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Avaliação da atividade antibacteriana e esporicida dos óleos essenciais de *Copaifera multijuga, Thymus vulgaris* e nisina frente à *Alicyclobacillus acidoterrestris*

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

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Orientador

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BIOGRAFIA

Angela Aparecida da Silva nasceu em 26 de outubro de 1980 na Cidade de Taboão da Serra-SP. Possui graduação em Ciências Biológicas pela Faculdade de Filosofia, Ciências e Letras de Mandaguari – FAFIMAN, no ano de 2012.

Dedico...

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"*Todo mundo ama um dia*
Todo mundo chora
Um dia a gente chega
E no outro vai embora

Cada um de nós compõe a sua história
Cada ser em si
Carrega o dom de ser capaz
E ser feliz."

Almir Sater

APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um (01) artigo científico.

Angela Aparecida da Silva, Idinea Fernandes dos Santos, Márcia Maria dos Anjos, Suelen Pereira Ruiz, Jane Martha Graton Mikcha, Miguel Machinski Junior, Tânia Ueda Nakamura, Celso Vataru Nakamura, Benício Alves de Abreu Filho, artigo intitulado por “Avaliação da atividade antibacteriana e esporicida dos óleos essenciais de *Copaifera multijuga, Thymus vulgaris* e nisina frente à *Alicyclobacillus acidoterrestris*”. Revista Food Control.

GENERAL ABSTRACT

INTRODUCTION. *Alicyclobacillus acidoterrestris* was first isolated in 1980 from apple juice. The name *A. acidoterrestris* was associated with the cyclic fatty acids that form its cell membrane and increase the resistance of its spores to thermal treatments, this behavior may also be related to the reduced membrane permeability in this species. *A. acidoterrestris* is a Gram-positive bacillus that is thermoacidophilic, aerobic, food spoiling, non-pathogenic and catalase positive with terminal or subterminal spores. Develops well in acid media (pH from 2.5 to 6.0) and at high temperatures (from 20 to 60 ºC). One of the important characteristics of *A. acidoterrestris* is its ability to spoil acidic fruit juices, such as orange juice. Spoilage caused by *A. acidoterrestris* occurs because of the production of 2-methoxyphenol (guaiacol), 2,6-dibromophenol and 2,6 dichlorophenol, which are substances associated with the unpleasant taste and odor of contaminated food. This spoilage process is characterized by a lack of gas production, low turbidity and sedimentation; thus, it is difficult to detect. The pasteurization process contributes to reducing pathogens and spoilage microorganisms, improving food safety and reducing chemical preservatives. However, spore-forming microorganisms can resist the highest temperatures used in pasteurization, and under appropriate conditions, these microorganisms develop inside packaged products, thus reducing the shelf life and profitability of affected products. Brazil is a major exporter of orange juice, and it accounts for 50% of the global production. To ensure orange juice quality, research is required to develop new antimicrobial agents that can contribute to food preservation by increasing the shelf life and preserving the sensory and nutritional characteristics of the juice, making it healthier. The demand for natural antimicrobial agents has increased because of the popularity of green consumerism and related concerns by consumers and regulatory agencies for the health and safety of food products. Several studies have indicated the potential of essential oils (EOs) and their isolates and nisin alone or in combination as antibacterial agents because of their bacteriostatic, bactericidal and/or sporicidal properties.

AIMS. Therefore, this study aimed to evaluate the antibacterial activity of the essential oils of *Copaifera multijuga* and *Thymus vulgaris* against the vegetative and sporulated forms of *Alicyclobacillus acidoterrestris* and assess the combined effects of the EOs and nisin.

MATERIAL AND METHODS. Bacterial strain and growth conditions: The *A. acidoterrestris* strains (CBMAI 0244^{T} – source: soil). *Bacillus acidoterrestris* (BAT) culture medium with a final pH adjusted to 4.0. Inoculum preparation: The vegetative cells of *A. acidoterrestris* procedure was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) M7-A9. *A. acidoterrestris* spores was performed and stored in sterile deionized water at 4 ºC until used. Essential oils: The *T. vulgaris* (thyme) EO was provided by the Laboratory of Toxicology (Laboratório de Toxicologia) of UEM, state of Paraná, Brazil. The EO of *C. multijuga* (Amazonas) was provided by the Laboratory of Microbiology of Natural and Synthetic Products (Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos) of the Department of Basic Health Sciences (Departamento de Ciências Básicas da Saúde) of UEM. Nisin: Nisin was commercially purchased, and a stock solution was prepared in 0.02 M hydrochloric acid (HCl) and sterilized in a 0.22 µm membrane. Determination of the antibacterial activity of the essential oils: The antibacterial

activity of the EOs was determined as recommended by the CLSI M7-A9. It was possible to evaluate the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum sporicidal concentration (MSC). Checkerboard method: The checkerboard method is widely used for in vitro evaluations of the combined antibacterial activity of two drugs as antibacterial agent. The microdilution was performed as recommended by Schelz, Molnar and Hohmann (2006). Interpretation of the results was performed as Gutierrez, Barry-Ryan and Bourke (2008). Death time curve: This assay was performed as Ruiz et al. (2013) and shows the bacterial decay time in contact with antimicrobial agents. Dose-response effect: The determination of 50% inhibitory concentration (IC_{50}) of vegetative cells of *A. acidoterrestris* has been accomplished through serial as microdiluições Tanaka et al. (2006). Cell viability assay: The cytotoxic activity of the EOs was evaluated by the MTT colorimetric method as described by Mosmann (1983). The EOs' cytotoxicity to the Vero cells was compared to the selectivity index (SI), which was determined by dividing the 50% cytotoxic concentration to the Vero cells (CC_{50}) by the 50% inhibitory concentration to the bacteria (IC_{50}). Scanning electron microscopy: In this assay, it was possible to determine the morphological alterations of the vegetative cells and spores of *A. acidoterrestris* by comparison with a negative control (no treatment). This methodology was performed as Haddad et al. (2007). Flow cytometry: In this test was determined and the survival rates of cell membrane integrity as Anjos et al. (2013).

