosa*. *E. coli* is a fecal coliform, so it is very important that MPsQTO has a bactericidal activity against this bacteria, otherwise its possibility of use as a sanitizer becomes more limited. An efficient sanitizer must quickly eliminate mainly bacteria related to food poisoning and fecal coliforms that may be present in the food, ensuring the safety of the food in addition to preserving its main nutritional characteristics (Dilarri et al. 2022). Dávila-Rodríguez et al. (2019) found that a nanoemulsion of cinnamon EO at a concentration of 100 $\mu\text{L mL}^{-1}$ effectively inhibited *Listeria monocytogenes* and *E. coli*. Azizkhani et al. (2013) achieved positive results in inhibiting *E. coli* (O157:H7) from baby leaves (baby red and green Batavia lettuce) by using pure EO of clove and oregano at a concentration of 100 $\mu\text{L mL}^{-1}$. Although Azizkhani et al. (2013)

Fig. 2 Spectra of GC–MS/MS and the identification of the compounds present in the EO of *P. anisum*



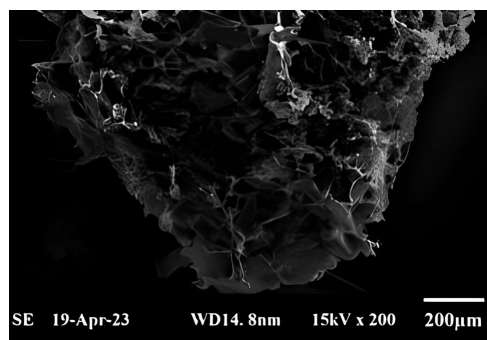


Fig. 3 Surface morphology of MPsQTO by SEM, with 200 µm of scale bar

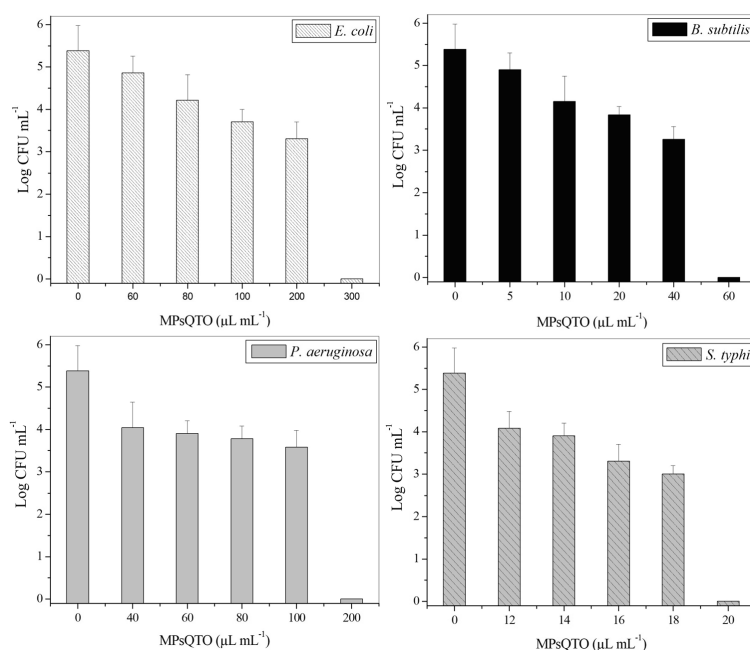
and Dávila-Rodríguez et al. (2019) studied pure EO applications, their inhibitory concentration results for *E. coli* are comparable to our findings. Therefore, the results obtained confirmed the efficiency of MPsQTO in eliminating pathogenic bacteria, in addition to being efficient against the fecal coliform evaluated in the present work.

