UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS
Programa de Pós-Graduação em Ciência de Alimentos

COMBINAÇÃO DE CINAMALDEÍDO COM SORBATO DE POTÁSSIO E COM NANOPARTÍCULAS DE PRATA BIOGÊNICAS: EFEITO ANTIBACTERIANO *IN VITRO* E EM ALIMENTOS

ANDREIA FARIAS PEREIRA BATISTA

Maringá
2023

ANDREIA FARIAS PEREIRA BATISTA

COMBINAÇÃO DE CINAMALDEÍDO COM SORBATO DE POTÁSSIO E COM NANOPARTÍCULAS DE PRATA BIOGÊNICAS: EFEITO ANTIBACTERIANO *IN VITRO* E EM ALIMENTOS

Tese apresentada ao programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de Doutora em Ciência de Alimentos

Maringá

2023

Orientadora

Profª. Drª. Jane Martha Graton Mikcha

ANDREIA FARIAS PEREIRA BATISTA

"COMBINAÇÃO DE CINAMALDEÍDO COM SORBATO DE POTÁSSIO E COM NANOPARTICULAS DE PRATA BIOGÊNICAS: EFEITO ANTIBACTERIANO *IN VITRO* E EM ALIMENTOS"

Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

Prof. Dr. Alex Fiori da Silva

Profa. Dra. Adriele Rodrigues dos Santos

Prof. Dr. Benício Alves de Abreu Filho

Profa. Dra. Paula Aline Zanetti Campanerut-Sá

Profa. Dra. Janie Martha Graton Mikcha
Orientadora

Maringá – 2023

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá - PR, Brasil)

B333c

Batista, Andreia Farias Pereira
Combinação de cinamaldeído com sorbato de potássio e com nanopartículas de prata biogênicas : efeito antibacteriano in vitro e em alimentos / Andreia Farias Pereira Batista. -- Maringá, PR, 2023.
65 f.: il. color., figs., tabs.

Orientadora: Profa. Dra. Jane Martha Graton Mikcha.
Tese (Doutorado) - Universidade Estadual de Maringá, Centro de Ciências Agrárias, Departamento de Ciências Agronômicas, Programa de Pós-Graduação em Ciência de Alimentos, 2023.

1. Atividade antibacteriana. 2. Patógenos de origem alimentar. 3. Efeitos sinérgicos. 4. Nanopartícula de prata biogênica. I. Mikcha, Jane Martha Graton, orient. II. Universidade Estadual de Maringá. Centro de Ciências Agrárias. Departamento de Ciências Agronômicas. Programa de Pós-Graduação em Ciência de Alimentos. III. Título.

CDD 23.ed. 579.165

Rosana de Souza Costa de Oliveira - 9/1366

BIOGRAFIA

Andreia Farias Pereira Batista nasceu em 1984, na cidade de Mandaguari, Paraná. Graduou-se em Nutrição pelo Centro Universitário Estácio de Sá de Santa Catarina, SC, no ano de 2014. Em fevereiro de 2018 concluiu o mestrado em Ciência de Alimentos pela Universidade Estadual de Maringá. Em março de 2018 iniciou o doutorado no mesmo programa. Concluiu especialização em Nutrição Clínica e cursos de formação em Modulação Intestinal, Saúde da Mulher e Fertilidade entre os anos de 2019 e 2022. É Pós-Graduanda em Nutrição Clínica em Gastroenterologia e atualmente trabalha como nutricionista, atuando na área de Nutrição Clínica, com ênfase em Saúde da Mulher e Fertilidade, doenças autoimunes e do trato gastrointestinal.

Dedico

À minha família, em especial ao meu filho Gabriel e meu esposo Everton pelo apoio incondicional em todos os momentos, principalmente nos de incerteza, muito comuns para quem tenta trilhar novos caminhos. Sem vocês nenhuma conquista valeria a pena.

AGRADECIMENTOS

Agradeço primeiramente à DEUS por me guiar, iluminar e me dar sabedoria para seguir em frente com os meus objetivos e não desanimar com as dificuldades.

À Universidade Estadual de Maringá, e ao Programa de Pós-graduação em Ciência de Alimentos, pela oportunidade.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa durante o período cedido.

À professora Drª. Jane Martha Graton Mikcha, só tenho a agradecer pela sua orientação, aos seus ensinamentos, palavras de incentivo, "puxões" de orelha, paciência e dedicação. Agradeço também a disponibilidade e total colaboração no solucionar dúvidas e problemas que foram surgindo ao longo da realização deste trabalho.

Aos professores Drª. Renata Katsuko Takayama Kobayashi e Dr. Gerson Nakazato pela parceria e compartilhar seus conhecimento e experiências.

Ao professor Benício por todo carinho, incentivo e suporte durante todo caminho.

Ao Neurocientista e Prof. Dr. Fabrício Assini, pelo incentivo e por acreditar em meu crescimento acadêmico e profissional. Você me fez entender que sou capaz de chegar onde eu almejar.

Aos amigos Daliah Trevisan, Adriele Santos, Alex Fiori e Victor Hugo pela amizade, força e ombro para dividir as dificuldades, angústias e também as alegrias.

Aos companheiros de laboratório Daliah Trevisan, Alex Fiori, Adriele Santos, Victor Hugo, Maíra Formaggio, João Vitor, Luana Rosa, Letícia Zavadizk e à Prof. Paula Campanerut-Sá pela amizade e toda ajuda.

À Eliana Guilhermeti que sempre me auxiliou quando precisei e pela amizade.

Aos meus pais, José e Cléria, meu infinito agradecimento. Mesmo distante, sempre acreditaram em minha capacidade. Isso só me fortaleceu e me fez tentar fazer o melhor de mim. Obrigada pelo amor incondicional!

Aos meus irmãos, Daiane e Anderson, e aos meus sogro e sogra João e Maria de Lourdes meu agradecimento especial, pois, a seu modo, sempre se orgulharam de mim e confiaram em meu trabalho. Obrigada pela confiança!

A meu querido esposo, Everton, por ser tão importante na minha vida. Sempre ao meu lado, me incentivando e fazendo acreditar que posso mais do que imagino. Devido a seu companheirismo, amizade, paciência, compreensão, apoio, alegria e amor, este trabalho pôde ser concretizado. Obrigada por ter feito do meu sonho o nosso sonho!

Ao meu "pequeno grande homem", Gabriel, que desde muito pequeno me acompanhou na vida acadêmica, sempre tão compreensivo, comportado e amoroso. Você me inspira a querer ser mais que fui até hoje!

Agradeço a todos que passaram pelo meu caminho e que com certeza deixaram um pouco de si. Os momentos de alegria serviram para me permitir acreditar na beleza da vida, e os de desafios, serviram para um crescimento pessoal único. É muito difícil transformar sentimentos em palavras, mas serei eternamente grata a vocês, pessoas imprescindíveis para a realização e conclusão deste trabalho.

MUITO OBRIGADA!

APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de dois artigos científicos redigidos de acordo com normas de publicação dos periódicos Food Science and Technology International (ISSN: 1082-0132) e Journal of Food Science and Technology (Eletronic ISSN 0975-8402 / Print ISSN 0022-1155).

Artigo 1:
Andreia Farias Pereira Batista, Daliah Alves Coelho Trevisan; Adriele Rodrigues dos Santos; Alex Fiori Silva; Paula Aline Zanetti Campanerut-Sá; Benício Alves de Abreu Filho; Miguel Machinski Junior; Jane Martha Graton Mikcha.
Artigo: Synergistic inhibition of *Salmonella* Typhimurium and *Staphylococcus aureus* in apple jam by cinnamaldehyde and potassium sorbate.
Revista: Food Science and Technology International.

Artigo 2:
Andreia Farias Pereira Batista; Luana Carolina Martins Rosa; Jessica Santos Pizzo; Alex Fiori da Silva; Jesuí Vergílio Visentainer; Benício Alves de Abreu Filho; Renata Katsuko Takayama Kobayashi; Gerson Nakazato; Jane Martha Graton Mikcha.
Artigo: Biogenic silver nanoparticles and cinnamaldehyde as an effective sanitizer for fresh sweet grape tomatoes.
Revista: Journal of Food Science and Technology.

GENERAL ABSTRACT

INTRODUCTION. Microbial contamination is a worldwide problem that causes enormous losses to both the food industry and public health. Foodborne illness has been perceived as a serious problem and foodborne pathogens commonly involved in foodborne outbreaks include *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* sp. Although there are methods to control microbial growth in foods, there remains a need for novel techniques that prove to be effective for microbial inactivation and also contribute to the maintenance of sensorial characteristics of foods. For years the food industry has used synthetic preservatives to control the growth of pathogens, however, the production of preservative-free food has been the target of large food industries due to the growing change in the population's eating style. The use of generally recognized as safe (GRAS) natural compounds is a promising alternative to maintaining food safety and is also perceived by consumers as a natural method of food preservation method. However, the concentrations of natural agents required to inhibit bacterial growth in foodstuffs may modify the sensorial properties or exceed the acceptable flavor limit of food products. The combined use of natural agents with synthetic antimicrobial agents to improve their antibacterial efficacy and to reduce their concentration level when applied to food. Green nanotechnology has also received considerable attention in the scientific community due to its eco-friendly and low-cost nature. In the food sector, silver nanoparticles have been applied to food processing, packaging, and sanitation. However, some works have reported microbial resistance to silver and toxicity when applied directly to food. Natural compounds can be incorporated into nanoparticles, making it possible to assess the effect of several substances simultaneously, and the combined use of nanoparticles and antimicrobials provides more potent antimicrobial activity than that of a single compound.

AIM. This work aimed to assess the antibacterial activity of cinnamaldehyde (CIN) and potassium sorbate (P.S.) alone and in combination against *Salmonella* Typhimurium and *Staphylococcus aureus in vitro* and in apple jam. The antibacterial activity and association of CIN with biogenic silver nanoparticles (BioAgNP) were also investigated against *Escherichia coli*, *S.* Typhimurium and *S. aureus* and their effects as a sanitizer for fresh sweet grape tomato.

MATERIALS AND METHODS. The compounds used in this work were CIN, P.S. and BioAgNP and their antibacterial activities were tested against *Escherichia coli* ATCC 25922, *Salmonella enterica* serotype Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 25923. The antibacterial activity of these compounds was determined by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), following the recommendations of the Clinical and Laboratory Standards Institute. The effects of the combination of CIN+P.S. and CIN+BioAgNP were determined by the Checkerboard method and their interactions were analyzed by Combenefit software. Time-kill assays were performed to evaluate the antibacterial activity of the compounds alone and in combination against the tested bacteria. In the first paper, the application of CIN and P.S. alone and in combination was performed in experimentally inoculated apple jam with a pool of *S.* Typhimurium and *S. aureus* at a final concentration of 10^6 CFU/mL. Samples of apple jam without artificial inoculation were submitted to microbiological analysis for *Salmonella* spp., Enterobacteriaceae, molds and yeasts according to Brazilian legislation on microbiological food standards. For the analyses, four sample groups of apple jam were prepared: control samples (without antimicrobials), samples with CIN at 78 μg/mL, samples with P.S. at 78 μg/mL and samples with CIN+P.S. at 78 μg/mL+78 μg/mL. The effect of CIN and P.S. alone and in combination on the survival of bacterial strains in apple jam was evaluated by counting viable cells on days 0, 1, 2, 3, 4, 5, 8, and 10. To this end, 10 g of the apple jam in 90 mL of sterile peptone water (1 g/L). Serial dilutions were performed and plated on Hektoen agar plates or Baird–Parker agar. All plates were incubated at 35 °C for 24 or 48 h before counting. The physicochemical analysis (pH, soluble solids, titratable acidity, reducing sugars and non-reducing sugars) of the apple jam samples were analyzed on days 0, 5 and 10 of shelf life. Sensory analysis was performed by 124 untrained panelists to evaluate the acceptability of the apple jam samples. In the second paper, *E. coli* was

chosen for the evaluation of the antibacterial effect of CIN and BioAgNP alone and in combination as a sanitizer in fresh sweet grape tomatoes. Before evaluating the sanitizing action of the compounds on artificially inoculated tomatoes, the samples were submitted to microbiological analysis (*Salmonella* sp. and *E. coli*), following the standards required by Brazilian legislation. To evaluate the antibacterial effect of CIN and BioAgNP alone and in combination, the tomatoes were submerged in *E. coli* suspensions standardized at 10^8 CFU/mL in sterile 0.1% peptone water supplemented with 0.1% agar for 30 minutes. Afterwards, the samples were air dried for 2 hours to facilitate bacterial adhesion before exposure to disinfection treatments. To assess the antibacterial activity of CIN and BioAgNP alone and in combination, four samples were defined: a control sample (without antimicrobials), a CIN sample (156 μg/mL), a BioAgNP sample (31.25 μM), and a CIN+BioAgNP sample (156 μg/mL+31.25 μM). The samples were treated for 0, 5, 10, 15, 30, and 60 min. After treatments, inoculated bacteria were counted by diluting 10 g of tomato in 90 mL of sterile peptone water (1 g/L). Serial dilutions were performed, plated on Eosin Methylene Blue Agar plates, and incubated at 35 °C for 24 h before counting. The survival of *E. coli* in tomatoes treated with CIN and BioAgNP alone and in combination during the shelf life was also evaluated. The samples were treated for 5 min, air dried, and packaged in sealed bags for 7 days. The samples were stored at room temperature and analyzed on days 0, 1, 3, 5 and 7. Physicochemical analysis regarding pH, titratable acidity, and soluble solids of the tomato samples were also analyzed at 0, 4 and 7 days of shelf life.

RESULTS. The MIC and MBC of CIN were 312-624 μg/mL for the bacteria evaluated and P.S. showed MIC of 2,500 μg/mL for *S.* Typhimurium and 5,000 μg/mL for *S. aureus*. The MBC of P.S. was 10,000 μg/mL for *S.* Typhimurium and 20,000 μg/mL for *S. aureus*. BioAgNPs also exhibited activity against *E. coli*, *S.* Typhimurium and *S. aureus* showing MIC of 125 μM for all bacteria investigated. The combination of CIN+P.S. and CIN+BioAgNP exhibited a synergistic effect for the bacteria evaluated. The association between CIN+P.S. showed an FIC index of 0.25 for *S.* Typhimurium and 0.37 for *S. aureus.* An FIC value of 0.49 was found for CIN+BioAgNP against *E. coli*, *S.* Typhimurium and *S. aureus*. The synergic effect was validated by the results of Bliss independence surface analysis showing a predominance of blue areas, confirming the synergism. In the Time-kill curves, the concentrations used were determined according to the results obtained from the FIC index. *Salmonella* Typhimurium, *S. aureus* and *E. coli* were able to grow at sub-inhibitory concentrations during all intervals evaluated when the CIN was evaluated alone. On the other hand, P.S. alone was able to completely inhibited *S.* Typhimurium and *S. aureus* after 24 and 72 h, respectively. The association of CIN+P.S. eradicated *S.* Typhimurium and *S. aureus* counts, and no recovery of viable cells was noted after 12 h and 24 h of incubation, respectively. BioAgNPs alone completely inactivated the growth of *E. coli* after 1 h of incubation. On the other hand, *S.* Typhimurium showed a reduction of approximately 2.5-3 log CFU/mL after 12 h and *S. aureus* counts were reduced by ~2 log CFU/mL after 24 h of incubation; however, for *S. aureus* it was possible to observe partial cellular recovery with 48 h of incubation. CIN+BioAgNP in combination inactivated *E. coli*, *S.* Typhimurium and *S. aureus* after 45 min, 6 h and 48 h of incubation, respectively. In the first paper, the effects of CIN and P.S. alone and in combination against *S.* Typhimurium and *S. aureus* experimentally inoculated in apple jam were evaluated. The results showed that *S.* Typhimurium and *S. aureus* populations reached 5.96 and 5.68 log CFU/g, respectively, on the 10th day. CIN alone slight reduced ($p < 0.05$) the populations of *S.* Typhimurium and *S. aureus*, showing a slight reduction in counts. On the other hand, *S.* Typhimurium and *S. aureus* counts were gradually decreased to undetectable levels on the fourth day when P.S. was evaluated alone. The application of CIN+P.S. significantly reduced *S.* Typhimurium and *S. aureus* on the first day of storage when compared to the control group. Total inhibition of bacterial growth was observed on the second day of storage. Regarding the physicochemical analysis of the apple jam, no significant difference in pH values, soluble solids and titratable acidity was found in the treated groups compared to the control one for all days of storage. Reducing sugars showed a significant difference in the CIN and CIN+P.S. groups on the first day of storage. In the CIN group, a significant difference was also observed on the fifth day of shelf life. Non-reducing sugars showed a significant difference in the P.S. sample on the first day and in CIN samples on the fifth day of storage when compared to the control group. In the

sensory analysis of the apple jam it was observed that the addition of CIN and P.S. in combination did not affect the panelists' sensory opinion for the parameters evaluated. When compared to the control group, only the samples containing CIN+P.S. showed no significant difference for the parameters evaluated (color, aroma, flavor, texture and overall acceptance). The apple jam containing CIN alone presented a significant difference in the parameters aroma, flavor and global acceptance and the samples added with P.S. alone presented a significant difference in the parameters aroma, flavor, texture and global acceptance. In the second paper, the sanitizing effect of CIN and BioAgNP alone and in combination against *E. coli* experimentally inoculated in fresh sweet grape tomatoes was evaluated. The microbiological quality of sweet grape tomatoes was analyzed and it was in accordance with the current standards established by Brazilian legislation. Regarding the sanitization of tomatoes, it was possible to observe that in the control groups, *E. coli* counts ranged from 4.15 to 5.37 log CFU/g. CIN alone reduced the bacterial load by only ~1 log CFU/g after 60 min of treatment compared to the control at time zero and ~1.5 CFU/g compared to the control after 60 min. No viable cells were observed after 15 min in the treatment with BioAgNP alone and after 5 min for the mixture. In the shelf life evaluation of the tomatoes, in the control group, *E. coli* reached ~6.12 log CFU/g on seventh day. *E. coli* counts treated with CIN were reduced less than 1 log CFU/g after 7 days of storage. The samples sanitized with BioAgNP alone and in association with CIN showed no growth of *E. coli* during the shelf life. In the physicochemical analysis of tomatoes sanitized with CIN, BioAgNP and CIN+BioAgNP, no significant difference in pH, soluble solids, and titratable acidity was found between the control and treatment groups on the analyzed days.