RESULTS AND DISCUSSION. Determination of the antibacterial activity of the essential oils: The MIC and MBC of the EOs of *C. multijuga* and *T. vulgaris* were determined for the vegetative cells and spores of *A. acidoterrestris*. For both EOs, the best effectiveness was obtained against the vegetative cells, resulting in moderate antibacterial activity (concentrations ranging from 100 to 500 µg mL^{-1}). The EOs had a weak antibacterial activity against *A. acidoterrestris* spores (concentrations ranged from 500 to 1,000 µg mL^{-1}). The resistance of spores to treatment with EOs may be related to the presence of dipicolinic acid found in the endospores. This characteristic confers high resistance to the spores to thermal and chemical treatments (Paredes-Sabja, Setlow and Sarker, 2011). The MIC and MBC of the EO of *C. multijuga* against the vegetative forms of *A. acidoterrestris* were 300 µg mL^{-1} and > 1,000 µg mL^{-1}, respectively. For the spores, a reduction of 3.07 log CFU mL^{-1} was observed when they were treated with 500 µg mL^{-1}. The MIC and MBC of the EO of *T. vulgaris* against the vegetative forms of *A. acidoterrestris* were 500 µg mL^{-1} and > 1,000 mL^{-1}, respectively. For treatments using spores, reductions of 0.64 log CFU mL^{-1} and 2.05 log CFU mL^{-1} were observed when 500 µg mL^{-1} and 1,000 µg mL^{-1} EO were used, respectively. Nisin exhibited good bacteriostatic, bactericidal and sporicidal activity against *A. acidoterrestris*. The MIC and MBC for the vegetative forms were 15.60 µg mL^{-1} and 31.25 µg mL^{-1}, respectively. The MSC with total elimination of the spores was reached at 62.50 µg mL^{-1}. The results observed in our assays were similar to the results of Ruiz et al. (2013). Checkerboard method: The EO evaluations (*C. multijuga* and *T. vulgaris*) in combination with nisin were performed through the checkerboard method. For both treatments (*C. multijuga* + nisin or *T. vulgaris* + nisin), the FIC index was 0.75, and the combination of EOs + nisin produced an additive effect. Nisin and EOs have great potential for use as antibacterial agents of natural origin, and their combined effect provides a promising alternative for controlling several microorganisms and for use in applications in food matrices as preservatives, can be used as food preservatives. Death time curve: The

death time curve method was conducted to assess the antibacterial activity of the EOs. At a concentration of 8x MIC *C. multijuga* EO, there was complete reduction of the bacterial load of *A. acidoterrestris* in the first three hours after treatment. With concentrations of 4x MIC and 2x MIC, a longer treatment was necessary, with 48 h required to eliminate the vegetative forms of *A. acidoterrestris*. With 1x MIC for 24 h, a reduction of approximately 4.65 log CFU mL^{-1} was obtained. The treatment with *T. vulgaris* EO exhibited a smaller reduction compared with the treatment using the *C. multijuga* EO. Total bacterial elimination was reached after 48 h of treatment using the concentration of 8x MIC. In the treatment using 1x MIC for 24 h and 48 h, there was a reduction of 3.81 log CFU mL^{-1} and approximately 4.11 Log CFU $mL^{-1,}$ respectively. Dose-response effect and cell viability: The results obtained in this assay revealed that the EOs of *C. multijuga* and *T. vulgaris* had CC_{50} values of 54.2 µg mL^{-1} and 142.3 µg mL^{-1}, respectively, causing a reduction of 50% of the viable Vero cells at these concentrations. The IC_{50} results of *A. acidoterrestris* using the EOs of *C. multijuga* and *T. vulgaris* were 500 µg mL^{-1} and 1,000 µg mL^{-1}, respectively, and there was 50% inhibition of bacterial growth with these concentrations. A comparison of the CC_{50} with IC_{50} showed that the EOs were less selective to *A. acidoterrestris* and more toxic to Vero cells. The selectivity index (SI) of the EO of *C. multijuga* and *T. vulgaris* were 0.11 and 0.14, respectively. Scanning electron microscopy: In this trial we observed morphological changes in both external and cellular forms treatments compared to the control. Flow cytometry: In this assay, the vegetative cells of *A. acidoterrestris* treated with the EOs of *C. multijuga* and *T. vulgaris* at their respective MICs had a higher percentage of cells with alterations in the cell membrane integrity compared with cells of the negative control. Therefore, we cannot conclude that the main mechanism of action of the EOs is changes in the cell membrane because the percentage of cells with membrane alterations was low in all treatments, with values ranging from 29.94% to 4.24%.

CONCLUSIONS. Our studies showed that the tested substances had good antibacterial properties because they reduced the viability of *A. acidoterrestris*. Thus, it is evident that the EOs of *C. multijuga* and *T. vulgaris* have potential for use as antibacterial agents against *A. acidoterrestris*.

Key words: Antibacterial, *Copaifera multijuga*, *Thymus vulgaris*, *Alicyclobacillus acidoterrestris*, orange juice.