The SD exhibited acceptable variation values, supporting the bactericidal doses of MPsQTO for all tested bacterial

species. Furthermore, an important factor in Fig. 4 was the sudden drop in the number of CFU mL⁻¹ after reaching the bactericidal concentration. This phenomenon is observed in all four different bacterial species tested. As a result, MPsQTO has no dose–response effect, but rather causes cell death once the bactericidal concentrations are reached. When a compound inhibits a cell without generating a dose–response graph, it indicates that the antimicrobial agent is affecting the surface area of the cell, leading to its immediate death upon contact. Andrade-Ochoa et al. (2021) evaluated the EO of *P. anisum*, which contained 88.9% anethole, being a concentration similar to the EO used in the present work. Although Andrade-Ochoa et al. (2021) found that *P. anisum* EO affects exterior structures, such as cytoplasmic membrane and cell wall of bacteria cells, they were unable to verify whether this is the only mechanism of bactericidal activity. Therefore, fluorescence microscopy assays were used to elucidate the action mechanism of the material produced with the EO of *P. anisum*.

Membrane integrity studies of *S. typhi*, *B. subtilis*, *E. coli*, and *P. aeruginosa* cells were investigated through fluorescence microscopy assays after each bacteria species was exposed to MPsQTO at its bactericidal concentration. It was verified that MPsQTO disrupted the cytoplasmic membranes of all evaluated bacteria strains after 15 min of exposure to MPsQTO at the bactericidal concentration

Fig. 4 Antimicrobial activity of MPsQTO for each different bacterium species evaluated in the present work. The living cell numbers at Log CFU mL⁻¹ after contact with different concentrations of MPsQTO are represented as bars. The whiskers indicate the SD of the mean of three independent experiments used to verify experimental errors



(Fig. 5). The Tx Red and DAPI Blue filters were applied to visualize PI and SYTO9 in the bacteria cells. Cells with intact membranes are stained in blue since the PI cannot pass through the cytoplasmic membrane. The cells with pores or ruptures in the membrane are stained in red; due to the damages in the membrane, the IP managed to enter and stain the cell from the inside (Savietto et al. 2018). These results are in line with the anethole molecules identified through GC–MS spectrometry assays, since the anethole, benzaldehyde, α -terpinol, and α -longipinene were already described as antimicrobial compounds that disrupt the cytoplasmic membrane of the bacteria cells (Gharib et al. 2017; Pisoschi et al. 2018; Chaudhari et al. 2020; Yang et al. 2023). Chaudhari et al. (2020) carried out a study very similar to the present work, where it was evaluated the antifungal potential of the anethole immobilized in chitosan nanoemulsion, and

they proved that the anethole immobilized in its biopolymer eliminated the fungus through damage of the cytoplasmic membrane. Furthermore, the material proposed by Chaudhari et al. (2020) showed fungicidal activity with concentrations close to $180 \mu\text{L mL}^{-1}$, a similar value to the bactericidal concentrations of the material synthesized in the present work. Although our chitosan biopolymer was complexed on the micro-scale, unlike Chaudhari et al. (2020) which achieved the nano-scale with the chitosan, the results obtained proved that its mechanism of action and biocidal potential/concentration are similar.

The carvacrol, identified in less concentration than anethole molecules, where also described as a bactericidal compound, causes damages in the bacterial membrane (Zamuner et al. 2023). Therefore, the antimicrobial action mechanisms of MPsQTO, which belong to the EO