CONCLUSION. The combination of CIN+P.S. and CIN+BioAgNP showed synergistic effect for the bacteria evaluated. The combination between CIN and P.S. at sub-inhibitory concentrations were effective to inhibit the growth of *S.* Typhimurium and *S. aureus* in apple jam during shelf life. Sensory evaluation suggested that addition of the mixture of CIN and P.S. to apple jam is acceptable to the consumers and there were no changes in physicochemical properties. The use of a sanitizer based on CIN and BioAgNP combined inhibited the growth of *E. coli* in fresh sweet grape tomatoes after 5 min of treatment. The antibacterial activity of the compounds in combination in sweet grape tomatoes was maintained during their shelf life. The combination of these compounds did not change the physicochemical properties of sweet grape tomatoes. The antibacterial activity of compounds combined could pave the way for a new generation of products to reach a balance between the demand for microbial safety and sensorial acceptability.

Keywords: Antibacterial activity; Foodborne pathogens; Foods; Natural compounds; Synergistic effect.

RESUMO GERAL

INTRODUÇÃO. A contaminação microbiana é um problema mundial que causa enormes prejuízos, tanto para a indústria alimentícia quanto para a saúde pública. As doenças transmitidas por alimentos têm sido percebidas como um grave problema e os patógenos de origem alimentar comumente envolvidos em surtos alimentares incluem *Escherichia coli*, *Staphylococcus aureus* e *Salmonella* sp.. Embora existam métodos para controlar o crescimento microbiano em alimentos, ainda há necessidade de novas técnicas que se mostrem eficazes na inativação microbiana e na manutenção das características organolépticas dos alimentos. Por anos, a indústria de alimentos tem usado de conservantes sintéticos com o intuito de controlar o crescimento de patógenos, entretanto, a produção de alimentos sem conservantes tem sido alvo de grandes indústrias alimentícias devido à crescente mudança no estilo alimentar da população. O uso de compostos naturais geralmente reconhecidos como seguros (GRAS - Generally Recognized As Safe) é uma alternativa promissora para manter a segurança dos alimentos e também é percebido pelo consumidor como um método natural de preservação de alimentos. No entanto, as concentrações de agentes naturais necessárias para inibir o crescimento bacteriano em alimentos podem modificar as propriedades sensoriais ou exceder o limite de sabor aceitável de produtos alimentícios. Nesse sentido, o uso combinado de agentes naturais com agentes antimicrobianos sintéticos pode ser uma alternativa eficaz para melhorar sua ação antibacteriana e reduzir suas concentrações quando aplicados aos alimentos. A nanotecnologia verde também recebeu atenção considerável na comunidade científica devido à sua natureza ecológica e de baixo custo. No setor de alimentos, as nanopartículas de prata são aplicadas ao processamento, embalagem e sanitização. No entanto, alguns trabalhos relataram resistência microbiana à prata e toxicidade quando aplicada como conservante em alimentos. Os compostos associados às nanopartículas possibilitam avaliar o efeito de várias substâncias simultaneamente e o uso combinado de nanopartículas e antimicrobianos fornece atividade antimicrobiana mais potente do que a de um único composto.

OBJETIVO. O objetivo deste trabalho foi avaliar a atividade antibacteriana de cinamaldeído (CIN) e sorbato de potássio (S.P.) sozinhos e em combinação contra *Salmonella* Typhimurium e *Staphylococcus aureus in vitro* e em geleia de maçã. Também foi investigada a atividade antibacteriana e associação de CIN com nanopartículas biogênicas de prata (BioAgNP) contra *Escherichia coli*, *S.* Typhimurium e *S. aureus* e seus efeitos como sanitizante em tomates *sweet grape*.

MATERIAIS E MÉTODOS. Os compostos utilizados neste trabalho foram CIN, S.P. e BioAgNP e suas atividades antibacterianas foram testadas contra *Escherichia coli* ATCC 25922, *Salmonella enterica* serotipo Typhimurium ATCC 14028 e *Staphylococcus aureus* ATCC 25923. A atividade antibacteriana destes compostos foi determinada pela concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM), seguindo as recomendações do *Clinical and Laboratory Standards Institute*. Os efeitos da combinação de CIN+S.P. e CIN+BioAgNP foram determinadas pelo método de *Checkerboard* e suas interações foram analisadas pelo software Combenefit. O ensaio de curva de morte foi utilizado para avaliar a atividade antibacteriana dos compostos sozinhos e em combinação contra as bactérias testadas. No primeiro artigo foi realizada a aplicação de CIN e S.P. sozinhos e em combinação em geleia de maçã experimentalmente contaminadas com um *pool* de *S.* Typhimurium e *S. aureus* com uma concentração final de 10^6 UFC/mL. Amostras de geleia de maçã sem a contaminação artificial foram submetidas à análise microbiológica para *Salmonella* sp., *Enterobacteriaceae*, bolores e leveduras seguindo as exigências da legislação brasileira. Para as análises, foram preparados quatro grupos de amostra de geleia de maçã: amostras controle (sem antimicrobianos), amostras contendo CIN a 78 μg/mL, S.P. a 78 μg/mL e CIN+S.P. a 78 μg/mL+78 μg/mL. O efeito de CIN e S.P. sozinhos e em combinação na sobrevivência das cepas bacterianas em geleia de maçã foi avaliada contando-se células viáveis nos dias 0, 1, 2, 3, 4, 5, 8 e 10. As bactérias inoculadas foram contadas diluindo 10 g da geleia de maçã em 90 mL de água peptonada estéril (1 g/L). Diluições seriadas foram realizadas e semeadas em placas de ágar Hektoen ou ágar Baird-Parker. Todas as

placas foram incubadas a 35 °C por 24 ou 48 horas antes da contagem. As análises físico-químicas (pH, sólidos solúveis, acidez titulável, açúcares redutores e não redutores) das amostras de geleia de maçã foram analisadas nos dias 0, 5 e 10 de vida de prateleira. A análise sensorial foi realizada por 124 provadores não treinados para avaliar a aceitabilidade das amostras de geleia de maçã. No segundo artigo, *E. coli* foi escolhida para avaliação do efeito antibacteriano de CIN e BioAgNP isolados e em combinação como sanitizante em tomates *sweet grape* frescos. Antes de avaliar a ação sanitizante dos compostos nos tomates artificialmente inoculados, as amostras foram submetidas à análise microbiológica (*Salmonella* sp. e *E. coli*), seguindo os padrões exigidos pela legislação brasileira. Para avaliar o efeito antibacteriano de CIN e BioAgNP isoladamente e em combinação, os tomates foram submersos em suspensões de *E. coli* padronizadas a 10^8 UFC/mL em água peptonada 0,1% estéril suplementada com ágar 0,1% por 30 min. Em seguida, as amostras foram secas por 2 h para facilitar a adesão bacteriana antes da exposição aos tratamentos de desinfecção. A atividade antibacteriana de CIN e BioAgNP sozinhos e em combinação foi avaliada a partir de quatro amostras: Controle (sem antimicrobianos), CIN (156 μg/mL), BioAgNP (31,25 μM) e CIN+BioAgNP (156 μg/mL+31,25 μM). As amostras foram tratadas por 0, 5, 10, 15, 30 e 60 min e após os tratamentos, as bactérias inoculadas foram contadas diluindo 10 g de tomate em 90 mL de água peptonada estéril (1 g/L). Diluições seriadas foram semeadas em placas de ágar Eosina Azul de Metileno e incubadas a 35 °C por 24 h antes da contagem. A sobrevivência de *E. coli* em tomates tratados com CIN e BioAgNP sozinhos e em combinação durante o prazo de validade também foi avaliada. Nesta análise as amostras foram tratadas por 5 minutos, secas e embaladas em sacos lacrados por 7 dias. Então, as amostras foram armazenadas em temperatura ambiente e analisadas nos dias 0, 1, 3, 5 e 7. A análise físico-química referente ao pH, acidez titulável e sólidos solúveis das amostras de tomate também foram analisadas nos intervalos de 0, 4 e 7 dias de vida de prateleira.

RESULTADOS. A CIM e CBM de CIN variou entre 312-624 μg/mL para as bactérias avaliadas e S.P. apresentou CIM de 2.500 μg/mL contra *S.* Typhimurium e 5.000 μg/mL contra *S. aureus.* Já a CBM de S.P. foi 10.000 μg/mL contra *S.* Typhimurium e 20.000 μg/mL contra *S. aureus*. As BioAgNP também exibiram atividade contra *E. coli*, *S.* Typhimurium e *S. aureus* apresentando CIM de 125 μM contra todas as bactérias investigadas. A combinação de CIN+S.P. e CIN+BioAgNP exibiram um efeito sinérgico para as bactérias avaliadas. A associação entre CIN+S.P. apresentou uma CIF de 0,25 para *S.* Typhimurium e 0,37 para *S. aureus* e a CIF da combinação de CIN+BioAgNP foi de 0,49 contra *E. coli*, *S.* Typhimurium e *S. aureus*. O efeito sinérgico foi validado pela análise da superfície de independência de Bliss demonstrando que as combinações apresentaram predominância de áreas azuis, indicando sinergismo. No ensaio de curva de morte, as concentrações utilizadas foram determinadas de acordo com os resultados obtidos pelo método de *Checkerboard*. *Salmonella* Typhimurium, *S. aureus* e *E. coli* foram capazes de crescer em concentrações subinibitórias durante todos os intervalos avaliados quando o CIN foi avaliado sozinho. Por outro lado, S.P. sozinho foi capaz de inativar completamente a população de *S.* Typhimurium e *S. aureus* após 24 e 72 h, respectivamente, e a associação de CIN+S.P. erradicou *S.* Typhimurium e *S. aureus*, e nenhuma recuperação de células viáveis foi observada após 12 h e 24 h de incubação, respectivamente. As BioAgNP sozinhas inativaram completamente o crescimento de *E. coli* após 1 h de incubação. Enquanto, *S.* Typhimurium apresentou uma redução de aproximadamente 2,5–3 log UFC/mL após 12 h e as contagens de *S. aureus* foram reduzidas em ~2 log UFC/mL após 24 h de incubação; entretanto, para *S. aureus* foi possível observar recuperação celular parcial com 48 h de incubação. Quando associados, CIN+BioAgNP foram capazes de inativar *E. coli*, *S.* Typhimurium e *S. aureus* após 45 min, 6 h e 48 h de incubação, respectivamente. No primeiro artigo foram avaliados os efeitos de CIN e S.P. sozinhos e em combinação contra *S.* Typhimurium e *S. aureus* inoculados experimentalmente em geleia de maçã. Os resultados demonstraram que as populações de *S.* Typhimurium e *S. aureus* atingiram 5,96 e 5,68 log UFC/g, respectivamente, no 10º dia. O CIN sozinho não causou redução significativa nas populações de *S.* Typhimurium e *S. aureus*, apresentando discreta redução nas contagens. Por outro lado, as contagens de *S.* Typhimurium e *S. aureus* diminuíram gradualmente para níveis indetectáveis no quarto dia, quando S.P. foi testado sozinho. A aplicação do CIN+S.P. reduziu significativamente as contagens de *S.* Typhimurium e *S. aureus* no primeiro dia de

armazenamento quando comparado ao grupo controle. A inibição total do crescimento bacteriano foi observada no segundo dia de armazenamento. Quanto à análise físico-química da geleia de maçã não houve diferença significativa nos valores de pH, de sólidos solúveis e nos índices de acidez titulável quando comparados ao grupo controle. Os açúcares redutores apresentaram diferença significativa nos grupos CIN e CIN+S.P. no primeiro dia de armazenamento. No grupo CIN, uma diferença significativa também foi observada no quinto dia de vida útil. Os açúcares não redutores apresentaram diferença significativa na amostra S.P. no primeiro dia e na amostra CIN no quinto dia de armazenamento quando comparados ao grupo controle. Na análise sensorial da geleia de maçã foi observado que a adição de CIN e S.P. em combinação não afetou a opinião sensorial dos provadores para os parâmetros avaliados. Quando comparado ao grupo controle, apenas as amostras contendo CIN+S.P. não apresentou diferença significativa para os parâmetros avaliados (cor, aroma, sabor, textura e aceitação global). A geleia contendo CIN sozinho apresentou diferença significativa para os parâmetros aroma, sabor e aceitação global e as amostras adicionadas de S.P. sozinho apresentou diferença significativa para os parâmetros aroma, sabor, textura e aceitação global. No segundo artigo, foi avaliado o efeito sanitizante de CIN e BioAgNP isolados e em combinação contra *E. coli* experimentalmente inoculada em tomates *sweet grape* frescos. A qualidade microbiológica dos tomates *sweet grape* foram analisadas e os resultados demonstram que as amostras atenderam aos padrões estabelecidos pela legislação brasileira. Em relação a sanitização de tomates, foi possível observar que nos grupos controle, as contagens de *E. coli* variaram de 4,15 a 5,37 log UFC/g. O CIN sozinho reduziu a carga bacteriana em apenas ~1 log UFC/g após 60 min de tratamento comparado com o controle no tempo zero e ~1,5 UFC/g comparado com o controle após 60 min. Nenhuma célula viável foi observada após 15 min no tratamento apenas com a BioAgNP e após 5 min para a mistura. Na avaliação do tempo de prateleira dos tomates, no grupo controle, *E. coli* atingiu ~6,12 log UFC/g no sétimo dia. Nos grupos tratados, as contagens de *E. coli* apresentou uma redução de menos de 1 log UFC/g após 7 dias de armazenamento e as amostras higienizadas apenas com a BioAgNP e com a combinação de CIN+BioAgNP não apresentaram crescimento de *E. coli* durante o armazenamento. Na análise físico-química dos tomates sanitizados com CIN, BioAgNP e CIN+BioAgNP, não foi encontrada diferença significativa no pH, sólidos solúveis e acidez titulável entre os grupos controle e tratamento nos dias analisados.

CONCLUSÃO. A combinação CIN+S.P. e CIN+BioAgNP apresentaram efeito sinérgico para as bactérias avaliadas. A combinação entre CIN e S.P. em concentrações subinibitórias foram eficazes para inibir o crescimento de *S.* Typhimurium e *S. aureus* em geleia de maçã durante o tempo de armazenamento. A avaliação sensorial sugeriu que a adição da mistura de CIN e S.P. à geleia é aceitável pelos consumidores e não houve alterações nas propriedades físico-químicas. Além disso, o uso de um sanitizante à base de CIN e BioAgNP combinados inibiu o crescimento de *E. coli* em tomates *sweet grape* frescos após 5 min de tratamento. A atividade antibacteriana dos compostos em combinação nos tomates foi mantida durante sua vida útil e a combinação desses compostos não alterou as propriedades físico-químicas dos tomates *sweet grape*. A atividade antibacteriana de compostos combinados pode abrir caminho para uma nova geração de produtos para alcançar um equilíbrio entre a demanda por segurança microbiana e aceitabilidade organoléptica.

Palavras Chave: Alimentos; Atividade antibacteriana; Compostos naturais; Efeito sinérgico; Patógenos de origem alimentar.