RESUMO GERAL

INTRODUÇÃO. *Alicyclobacillus acidoterrestris* foi isolado primeiramente em 1980 do suco de maçã. Seu nome foi associado aos ácidos graxos cíclicos constituintes de sua membrana celular, característica que tornam seus esporos mais resistentes ao tratamento térmico, este fator pode estar relacionado a redução da permeabilidade da membrana. *A. acidoterrestris* é um bacilo Gram-positivo, ácido-termofílico, aeróbico, catalase positiva, formador de esporos terminal ou subterminal, deteriorante de alimentos e não patogênico. Possui bom desenvolvimento em ambientes acidificados (com pH entre 2,5 a 6,0) e altas temperatura (entre 20 a 60 ºC). Uma das características importante do *A. acidoterrestris* é deteriorar sucos de frutas ácidas, como o de laranja. A deterioração por *A. acidoterrestris* ocorre devido a produção de 2-metoxifenol (guaiacol), 2,6-dibromofenol e 2,6 diclorofenol, substâncias associadas ao sabor e odor desagradáveis presentes em alimentos contaminados. Sua deterioração é caracterizada pela falta de produção de gases, baixa turbidez e sedimentação, sendo assim difícil de ser detectada. O processo de pasteurização contribui com a redução de micro-organismos patogênicos e deteriorantes, auxiliando na segurança alimentar e favorecendo uma redução na aplicação de conservantes químicos. No entanto, os micro-organismos esporulados conseguem resistir às temperaturas mais elevadas aplicadas neste processo, e em condições adequadas se desenvolvem dentro dos produtos já embalados, diminuindo a vida de prateleira, causando perdas e prejuízos econômicos. O Brasil é um grande exportador de suco de laranja, responsável por 50% da produção. Para garantir a qualidade do suco de laranja, há uma grande necessidade de pesquisas que favoreçam o desenvolvimento de novos agentes antimicrobianos que possam contribuir com a preservação do alimento, aumentando sua vida de prateleira, garantindo a conservação de suas características sensoriais e nutricionais tornando-os mais saudáveis para o consumo. A procura de agentes antimicrobianos naturais aumentou por causa da popularidade do consumismo verde e preocupações relacionadas por parte dos consumidores e agências reguladoras para a saúde e segurança dos produtos alimentares. Muitos trabalhos mostram a eficiência dos óleos essenciais (OEs) e seus isolados, assim como o emprego da nisina, com grande potencialidade como agente antibacteriano, aplicados sozinhos ou em ação conjunta, exercendo efeitos bacteriostático, bactericida e/ou esporicida.

OBJETIVOS. Diante do exposto, este trabalho teve como principal objetivo avaliar a atividade antibacteriana dos óleos essenciais de *Copaifera multijuga* e *Thymus vulgaris* frente às formas vegetativa e esporulada de *Alicyclobacillus acidoterrestris*, assim como verificar sua ação combinada à nisina.

MATERIAL E MÉTODOS. Cepa bacteriana e condições de cultura: A linhagen-tipo utilizada nos ensaios foram *A. acidoterrestris* (CBMAI 0244^{T} – origem: Solo). O meio de cultura utilizado foi o BAT (*Bacillus acidoterrestris*) com pH final ajustado em 4,0. Preparação dos inóculos: A suspensão da célula vegetativa de *A. acidoterrestris* foi preparada conforme preconiza o CLSI M7-A9. Os esporos de *A. acidoterrestris*, foram preparados e armazenados em água deionizada estéril em ambiente refrigerado a 4 ºC até sua utilização. Óleos essenciais: O óleo essencial (OE) de *T. vulguris* (tomilho) foi obtido do Laboratório de Toxicologia da Universidade Estadual de Maringá – Paraná – Brasil. O OE de *C. multijuga* (Amazonas) foi obtido do Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos Ciências

Básicas da Saúde da Universidade Estadual de Maringá – Paraná – Brasil. Nisina: A solução estoque de nisina purificada adquirida comercialmente, foi preparada em ácido clorídrico (HCl) na concentração de 0,02 M e esterilizada em membrana de 0.22 µm. Determinação da atividade antibacteriana dos óleos essenciais: A atividade antibacteriana dos OEs foram determinadas por meio de microdiluições seriadas conforme preconiza a CLSI M7-A9. Foi possível avaliar a Concentração Inibitória Mínima (CIM), a Concentração Bactericida Mínima (CBM) e a Concentração Esporicida Mínima (CEM). Método *checkerboard*: Este método é muito utilizado em avaliações *in vitro* da combinação de duas drogas como agente antibacteriano. As microdiluições foram realizadas conforme Schelz, Molnar e Hohmann (2006). A interpretação dos resultados foi realizada conforme Gutierrez, Barry-Ryan e Bourke (2008). Curva de tempo de morte: Este ensaio foi realizado conforme Ruiz et al. (2013) e mostra o tempo de declínio da bactéria em contato com os agentes antimicrobianos. Efeito dose resposta: A determinação da concentração inibitória de 50% (IC_{50}) das células vegetativa de *A. acidoterrestris* foi realizada por meio de microdiluições seriadas conforme Tanaka et al. (2006). Ensaio da viabilidade celular: A atividade citotóxica dos OEs foram avaliados pelo método colorimétrico MTT conforme descrito por Mosmann (1983). A citotoxicidade perante às células VERO foi comparada ao Índice de seletividade (SI) determinada pela razão entre a concentração citotóxica de 50% (CC_{50}) para as células VERO e a concentração inibitória de 50% (IC_{50}) das bactérias. Microscopia eletrônica de varredura: Neste ensaio foi possível verificar as alterações morfológicas das células vegetativas e dos esporos de *A. acidoterrestris* por meio da comparação com o controle negativo (sem tratamento). Esta metodologia foi realizada conforme Haddad et al. (2007). Citometria de fluxo: Neste ensaio foi determinada as taxas de sobrevivência e integridade da membrana celular conforme Anjos et al. (2013).