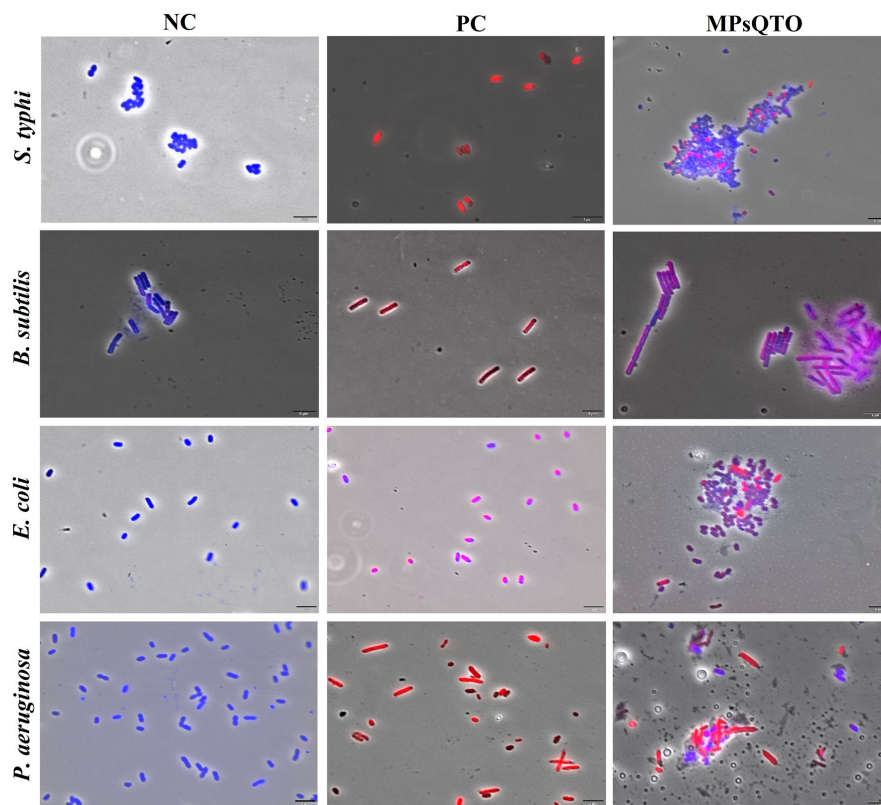


Fig. 5 Fluorescence microscopy in cells stained with SYTO9 and PI after 15 min of exposure to MPsQTO. Cells with intact membranes are stained in blue; while cells with damaged membranes are stained in red. The test was considered valid when $90 \pm 10\%$ of the cells from

NC were alive and without any damage at the cytoplasmic membrane, and $90 \pm 10\%$ of PC were dead with damaged membranes. The experiment was performed three times and at least 100 cells were evaluated per experiment ($N > 300$). Scale bar $5 \mu\text{m}$; $\times 100$ magnification

of anise, target the cytoplasmatic membrane of the bacteria cell disrupting it, which corroborates with the organic compounds found in the composition of the anise oil.

The microscopies also showed an unusual characteristic of the MPsQTO that helps in the antimicrobial action due to its capacity for aggregation/agglomeration of the bacteria cells in the microparticles of the biopolymer (Fig. 5). In addition to aggregating the cells, causing agglutination, the MPsQTO ends up preventing the cellular movement of the bacteria, immobilizing, and further increasing their contact with the EO of anise present in the biopolymer. This proves the efficiency of immobilizing anise EO on chitosan microparticles, increasing the delivery of the antimicrobial compound, making it soluble and easier to disperse, in addition to agglutinating microbial cells on the applied surface. All cells showed the same clustering behavior due to the positive charges of the NH_2 groups that are easily protonated to NH_3^+ . In addition, chitosan is formed by individual polymer chains that are held together by dipole–dipole bonds and Van der Waals forces, which generate an electrostatic attraction with the cell surface. Electrostatic attraction is inevitable because MPsQTO has positive charges, and the cell surface has a negative surface charge influenced by diffuse anions and cations in the electrical double layer with phosphoric groups of the cell membrane. This region of electrostatic interface between the aqueous medium and the cell surface is called the zeta potential, which generates a long-range interaction between the cells and the microparticles due to the opposite charges (Mendes et al. 2021). After cell attraction in the microparticles, the bactericidal compounds present in the EO target damage to the bacteria membrane.