LISTA DE ILUSTRAÇÕES

ARTIGO 1

ARTIGO 2

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

LISTA DE ABREVIATURAS E SIGLAS

°Bx	degrees Brix
ºC	degrees Celsius
µg/mL	microgram per milliliter
µL	microliter
µM	micromolar
ΣFIC	Fractional Inhibitory Concentration Index
AgNPs	silver nanoparticles
AOAC	Association of Oficial Analytical Chemistral
ATCC	American Type Culture Collection
bacteria/mL	bacteria per milliliter
BHI	Brain Heart Infusion
BioAgNP	biogenic silver nanoparticles
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CDC	Centers for Disease Control and Prevention
CFSAN	Center for Food Safety and Applied Nutrition
CFU	colony-forming units
CFU/mL	colony-forming units per milliliter
CFU/g	colony-forming units per grams
CIN	Cinnamaldehyde
CIN+BioAgNP	Cinnamaldehyde plus silver nanoparticles
CIN+P.S	Cinnamaldehyde plus potassium sorbate
CLSI	Clinical and Laboratory Standards Institute
DMSO	dimethyl sulfoxide
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
Fig	figure
g	grams
g/L	grams per liter
GRAS	Generally Recognized As Safe
h	hour
HCl	hydrochloric acid

L	liter
log	logarithm
log CFU/mL	logarithm colony-forming units per milliliter
MBC	Minimum Bactericidal Concentration
mg/mL	miligram per milliliter
MIC	Minimum Inhibitory Concentration
min	minute
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
mL	milliliter
mol/L	mole per liter
N	normal
OEO	oregano essential oil
P.S.	Potassium sorbate
pH	potential of Hydrogen
ppm	parts per million
TSB	Trypic Soy Broth

SUMÁRIO

ARTICLE 1

SYNERGISTIC INHIBITION OF *Salmonella* TYPHIMURIUM AND *Staphylococcus aureus* IN APPLE JAM BY CINNAMALDEHYDE AND POTASSIUM SORBATE

Synergistic inhibition of *Salmonella* Typhimurium and *Staphylococcus aureus* in apple jam by cinnamaldehyde and potassium sorbate

Andreia Farias Pereira Batista[1]*, Daliah Alves Coelho Trevisan[2]; Adriele Rodrigues dos Santos[1]; Alex Fiori Silva[2]; Paula Aline Zanetti Campanerut-Sá[3]; Benício Alves de Abreu Filho[1,4]; Miguel Machinski Junior[1,4]; Jane Martha Graton Mikcha [1, 2, 3]

[1] Center for Agricultural Sciences, Postgraduate Program of Food Science, State University of Maringá, Maringá, PR, Brazil
[2] Center for Health Sciences, Postgraduate Program in Health Sciences, State University of Maringá, Maringá, PR, Brazil
[3] Department of Clinical Analyses and Biomedicine, State University of Maringá, Maringá, PR, Brazil
[4]Department of Basic Health Sciences, State University of Maringá, Maringá, PR, Brazil
* Corresponding author: Andreia Farias Pereira Batista. Postgraduate Program of Food Science, State University of Maringá, Av. Colombo, 5790. CEP: 87020-900, Maringá, PR, Brazil. Phone: +55 44 3011-4959. E-mail address: andreia.farias04@hotmail.com

Abbreviated running headline: Synergistic antimicrobial action of CIN and P.S.

Significance and Impact of Study

The association between natural and synthetic antimicrobials could be applied to food as an alternative to control foodborne pathogens.

ABSTRACT

The objective of this study was to evaluate the antimicrobial effectiveness of cinnamaldehyde (CIN) and potassium sorbate (P.S.), alone and in combination, against *Salmonella* Typhimurium and *Staphylococcus aureus in vitro* and in apple jam. The antimicrobial activity *in vitro* was investigated by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), time-kill assay and determination of fractional inhibitory concentration index. CIN MIC and MBC values was 312 μg/mL. P.S. MIC and MBC were 2500 and 5000 μg/mL, respectively, against *S.* Typhimurium; and 10000 and 20000 μg/mL, respectively, against *S. aureus*. The compounds combined exhibited a synergistic effect (FIC < 0.5), inhibiting *S.* Typhimurium growth after 12 h and *S. aureus* after 24 h. The effect of CIN and P.S., at sub-inhibitory concentrations, against bacterial strains in apple jam was evaluated during storage. Physicochemical and sensory analysis were also performed. No cultivable *S.* Typhimurium or *S. aureus* cells were recovered in apple jam supplemented with CIN+P.S. on the third day of storage. The addition of CIN and P.S. did not affect the physicochemical properties and sensory evaluation showed a score above 7.0. CIN and P.S. association at sub-inhibitory concentrations was effective in controlling of *Salmonella* sp. and *S. aureus*.

Keywords: Antibacterial activity; Foodborne pathogens; Natural antimicrobials; Synergistic effect; Synthetic preservatives.

1. INTRODUCTION

Control of foodborne pathogens is one of the major challenges for food companies as they can cause millions of gastrointestinal illnesses worldwide each year, with more than 420000 deaths (CDC, 2021). *Salmonella* sp. and *Staphylococcus aureus* are among the top five bacteria involved in foodborne illnesses in the United States; *Salmonella enterica* serovar Typhimurium is one of the most common serovars associated with human infection and *S. aureus* is mostly accountable for food poisoning (CDC, 2021).

These pathogens may be present in a wide variety of foods, including vegetables and processed foods, causing outbreaks of foodborne diseases (CDC, 2021). In order to ensure food safety and control pathogen growth, the food industry has used potassium sorbate (P.S.) as a synthetic preservative for a wide variety of food products (Jaiswal and Jaiswal, 2014). In the meantime, the production of preservative-free food has been the target of large food industries due to the growing change in the population's eating style (Rao et al., 2019).

The use of generally recognized as safe (GRAS) natural compounds, such as cinnamaldehyde (CIN), is a promising alternative to maintain food safety and it is also perceived by the consumer as a natural food preservation method (Calo et al., 2015; Burt, 2016). CIN is the main component isolated from cinnamon oil (Ribeiro-Santos et al., 2017). Researchers have already demonstrated its antibacterial activity against a wide range of pathogenic bacteria, including *Salmonella* sp. and *S. aureus* (Piovezan et al., 2014; Shen et al., 2015; Burt et al., 2016; Klangpetch et al., 2018; Silva et al., 2018; Malheiro et al., 2019; Rao et al., 2019). However, the concentrations of natural agents required to inhibit bacterial growth in foodstuffs may modify the organoleptic properties or exceed the acceptable flavor limit of food products (Requena et al., 2019).

Some researchers have suggested the combined use of natural agents with synthetic antimicrobial agents to improve their antibacterial efficacy and to reduce their concentration level when applied to food (Klangpetch et al., 2018; Rao et al., 2019; Chen et al., 2020; González-Fandos et al., 2021; Zhan et al., 2021). Recently, we reported that combined treatment using low concentrations of carvacrol and P.S. shows bactericidal effects against *S.* Typhimurium (Batista et al., 2019).

The purpose of this study was to assess the antibacterial activity of CIN and P.S. alone and in combination against *S.* Typhimurium and *S. aureus in vitro* and in apple jam. These effects *in vitro* were determined by the minimum inhibitory concentration, minimum bactericidal concentration, fractional inhibitory concentration index and time-kill curve. The antibacterial effects of CIN and P.S. alone and combined in apple jam were evaluated. Moreover, the influence of these compounds on the physicochemical and sensory attributes of apple jam during storage was investigated.

2 MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

Salmonella enterica serotype Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 25923 strains were provided by the Laboratory of Food Microbiology, State University of Maringá, Paraná, Brazil. The cultures were maintained in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA), supplemented with 20% glycerol at −20 °C. Before use, an aliquot was transferred to brain heart infusion broth (BHI; Difco, Becton Dickinson, Sparks, MD, USA) and incubated for 24 h at 35 °C. The culture was transferred to appropriate selective media: Hektoen enteric agar (Difco, Becton Dickinson, Sparks, MD, USA) for *S.* Typhimurium and Baird–Parker agar base (Difco, Becton Dickinson, Sparks, Le Pont de Claix, France) for *S. aureus*. Plates were incubated at 35 °C for 24 h or 48 h, respectively.

2.2 Minimum inhibitory concentration and minimum bactericidal concentration

The MIC and MBC of CIN (93% purity, Sigma Aldrich, Buchs, Switzerland) and P.S. (Vetec, Jaraguá do Sul, Brazil) were determined following recommendations by the Clinical and Laboratory Standards Institute (CLSI, 2018), using the broth microdilution method in 96-well microtiter plates (TPP, Trasadingen, Switzerland). For MIC determination, the pH of Mueller–Hinton broth (MHB) (Difco, Becton Dickinson, Sparks, MD, USA) was adjusted to 4.5 with HCl. CIN was initially diluted in 0.5% dimethyl sulfoxide (DMSO) and P.S. was prepared in MHB; 100 μL. The compounds were added to each well containing MHB at concentrations ranging between 5000 and 19 μg/mL for CIN, and between 320 and 0.312 mg/mL for P.S. Bacterial suspensions were standardized to McFarland scale 0.5 and diluted at 1:20; 10 μL was inoculated in each microplate well. After 48 h of incubation at 35 °C, the MIC was defined as the lowest concentration of antimicrobial that inhibited bacterial growth by visual reading. Bacterial growth control in MHB and a control with CIN or P.S. in MHB were included. After MIC determination, 20 μL was removed from the wells in which bacterial growth was not observed and inoculated into Hektoen agar plates or Baird–Parker agar, and incubated at 35 °C for 24 h or 48 h. The MBC was determined as the lowest concentration where no bacterial growth was observed on agar plates. Each test was performed in duplicate. The results correspond to three experiments.

2.3 Determination of synergistic activity

The synergistic effects of the CIN and P.S. combination were determined using the checkerboard method (Doern, 2014). P.S. was added to 96-well microtiter plates with MHB at pH 4.5 and diluted on the x-axis. CIN was added and diluted on the y-axis in the same way. Bacterial suspensions with 6×10^5 colony-forming units per milliliter (CFU/mL) were inoculated in each microplate well. The plates were incubated at 35 °C for 48 h. The combination of the

substances was carried out after calculating the fractional inhibitory concentration (FIC) index (ΣFIC) which was determined as follows:

$$\sum FIC = \frac{MIC\ of\ cinnamaldehyde\ in\ combination}{MIC\ of\ cinnamaldehyde\ alone} + \frac{MIC\ of\ P.S.\ in\ combination}{MIC\ of\ P.S.\ alone}$$

The interaction between CIN and P.S. was defined as synergistic (FIC < 0.5), additive (0.5 ≤ FIC ≤ 1), indifferent (1 < FIC ≤ 4) or antagonistic (FIC > 4) (Pillai et al., 2005). The Bliss-independent interactions were analyzed by Combenefit software (Di Veroli et al., 2016). All the experiments were repeated thrice.

2.4 Time-kill assay

Time-kill assays were performed according to Isenberg (2004), with modifications. Overnight cultures of *S.* Typhimurium ATCC 14028 and *S. aureus* ATCC 25923 were standardized using McFarland scale 1, transferred to MHB at pH 4.5 and supplemented with CIN and P.S. alone and in combination, obtaining a final inoculum of 6×10^5 CFU/mL. CIN was tested at 78 μg/mL (1/4 MIC) and P.S. at 78 μg/mL (1/32 MIC), according to the checkerboard results. Aliquots of 100 μL were withdrawn at intervals of 0, 3, 6, 12, 24 and 48 h, serially diluted and plated on Mueller–Hinton agar (MHA) (Difco, Becton Dickinson, Sparks, MD, USA). Plates were incubated at 35 °C for 24 h and CFUs were counted. Each test was performed in duplicate and repeated three times.

2.5 Preparation of apple jam

Apple jam was prepared by hand following the methodology proposed by Krolow et al. (2005), with modifications, and considering the Brazilian legislation on Good Manufacturing Practices (Brasil, 2004). Apples (*Malus domestica*) were purchased in a local market and were selected during their mature phase and without any mechanical blemish. After selection, the apples were washed in running water, immersed in cold water with 200 ppm chloride for 10 min and washed. The sanitized apples were pulped and ground mechanically. Sugar was added to the pulp and the mixture processed by evaporation to achieve the jam concentration. Aliquots of 40 mL of apple jam were packed in sterile glass jars and pasteurized.

Samples of apple jam were submitted to microbiological analysis for *Salmonella* sp., *Enterobacteriaceae*, molds and yeasts according to Brazilian legislation on microbiological food standards (Brasil, 2019).

Four sample groups of apple jam were prepared (glass jars with 40 mL each): control samples (apple jam without antimicrobials); samples with CIN at 78 μg/mL (1/4 MIC) (cinnamaldehyde); samples with P.S. at 78 μg/mL (1/32 MIC) (potassium sorbate); samples with CIN+P.S. (1/4 MIC

+ 1/32 MIC) (cinnamaldehyde + potassium sorbate). Compound concentrations were selected by the checkerboard method. Glass jars were stored at room temperature for 10 days.

2.6 Effect of cinnamaldehyde and potassium sorbate on survival of *Salmonella* Typhimurium and *Staphylococcus aureus* in apple jam

The effect of CIN and P.S. alone and in combination on survival of the bacterial strains in apple jam was evaluated using the viable cell count procedure. Bacterial suspension was prepared with McFarland scale 1. An inoculum cocktail was prepared by mixing equal amounts of each *S.* Typhimurium ATCC 14028 strain and *S. aureus* ATCC 25923 at a final concentration of 10^6 CFU/mL. Control and treatment samples were inoculated with 400 µL of each standardized inoculum, stored at room temperature and analyzed on days 0, 1, 2, 3, 4, 5, 8 and 10 of shelf life. Inoculated bacteria were counted by diluting 10 g of the apple jam in 90 mL of sterile peptone water (1 g/L). Serial dilutions were performed and plated on Hektoen agar plates or Baird–Parker agar. All plates were incubated at 35 °C for 24 h or 48 h before counting. The analyses were repeated twice and the results are expressed in log CFU per milliliter (CFU/mL).

2.7 Physicochemical analysis

Apple jam samples were analyzed on days 0, 5 and 10 of shelf life with regard to pH, soluble solids, titratable acidity, reducing sugars and non-reducing sugars following AOAC methodology (1992). Analyses were done in triplicate, with two replicates. Hydrogen ionic potential (pH) was determined in apple jam by digital potentiometer. The soluble solids were determined using a refractometer and the results are given as degrees Brix (°Bx). Total titratable acidity was determined in three samples from the titration of 10 g of homogenate pulp diluted in 100 mL of distilled water and a standard solution of 0.1 N sodium hydroxide. Reducing and non-reducing sugars were determined using the Lane–Eynon method, which is based on the reduction power of the simplest glycides. In this method, the copper II of the Fehling reagents is reduced to cuprous oxide by the reducing sugars present in the sample. All analyses were done in triplicate with two replicates.

2.8 Sensory evaluation

Sensory analysis was carried out in a standard and authorized sensory laboratory, provided with three testing booths, under normal lighting conditions, according to the international standard ISO 8589 (2015). The panel consisted of 124 untrained assessors comprising students and employees of the State University of Maringá, Maringá, PR, Brazil. Only four samples were tested during the same session. All apple jam samples were presented to panelists at the same time at room temperature and in randomly three-digit-coded cups.

Four samples: control, CIN, P.S. and CIN+P.S. were evaluated by applying a 9-point hedonic scale (1 = highly disliked to 9 = highly liked). The following attributes were evaluated: color, texture, taste, aroma and overall acceptability. Mean scores of all sensory evaluations were used in the analysis.

2.9 Statistical analysis

The results were analyzed with GraphPad Prism 7.0 Software. *In vitro* analyses were performed in duplicate, with three replications, and apple jam analysis was repeated twice. Results were expressed as the mean and standard deviation. The data were analyzed by ANOVA at 5% significance level. Post-hoc comparisons were performed by Tukey's test.

3. RESULTS AND DISCUSSION

3.1 Minimum inhibitory concentration and minimum bactericidal concentration

The *in vitro* antibacterial activity of CIN and P.S. against *S.* Typhimurium and *S. aureus* was determined at pH 4.5; CIN presented a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 312 μg/mL for both bacteria. Other studies have evaluated the antibacterial activity of CIN against *S.* Typhimurium or *S. aureus* at neutral pH (Ye et al., 2013; Burt et al., 2016; Shi et al., 2017; Klangpetch et al., 2018; Silva et al., 2018). Burt et al. (2016) obtained MIC of 156 μg/mL for *S.* Typhimurium while Ye et al. (2013) and Shi et al., (2017) reported CIN MIC ranging from 250 to 500 μg/mL against *S. aureus*, which is consistent with the values obtained in the present study. Klangpetch et al. (2018) reported higher a CIN MIC values: 6.25 mg/mL against *S.* Typhimurium and 0.78 mg/mL for *S. aureus*. To our knowledge, no articles were found that tested CIN at acidic pH.