RESULTADOS E DISCUSSÃO. Determinação da atividade antibacteriana dos óleos essenciais: A CIM e a CBM dos OEs de *C. multijuga* e *T. vulgaris* foram determinadas frente às formas vegetativas e esporuladas de *A. acidoterrestris*. Para ambos os tratamentos, os melhores resultados foram obtidos frente às formas vegetativas com atividade antibacteriana moderada (valores entre 100 a 500 µg mL^{-1}). Os EOs apresentaram uma atividade antibacteriana fraca contra os esporos de *A. acidoterrestris* (valores entre 500 a 1000 µg mL^{-1}). A resistência dos esporos ao tratamento com os OEs, podem estar relacionada a presença do ácido dipicolínico encontrados nos endoesporos, essa característica lhes confere alta resistência a tratamentos térmicos e químicos (Paredes-Sabja, Setlow e Sarker, 2011). A CIM e a CBM do OE de *C. multijuga* frente às formas vegetativas de *A. acidoterrestris* foram de 300 µg mL^{-1} e >1.000 µg mL^{-1}, respectivamente. Para os esporos houve uma redução de 3,07 Log UFC mL^{-1} quando tratados em concentrações de 500 µg mL^{-1}. A CIM e a CBM para o óleos essencial de *T. vulgaris* frente as formas vegetativas de *A. acidoterrestris* foi determinada em 500 µg mL^{-1} e >1.000 mL^{-1} respectivamente. Para tratamentos utilizando os esporos foram observadas reduções de 0,64 Log UFC mL^{-1} e 2,05 Log UFC mL^{-1}, quando tratados em concentrações de 500 µg mL^{-1} e 1.000 µg mL^{-1}, respectivamente. A nisina exibiu boa atividade bacteriostática, bactericida e esporicida frente à *A. acidoterrestris*. O CIM e a CBM para as formas vegetativa foram de 15,60 µg mL^{-1} e 31,25 µg mL^{-1} respectivamente. A CEM foi alcançada na concentração de 62,50 µg mL^{-1} com eliminação completa dos esporos. Os resultados presente em nossos ensaios foram semelhantes aos encontrados por Ruiz et al. (2013). Método *checkerboard*: A avaliação dos OEs (*C. multijuga* e *T.*

vulgaris) em combinação com a nisina foram realizados por meio da microdiluição pelo método *checkerboard*. Para ambos os tratamentos o índice FIC obtido foi de 0,75, no qual produziu um efeito aditivo. A nisina e os OEs possuem grande potencialidade como agentes antibacterianos de origem naturais, e seus efeitos combinados são alternativas promissoras frente a diversos micro-organismos e com aplicações em matrizes alimentares, podendo ser utilizados como conservantes de alimentos. Curva de tempo de morte: A curva de morte foi realizada para investigar a atividade antibacteriana dos OEs. Na concentração de 8x a CIM do OE de *C. multijuga* houve redução completa da carga bacteriana de *A. acidoterrestris* nas três primeiras horas de tratamento. Em valores de 4x e 2x a CIM houve a necessidade de um tratamento mais prolongado, com duração de 48 h para eliminar as formas vegetativas de *A. acidoterrestris*. Tratamento empregando 1x a CIM após 24 h foi possível reduzir aproximadamente 4,65 Log UFC mL^{-1}. Para o tratamento utililizando o OE de *T. vulgaris* houve uma menor redução quando comparado ao tratamento utilizando o OE de *C. multijuga*. A redução bacteriana completa foi obtida após 48 h de tratamento na concentração de 8x o CIM. No tratamento empregando a concentração de 1x o CIM após 24 h houve uma redução de 3,81 Log UFC mL^{-1} e após 48 h a redução foi de aproximadamente 4,11 Log UFC mL^{-1}. Efeito dose resposta e viabilidade celular: Os resultados obtidos neste ensaio revelaram que os OEs de *C. multijuga* e *T. vulgaris* apresentaram valores de CC_{50} de 54,20 µg mL^{-1} e 142,3 µg mL^{-1} respectivamente, provocando uma redução de 50% das células VERO viáveis nestas concentrações. O resultado do IC_{50} para *A. acidoterrestris* utilizando os tratamentos com os OEs de *C. multijuga* e *T. vulgaris* foi de 500 µg mL^{-1} e 1.000 µg mL^{-1}, respectivamente, sendo que nestas concentrações houve uma inibição do crescimento bacteriano em 50%. Quando comparado o CC_{50} e o IC_{50} os resultados foram menos seletivos para *A. acidoterrestris* e mais tóxicos para as células VERO. O índice de seletividade (IS) para o OE de *C. multijuga* foi de 0,11 e para *T. vulgaris* foi de 0,14, respectivamente. Microscopia eletrônica de varredura: Neste ensaio foi possível observar alterações morfológicas externas em ambos tratamentos e formas celulares quando comparadas ao controle. Citometria de fluxo: Neste ensaio foi possível observar que as células vegetativas de *A. acidoterrestris* tratadas com os OEs de *C. multijuga* e *T. vulgaris* em suas respectivas CIMs tiveram uma porcentagem maior de células com alterações na integridade da membrana celular quando comparadas ao controle negativo. Enquanto que os esporos tratados com os OEs de *C. multijuga* e *T. vulgaris* nas concentrações de 500 µg mL^{-1} e 1.000 mL^{-1}, respectivamente, não apresentaram alterações quando comparados ao controle negativo. No entanto, não podemos concluir que o principal mecanismo de ação dos OEs seja alterações na membrana celular, pois a porcentagem de células com alterações na membrana foram baixas em todos os tratamentos, onde a variação foi de 29,94% a 4,24%.

CONCLUSÕES. Nossos estudos mostraram que as substâncias testadas apresentaram boas propriedades antibacterianas porque reduziram a viabilidade de *A. acidoterrestris*. Desta maneira, fica evidente que os CEs da *C. multijuga* e *T. vulgaris* têm potencial para utilização como agentes antibacterianos frente *A. acidoterrestris*.

Palavras-chave: Antibacterianos, *Copaifera multijuga*, *Thymus vulgaris*, *Alicyclobacillus acidoterrestris*, Suco de laranja.