The fluorescence microscopy also proved the fast bactericidal effect of the MPsQTO, since after 15 min of contact with each bacteria species, more than 50% of all cells were dead. *B. subtilis* and *E. coli* were the most susceptible species in relation to death due to contact time, wherein after 15 min of contact with MPsQTO, $72.60 \pm 9.04\%$ and $68.93 \pm 6.11\%$ of the cells were dead with the ruptured cytoplasmatic membrane, respectively. On the other hand, *S. typhi* and *P. aeruginosa* were the most resistant specie, with $52.10 \pm 5.80\%$ and $59.55 \pm 7.87\%$ of dead cells after the first 15 min of exposure to MPsQTO, respectively. *P. aeruginosa* is an opportunistic pathogen with a great capacity to resist antibiotics, and formatting of multi-drug-tolerant persisted cells (Pang et al. 2019). Therefore, even with the most resistance capacity, it is possible to highlight the fast effect of the proposed material in eliminating more than half of the cell population of *P. aeruginosa* in the first minutes of contact. It is important to note that the amount of cells used in this test is higher than the usual naturally found on contaminated surfaces and in food, so this inhibitory effect and population reduction will be greater in a real application.

Although being the bacterial species that presented the lowest bactericidal concentration of MPsQTO compared to the others, the microscopy analyses proved that *S. typhi* has the highest number of live cells after 15 min of contact with MPsQTO, showing that the compound takes longer to have its effect on *S. typhi* cells. Despite this, the *S. typhi* cells were agglutinated even though they still lived due to the effect of MPsQTO. The loss of mobility, together with the constant contact with the EO of anise, due to the agglutination carried out by the MPsQTO, is probably what generates such a low concentration to have a bactericidal effect on *S. typhi* cells, even though they take longer to die after contact with MPsQTO.

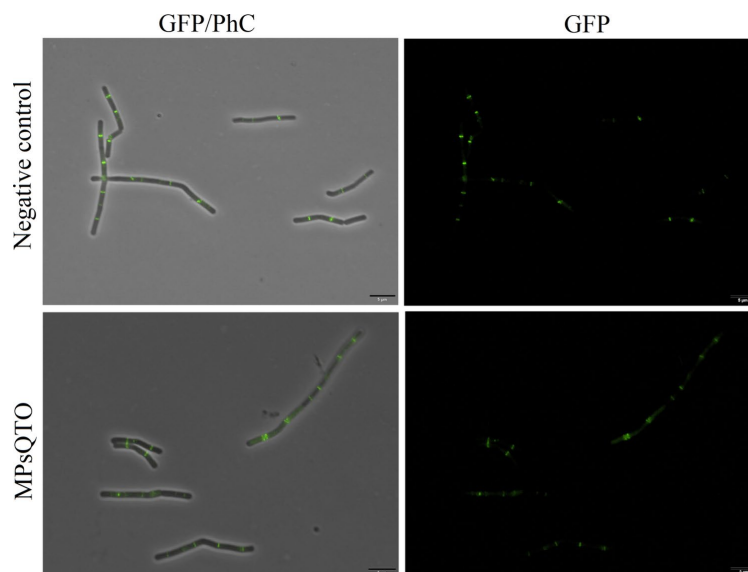
Some compounds can target more than one structure in the bacteria cell, presenting in this way more than one mechanism of action against the microorganism. Therefore, it is important to evaluate other possible structures that can be affected by the antimicrobial compounds. Understanding the mechanisms of action of MPsQTO is essential to predict the possible selection of resistant microorganisms by the use of new bactericidal products. FtsZ is a protein (the ancestral tubulin conserved in bacteria) used to assemble the divisome, being its function dependent on the nucleotide guanosine triphosphate (GTP) (Du et al. 2016; Hurley et al. 2016; Xiao and Goley 2016; Tripathy and Sahu 2019; Mendes et al. 2022). Therefore, any disturbance in this protein will prevent cell division, in addition to other actions such as GTPase inhibition will also prevent the FtsZ functional activity. If the bacterial nucleic acid is being affected, FtsZ will also not be able to form the bacterial divisional ring. This way, it was used the mutant *B. subtilis* expressing FtsZ-GFP exposed to MPsQTO at its bactericidal concentration for 15 min, and subsequently, observed under the fluorescence microscope.

The results showed that, even after MPsQTO exposure, the *B. subtilis* still has an intact divisional septum (Fig. 6). These results were equal to NC, where was not observed any disruption of the divisional ring (Fig. 6). Thus, MPsQTO does not target to block the formation of the divisional ring. MPsQTO also do not act against the nucleic acid or GTPase from the bacteria cell. In this way, it was possible to conclude that the mechanism of action of MPsQTO only targets the bacteria cytoplasmatic membrane, which leads the cell to death.