The mechanisms of action of natural compounds are not completely understood. However, some studies have attempted to explain how inhibition of microbial growth occurs. The main mechanism for the antimicrobial activity of CIN has been attributed to disruption of the cytoplasmic membrane and inhibition of active transport across it (Zhang et al., 2016; Vasconcelos et al., 2018; Khameneh et al., 2019).

Some authors have reported that P.S. is able to inhibit a wide spectrum of foodborne pathogens (Sofos and Busta, 1986; Santiesteban-López et al., 2007; Zhang et al., 2019; Zhu et al., 2019; González-Fandos et al., 2021). In this study, the MIC of P.S. was 2,500 μg/mL for *S.* Typhimurium whereas for *S. aureus* the MIC was 5,000 μg/mL. Santiesteban-López et al. (2007) determined only the MIC and showed a P.S. MIC of 600 and 500 μg/mL at pH 5.5 and 4.5, respectively, against *S.* Typhimurium. MIC of 800 and 600 μg/mL, at the same pH, were obtained against *S. aureus*. In another study, P.S. at pH 4 showed MIC of 800 μg/mL against *S.* Typhimurium; and 1.6 and 6.25 mg/mL against *S. aureus* (Zhang et al., 2019). The MBC of P.S.,

in our study, was 10,000 μg/mL for *S.* Typhimurium and 20,000 μg/mL for *S. aureus*. Zhang et al. (2019) also found MBC until 5-fold greater than MIC. According Sofos and Busta (1981) P.S. prevents or delays microbial growth, and its bactericidal or bacteriostatic effect is dependent of factors such as concentration and pH of the medium.

Our results demonstrated that the P.S. MIC at acidic pH is six times lower when compared to that at neutral pH for *S. aureus* (data not shown). According to van Beilen et al. (2014), sorbic acid is most effective at low pH. Moreover, some papers have demonstrated the influence of sorbate on cell walls and membranes, which may alter their integrity and permeability (Sofos and Busta, 1981; Hwang and Huang, 2013; van Beilen et al., 2014). Other actions can be explained by considering the pKa (4.76) of sorbic acid. In this case, a larger amount of sorbic acid enters the cell and dissociates in the cytoplasm when the extracellular pH is lower than the intracellular pH. That action increases the cytoplasmic hydrogen ion concentration, which acidifies the cytoplasm, inhibits metabolic processes and diffuses the proton gradient across the cytoplasmic membrane (Sofos and Busta, 1981; van Beilen et al., 2014).

3.2 Synergistic activity of cinnamaldehyde and potassium sorbate

The interaction of CIN and P.S. against *S.* Typhimurium and *S. aureus* was determined by calculating the fractional inhibitory concentration (FIC) index. The compounds in combination exhibited a synergistic effect for both bacteria: an FIC index of 0.25 for *S.* Typhimurium and 0.37 for *S. aureus* was found. The combination reduced the MIC of CIN from 312 μg/mL to 78 μg/mL (1/4 MIC) for both bacteria, while the MIC of P.S. was reduced from 2500 μg/mL to 78 μg/mL (1/32 MIC) against *S.* Typhimurium and from 5000 μg/mL to 78 μg/mL (1/64 MIC) against *S. aureus*. Santiesteban-López et al. (2007) also reported additive and synergistic interactions when combining P.S. with thymol, with carvacrol or with eugenol against *S.* Typhimurium and *S. aureus*. An additive interaction of carvacrol and P.S. against *S.* Typhimurium was previously observed by our research team (Batista et al., 2019). Recently, Zhan et al. (2021) observed a synergistic interaction for P.S. + nisin against *Staphylococcus epidermidis*. Additive interactions between CIN and cinnamic acid, lactic acid and propionic acid against *S.* Typhimurium were reported by Burt et al. (2016). Klangpetch et al. (2018) observed synergism between CIN and nisin when tested against *S.* Typhimurium and *S. aureus*; however, there is limited information regarding the antimicrobial effects of CIN and P.S. in combination against foodborne bacteria. Our results demonstrated that mixtures of these compounds are able to inhibit the growth of *S.* Typhimurium and *S. aureus*, at lower concentrations than those needed when they are individually used.

The synergic effect revealed by FIC values was validated by the result of the Bliss independence surface analysis (Fig 1). Combenefit software enables systematic quantification of high-throughput screens, incorporating metrics to describe synergy distributions and advanced

graphical visualization of the combination between CIN and P.S. *in vitro* against *S.* Typhimurium and *S. aureus*. In this way, CIN and P.S. combined showed a predominance of blue areas, indicating synergism.

3.3 Time-kill assay

The antibacterial activity of CIN and P.S., alone and in association, was also determined by time-kill assay (Fig 2). The concentrations used were determined according to the results obtained from the FIC index. The control group (without antimicrobials) reached a bacterial population of 6 log CFU/mL after 48 and 72 h of incubation at 35 °C, for *S.* Typhimurium and *S. aureus*, respectively.

Salmonella Typhimurium and *S. aureus* were able to grow at sub-inhibitory concentrations (78 μL/mL) during all the intervals evaluated when CIN was applied alone. On the other hand, P.S. tested alone at sub-inhibitory concentrations (78 μL/mL) inhibited *S.* Typhimurium and *S. aureus* after 24 and 72 h, respectively. The association of CIN (78 μg/mL) and P.S. (78 μg/mL) gradually reduced *S.* Typhimurium and *S. aureus* counts, and no recovery of viable cells was noted after 12 h and 24 h of incubation, respectively.

A previous study by our research group reported an additive effect of carvacrol and P.S., and more rapid killing of *S.* Typhimurium was observed by carvacrol and P.S. in combination, when compared to those agents alone at the same concentrations (Batista et al., 2019). In the current study, time-kill assay results are in accordance with those observed for the checkerboard assay, in which CIN+P.S. inhibited *S.* Typhimurium and *S. aureus* at lower concentrations than those needed when antimicrobials were used alone. There are limited studies dealing with the mechanisms of action of the combination of compounds. However, there are some generally accepted mechanisms of antimicrobial interaction that produce synergism. They include the sequential inhibition of a common biochemical pathway, inhibition of protective enzymes and the use of cell membrane active agents to enhance the uptake of other antimicrobials (Bassolé and Juliani, 2012; Yuan et al., 2019).

3.4 Antimicrobial effect of cinnamaldehyde and potassium sorbate against bacteria in experimentally inoculated apple jam

The results of the antibacterial effects of CIN and P.S. alone and in combination against foodborne bacteria in experimentally inoculated apple jam are shown in Table 1. *S.* Typhimurium and *S. aureus* populations reached 5.96 and 5.68 log CFU/g, respectively, on the 10th day. CIN alone did not cause a significant reduction ($p < 0.05$) in the populations of *S.* Typhimurium and *S. aureus*, showing a slight reduction in the counts. On the other hand, *S.* Typhimurium and *S. aureus* counts were gradually decreased to undetectable levels on the fourth day when P.S. was used ($p < 0.05$). The application of CIN+P.S. significantly reduced *S.* Typhimurium (by 3 log

CFU/g) and *S. aureus* counts (by 1.6 log CFU/g) on the first day of storage when compared to the control group ($p < 0.05$). Total inhibition of bacterial growth was observed on the second day of storage.

Brazilian legislation allows the use of 1000 μg/mL of P.S. alone and in combination with other additives in fruit jam (Brasil, 2009). In our work, P.S. at 78 μg/mL when combined with CIN, that is 92.5% less than allowed, completely inhibited the bacterial group.

Klangpetch et al. (2018) tested CIN (1.56 mg/mL) combined with nisin (1.25 IU/mL) against *S.* Typhimurium and *S. aureus* in sandwich spread (pH 4.5) and obtained a reduction of 4 log CFU/g from the 6th to 14th days of storage. Previous studies have demonstrated the antimicrobial efficacy of CIN alone in food matrices (Yuste and Fung, 2002) and food packaging (Raybaudi-Massilia et al., 2008); however, to our knowledge, there are no studies reporting the antimicrobial action of the association of CIN and P.S. applied to food.

The use of natural antimicrobials in foods as preservatives is often limited due to the strong smell and taste they impart to these foods (Gyawali and Ibrahim, 2014). In view of these limitations, the use of CIN and P.S. combined at lower concentrations becomes an interesting option for the control of *S.* Typhimurium and *S. aureus* in apple jam.

Is noteworthy that the microbiological quality of apple jam was also evaluated and the samples complied with the current standards established by Brazilian legislation (Brasil, 2019): *Enterobacteriaceae* < 1 log CFU/g, yeasts and molds < 2 log CFU/g and the absence of *Salmonella* sp./25 g.

3.5 Physicochemical analysis

Apple jam samples were prepared only with apples and sugar; after addition of CIN, P.S. or CIN+P.S. they were submitted to physicochemical analysis of pH, soluble solids, titratable acidity, reducing sugars and non-reducing sugars during shelf life as shown in Table 2. No significant difference (p <0.05) in pH values was found in the treated groups compared to the control one for all days of storage (from 4.78 to 5.06). Slight differences were found by Moreira et al. (2019), who reported pH values of 3.9 and 4.04 in mixed jams of rose petals and apple, respectively. This can be explained by the addition of citric acid by these authors, which was not done in the current study.

Soluble solids values ranged between 34.43% and 35.73% ($p < 0.05$) over the storage period in all jam groups. The Brazilian law recommends minimum total soluble solids of 62% for jams (Brasil, 1978). In the current study, the apple jam was composed only of fruit pulp and a small amount of sugar (~ 10%), which does not follow the industrial production standards (35 parts of fruit and 65 parts of sugar) (Brasil, 1978). Perhaps this is the reason why we did not reach the minimum values of soluble solids.

The apple jam presented titratable acidity rates ranging from 0.35% to 0.46% and no significant difference ($p < 0.05$) was observed during the storage period. Similar results were reported by Aguiar et al. (2016), showing titratable acidity rates of 0.36% in apple and honey mixed jam. These values are recommended for maintaining the texture of the jam (Aguiar et al., 2016). According to Torrezan (1998), in the production of jams, the acidity must be controlled and remain between 0.3% and 0.8%.

Reducing sugars showed values ranging between 11.58% and 14.32% and a significant difference ($p < 0.05$) was observed in CIN and CIN+P.S. groups on the first day of storage when compared to the control group. In the CIN-treated group, a significant difference ($p < 0.05$) was also observed on the fifth day of shelf life. Control and CIN groups presented a gradual increase in reducing sugars over the period evaluated. Non-reducing sugars presented a significant difference ($p < 0.05$) in P.S. samples on the first day and in CIN samples on the fifth day of storage. Apple jam samples presented non-reducing sugar levels ranging from 10.33% to 12.01%. These results indicate that the levels of reducing sugars were close to those of non-reducing sugars. These findings agree with the small amount of sugar used in the apple jam production process. Unlike our method, Mendonça et al. (2000) prepared apple jam using different levels of brown sugar (35%, 50% and 65%), and found non-reducing sugar values (35.1%, 36.9% and 41.6%, respectively) higher than those for reducing sugars (21.1%, 22.6% and 18.9%, respectively).

3.6 Sensory evaluation

The results of the sensory analysis of apple jam containing CIN and P.S. alone and in combination are shown in Table 3. Apple jam with CIN alone received the highest scores for color, taste and overall acceptance. The acceptance index of these parameters was 88%, 87% and 86%, respectively. On the other hand, P.S. alone obtained the lowest scores for the same parameters, presenting an acceptance index of 83%, 85% and 84%, respectively. The CIN+P.S. samples presented scores lower than those for CIN alone, showing 83% acceptance for color, 85% for taste and 82% for overall acceptance. These results indicate that, for these attributes, addition of the antimicrobial agents evaluated did not affect the panelists' sensory opinion. There are few studies concerning the addition of CIN to food matrices (Higueras et al., 2015; Fadel et al., 2019). Fadel et al. (2019) showed good sensory scores for biscuits flavored with cinnamon essential oil encapsulated in maltodextrin and propylene glycol. Higueras et al. (2015) reported that milk samples in contact with films containing CIN were preferred to the control without CIN. In this sense, the results obtained in the presented study corroborate the earlier studies in that CIN added to food can promote good acceptance.

In our study, panelists opined on aroma, taste and overall acceptance and attributed "I liked moderately" to P.S. samples. Apple jam with CIN and CIN+P.S. was acceptable for all sensory

attributes including aroma, after taste and overall acceptability. The panelists attributed “I liked very much” to these attributes. The main challenge for using essential oils or their compounds as food preservatives is that they may cause negative organoleptic effects when added in sufficient amounts to provide an antimicrobial effect (Jouki et al., 2014). Therefore, combinations of substances may reduce the amount of antimicrobial preservatives needed to lower potential impacts on sensory quality. In this study, apple jam was well accepted by panelists, confirming the theory that the use of CIN and P.S. at lower concentrations does not interfere with the organoleptic properties of the jam so that good acceptance of the product is obtained.

In conclusion, the results presented in this study show a synergistic effect between CIN and P.S. based on FIC index, kill-time assay and application in apple jam. These compounds combined at sub-inhibitory concentrations were effective in inhibiting the growth of *S.* Typhimurium and *S. aureus*, although the underlying mode of action remains to be explored in the future. Sensory evaluation suggested that addition of the mixture of CIN and P.S. to apple jam is acceptable to consumers and there were no changes in physicochemical properties. Our findings demonstrate that mixtures of natural and synthetics compounds at sufficiently low concentrations could arise as an alternative to replace synthetic preservatives classically applied in the food industry, and to reach a balance between the demand for microbial safety and organoleptic acceptability.

4. CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

5. REFERENCES

Aguiar VF, da Silva JMM, Cavalcante CEB and Ribeiro ETS. (2016). Desenvolvimento de geleia mista de maçã e mel: análise da viabilidade através da aceitação sensorial. *Conexões - Ciência e Tecnologia* 10: 78–84.

AOAC (1992). *Association of official analytical chemistral. Official methods of analysis of the association of official analytical chemistry* (11th ed.) 1115 (Washington).

Bassolé IHN, Juliani HR. (2012). Essential oils in combination and their antimicrobial properties. *Molecules* 17: 3989–4006.

Batista AFP, dos Santos AR, da Silva AF, Trevisan DAC, Ribeiro LH, Campanerut-Sá PAZ, Abreu Filho BA, Machinski Junior M and Mikcha JMG. (2019). Inhibition of *Salmonella enterica*

serovar Typhimurium by combined carvacrol and potassium sorbate *in vitro* and in tomato paste. *LWT - Food Science and Technology* 100: 92–98.

Brasil (1978). Resolução n.12 - CNNPA, de 24 julho de 1978. A CNNPA do Ministério da Saúde aprova 47 padrões de identidade e qualidade relativos a alimentos e bebidas para serem seguidos em todo território brasileiro. Diário Oficial da União. Brasília.

Brasil (2004). Ministério da saúde, Agência Nacional de Vigilância Sanitária - ANVISA. Resolução RDC nº 216 de 15 de outubro de 2004. Estabelece procedimentos de Boas Práticas para serviços de alimentação. Diário Oficial da União. Brasília.

Brasil (2009). Ministério da saúde, Agência Nacional de Vigilância Sanitária - ANVISA. Resolução nº 28 de 26 de maio de 2009. Dispõe sobre o uso de aditivos alimentares para geleias. Diário Oficial da União. Brasília.

Brasil (2019). Ministério da saúde, Agência Nacional de Vigilância Sanitária - ANVISA. Instrução Normativa n° 60, de 23 de dezembro de 2019. Estabelece as listas de padrões microbiológicos para alimentos. *Diário Oficial da União*. Brasília.

Burt SA, Adolfse SJM, Ahad DSA, Tersteeg-Zijderveld MHG, Jongerius-Gortemaker BGM, Post JA, Brüggemann H and Santos RR. (2016). Cinnamaldehyde, carvacrol and organic acids affect gene expression of selected oxidative stress and inflammation markers in IPEC-J2 cells exposed to *Salmonella* Typhimurium. *Phytotherapy Research* 30: 1988–2000.

Calo JR, Crandall PG, O'Bryan CA and Ricke SC. (2015). Essential oils as antimicrobials in food systems - A review. *Food Control* 54: 111–119.

CDC (2021). Department of health & human services, national center for emerging and zoonotic infectious diseases, division of foodborne, waterborne, and environmental diseases [online]. Available: https://www.cdc.gov/ [May, 2021].

Chen X, Yu F, Li Y, Lou Z, Toure SL and Wang H. (2020). The inhibitory activity of p-coumaric acid on quorum sensing and its enhancement effect on meat preservation. *CyTA - Journal of Food* 18: 61–67.

CLSI (2018). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard CLSI document M07-A10 (10th ed.). Wayne, PA: Clinical and Laboratory Standards Institute.