ARTICLE

Evaluation of the antibacterial and sporicidal activity of the essential oils of *Copaifera multijuga and Thymus vulgaris* and nisin against *Alicyclobacillus acidoterrestris*

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Abstract

This study aimed to evaluate the bactericidal, bacteriostatic and sporicidal activity of the essential oils (EOs) of *Copaifera multijuga* and *Thymus vulgaris* against *Alicyclobacillus acidoterrestris*. The combined activity of the EOs and nisin was also assessed by determining the fractional inhibitory concentration (FIC). In addition, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum sporicidal concentration (MSC) were evaluated by serial microdilution in Bacillus acidoterrestris (BAT) broth with subcultures in BAT agar. The MIC of the *C. multijuga* and *T. vulgaris* EOs were 300 µg mL^{-1} and 500 µg mL^{-1}, respectively, and the MBC of both EOs was >1,000 µg mL^{-1}. The best MSC of *C. multijuga* was reached at a concentration of 500 µg mL^{-1} with a reduction of 3.07 log CFU mL^{-1}. For the EO *of T. vulgaris*, better results were observed at concentrations higher than 500 µg mL^{-1}, and a reduction of 2.05 log CFU mL^{-1} was achieved at 1,000 µg mL^{-1}. The checkerboard method showed that the combination of EOs and nisin had an additive interaction (FIC index of 0.75). The bactericidal activity was confirmed by the death curve. According to the selectivity index, the treatment was less selective for *A. acidoterrestris* than for Vero cells. The flow cytometry results indicated that the vegetative forms of *A. acidoterrestris* had a higher incidence of vegetative cells with alterations in cell membrane integrity compared to their spores; however, we cannot conclude that this is the main mechanism of action of the EOs. Our studies showed that the tested substances had good antibacterial properties because they reduced the viability of *A. acidoterrestris*. Thus, it is evident that the EOs of *C. multijuga* and *T. vulgaris* have potential for use as antibacterial agents against *A. acidoterrestris*.

Keywords: Antibacterial, *Copaifera multijuga*, *Thymus vulgaris*, *Alicyclobacillus acidoterrestris*, orange juice.

1. Introduction

Alicyclobacillus acidoterrestris, which is a species of spore-forming *Bacillus* acidophilic originally named *Bacillus acidoterrestris,* was first isolated in 1980 from apple juice. Recently, it has been reclassified to the genus *Alicyclobacillus* (Deinhard et al., 1987; Wisotzkey et al., 1992). The name *A. acidoterrestris* was associated with the cyclic fatty acids that form its cell membrane and increase the resistance of its spores to thermal treatments; this behavior may also be related to the reduced membrane permeability in this species (Kawase et al., 2008; Komitopoulou et al., 1999).

A. acidoterrestris is a Gram-positive bacillus that is thermoacidophilic (Karavaiko et al., 2005), aerobic, food spoiling, non-pathogenic and catalase positive with terminal or subterminal spores. *A. acidoterrestris* develops well in acid media (pH from 2.5 to 6.0) and at high temperatures (from 20 to 60 ºC) (Kawase et al., 2008; Walker and Philips, 2008). It preferably develops in foods with a low pH, such as sports drinks, ice tea and fruit juice. There are reports of isolation of *Alicyclobacillus* spp. from banana, guava, papaya and passion fruit puree, pineapple and grape concentrate, coconut cream, and mango concentrate and puree (Danyluk et al., 2011; Sokolowska, Niezgoda and Chotkiewicz, 2013).

One of the important characteristics of *A. acidoterrestris* is its ability to spoil acidic fruit juices, such as orange juice. Its sporulated form is naturally found in cultivation environments (orchards), and this bacterium can be easily introduced into the industrial processes. The forms of contamination of *A. acidoterrestris* are associated with processing, transportation or handling of products; thus strict control of the raw material in food processing is required because even small quantities are enough to cause product spoilage (Danyluk et al., 2011; Walker and Philips, 2008).

Spoilage caused by *A. acidoterrestris* occurs because of the production of guaiacol (2-methoxyphenol), 2,6-dibromophenol and 2,6 dichlorophenol, which are substances associated with the unpleasant taste and odor of contaminated food. This spoilage process is characterized by a lack of gas production, low turbidity and sedimentation; thus, it is difficult to detect (Bevilacqua; Sinigaglia; Corbo, 2008; Danyluk et al., 2011; Komitopoulou et al., 1999; Sokolowska, Niezgoda and Chotkiewicz, 2013; Witthuhn et al., 2012).

Several studies have been performed to reduce contamination by *A. acidoterrestris* during the early stages of orange juice processing (Anjos et al., 2013; Walker and Philips, 2008).

The pasteurization process contributes to reducing pathogens and spoilage microorganisms, improving food safety and reducing chemical preservatives (Silva et al., 2014). However, spore-forming microorganisms can resist the highest temperatures used in pasteurization, and under appropriate conditions, these microorganisms develop inside packaged products, thus reducing the shelf life and profitability of affected products.

Brazil is a major exporter of orange juice with 98% of its orange juice production exported, and it accounts for 50% of the global production and 85% of the global market share of orange juice. Orange juice accounted for 56% of the world production of agricultural products in 2009 (Citrus, 2014).

To ensure orange juice quality, research is required to develop new antimicrobial agents that can contribute to food preservation by increasing the shelf life and preserving the sensory and nutritional characteristics of the juice, making it healthier.

The demand for natural antimicrobial agents has increased because of the popularity of green consumerism and related concerns by consumers and regulatory agencies for the health and safety of food products.

Several studies have indicated the potential of essential oils (EOs) and their isolates and nisin alone or in combination as antibacterial agents because of their bacteriostatic, bactericidal and/or sporicidal properties (Bevilacqua, Sinigaglia and Corbo, 2008; Ruiz et al., 2013; Tajkarimi, Ibrahim and Cliver, 2010; Walker and Philips, 2008). However no data were found in the literature that reported the use of essential oils of *T. vulgaris* and *C. multijuga*, alone or combined nisin against *A. acidoterrestris*.

EOs have specific functions in plant development and growth, and they can be extracted from different plant parts (Tajkarimi, Ibrahim and Cliver, 2010). The genus *Copaifera* is found throughout Brazil. *Copaifera multijuga* belongs to the family *Leguminosae* and is a large tree found in the states of Amazonas, Acre, Rondônia, Roraima and Mato Grosso (Mendonça and Onofre, 2009). *Thymus vulgaris* is a medicinal aromatic plant native to the western Mediterranean, and it belongs to the family *Lamiaceae*, which is economically important in North America, Europe, North

Africa and Asia. In addition, it is widely cultivated as a spice in temperate regions (Alçiçek, 2011; Letchamo and Gosselin, 1996).