Mutagenic and carcinogenic assays with *S. cerevisiae* D7

Mutagenic and carcinogenic assays with *S. cerevisiae* D7 were performed to assess possible harmful effects on human health. This mutant yeast strain was constructed and described by Zimmermann et al. (1975) and is widely used as a model organism to assess the genotoxicity of

Fig. 6 MPsQTO does not target the bacterial divisome. The mutant strain *B. subtilis* expressing FtsZ-GFP, labelled for the divisomal septum, was exposed to $60 \mu\text{L mL}^{-1}$ of MPsQTO for 15 min before microscope observation. The divisomal septum corresponds to the green bar perpendicular to the long axis of the rods. The scale bar corresponds to $5 \mu\text{m}$; magnification of $100\times$; GFP/PhC is the phase contrast images superimposed on the GFP fluorescence images



compounds according to the EPA-Environmental Protection Agency (EPA-USA 1996). This yeast is genetically engineered to exhibit a mutation or repair mechanism in its chromosomes. Zimmermann et al. (1975) describe the yeast *ade2* heteroallelic locus, which produces the phenotype of white colonies in the YPD culture medium as shown in the NC (Fig. 7). However, mutagenic compounds induce mitotic crossovers and then recombination repair mechanisms at this heteroallelic site. This mutation results in the inactivation of the homoallelic *ade2* locus and therefore presents the phenotype of pink or red colonies in the YPD culture medium (Fig. 7). This phenotypic alteration is shown in the PC with the mutagenic agent ethyl methanesulfonate at 1% ($v v^{-1}$) (Fig. 7). Thus, to evaluate the potential mutagenic/carcinogenic effect of MPsQTO, mitotic gene conversion tests were performed with it.

The results of *S. cerevisiae* D7 strain growth, after contact with MPsQTO at $300 \mu\text{L mL}^{-1}$ (being the higher bactericidal concentration determined previously) were compared with the PC and NC. The yeast colonies did not change from white to pink/red in a significant amount after contact with MPsQTO (Fig. 7). The NC showed $13.8 \pm 5.3\%$ of pink or red colonies, being near to the values of the MPsQTO ($14.42 \pm 7.14\%$ of pink or red colonies), being similar results and statistically the same. Therefore, the MPsQTO did not generate genetic divergences in the *ade2* loci. Consequently, it is possible to conclude that MPsQTO at the concentration tested in the present studies has no mutagenic effect in the cells. This result is mainly corroborated by the PC values,

where $94.33 \pm 4.1\%$ of red or pink colonies could be counted, indicating that MPQTO does not have the same mutagenic potential as the PC. On the other hand, a reduction in the number of CFU was observed, an expected result since the composition of the EO of *P. anisum* presents aromatic ring compounds, determined in studies with GC-MS/MS, which has a biocidal effect against the yeast cells. The antifungal effects of anethole molecules immobilized in chitosan nanoemulsion have also already been described (Chaudhari et al. 2020), which corroborate the results obtained showing an inhibitory effect on *S. cerevisiae* D7, but without generating mutagenic effects on the microorganism. Probably if the concentration of MPsQTO or its contact time was increased, it would have a fungicidal effect with the total elimination of *S. cerevisiae* D7 cells. These results also corroborate that the action of the material produced in the present work is by damage to the cell membrane since the yeast chromosomes did not undergo any type of change.