Di Veroli GY, Fornari C, Wang D, Mollard S, Bramhall JL, Richards FM and Jodrell DI. (2016). Combenefit: an interactive platform for the analysis and visualization of drug combinations. *Bioinformatics* 32: 2866–2868.

Doern CD. (2014). When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. *Journal of Clinical Microbiology* 52: 4124–4128.

Downes FP, and Ito K. (2001). *Compendium of methods for the microbiological examination of foods* (4th ed.). Washington, DC: American Public Health Association.

Fadel HHM, Hassan IM, Ibraheim MT, Abd El Mageed MA and Saad R. (2019). Effect of using cinnamon oil encapsulated in maltodextrinas exogenous flavouring on flavour quality and stability of biscuits. *Journal of Food Science and Technology* 56: 4565–4574.

González-Fandos L, Martínez-Laorden A and Perez-Arnedo I. (2021). Efficacy of combinations of lactic acid and potassium sorbate against *Listeria monocytogenes* in chicken stored under modified atmospheres. *Food Microbiology* 93: 103596.

Gyawali R and Ibrahim SA. (2014). Natural products as antimicrobial agents. *Food Control* 46: 412–429.

Higueras L, López-Carballo G, Gavara R and Hernández-Muñoz P. (2015). Reversible Covalent Immobilization of Cinnamaldehyde on Chitosan Films via Schiff Base Formation and Their Application in Active Food Packaging. *Food and Bioprocess Technology* 8: 526–538.

Hwang C and Huang L. (2014). The effect of potassium sorbate and pH on the growth of *Listeria monocytogenes* in ham salad. *Journal of Food Processing and Preservation* 38: 1511–1516.

Isenberg HD. (2004). *Clinical Microbiology procedures handbook*. Washington: ASM Press (p. 5.10.2).

ISO 8589 (2015). *Sensory analysis – general guidance for the design of test rooms*. Geneva: International Organization for Standardization.

Jaiswal AK and Jaiswal S. (2014). Modelling the effects of natural antimicrobials as food preservatives. In: Taylor M. (ed.) *Handbook of Natural Antimicrobials for Food Safety and Quality*. (pp. 259–284). Woodhead Publishing Limited, Cambridge, UK.

Jouki M, Yazdi FT, Mortazavi SA, Koocheki A and Khazaei N. (2014). Effect of quince seed mucilage edible films incorporated with oregano or thyme essential oil on shelf life extension of refrigerated rainbow trout fillets. *International Journal of Food Microbiology* 174: 88–97.

Kadariya J, Smith TC and Thapaliya D. (2014). *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public Health. *BioMed Research International* 2014: 1–9.

Klangpetch W and Noma S. (2018). Inhibitory effects of nisin combined with plant-derived antimicrobials on pathogenic bacteria and the interaction with complex food systems. *Food Science and Technology Research* 24: 609–617.

Khameneh B, Iranshahy M, Soheili V and Bazzaz BSF. (2019). Review on plant antimicrobials: a mechanistic viewpoint. *Antimicrobial Resistance and Infection Control* 8:118.

Krolow ANR. Preparo artesanal de geleias e geleiadas. Pelotas: Embrapa Clima Temperado, 2005, 29 p. (Embrapa Clima Temperado. Documentos, 138). ISSN 1806–9193.

Malheiro JF, Maillard JY, Borges F and Simões M. (2019). Evaluation of cinnamaldehyde and cinnamic acid derivatives in microbial growth control. *International Biodeterioration and Biodegradation* 141: 71–78.

Mendonça CR, Rodrigues RS and Zambiazi RC. (2000). Brown sugar in apple jellies. *Ciência Rural* 30: 1053–1058.

Moreira MCND, de Almeida GL, Carvalho EEN, Garcia JAD, Nachtigall AM and Vilas Boas BM. (2019). Quality parameters, antioxidant activity, and sensory acceptability of mixed jams of rose petals and apple. *Journal of Food Processing and Preservation* 43: 1–6.

Pierson MD, Zink DL and Smoot LM. (2007). Indicators microorganisms and microbiological criteria. *Food Microbiology: Fundamentals and frontiers* (pp. 69–83). (3rd ed.). Washington, D.C: ASM Press.

Piovezan M, Uchida NS, Silva AF, Grespan R, Santos PR, Silva EL, Cuman RKN, Machinski Junior M and Mikcha JMG. (2014). Effect of cinnamon essential oil and cinnamaldehyde on *Salmonella* Saintpaul biofilm on a stainless steel surface. *Journal of General and Applied Microbiology* 60: 119–121.

Pillai SK, Moellering RC and Eliopoulos GM. (2005). Antimicrobial combinations. In V. Lorian (Ed.). *Antibiotics in laboratory medicine* (pp. 365–440). (5th ed.). Philadelphia: Lippincott Williams & Wilkins Co.

Rao J, Chen B and McClements DJ. (2019). Improving the Efficacy of Essential Oils as Antimicrobials in Foods: Mechanisms of Action. *Annual Review of Food Science and Technology* 10: 365–87.

Raybaudi-Massilia RM, Rojas-Grau MA, Mosqueda-Melgar J and Martin-Belloso O. (2008). Comparative study on essential oils incorporated into an alginate-based edible coating to assure the safety and quality of fresh-cut fuji apples. *J Food Protection* 71: 1150–1161.

Requena R, Vargas M and Chiralt A. (2019). Study of the potential synergistic antibacterial activity of essential oil components using the thiazolyl blue tetrazolium bromide (MTT) assay. *LWT - Food Science and Technology* 101: 183–190.

Ribeiro-Santos R, Andrade M, Madella D, Martinazzo AP, de Aquino GML, de Melo NR, et al. (2017). Revisiting an ancient spice with medicinal purposes: Cinnamon. *Trends in Food Science & Technology* 62: 154–169.

Santiesteban-López A, Palou E and López-Malo A. (2007). Susceptibility of food-borne bacteria to binary combinations of antimicrobials at selected aw and pH. *Journal of Applied Microbiology* 102: 486–497.

Shen S, Zhang T, Yuan Y, Lin S, Xu J and Ye H. (2015). Effects of cinnamaldehyde on *Escherichia coli* and *Staphylococcus aureus* membrane. *Food Control* 47: 196–202.

Shi C, Zhang X, Zhao X, Meng R, Liu Z, Chen X and Guo N. (2017). Synergistic interactions of nisin in combination with cinnamaldehyde against *Staphylococcus aureus* in pasteurized milk. *Food Control* 71: 10–16.

Silva AF, dos Santos AR, Trevisan DAC, Ribeiro AB, Campanerut-Sá PAZ, de Souza EM, et al. (2018). Cinnamaldehyde induces changes in the protein profile of *Salmonella* Typhimurium biofilm. *Microbiological Research* 169: 33–43.

Sofos JN and Busta FF. (1981). Antimicrobial activity of sorbate. *Journal of Food Protection* 44(8): 614–622.

Sofos JN, Pierson MD, Blocher JC and Busta FF. (1986). Mode of action of sorbic acid on bacterial cells and spores. *International Journal of Food Microbiology* 3: 1–17.

Torrezan R. *Manual para a produção de geleias de frutas em escala industrial*. Rio de Janeiro: EMBRAPA - CTAA, 1998. 27 p. (EMBRAPA-CTAA. Documentos, 29).

van Beilen JWA, Teixeira de Mattos MJ, Hellingwerf KJ and Brul S. (2014). Distinct effects of sorbic acid and acetic acid on the electrophysiology and metabolism of *Bacillus subtilis. Applied and Environmental Microbiology* 80: 5918 –5926.

Vasconcelos NG, Croda J and Simionatto S. (2018). Antibacterial mechanisms of cinnamon and its constituents: A review. *Microbial Pathogenesis* 120: 198–203.

Ye H, Shen S, Xu J, Lin S, Yuan Y and Jones GS. (2013). Synergistic interactions of cinnamaldehyde in combination with carvacrol against food-borne bacteria. *Food Control* 34: 619–623.

Yuan, W, Teo CHM and Yuk H. (2019). Combined antibacterial activities of essential oil compounds against *Escherichia coli* O157:H7 and their application potential on fresh-cut lettuce. *Food Control* 96: 112–118.

Yuste J and Fung DYC. (2002). Inactivation of *Listeria monocytogenes* Scott A 49594 in apple juice supplemented with cinnamon. *Journal of Food Protection* 65: 1663–1666.

Zhang Y, Liu X, Wang Y, Jiang P and Quek S. (2016). Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus. Food Control* 59: 282–289.

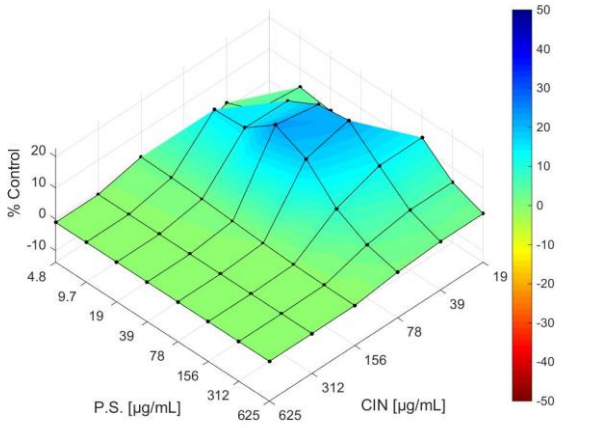
Zhang S, Xiong J, Lou W, Ning Z, Zhang D and Yang J. (2019). Antimicrobial activity and action mechanism of triglycerol monolaurate on common foodborne pathogens. *Food Control* 98: 113–119.

Zhan G, Pan D, Zhou C, Wang Y, He J, Zhang J, Li J and Cao J. (2021). Characterizing bacterial strains of spoiled Jinhua ham and evaluating the effect of antimicrobial agents on these isolated bacterial strains. *LWT - Food Science and Technology* 136: 110351.

Zhu H, Chen G, Shen S, Wang Q, Wan L and Jian S. (2019). Characterization of polyphenolic constituents from *Sanguisorba officinalis* L. and its antibacterial activity. *European Food Research and Technology* 245: 1487–1498.

Figure 1. The Bliss independence surface analysis for *in vitro* combinations of CIN and P.S. against *Salmonella* Typhimurium (A) and *Staphylococcus aureus* (B)

A



B

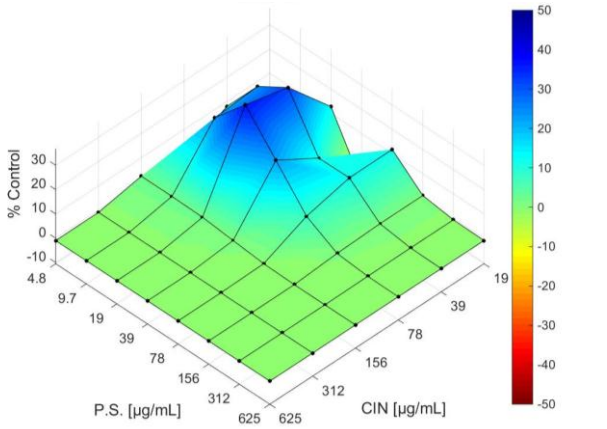
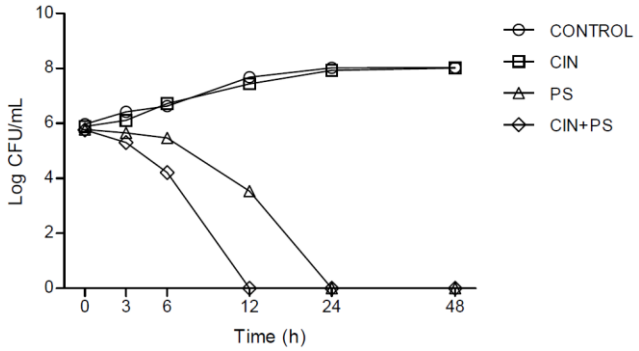


Figure. 2. Time-kill curve assay of CIN (78 µg/mL), P.S. (78 µg/mL) and CIN + P.S. (78 µg/mL+78 µg/mL). (A) *Salmonella* Typhimurium ATCC 14028; (B) *Staphylococcus aureus* ATCC 25923.

A



B

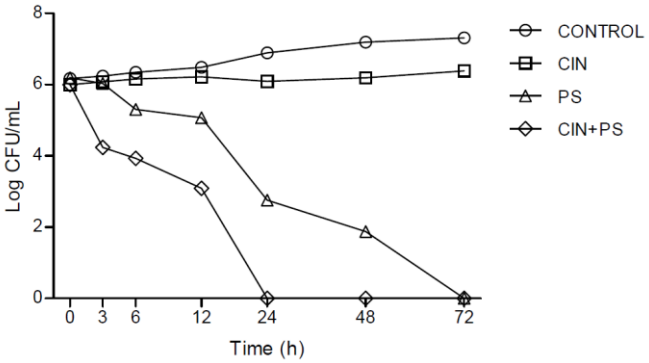


Table 1. Counts of *Salmonella* Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 25923 in apple jam added from CIN and P.S. alone and in combination

***Salmonella* Typhimurium**								
Group	**Day**							
	0	**1**	**2**	**3**	**4**	**5**	**8**	**10**
Control	5.88 ± 0.05^{A}	5.88 ± 0.04^{A}	5.92 ± 0.07^{A}	5.90 ± 0.11^{A}	5.92 ± 0.07^{A}	5.93 ± 0.07^{A}	5.95 ± 0.06^{A}	5.96 ± 0.06^{A}
CIN	5.90 ± 0.08^{A}	5.76 ± 0.04^{A}	5.72 ± 0.06^{A}	5.72 ± 0.18^{A}	5.60 ± 0.12^{A}	5.58 ± 0.16^{A}	5.38 ± 0.07^{A}	5.32 ± 0.07^{A}
P.S.	5.89 ± 0.04^{A}	4.78 ± 0.06^{B}	3.77 ± 0.10^{B}	2.49 ± 0.19^{B}	ND	ND	ND	ND
CIN + P.S.	5.80 ± 0.09^{A}	2.85 ± 0.06^{B}	ND	ND	ND	ND	ND	ND

Staphylococcus aureus								
Group	**Day**							
	0	**1**	**2**	**3**	**4**	**5**	**8**	**10**
Control	5.53 ± 0.06^{A}	5.56 ± 0.27^{A}	5.53 ± 0.33^{A}	5.53 ± 0.33^{A}	5.52 ± 0.29^{A}	5.52 ± 0.38^{A}	5.57 ± 0.36^{A}	5.68 ± 0.13^{A}
CIN	5.49 ± 0.05^{A}	5.17 ± 0.14^{A}	5.14 ± 0.20^{A}	5.08 ± 0.19^{A}	4.98 ± 0.18^{A}	4.90 ± 0.03^{A}	4.84 ± 0.15^{A}	4.89 ± 0.05^{A}
P.S.	5.49 ± 0.10^{A}	4.77 ± 0.13^{B}	4.38 ± 0.07^{B}	3.15 ± 0.05^{B}	ND	ND	ND	ND
CIN + P.S.	5.46 ± 0.04^{A}	3.89 ± 0.06^{B}	ND	ND	ND	ND	ND	ND

Values are mean log CFU/g followed by standard deviation. Means in the same column with different letters are significantly different ($p < 0.05$; Tukey's test). Control (without treatment); CIN (78 µg/mL); P.S. (78 µg/mL) and CIN + P.S. (78 µg/mL + 78 µg/mL). ND: not detected.

Table 2. pH, soluble solids, titratable acidity, reducing sugar and non-reducing sugar in apple jam with CIN and P.S. alone and combined

	pH			Soluble solids (%)			Titratable acidity (%)			Reducing sugar (%)			Non-reducing sugar (%)		
Group	**Day**														
	0	**5**	**10**	**0**	**5**	**10**	**0**	**5**	**10**	**0**	**5**	**10**	**0**	**5**	**10**
Control	4.92 ±0.56^{A}	4.9 ±0.49^{A}	5.06 ±0.47^{A}	34.62 ±0.87^{A}	34.77 ±0.59^{A}	35.05 ±1.04^{A}	0.41 ±0.07^{A}	0.42 ±0.06^{A}	0.45 ±0.03^{A}	11.58 ±0.31^{A}	13.01 ±0.24^{A}	13.8 ±0.30^{A}	12.27 ±1.15^{A}	10.78 ±0.21^{A}	11.00 ±0.39^{A}
CIN	4.78 ±0.14^{A}	4.85 ±0.46^{A}	4.87 ±0.57^{A}	35.12 ±0.44^{A}	34.43 ±0.96^{A}	35.15 ±0.64^{A}	0.36 ±0.02^{A}	0.35 ±0.03^{A}	0.38 ±0.02^{A}	13.72 ±0.24^{B}	13.99 ±0.38^{B}	13.43 ±0.31^{A}	11.43 ±0.35^{A}	11.72 ±0.38^{B}	11.16 ±0.14^{A}
P.S.	5.0 ±0.60^{A}	4.96 ±0.53^{A}	4.88 ±0.51^{A}	34,95 ±0.85^{A}	35.73 ±0.48^{A}	35.15 ±0.94^{A}	0.41 ±0.05^{A}	0.37 ±0.10^{A}	0.42 ±0.04^{A}	11.92 ±0.22^{A}	12.63 ±0.38^{A}	14.32 ±0.34^{A}	10.33 ±0.32^{B}	10.62 ±0.33^{A}	11.57 ±0.36^{A}
CIN+P.S.	4,93 ±0.42^{A}	4.97 ±0.37^{A}	4.99 ±0.30^{A}	34.83 ±0.49^{A}	34.57 ±0.84^{A}	35.05 ±0.77^{A}	0.42 ±0.06^{A}	0.41 ±0.07^{A}	0.46 ±0.02^{A}	13.42 ±0.21^{B}	13.45 ±0.29^{A}	13.41 ±0.18^{A}	12.01 ±0.25^{A}	11.04 ±0.28^{A}	11.32 ±0.21^{A}

Values are mean followed by standard deviation. Means in the same column with different letters are significantly different ($p < 0.05$; Tukey's test). Control (without treatment); CIN (78 µg/mL); P.S. (78 µg/mL) and CIN + P.S. (78 µg/mL + 78 µg/mL).