Nisin is a bacteriocin produced by *Lactococcus lactis* subsp *lactis,* and it has antimicrobial effects against a wide range of Gram-positive bacteria, including *A. acidoterrestris*. Nisin is approved by the Food and Drug Administration (FDA) as a food preservative, and it is generally recognized as safe (GRAS) (Komitopoulou et al., 1999; Stevens et al., 1991; Walker and Philips, 2008; Yamazaki et al., 2000).

Therefore, this study aimed to evaluate the antibacterial activity of the EOs of *C. multijuga* and *T. vulgaris* against the vegetative and sporulated forms of *A. acidoterrestris* and assess the combined effects of the EOs and nisin.

2. Material and Methods

2.1. Bacterial strain and growth conditions

The *A. acidoterrestris* strains (CBMAI 0244^{T} – source: soil) were provided by the CBMAI (Brazilian Collection of Environment and Industry Microorganisms). *Bacillus acidoterrestris* (BAT) culture medium (Deinhard et al., 1987) with a final pH adjusted to 4.0 with 1 M NaOH and 1 M HCl solutions was used in our assays. The strains were stored in cryovials containing BAT medium with 30% glycerol (Thermo Fisher Scientific, Waltham, MA) at -20 ºC in the Laboratory of Water, Environment and Food Microbiology (Laboratório de Microbiologia da Água, Ambiente e Alimentos) of Maringá State University (Universidade Estadual de Maringá – UEM). The bacterium/stock used in the assays were maintained in petri dish (Inlab, Interlab, São Paulo, Brazil) with BAT agar at 4 ºC, and a subculture was maintained in BAT medium at 45 ºC to assess the bacterial viability in every test performed.

2.2. Inoculum preparation

The vegetative cells of *A. acidoterrestris* were suspended in 0.85% sterile saline solution, until turbidity equivalent 0.5 McFarland scale with subsequent dilution to obtain an inoculum with a final concentration of 10^4 CFU mL^{-1} in each well of the microplate. This procedure was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) M7-A9 (2012).

For the preparation of *A. acidoterrestris* spores, the inoculum was cultured in test tubes containing BAT broth (5 mL) and incubated at 45 ºC for 120 h for spore formation until 80% sporulation. The sporulation index was evaluated by direct counting using a phase contrast microscope. Subsequently, the spores were transferred to microtube, centrifuged at 9,500 g for 3 minutes, and then rinsed in sterile deionized water three times. Serial microdilution was then performed followed by thermal shock in a hot bath (Nova Técnica, Piracicaba, Brazil) at 80 ºC for 10 min to count the viable cells of the spore suspension using the spread plate method to determine the CFU mL^{-1}. The spores were stored in sterile deionized water at 4 ºC until used.

For the minimum sporicidal concentration (MSC) assays, the spore suspension was adjusted to 10^7 CFU mL^{-1} in 0.85% sterile saline solution, whereas a concentration of 10^4 CFU mL^{-1} was maintained in microplate wells.

2.3. Essential oils

The *T. vulgaris* (thyme) EO was provided by the Laboratory of Toxicology (Laboratório de Toxicologia) of UEM, state of Paraná, Brazil. The EO of *C. multijuga* (Amazonas) was provided by the Laboratory of Microbiology of Natural and Synthetic Products (Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos) of the Department of Basic Health Sciences (Departamento de Ciências Básicas da Saúde) of UEM.

The EO of *T. vulgaris* was characterized by nuclear magnetic resonance (NMR) and gas chromatography coupled to a mass spectrometer (GC-MS) in the Laboratory of Toxicology (Laboratório de Toxicologia) of UEM. The two major components in the EO of *T. vulgaris* were borneol (40.6%) and α-terpineol (19.9%) (Kohiyama et al., 2015).

The EO of *C. multijuga* were collected from the trunks of the tree copaiba, was located at Manaus, Amazonas State. Specimens of plant were deposited in the Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA-Manaus) under the numbers INPA 82.418. The EO was analyzed by high-resolution chromatography (Hewlett-Packard model 5890) equipped with flame ionization detectors. The major constituents of the *C. multijuga* EO belonged to the sesquiterpene and diterpene groups (Santos et al., 2008a).

Because the EOs are unstable and volatile, they were stored in a sealed container under refrigeration at 4 ºC to retain their characteristics (Burt, 2004). The EOs were prepared prior to performing the assays, solubilized in 2.5% dimethyl sulfoxide (DMSO, Sigma-Aldrich®, St. Louis, Mo., U.S.A.) and diluted in BAT broth. To evaluate the toxicity of DMSO was performed a positive control (free of EOs) against *A. acidoterrestris* (CBMAI 0244^{T}) under the same experimental conditions.

2.4. Nisin

Nisin (Sigma-Aldrich®, St. Louis, Mo., U.S.A.) was commercially purchased, and a stock solution was prepared in 0.02 M hydrochloric acid (HCl) and sterilized in a 0.22 µm membrane (Millipore, São Paulo, Brazil). Nisin concentrations of 1,000 to 0.49 µg mL^{-1} were evaluated in the assays. Based on the results were determined concentrations bacteriostatic, bactericidal and sporicidal against *A. acidoterrestris*.

2.5. Determination of the antibacterial activity of the essential oils and nisin

The antibacterial activity of the Eos and nisin was determined by serial microdilution in 96-well microplates (TPP® – Techno Plastic Products, Trasadingen, Switzerland) as recommended by the CLSI M7-A9 (2012). It was possible to evaluate the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum sporicidal concentration (MSC).