Sanitization assays

The effectiveness in prolonging the durability of the MPF was evaluated through the sanitization assays. The fresh-cut pumpkin, chosen for this assay, showed fungal mycelium growth after 6 days in the NC and 8 days in the PC. Both controls also showed a color change, and the pumpkin dried out at the end of the 12 days of the experiment. One difference was the microbial growth and formation of bacterial biofilm after 5 days in the NC, leaving the pumpkin with a

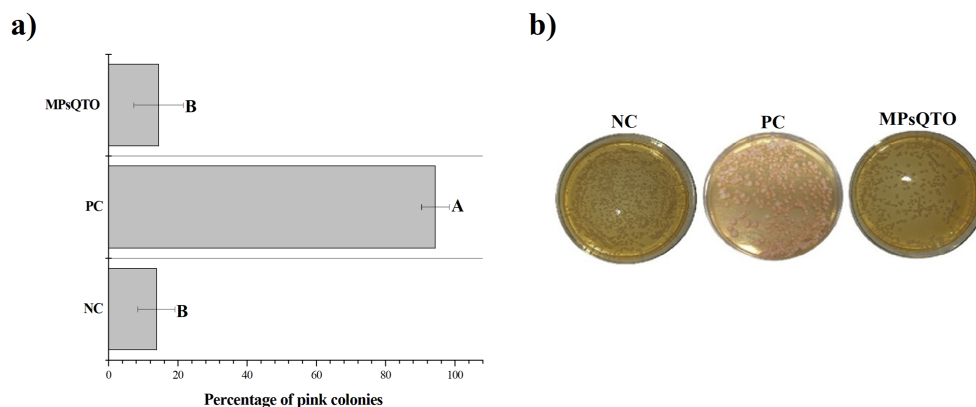


Fig. 7 MPsQTO did not have a mutagenic or carcinogenic effect on *S. cerevisiae* D7 cells. **a** The graph of the percentage of *S. cerevisiae* D7 colonies counted that presented phenotypic alteration due to genetic changes in the yeast cell chromosome after contact with each treatment. The axis represents the SD of the samples. Different let-

ters show the statistical difference between the treatments ($P < 0.05$) by the non-parametric statistical analysis of Kruskal–Wallis. **b** The photos of the respective plates to illustrate the phenotypic change observed in the experiment

viscous appearance, and this characteristic was not observed at any time in the treatments with MPsQTO and PC.

Treatment with MPsQTO at $300 \mu\text{L mL}^{-1}$ showed less drying due to the protective layer of the biopolymer that reduces the degradative oxidation of the organic matter. The color consequently did not show major changes. It was also observed molds on the pumpkin surface, but only after 10 days of the experiments (Fig. 8). These results are similar to the efficiency of protection of EO on the surface of foods observed by Falcó et al. (2019). Chaudhari et al. (2020) also made a material similar to that produced in the present work and obtained positive results in the conservation of maize, managing to have antifungal action, thus increasing the storage time of the maize. Furthermore, Chaudhari et al. (2020) managed to relate this increase in storage time with the antioxidant action belonging to anethole, which ended up being prolonged even further by the residual effect of chitosan. This study by Chaudhari et al. (2020) corroborates the results obtained in the present sanitization assays, since in addition to prolonging shelf life and inhibiting the appearance of fungi for a longer time, MPsQTO showed antioxidant activity (belonging to the anethole), reduction of the drying effect, and maintaining the color and texture of the tested food for much longer than PC (200 ppm of free chlorine).

For the conservation of an MPF, it is very important that the conservation treatment does not affect the sensory properties of the food, as reported by Alvarez et al. (2015), where the gallic acid treatment reduced vegetable sample quality, especially under high temperature, causing celery browning, off-odors, and texture deterioration in pumpkin cubes.