Table 3. Sensory analysis of apple jam treated with CIN and P.S. alone and combined using a 9-Point affection hedonic scale with 5 parameters

Parameters	Control	CIN	P.S.	CIN + P.S.
Color	7.8^{A}	8.0^{A}	7.5^{A}	7.5^{A}
Aroma	7.6^{A}	7.5^{B}	7.6^{B}	7.6^{A}
Taste	7.4^{A}	7.9^{B}	7.7^{B}	7.7^{A}
Texture	7.7^{A}	7.7^{A}	7.5^{B}	7.4^{A}
Overall acceptance	7.5^{A}	7.8^{B}	7.6^{B}	7.4^{A}

Control (without treatment); CIN (78 µg/mL); P.S. (78 µg/mL) and CIN + P.S. (78 µg/mL + 78 µg/mL). Values represent mean ± standard deviation (n=120). Means in the same line with different letters are significantly different ($p < 0.05$; Tukey's test).

ARTICLE 2

BIOGENIC SILVER NANOPARTICLES AND CINNAMALDEHYDE AS AN EFFECTIVE SANITIZER FOR FRESH SWEET GRAPE TOMATOES

Biogenic silver nanoparticles and cinnamaldehyde as an effective sanitizer for fresh sweet grape tomatoes

Andreia Farias Pereira Batista[1]*; Luana Carolina Martins Rosa[1]; Jessica Santos Pizzo[5]; Alex Fiori da Silva[6]; Jesuí Vergílio Visentainer[5]; Benício Alves de Abreu Filho[1,4]; Renata Katsuko Takayama Kobayashi[7]; Gerson Nakazato[7]; Jane Martha Graton Mikcha [1, 2, 3]

[1]Center for Agricultural Sciences, Postgraduate Program of Food Science, State University of Maringá, Maringá, PR, Brazil
[2]Center for Health Sciences, Postgraduate Program in Health Sciences, State University of Maringá, Maringá, PR, Brazil
[3]Department of Clinical Analyses and Biomedicine, State University of Maringá, Maringá, PR, Brazil
[4]Department of Basic Health Sciences, State University of Maringá, Maringá, PR, Brazil
[5]Department of Chemistry, State University of Maringá, Maringá, PR, Brazil
[6]Department of Agricultural and Natural Sciences, State University of Minas Gerais, Ituiutaba, MG, Brazil
[7]Laboratory of Basic and Applied Bacteriology, Department of Microbiology, Center of Biological Sciences, State University of Londrina, Londrina, Brazil
*Corresponding author: Andreia Farias Pereira Batista. Postgraduate Program of Food Science, State University of Maringá, Av. Colombo, 5790. CEP: 87020-900, Maringá, PR, Brazil. Phone: +55 44 3011-4959. E-mail address: andreia.farias04@hotmail.com

ABSTRACT

This study evaluated the antibacterial activity of cinnamaldehyde (CIN) and biogenic silver nanoparticles (BioAgNP), alone and in combination, against *Escherichia coli*, *Salmonella* Typhimurium, and *Staphylococcus aureus* in vitro. Their sanitation activities on fresh sweet grape tomatoes were also evaluated. CIN and BioAgNP inhibited the growth of the tested bacteria, and at low concentrations, their combinations presented a synergistic effect. In the sanitization of fresh sweet grape tomatoes, the combination of sub-inhibitory concentration of CIN (156 µg/mL) and BioAgNP (31.25 µM) inhibited the growth of *E. coli* after only 5 min of contact. In addition, *E. coli* did not grow during the shelf life period. The combination of these compounds did not change the physicochemical properties of sweet grape tomatoes. CIN combined with BioAgNP represents an effective method for decontaminating fruits and vegetables. This combination has great potential for application in the prevention of foodborne diseases.

Keywords: antibacterial; natural compounds; sanitizers; silver nanoparticles; sweet grape tomato

1. INTRODUCTION

Microbial contamination is a worldwide problem that causes enormous losses for the food industry and generates high healthcare costs (US$ 15.6 billion each year) (CDC, 2022). Foodborne diseases have been perceived as a serious public health problem worldwide. Centers for Disease Control and Prevention (CDC) estimates that each year one in six Americans become ill from contaminated food or beverages, and 3,000 die from foodborne illness (CDC, 2022). The foodborne pathogens commonly involved in food safety incidents include *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., and *Listeria monocytogenes* (CDC, 2022). Although there are methods to control microbial growth in foods, there remains a need for novel techniques that prove to be effective for microbial inactivation and the maintenance of sensorial characteristics of foods (Nile *et al.*, 2020).

Natural compounds are promising alternative food preservatives (Batista *et al.*, 2019). Among them, cinnamaldehyde (CIN) has been studied extensively due to its antimicrobial properties (Malheiro *et al*., 2019; Burt *et al.*, 2016). CIN is the major component in cinnamon essential oil and can be used as a food additive and flavoring agent (Barceloux, 2009). Furthermore, it qualifies as 'generally recognized as safe' (GRAS) according to the Food and Drug Administration (21 CFR 182.60) (FDA, 2018). However, its strong taste and aroma limit its use; therefore, novel alternatives are needed to minimize or eliminate these undesirable organoleptic effects (Li *et al*., 2022).

Green nanotechnology has also received considerable attention in the scientific community due to its eco-friendly and low-cost nature (Kobayashi and Nakazato, 2020). Among the engineered nanomaterials, silver nanoparticles (AgNPs) have gained increased interest due to their strong antimicrobial activities and antiviral properties (Chue-Gonçalves *et al.*, 2021; Kobayashi and Nakazato, 2020). In the food sector, silver nanoparticles have been applied to food processing, packaging, and sanitation (Nile *et al.*, 2020). Among the commercially available nanotechnology-based disinfectants, silver nanoparticles are the most used active constituent (Nile *et al.*, 2020). However, some works have reported microbial resistance to silver (Graves *et al.*, 2015) and toxicity when applied directly to food (Li *et al.*, 2022).

Interestingly, many compounds can be incorporated into nanoparticles, making it possible to assess the effect of several substances simultaneously (Nile *et al.*, 2020). The combined use of nanoparticles and antimicrobials provides more potent antimicrobial activity than that of a single compound. In this way, the combination of CIN and AgNPs is a potential strategy to increase the antibacterial activity and reduce the effective concentration of both compounds, thus reducing the impact of undesirable characteristics of natural compounds on food (Scandorieiro *et al.*, 2016; Ghosh *et al.*, 2013).

To this end, we aimed to investigate the association of CIN with biogenic silver nanoparticles (BioAgNP) and their effects as a sanitizer for fresh sweet grape tomato. The first part of this study evaluated the antibacterial activity of CIN and BioAgNP alone and in combination against *E. coli*, *Salmonella* Typhimurium, and *S. aureus*. The second part was conducted to investigate the effect of CIN and BioAgNP alone and in combination as a sanitizer for fresh sweet grape tomato.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Escherichia coli ATCC 25922, *Salmonella enterica* serotype Typhimurium ATCC 14028, and *Staphylococcus aureus* ATCC 25923 were used in this study. The cultures were maintained in Tryptic Soy Broth (TSB) (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 20% glycerol at −20 °C.

2.2. Antimicrobial agents

Cinnamaldehyde (CIN) with 93% purity was obtained from Sigma Aldrich, Buchs, Switzerland. Biogenic silver nanoparticles (BioAgNP) were obtained from GRAL Bioativos®, Londrina, Brazil. These BioAgNP were produced from plant extract and showed an average bioAgNP diameter of 82.73 nm, zeta potential of −23.27 mV, and polydispersity index (PI) of 0.17.

2.3. Antibacterial activity

2.3.1. Antibacterial activity of cinnamaldehyde and BioAgNP

The minimum inhibitory concentration (MIC) of CIN and BioAgNP were determined using the broth microdilution method following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2018). CIN was initially diluted in 0.5% dimethyl sulfoxide (DMSO), and BioAgNP was prepared in Mueller Hinton Broth (MHB, Difco, Becton Dickinson, Sparks, MD, USA). Tested concentrations of CIN and BioAgNP ranged from 19 to 5,000 μg/mL and 0.97 to 500 μM, respectively. Bacterial suspensions were standardized by 0.5 McFarland scale (1.5×10^8 bacteria/mL) and diluted at 1:20, and 10 μL were inoculated in each microplate well. After 24 h of incubation at 35 °C, the MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth. Bacterial growth control in MHB and 0.5% DMSO and a control with CIN in MHB and BioAgNP in MHB were included. All assays were carried out in triplicate and on at least three different occasions.

2.3.2. Antibacterial combination assay

The interaction of CIN and BioAgNP was determined by the checkerboard method (Doern, 2014).

BioAgNP was added to 96-well microtiter plates with MHB and diluted along the x-axis. CIN was added and diluted along the y-axis in the same way. Bacterial suspensions with approximately 10^6 colony-forming units per milliliter (CFU/mL) were inoculated in each microplate well. The plates were incubated at 35 °C for 24 h. Fractional inhibitory concentration (FIC) index (ΣFIC), was calculated using the formula below:

$$\sum FIC = \frac{MIC\ of\ CIN\ in\ combination}{MIC\ of\ CIN\ alone} + \frac{MIC\ of\ BioAgNPin\ combination}{MIC\ of\ BioAgNP\ alone}$$

The interaction between CIN and BioAgNP was defined as synergistic (FI0 < 0.5), additive (0.5 ≤ FIC ≤ 1), indifferent (1 < FIC ≤ 4), or antagonistic (FIC > 4) (Pillai *et al.*, 2005). The Bliss-independent interactions were analyzed with Combenefit software (Di Veroli *et al.*, 2016). All the experiments were repeated in triplicate.

2.3.3. Time-kill assay

Time-kill assays were performed according to Isenberg (2004), with modifications. Overnight cultures of *E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028, and *S. aureus* ATCC 25923 were standardized according to 1.0 McFarland (3.0 x 10^8 bacteria/mL) and transferred to MHB supplemented with CIN and BioAgNP, alone and in combination, to obtain a final inoculum of 6 × 10^5 CFU/mL. CIN was tested at 156 μg/mL (1/4 MIC) and BioAgNP at 31.25 μM (1/2 MIC), according to the checkerboard results. Aliquots of 100 μL were withdrawn at different time intervals, serially diluted, and plated on Mueller–Hinton agar (MHA) (Difco, Becton Dickinson, Sparks, MD, USA). Plates were incubated at 35 °C for 24 h, and CFUs were counted. Each test was performed in duplicate and repeated three times.

2.4. Application in fresh sweet grape tomatoes

Escherichia coli was chosen for evaluation of the antibacterial effect of CIN and BioAgNP alone and in combination as sanitizers in fresh sweet grape tomatoes. The concentrations of evaluated compounds were selected by the checkerboard method.

2.4.1. Microbiological quality

Samples of tomatoes not subjected to sanitizing treatments and without the artificial inoculation step were also submitted to microbiological analysis (*Salmonella* sp. and *E. coli*), following the standards required by Brazilian legislation (Brasil, 2019).

For detection of *Salmonella* sp., 25 g of the samples were added to 225 mL of lactose broth (Difco, Becton Dickinson, Sparks, MD, USA) and incubated at 35 °C for 24 h. After the incubation

period, selective enrichment was performed in selenite cystine broth (Difco, Becton Dickinson, Sparks, MD, USA) and in Rappaport–Vassiliadis medium (Difco, Becton Dickinson, Sparks, MD, USA). Subsequently, samples were plated on Hektoen agar and incubated at 35 °C for 24 h. *Escherichia coli* enumeration was performed using Petrifilm™ EC plates (3M Company, St. Paul, MN, EUA). Aliquots of 1 mL of each sample were seeded in Petrifilm™ EC plates and incubated at 35 ºC for 24 and 48 h. *E. coli* colonies were enumerated according to the manufacturer's instructions.

2.4.2. Preparation and inoculation of sweet grape tomatoes

Sweet grape mini tomatoes (*Lycopersicum esculentum* Mill.) were purchased in a local market and selected during their mature phase without mechanical blemishes. The tomatoes were washed in running water, immersed in cold water with 200 ppm chloride for 10 min, washed, and dried. The sweet grape tomatoes were submerged in *E. coli* ATCC 25922 suspensions standardized at 10^8 CFU/mL in sterile 0.1% peptone water supplemented with 0.1% agar for 30 minutes. Afterwards, the samples were air dried for 2 hours to facilitate bacterial adhesion before exposure to disinfection treatments (Choi *et al*., 2018).

2.4.3. Sanitizing treatments and shelf life

To assess the antibacterial activity of CIN and BioAgNP alone and in combination against *E. coli* in sweet grape tomatoes, four samples were defined: a control sample (without antimicrobials), a CIN sample at 156 μg/mL (CIN), a BioAgNP sample at 31.25 μM (BioAgNP), and a CIN + BioAgNP sample at 156 μg/mL + 31.25 μM (CIN and BioAgNP). The samples were treated for 0, 5, 10, 15, 30, and 60 min. After treatments, inoculated bacteria were counted by diluting 10 g of tomato in 90 mL of sterile peptone water (1 g/L). Serial dilutions were performed, plated on Eosin Methylene Blue Agar (Difco, Becton Dickinson, Sparks, MD, USA) plates, and incubated at 35 °C for 24 h before counting. The analyses were repeated twice, and the results are expressed in log CFU/mL.

The survival of *E. coli* in sweet grape tomatoes treated with CIN and BioAgNP alone and in combination during the shelf life was also evaluated. The samples were treated for 5 min, air dried, and packaged in sealed bags for 7 days. The samples were stored at room temperature and analyzed on days 0, 4, and 7 of their shelf life.

2.4.5. Physicochemical analysis

The sweet grape tomato samples stored at room temperature were analyzed at intervals of 0, 4, and 7 days of shelf life with regard to pH, titratable acidity, and soluble solids (Instituto Adolfo Lutz, 1985). The hydrogenionic potential (pH) was determined in homogenized tomato pulp using

a digital potentiometer (MCA-150, Lucadema). The amount of soluble solids was determined by refractrometric analysis of homogenized tomato pulp samples. The results were expressed as degrees Brix (°Bx). Total titratable acidity was determined from the titration of 5 g of homogenate pulp diluted in 100 ml of distilled water and a standard solution of 0.1 mol/L sodium hydroxide. Analyses were performed in triplicate, with three replicates.

2.5. Statistical analysis

The results were analyzed with GraphPad Prism 7.0 Software. *In vitro* analyses were performed in duplicate, with three replications, and sanitizer analysis was repeated twice. Results were expressed as the mean and standard deviation. The data were analyzed by ANOVA at the 5% significance level. Post hoc comparisons were performed by Tukey's test.

3. RESULTS AND DISCUSSION

3.1. Antibacterial activity *in vitro*

3.1.1. Antibacterial activity of cinnamaldehyde and BioAgNP

Cinnamaldehyde and BioAgNP exhibited antimicrobial activity against *E. coli*, *S.* Typhimurium, and *S. aureus*. CIN presented an MIC of 624 µg/mL for all bacteria investigated. Other studies evaluating the activity of CIN against *E. coli* revealed MIC values between 100 and 310 µg/mL (Andrade-Ochoa *et al.*, 2021; Gosh *et al.*, 2013; Ye *et al.*, 2013). An MIC between 131 and 600 µg/mL was obtained against *S.* Typhimurium (Andrade-Ochoa *et al.*, 2021; Burt *et al.*, 2016), and MIC values between 58 and 500 µg/mL were observed for *S. aureus*. (Gosh *et al.*, 2013; Ye *et al.*, 2013; Al-Bayati and Mohammed, 2009).