A volume of 100 µL was added to each well. After the microdilution, 5 µL of the inoculum that contained vegetative cells of *A. acidoterrestris* previously adjusted to 10^4 CFU mL^{-1} was added to each well, and the plate was incubated at 45 ºC for 24 h to evaluate the antibacterial activity of the EOs (concentrations of 10,000 to 0.49 µg mL^{-1}) and nisin (concentrations of 1,000 to 0.49 µg mL^{-1}) against the vegetative cells of *A. acidoterrestris*. A negative control was performed to control the sterility of the culture medium and plate. A positive control was also conducted to evaluate the cell growth. The MIC was defined as the lowest concentration capable of inhibiting visible bacterial growth in the microplate. Microcultures of 10 µL suspensions (in triplicate) from the wells with no visible bacterial growth were performed in BAT agar to evaluate the bacterial viability and determine the MBC. The MBC was defined as the lowest concentration capable of inhibiting *A. acidoterrestris* growth after its

inoculation and incubation in a specific medium free of the antibacterial agent at 45 ºC for 24 h. The absence of bacterial colonies indicated that the concentration was effective as a bactericidal agent against vegetative cells of *A. acidoterrestris*.

To evaluate the antibacterial activity of the Eos and nisin against *A. acidoterrestris* spores, the serial microdilution technique used was similar to the previously described methodology. However, a thermal shock on a hot bath (Nova Técnica, Piracicaba, Brazil) at 80 ºC for 10 min was performed after incubation at 45 ºC for 24 h for spore activation. Then, 10 µL of the suspension (in triplicate) was cultured in BAT agar to assess sporicidal concentration determined with this assay. The absence or reduction of the number of colonies compared to the positive control indicated treatment effectiveness against *A. acidoterrestris* spores.

Concentration values below 100 µg mL^{-1} indicated good antibacterial activity of the EOs; values ranging from 100 to 500 µg mL^{-1} indicated moderate activity; values ranging from 500 to 1000 µg mL^{-1} indicated weak activity; and values above 1000 µg mL^{-1} indicated inactive activity (Holetz et al., 2002). All of the assays were performed in triplicate.

2.6. Checkerboard method

The checkerboard method is widely used for in vitro evaluations of the combined antibacterial activity of two drugs, and it was performed in 96-well microplates to obtain the fractional inhibitory concentration (FIC) of the EO of *C. multijuga* combined with nisin and the EO of *T. vulgaris* combined with nisin against vegetative cells of *A. acidoterrestris*. The microdilution method was performed as recommended by Schelz, Molnar and Hohmann (2006).

For this assay, a 1:2 serial microdilution with a final volume of 100 µL in each well was performed. Aliquots of the nisin stock solution were added along the x-axis, and aliquots of the stock solutions of the EOs were added along the y-axis. The concentrations of the bacterial agents were based on their respective MIC results. The nisin concentrations ranged from 500 to 0.25 µg mL^{-1}, the EO concentrations of *C. multijuga* ranged from 2,400 to 18.75 µg mL^{-1}, and the EO concentrations of *T. vulgaris* ranged from 4,000 to 31.25 µg mL^{-1}.

At the end of the microdilutions, 5 µL of the inoculum at 10^4 CFU mL^{-1} was added to each well, and the plates were incubated at 45 ºC for 24 h. The FICs of both

combinations (*C. multijuga* combined with nisin and *T. vulgaris* combined with nisin) were calculated based on the results of this assay. The FIC index for solution A (nisin) and B (essential oil) was calculated using the formula $FIC = FIC_A + FIC_B$, where the $FIC_A = MIC_A$ in combination divided by the MIC_A alone and the $FIC_B = MIC_B$ in combination divided by the MIC_B alone. The tests detected whether the concentrations of the antibacterial agents exerted a synergistic effect ($FIC_{total} \leq 0.5$), additive effect ($0.5 \leq FIC_{total} \leq 1$), indifferent effect ($1 < FIC_{total} \leq 4$) or antagonistic effect ($FIC > 4$) (Gutierrez, Barry-Ryan and Bourke, 2008). All of the assays were performed in triplicate.

2.7. Death time curve

To calculate the death time curve, the vegetative cells of *A. acidoterrestris* treated with the EOs of *C. multijuga* and *T. vulgaris* were prepared in test tubes, diluted in BAT media at final concentrations adjusted to 8x MIC, 4x MIC, 2x MIC, 1x MIC, 0.5x MIC, 0.25x MIC and a final volume of 5 mL per tube. A positive control was prepared in BAT broth free of the antibacterial agents. Then, 100 µL of the inoculum prepared in 0.85% sterile saline solution and adjusted to 10^5 CFU mL^{-1} was added and incubated at 45 ºC for 48 h. The bacterial growth evaluations were conducted at 0, 3, 6, 9, 12, 24 and 48 h (Ruiz et al., 2013). This assay was performed in triplicate.

2.8. Dose response effect

The dose response effect of the *A. acidoterrestris* vegetative cells was determined by serial microdilution in 96-well plates. The cells were treated with the EOs of *C. multijuga* and *T. vulgaris* at concentrations of 1,000 µg mL^{-1} to 31.25 µg mL^{-1}. An inoculum at a concentration of 10^4 CFU mL^{-1} was added to each microplate well, and the microplates were incubated at 45 ºC for 24 h (Tanaka et al., 2006). The IC_{50} defines the dose that causes the inhibition of 50% of the bacterial growth.

2.9. Cell viability assay

The cytotoxic activity of the EOs was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) colorimetric method as

described by Mosmann (1983). This method evaluates the ability of Vero cells (viable African green monkey kidney cells) to metabolize the tetrazolium salt of the formazan compound, thereby providing information on the cytotoxicity of the tested compound.

The Vero cells were cultivated in 96-well plates (TPP®) with 10% fetal bovine serum (Gibco Invitrogen Corporation, NY, USA) in DMEM (Dulbecco Modified Eagle Medium-GibcoR®) at a concentration of 2.5×10^5 in each well. The plates were then incubated at 37 ºC with 5% CO_2 until forming a confluent layer.