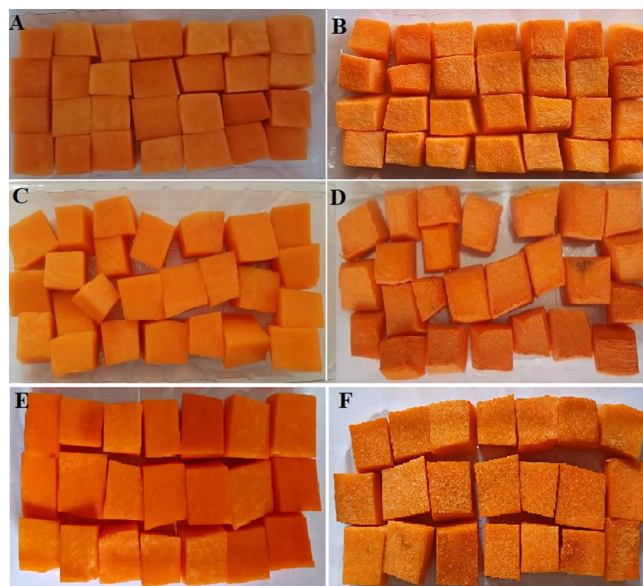
This was a positive observation following the sanitization tests, as the MPsQTO did not change the main characteristics of the pumpkin, including their color and odor. Soares et al. (2018) achieved favorable results using the biopolymer chitosan in combination with a vacuum process, whereas similar to our findings, chitosan facilitated a reduction in pumpkin dehydration and extended the food's shelf life. This validates the selection and effectiveness of using chitosan as a polymeric matrix for this application and the production of our antimicrobial material. Santos et al. (2016) utilized carvacrol coatings as the primary antimicrobial agent. Despite carvacrol's notable traits, such as its odor and other chemical characteristics, the experiments conducted by Santos et al. (2016) confirmed that there were no alterations in the physicochemical parameters analyzed in the pumpkins, a result consistent with the findings for the applied MPsQTO in the current study. It is worth highlighting that carvacrol is a component of many EO. Therefore, even containing EO of anise, MPsQTO also did not cause many changes in the physicochemical or visual parameters of the pumpkins after its application in the present work.

Conclusion

Chitosan proved to be an efficient polymeric matrix for the immobilization of *P. anisum* EO, maintaining the bactericidal properties of the EO, and facilitating its application while prolonging its residual antimicrobial effect.

The synthesized material also showed a micro-scale with a proximate size of $700 \mu\text{m}$ and many pores and cavities. The

Fig. 8 Comparison of fresh-cut pumpkin before and after sanitization with MP_sQTO. **A** The fresh-cut pumpkin before sanitization with MP_sQTO at 300 $\mu\text{L mL}^{-1}$ of concentration. **B** The fresh-cut pumpkin after sanitization with MP_sQTO at 300 $\mu\text{L mL}^{-1}$ in the final experiment time of 12 days. **C** The fresh-cut pumpkin before sanitization with any compound. **D** The fresh-cut pumpkin after sanitization with autoclaved tap water in the final experiment time of 12 days. **E** The fresh-cut pumpkin before sanitization with 200 ppm of free chlorine. **F** The fresh-cut pumpkin after sanitization with 200 ppm of free chlorine in the final experiment time of 12 days



main molecule identified through GC–MS/MS was anethole (~ 89.0% of concentration), the main antibacterial agent in the EO.

The MP_sQTO proved to be bactericidal against *S. typhi*, *B. subtilis*, *E. coli*, and *P. aeruginosa*, managing to eliminate all different species when used at a concentration of 300 $\mu\text{L mL}^{-1}$. MP_sQTO also did not show any mutagenic or carcinogenic effect in the toxicity tests with the mutant *S. cerevisiae* D7 when applied at the concentrations used in the present work.

Membrane integrity assays showed that MP_sQTO targets the cytoplasmic membrane, directly affecting its structure and causing rapid cell death. Fluorescence microscope analysis of *B. subtilis* expressing FtsZ-GFP revealed that MP_sQTO does not impact the divisional ring, DNA, or GTPase. Therefore, it was concluded that the only target of MP_sQTO is the bacterial cytoplasmic membrane. It was also confirmed that MP_sQTO has the ability to agglutinate bacterial cells, immobilizing them and facilitating their exposure to the EO of *P. anisum*.

The MP_sQTO at 300 $\mu\text{L mL}^{-1}$ was efficient as a sanitizer for pumpkin MPF, prolonging the shelf life of the food. Therefore, the proposed biomaterial produced can be considered as a substitute for NaOCl in the sanitization of food in packinghouses.

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Data availability No datasets were generated or analysed during the current study.

Declarations

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