BioAgNP showed MIC values of 125 µM against *E. coli*, *S.* Typhimurium, and *S. aureus*. Scandorieiro *et al.* (2016) analyzed the antimicrobial action of BioAgNP produced from fungi extract and reported MICs of 62.5, 125, and 250 µM against *E. coli* (ATCC 25922), *S.* Typhimurium (ATCC 68169), and *S. aureus* (ATCC 25923), respectively. Other studies revealed AgNP or BioAgNP MIC values between 3.12 and 50 µg/mL against *E. coli* (Dalir *et al.*, 2020; Al-Sharqi *et al.*, 2019; Kelkawi *et al.*, 2016; Zarei *et al.*, 2014) and *S.* Typhimurium (Dehkordi *et al.*, 2019; Zarei *et al.*, 2014), respectively. AgNP MIC values between 6.7 and 128 µg/mL were also found against *S. aureus* (Dalir *et al.*, 2020; Al-Sharqi *et al.*, 2019; Andrade *et al.*, 2017).

3.1.2. Synergistic effect between cinnamaldehyde and BioAgNP

The combination of CIN and BioAgNP against *E. coli*, *S.* Typhimurium, and *S. aureus* was synergistic, with an FIC value of 0.49. The combination of compounds reduced the MIC value of CIN two-fold (from 624 to 156 µg/mL) and that of BioAgNP (from 125 to 31.25 µM) for all bacteria tested. To our best knowledge, the only study reporting on a combination of CIN and

AgNP was performed by Gosh *et al.* (2013); however, those authors used a silver nanoparticle synthesized by silver nitrate. Gosh *et al.* (2013) showed that the effect of the combination of CIN and AgNP was additive (FIC 0.53) against *E. coli*, *Salmonella typhi*, and *S. aureus*. Scandorieiro *et al.* (2016) studied the antibacterial effect of oregano essential oil (OEO) and biological silver nanoparticles (BioAgNP) on *E. coli*, *S.* Typhimurium, and *S. aureus*. The FIC value (0.50) indicated that OEO and BioAgNP had a synergistic effect on *S. aureus*. The combination of OEO and BioAgNP significantly decreased the MIC of EOE (2-fold) and BioAgNP (2-fold), in agreement with our results. The combination of OEO and BioAgNP presented an additive interaction when tested against *E. coli* and *S.* Typhimurium. Dehkordi *et al.* (2019) also showed a synergistic effect on *S. aureus* (FIC 0.5) by combining eugenol and colloidal silver nanoparticles.

The synergic effect revealed by FIC values was validated by the results of Bliss independence surface analysis (Figure 1). In this way, CIN combined with BioAgNP showed a predominance of blue areas, confirming the synergism.

Silver nanoparticles have been widely studied because of their broad-spectrum antimicrobial effect, even at low concentrations (Dalir *et al.*, 2020; Dehkordi *et al.*, 2019; Kelkawi *et al.*, 2016; Scandorieiro *et al.*, 2016). In addition, the combination of these nanoparticles with several compounds, such as plant derivatives and bacteriocins, has shown potent antimicrobial activity in different microbial species, including foodborne bacteria (Al-Sharqi *et al.*, 2019; Dehkordi *et al.*, 2019).

3.1.3. Time-kill curve

Time-kill curves were used to assess the antibacterial activity of CIN and BioAgNP alone and combined against *E. coli*, *S.* Typhimurium, and *S. aureus* (Figure 2).

Escherichia coli (control group) reached 8.7 log CFU/mL after 6 h at 35 °C. CIN at a sub-inhibitory concentration (156 µg/mL) did not reduce the bacterial count during 6 h of incubation, while the treatment with BioAgNP alone (31.25 µM) completely inhibited bacterial growth ~~after~~ with 1 hour. The association between CIN at 1/4 MIC (156 µg/mL) and BioAgNP at 1/4 MIC (31.25 µM) inactivated *E. coli* in up to 45 min. Scandorieiro *et al.* (2016) also showed a 3.3 log CFU/mL reduction after 2 h, and no viable cells were detected after 4 h of incubation with BioAgNP at 62.5 µM. The combination of OEO (298 µg/mL) and BioAgNP (15.62 µM) decreased 2.3 log CFU/mL of *E. coli* within 10 min of treatment, and there were no viable cells after 20 min (Scandorieiro *et al.*, 2016).

The control group of *S.* Typhimurium reached approximately 9.0 log CFU/mL after 24 h at 35 °C. Treatment with CIN alone (156 µg/mL) failed to reduce the bacterial population during all intervals evaluated. On the other hand, BioAgNP alone (31.25 µM) showed a reduction of approximately 2.5–3 log CFU/mL in up to 12 hours. A mixture of CIN and BioAgNP inhibited

bacterial growth, and no cells were observed after 6 h of incubation. Dehkordi *et al.* (2019) also found a >2 log CFU/mL reduction in *S.* Typhimurium upon exposure to silver nanoparticles at 12.5 µg/mL during 3 h of treatment. These authors also evaluated the combined effects of silver nanoparticles and eugenol on the growth of *S.* Typhimurium and reported a ~6 log CFU/mL reduction after 9 h of treatment with eugenol at 1250 µg/mL combined with silver nanoparticles at 6.25 µg/mL.

Staphylococcus aureus reached 8.6 log CFU/mL after 48 h of incubation at 35 °C. CIN at 156 µg/mL did not reduce the bacterial counts. BioAgNP at 31.25 µM decreased bacterial counts by approximately 2 log CFU/mL after 24 hours; however, it was possible to observe partial cellular recovery with 48 h of incubation. No viable bacterial cells were observed after 48 h of treatment with CIN and BioAgNP in combination. The synergistic activity of silver nanoparticles and natural compounds on *S. aureus* was previously investigated (Dehkordi *et al.*, 2019; Scandorieiro *et al.*, 2016). Significant bactericidal activity was found for *S. aureus* treated with silver nanoparticles at 6.25 µg/mL and eugenol at 625 µg/mL after 6 h (Dehkordi *et al.*, 2019). Scandorieiro *et al.* (2016) showed that the combination of OEO and BioAgNP at 298 µg/mL and 125 µM, respectively, against *S. aureus* ATCC 25923 caused a 3.48 log CFU/mL decrease in the cell population in 2 h. ~~and resulted in~~ No viable bacterial cells after 7 h of incubation. Gosh *et al.* (2013) demonstrated synergism between CIN and AgNP against *Clostridium perfringens* and *Bacillus cereus* and showed a 2 log reduction of both bacteria after 1 hour of treatment. To our knowledge, there are no studies employing the time-kill assay to evaluate the antibacterial activity of CIN and BioAgNP alone and combined against *E. coli*, *S.* Typhimurium, and *S. aureus*.

3.2. Application in fresh sweet grape tomatoes

3.2.1. Microbiological quality

Sweet grape tomato samples were analyzed to evaluate their microbiological quality during their shelf life following the standards required by Brazilian legislation (Brasil, 2022). *Salmonella* sp. was absent in all groups on the different days evaluated. *Escherichia coli* enumeration was <2 log CFU/g for all days analyzed. These results demonstrate that sweet grape tomato samples complied with the current standards established by Brazilian legislation (Brasil, 2022).

3.2.2. Efficacy of CIN and BioAgNP in the sanitization of fresh sweet grape tomatoes

Table 1 shows the efficacy of CIN and BioAgNP at sub-inhibitory concentrations, alone and in combination, in the sanitization of fresh sweet grape tomatoes experimentally contaminated with *E. coli*. In the control groups, *E. coli* counts ranged from 4.15 to 5.37 log CFU/g. CIN alone reduced the bacterial load by only ~1 log CFU/g after 60 min of treatment, and no viable cells

were observed after 15 min in the treatment with BioAgNP alone. Sanitizing of sweet grape tomatoes with CIN and BioAgNP in combination was able to eradicate *E. coli* after 5 min.

Gopal *et al.* (2010) evaluated the effect of washing shredded lettuce with water containing low concentrations of silver (0.1 ppm) and hydrogen peroxide (0.4 ppm). A less than 1 log CFU/g reduction in *Pseudomonas* sp. counts and an approximately 1.5 log CFU/g reduction in *Enterobactericeae* were observed following treatment with silver and hydrogen peroxide in combination after 7 days of storage at 12 °C. Combinations of plant-derived antimicrobials and hydrogen peroxide reduced *L. monocytogenes* to undetectable levels in cantaloupes after a 10-min wash treatment (Upadhyay *et al*., 2014). To our best knowledge, this is the first study reporting the antimicrobial action of CIN and silver nanoparticles applied in combination as a sanitizer in sweet grape tomatoes.

Sanitization of vegetables is one of the important steps designed to reduce or eliminate microbial hazards in fresh vegetables (Ssemanda *et al.*, 2018). In this process, contact time with sanitizers is important to guarantee microbial and chemical safety and acceptability for consumption (Ssemanda *et al.*, 2018). In our work, only 5 min of contact with CIN + BioAgNP was sufficient to verify microbial control in fresh grape tomatoes. Bermúdez-Aguirre *et al.* (2013) showed total inactivation of *E. coli* in tomatoes after 15 min using 200 ppm of chlorine (8.06 log), which is considered the standard sanitizer in the decontamination of vegetables (BRASIL, 2004).

3.2.3. Shelf life of fresh sweet grape tomatoes treated with CIN and BioAgNP

The effects of CIN and BioAgNP alone and in combination in the sanitization of fresh sweet grape tomatoes contaminated by *E. coli*, on different days of shelf life are shown in Table 2. In control groups, *E. coli* counts were maintaining during storage and reached approximately 6.12 log CFU/g on the seventh day. *E. coli* counts in tomatoes treated with CIN were reduced less than 1 log CFU/g after 7 days of storage. The samples sanitized with BioAgNP alone and in association with CIN showed no growth of *E. coli* during the shelf life.

Upadhyay *et al.* (2014) reported on the reduction of *L. monocytogenes* on artificially contaminated cantaloupes. Combinations of plant-derived antimicrobials + hydrogen peroxide reduced *L. monocytogenes* to undetectable levels in cantaloupes after a 10-min wash treatment, and no bacterial cells were recovered after 7 days of storage.

Our study showed that BioAgNP alone presented antimicrobial activity; however, the prolonged use of silver nanoparticles can be toxic to humans (Li *et al*., 2022). Furthermore, resistance to silver nanoparticles has already been reported (Graves *et al.*, 2015). By contrast, natural compounds used as sanitizers are biodegradable and environmentally friendly and pose a lower risk to human health. In addition, to antimicrobial activity, CIN presents anti-inflammatory and antioxidant properties (Barceloux, 2009); however, the highly volatile nature of CIN can cause sensorial changes in foods (Li *et al*., 2022).

Therefore, the combination of antimicrobials is a potential strategy to minimize the undesirable effects of substances, since their combined use reduces the concentration of the compounds and the probability of selecting resistant bacteria (Nile *et al.*, 2020).

3.2.4. Physicochemical analysis

Sweet grape tomato samples sanitized with CIN, BioAgNP, and CIN + BioAgNP were subjected to physicochemical analysis for evaluation of pH, soluble solids, and titratable acidity during the shelf life (Table 3). No significant difference ($p < 0.05$) in pH was found between the control and treatment groups on the analyzed days. Sweet grape tomato samples presented pH values ranging from 4.16 to 4.26. According to the Center for Food Safety and Applied Nutrition (CFSAN, 2003), tomato pH levels should range between 4.3 and 4.9. In addition, a tomato pH below 4.3 prevents microorganism proliferation (CFSAN, 2003).

There were no significant differences in soluble solids in any sample during the 7 days of storage. Their rates ranged from 7.9 ºBrix to 8.5 ºBrix. These values are in agreement with Ribeiro *et al.* (2010), who found soluble solid values between 7.63 and 8.5 ºBrix in sweet grape tomatoes treated with coatings containing phenolic compounds. Sweet grape tomato is sweeter than traditional tomato and can reach 9 ºBrix (between 4 and 6 ºBrix) (Onoda, 2010).

No significant difference ($p < 0.05$) was observed in titratable acidity for all samples analyzed. The titratable acidity rates varied between 7.95% and 9.37% in the 7 days of storage. Acidity is an important determinant of tomato quality. According to Kader *et al.* (2002), tomatoes that present a titratable acidity greater than 0.32% are considered of good quality.

4. CONCLUSION

From this study, it is clear that CIN and BioAgNP in combination at sub-inhibitory concentrations effectively inhibited the *in vitro* growth of foodborne pathogenic bacteria. The use of a sanitizer based on CIN and BioAgNP combined inhibited the growth of *E. coli* in fresh sweet grape tomatoes after 5 min of treatment. The antibacterial activity of the compounds in combination in sweet grape tomatoes was maintained during their shelf life. The combination of these compounds did not change the physicochemical properties of sweet grape tomatoes. The disinfectant activity of plant-derived compounds combined with AgNPs could pave the way for a new generation of disinfection products to control and prevent further disease outbreaks.

5. ACKNOWLEDGEMENTS

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES for a scholarship granted to the first author.

6. CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

7. ETHICS STATEMENT

Ethics approval was not required for this research.

8. DATA AVAILABILITY STATEMENT

The data supporting the findings of this study will be available on request from the corresponding author. Due to privacy or ethical restrictions, the data are not publicly available.

9. REFERENCES

Al-Bayati FA and Mohammed MJ (2009). Isolation, identification, and purification of cinnamaldehyde from *Cinnamomum zeylanicum* bark oil. An antibacterial study. Pharmaceutical Biology, 47(1): 61–66. doi: 10.1080/13880200802430607.

Al-Sharqi A, Apun K, Vincent M, Kanakaraju D, Bilung LM (2019). Enhancement of the Antibacterial Efficiency of Silver Nanoparticles against Gram-Positive and Gram-Negative Bacteria Using Blue Laser Light. International Journal of Photoenergy, 2528490. https://doi.org/10.1155/2019/2528490.

Andrade PF, Nakazato G, Durán N (2017). Additive interaction of carbon dots extracted from soluble coffee and biogenic silver nanoparticles against bacteria. Journal of Physics Conference Series, 838, 012028. doi :10.1088/1742-6596/838/1/012028.

Andrade-Ochoa S, Chacón-Vargas KF, Sánchez-Torres LE, Rivera-Chavira BE, Nogueda-Torres B, Nevárez-Moorillón GV (2021). Differential Antimicrobial Effect of Essential Oils and Their Main Components: Insights Based on the Cell Membrane and External Structure. Membranes 2021, 11, 405. https://doi.org/10.3390/membranes11060405.

Barceloux DG (2008). Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Toxic Plants, and Venomous Animals. Hoboken, NJ: John Wiley & Sons, pp. 39-43. doi:10.1016/j.disamonth.2009.03.003.

Batista AFP, dos Santos AR, da Silva AF, Trevisan DAC, Ribeiro LH, Campanerut-Sá PAZ, *et al*. (2019). Inhibition of *Salmonella enterica* serovar Typhimurium by combined carvacrol and

potassium sorbate in vitro and in tomato paste. LWT - Food Science and Technology, 100, 92–98. doi.org/10.1016/j.lwt.2018.10.006.

Bermúdez-Aguirre D and Barbosa-Cánovas GV (2013). Disinfection of selected vegetables under nonthermal treatments: Chlorine, acid citric, ultraviolet light and ozone. Food Control, 29, 82e90. doi.org/10.1016/j.foodcont.2012.05.073.

Brasil (2004). Ministério da saúde, Agência Nacional de Vigilância Sanitária - ANVISA. Resolução – RDC Nº 216, de 15 de Setembro de 2004. Estabelece procedimentos de boas práticas para serviço de alimentação, garantindo as condições higiênico-sanitárias do alimento preparado. Diário Oficial da União, Brasília.

Brasil (2019). Ministério da saúde, Agência Nacional de Vigilância Sanitária - ANVISA. Instrução normativa n° 60, de 23 de dezembro de 2019. Listas de padrões microbiológicos para alimentos. Diário Oficial da União. Brasília.

Burt SA, Adolfse SJM, Ahad DSA, Tersteeg-Zijderveld MHG, Jongerius-Gortemaker BGM, Post JA, *et al*. (2016). Cinnamaldehyde, Carvacrol and Organic Acids Affect Gene Expression of Selected Oxidative Stress and Inflammation Markers in IPEC J2Cells Exposed to *Salmonella* Typhimurium. Phytotherapy Research, 30, 1988–2000. doi: 10.1002/ptr.5705.

Centers for Disease Control and Prevention (CDC) (2022). Department of health & human services, national center for emerging and zoonotic infectious diseases, division of foodborne, waterborne, and environmental diseases [online]. Available: https://www.cdc.gov/ Accessed date: August 2022.

Center for Food Safety and Applied Nutrition (CFSAN) (2003). Approximate pH of Foods and Food products. https://www.webpal.org/SAFE/aaarecovery/2_food_storage/Processing/lacf-phs.htm. Accessed date: August 2022.

Choi JI, Chae SJ, Kim JM, Choi JC, Park SJ, Choi HJ, Bae H, Park HJ (2018). Potential silver nanoparticles migration from commercially available polymeric baby products into food simulants. Food Additives & Contaminants: Part A, 35:5, 996–1005. doi.org/10.1080/19440049.2017.1411611.

Chue-Gonçalves M, Pereira GN, Faccin-Galhardi LC, Kobayashi RKT, Nakazato G (2021). Metal Nanoparticles against Viruses: Possibilities to Fight SARS-CoV-2. Nanomaterials, 11, 3118. https://doi.org/10.3390/nano11113118.

Clinical and Laboratory Standards Institute (CLSI) (2018). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard CLSI document M07-A10 (10th ed.). Wayne, PA: Clinical and Laboratory Standards Institute.

Dalir SJB, Djahaniani H, Nabati F, Hekmati M. (2020). Characterization and the evaluation of antimicrobial activities of silver nanoparticles biosynthesized from *Carya illinoinensis* leaf extract. Heliyon 6, e03624. doi.org/10.1016/j.heliyon.2020.e03624.

Dehkordi NH, Tajik H, Moradi M, Kousheh SA, Molaei R. (2019). Antibacterial Interactions of Colloid Nanosilver with Eugenol and Food Ingredients. Journal of Food Protection, 82, 1783–1792. doi.org/10.4315/0362-028X.JFP-19-174.

Di Veroli GY, Fornari C, Wang D, Mollard S, Bramhall JL, Richards FM, Jodrell DI (2016). Combenefit: an interactive platform for the analysis and visualization of drug combinations. Bioinf 32, 2866–2868. doi: 10.1093/bioinformatics/btw230.

Doern CD (2014). When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. J Clin Microbiol 52, 4124–4128. doi:10.1128/JCM.01121-14.

FDA/Food and Drug Administration (2018). Food additives permitted for direct addition to food for human consumption. U.S. Available from: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?FR=172.515, Accessed date: May 2022.

Ghosh IN, Deepak Patil S, Sharma TK, Srivastava SK, Pathania R, Navani NK (2013). Synergistic action of cinnamaldehyde with silver nanoparticles against spore-forming bacteria: a case for judicious use of silver nanoparticles for antibacterial applications. International Journal of Nanomedicine 2013:8 4721–4731. doi.org/10.2147/IJN.S49649.

Gopal A, Coventry J, Wan J, Roginski H, Ajlouni S (2010). Alternative disinfection techniques to extend the shelf life of minimally processed iceberg lettuce. Food Microbiology, 27(2):210-9. doi: 10.1016/j.fm.2009.10.006.

Graves JL Jr, Tajkarimi M, Cunningham Q, Campbell A, Nonga H, Harrison SH, Barrick JE (2015). Rapid evolution of silver nanoparticle resistance in Escherichia coli. Frontiers in Genetics, 17; 6:42. doi: 10.3389/fgene.2015.00042.

Isenberg HD (2004). Clinical Microbiology procedures handbook. Washington: ASM Press 488 (p. 5.10.2)

INSTITUTO ADOLFO LUTZ (1985). Normas Analíticas do Instituto Adolfo Lutz. v. 1: Métodos químicos e físicos para análise de alimentos. 3. ed. São Paulo: IMESP, p.181-182.

Kader AA, Morris MA, Stevens MA, Albright-Holton M (1978). Composition and flavor quality of fresh market as influenced by some postharvest handing procedures. Journal of the American Society for Horticultural Science. Alexandria, v. 103, n. 1, p. 6-11. doi.org/10.21273/JASHS.103.1.6.

Kelkawi AHA, Kajani AA, Bordbar A (2016). Green synthesis of silver nanoparticles using *Mentha pulegium* and investigation of their antibacterial, antifungal and anticancer activity. IET Nanobiotechnol., 11, 370-376. doi: 10.1049/iet-nbt.2016.0103.

Kobayashi RKT, Nakazato G (2020). Editorial: Nanotechnology for Antimicrobials. Front. Microbiol. 11:1421. doi: 10.3389/fmicb.2020.01421.

Li Q, Ren T, Perkins P (2022). The development and application of nanocomposites with pH-sensitive “gates” to control the release of active agents: Extending the shelf-life of fresh wheat noodles. Food Control, 132, 108563. doi.org/10.1016/j.foodcont.2021.108563.

Malheiro JF, Maillard JY, Borges F, Simões M (2019). Evaluation of cinnamaldehyde and cinnamic acid derivatives in microbial growth control. International Biodeterioration & Biodegradation, 141, 71–78. doi.org/10.1016/j.ibiod.2018.06.003

Nile SH, Baskar V, Selvaraj D, Nile A, Xiao J, Kai G (2020). Nanotechnologies in Food Science: Applications, Recent Trends, and Future Perspectives. Nano-Micro Letters, 12, 45. doi.org/10.1007/s40820-020-0383-9.

Onoda, Saulo Mitsuru. Pesquisa Qualitativa para o Tomate Sweet Hearts. Trabalho de Conclusão do Curso de MBA em Marketing, da Fundação Instituto de Administração - FIA, Universidade de São Paulo, 2010.

Pillai, SK, Moellering, RC, Eliopoulos, GM (2005). Antimicrobial combinations. In V. Lorian (Ed.). Antibiotics in laboratory medicine (pp. 365–440). (5th ed.). Philadelphia: Lippincott Williams & Wilkins Co.

Scandorieiro S, Camargo LC, Lancheros CAC, Yamada-Ogatta SF, Nakamura CV, Oliveira AG, Andrade CGTJ, Duran N, Nakazato G and Kobayashi RKT (2016). Synergistic and Additive Effect of Oregano Essential Oil and Biological Silver Nanoparticles against Multidrug-Resistant Bacterial Strains. Front. Microbiol. 7:760. doi: 10.3389/fmicb.2016.00760.

Ssemanda JN, Joosten H, Bagabe MC, Zwietering MH, Reij MW (2018). Reduction of microbial counts during kitchen scale washing and sanitization of salad vegetables. Food Control, 85, 495e503. doi.org/10.1016/j.foodcont.2017.10.004.

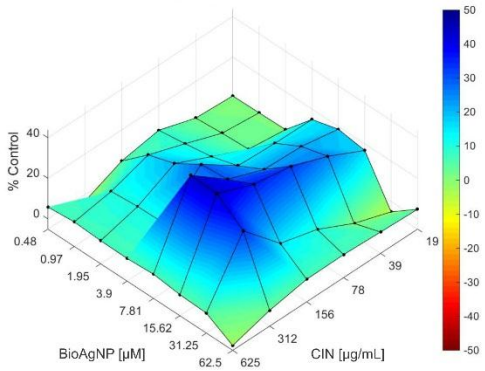
Upadhyay A, Upadhyay I, Mooyottu S, Kollanoor-Johny A, Venkitanarayanan K (2014). Efficacy of plant-derived compounds combined with hydrogen peroxide as antimicrobial wash and coating treatment for reducing *Listeria monocytogenes* on cantaloupes. Food Microbiology 44, 47e53. doi.org/10.1016/j.fm.2014.05.005.

Ye H, Shen S, Xu J, Lin S, Yuan Y, Jones G. (2013). Synergistic interactions of cinnamaldehyde in combination with carvacrol against food-borne bacteria. Food Control, 34, 619e623. doi.org/10.1016/j.foodcont.2013.05.032.

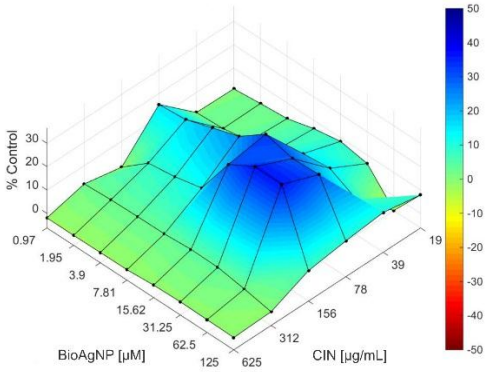
Zarei M, Jamnejad A, Khajehali E. (2014). Antibacterial Effect of Silver Nanoparticles Against Four Foodborne Pathogens. Jundishapur J Microbiol., 7(1): e8720. doi: 10.5812/jjm.8720.

Figure 1. The Bliss independence surface analysis for *in vitro* combinations CIN and BioAgNP against (A) *Escherichia coli* ATCC 25922; (B) *Salmonella* Typhimurium ATCC 14028; (C) *Staphylococcus aureus* ATCC 25923 by Bliss independence surface analysis

A.



B.



C.

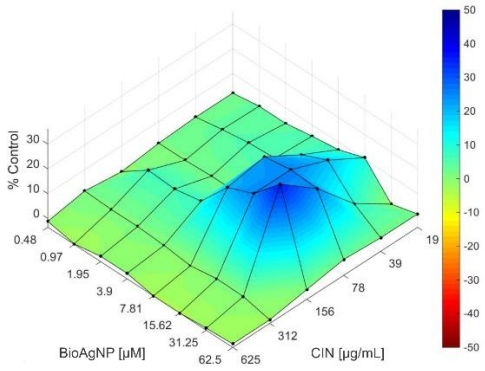
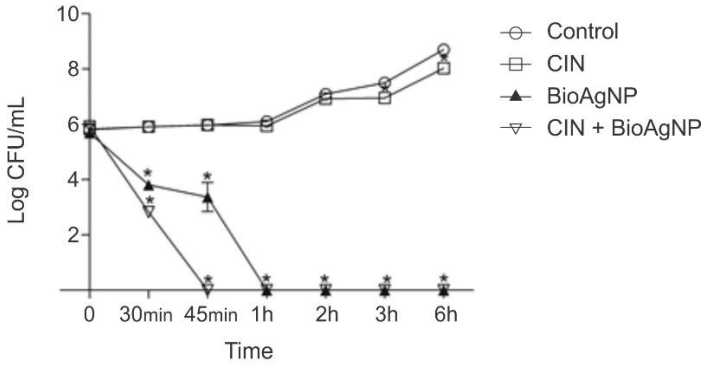
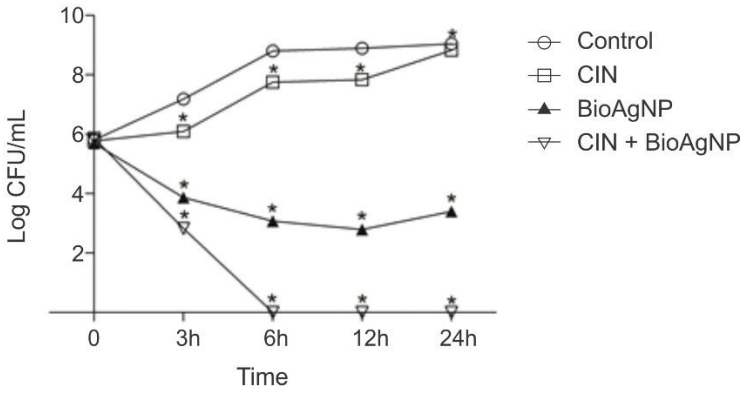


Figure 2. Time-kill curve assay of CIN (156 µg/mL), BioAgNP (31.25 µM) and CIN + BioAgNP (156 µg/mL + 31.25 µM). (A) *Escherichia coli* ATCC 25922; (B) *Salmonella* Typhimurium ATCC 14028; (C) *Staphylococcus aureus* ATCC 25923. *$p < 0.05$

A.



B.



C.

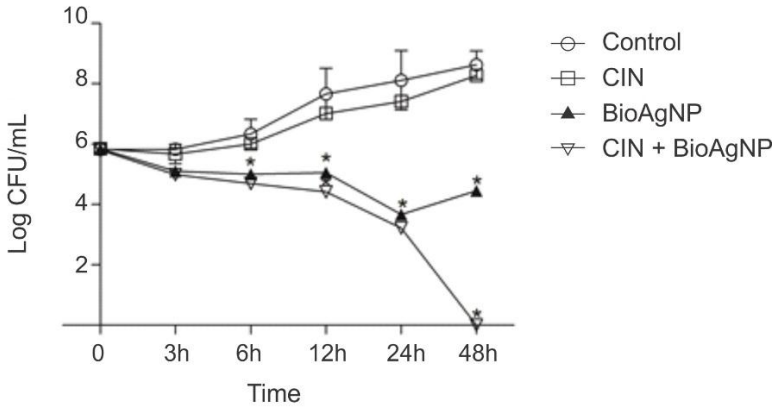


Table 1. Counts of *Escherichia coli* ATCC 25922 in sanitization of sweet grape tomatoes added from CIN and BioAgNP alone and in combination

Time (min)	Control	CIN	BioAgNP	CIN + BioAgNP
0	4.88 ± 0.00 ^{A}	4.85 ± 0.06 ^{A}	5.00 ± 0.00^{A}	4.70 ± 0.00 ^{A}
5	4.29 ± 0.07 ^{A}	3.85 ± 0.03 ^{B}	3.40 ± 0.05 ^{B}	ND
10	4.15 ± 0.21 ^{A}	3.77 ± 0.32 ^{A}	2.29 ± 0.57 ^{B}	ND
15	4.50 ± 0.04 ^{A}	3.79 ± 0.12 ^{B}	ND	ND
30	5.22 ± 0.05 ^{A}	3.58 ± 0.09 ^{B}	ND	ND
60	5.37 ± 0.03 ^{A}	3.81 ± 0.02 ^{B}	ND	ND

Values are mean log CFU/g followed by standard deviation. Means in the same line with different letters are significantly different ($p < 0.05$; Tukey's test). Control (without treatment); CIN (156 µg/mL); BioAgNP (31.25 µM) and CIN + BioAgNP (156 µg/mL + 31.25 µM). ND: not detected.

Table 2. Counts of *Escherichia coli* ATCC 25922 in shelf life of sweet grape tomatoes added from CIN and BioAgNP alone and in combination

Time (days)	Control	CIN	BioAgNP	CIN + BioAgNP
1	5.14 ± 0.04^{A}	3.71 ± 0.01^{B}	ND	ND
2	6.31 ± 0.02^{A}	2.89 ± 0.04^{B}	ND	ND
3	6.21 ± 0.04^{A}	2.36 ± 0.06^{B}	ND	ND
5	6.27 ± 0.17^{A}	2.28 ± 0.02^{B}	ND	ND
7	6.12 ± 0.03^{A}	2.84 ± 0.02^{B}	ND	ND

Values are mean log CFU/g followed by standard deviation. Means in the same line with different letters are significantly different ($p < 0.05$; Tukey's test). Control (without treatment); CIN (156 µg/mL); BioAgNP (31.25 µM) and CIN + BioAgNP (156 µg/mL + 31.25 µM). ND: not detected.

Table 3. Physicochemical analysis of sweet grape tomatoes added from CIN and BioAgNP alone and in combination

Group	pH			Soluble Solids (ºBrix)			Titratable acidity (%)		
	0	4	7	0	4	7	0	4	7
Control	4.19 ± 0.01^{A}	4.36 ± 0.01^{A}	4.19 ± 0.01^{A}	8.50 ± 0.00^{A}	8.30 ± 0.00^{A}	7.90 ± 0.00^{A}	9.26 ± 0.15^{A}	7.95 ± 0.00^{A}	9.22 ± 0.11^{A}
CIN	4.16 ± 0.01^{A}	4.33 ± 0.02^{A}	4.23 ± 0.02^{A}	8.50 ± 0.00^{A}	8.30 ± 0.00^{A}	7.90 ± 0.00^{A}	9.37 ± 0.17^{A}	8.60 ± 0.14^{A}	8.62 ± 0.15^{A}
BioAgNP	4.18 ± 0.01^{A}	4.34 ± 0.00^{A}	4.28 ± 0.01^{A}	8.50 ± 0.00^{A}	8.30 ± 0.00^{A}	7.90 ± 0.00^{A}	9.04 ± 0.17^{A}	8.36 ± 0.01^{A}	8.55 ± 0.21^{A}
CIN+BioAgNP	4.16 ± 0.01^{A}	4.33 ± 0.01^{A}	4.20 ± 0.02^{A}	8.50 ± 0.00^{A}	8.30 ± 0.00^{A}	7.90 ± 0.00^{A}	9.19 ± 0.05^{A}	9.17 ± 0.00^{A}	8.75 ± 0.01^{A}

Values are mean log CFU/g followed by standard deviation. Means in the same column with different letters are significantly different ($p < 0.05$; Tukey's test). Control (without treatment); CIN (156 µg/mL); BioAgNP (31.25 µM) and CIN + BioAgNP (156 µg/mL + 31.25 µM). ND: not detect