The evaluated concentrations of the EOs of *C. multijuga* and *T. vulgaris* ranged from 1,000 µg mL^{-1} to 31.25 µg mL^{-1}. A positive control (free of EOs) and blank were prepared. The plates were incubated for 72 h under the same conditions described above. After the incubation period, the culture media were removed, and 50 µL of MTT solution (2.0 mg mL^{-1} in distilled water) was added to each microplate well. Then, the plates were incubated at 37 ºC for 4 h. Subsequently, the MTT solution was discarded, and 150 µL of DMSO was added to solubilize the formazan. The plates were read in an ELISA reader (Bio-Tek Power Wave XS Microplate Fluorescence Reader) at an absorbance of 570 nm.

The EOs' cytotoxicity to the Vero cells was compared to the selectivity index (SI), which was determined by dividing the 50% cytotoxic concentration to the Vero cells (CC_{50}) by the 50% inhibitory concentration to the bacteria (IC_{50}). The experiment was performed in triplicate.

2.10. Scanning electron microscopy

In this assay, it was possible to determine the morphological alterations of the vegetative cells and spores of *A. acidoterrestris* by comparison with a negative control (no treatment). The vegetative cells and spores were treated with the EOs of *C. multijuga* and *T. vulgaris* at concentrations of 1x MIC and 0.5x MIC for vegetative cells and 500 µg mL^{-1} for spores. After incubation at 45 ºC for 24 h, the cells were washed three times with phosphate buffered saline (PBS) at pH 7.2 at room temperature and fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium cacodylate buffer (SEM, Hatfield, PA). The cells were maintained at room temperature for 1 h, and then, the fixing solution was removed and the cells were washed twice with 0.1 M sodium cacodylate buffer and placed on a specimen support with poly-L-lysine. Immediately after this procedure, the cells were

dehydrated in 50%, 70%, 80%, 90% and 100% alcohol (10 to 15 min at each alcohol concentration and twice in 100% alcohol), dried to the critical point in CO_2, coated with gold and observed under a Shimadzu SS-550 scanning electron microscope (Tokyo, Japan) (Haddad et al., 2007).

2.11. Flow cytometry

A. acidoterrestris spores standardized at a concentration of 10^4 CFU mL^{-1} were treated with 500 µg mL^{-1} and 1,000 µg mL^{-1} *C. multijuga* and *T. vulgaris* EOs, whereas the vegetative cells (10^4 CFU mL^{-1}) were treated with the MICs of the EOs. Then, the vegetative cells and spores were incubated at 45 ºC for 24 h. For the spores of *A. acidoterrestris*, a thermal shock at 80 ºC for 10 min was performed after incubation for spore activation. A negative control with no EOs was performed for both cellular forms (vegetative and sporulated). In addition, both cellular forms were neutralized, centrifuged, washed and stained with propidium iodide (PI; Invitrogen) in PBS (2 µg/mL). CellQuest Software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA) was used for the data analysis. The samples were counted in a Clibur FACS Flow Cytometer (BD Bioscience) until a total of 10,000 events were reached for each sample in the pre-set region. Survival and cell membrane integrity were determined by the fluorescence intensity (Anjos et al., 2013).

3. Results and Discussion

3.1 Determination of the antibacterial activity of the essential oils

A. acidoterrestris is problematic for the acidic beverage industry, including processed juices, because it is a spoilage microorganism. *A. acidoterrestris* is a spore-forming microorganism that develops after undergoing the pasteurization process, and it produces an unpleasant taste and odor (Danyluk et al., 2011). The EOs are substances extracted from plants that have bioactive principles with several applications, and they can be used as food preservatives against spoilage microorganisms such as *A. acidoterrestris* (Bakkali et al, 2008; Bevilacqua, Corbo and Sinigaglia, 2010; Tajkarimi, Ibrahim and Cliver, 2010).

The results of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) are shown in Table 1.

The MIC and MBC of the EOs of *C. multijuga* and *T. vulgaris* were determined for the vegetative cells and spores of *A. acidoterrestris*. For both EOs, the best effectiveness was obtained against the vegetative cells, resulting in moderate antibacterial activity (concentrations ranging from 100 to 500 µg mL^{-1}). The EOs had a weak antibacterial activity against *A. acidoterrestris* spores (concentrations ranged from 500 to 1,000 µg mL^{-1}). The resistance of spores to treatment with EOs may be related to the presence of dipicolinic acid found in the endospores. This characteristic confers high resistance to the spores to thermal and chemical treatments (Paredes-Sabja, Setlow and Sarker, 2011).

The MIC and MBC of the EO of *C. multijuga* against the vegetative forms of *A. acidoterrestris* were 300 µg mL^{-1} and > 1,000 µg mL^{-1}, respectively (Tab. 1). For the spores, a reduction of 3.07 log CFU mL^{-1} was observed when they were treated with 500 µg mL^{-1} *C. multijuga* EO (Fig. 1).

Antibacterial tests performed by Santos et al. (2008b) using EO of *C. multijuga* showed MICs from 125 µg mL^{-1} to 1,000 µg mL^{-1} for Gram-positive bacteria, however, the authors only evaluated vegetative forms in their study.

Mendonça and Onofre (2008) also observed that the EO of *C. multijuga* had an inhibitory effect on the bacterial growth of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 9027) with MICs of 1.56, 3.12 and 12.5 %, respectively. Several studies have shown that the EO of *C. multijuga* has different applications, including as an antileishmanial (Santos et al., 2008a), antibacterial (Lima et al., 2006), antinociceptive (Gomes et al., 2007), larvicidal (Trindade et al., 2013), antitumor and anticancer (Gomes et al., 2008) agent, and it also has great potential in the treatment of major infectious diseases (Santos et al., 2008b).

The MIC and MBC of the EO of *T. vulgaris* against the vegetative forms of *A. acidoterrestris* were 500 µg mL^{-1} and > 1,000 mL^{-1}, respectively (Tab. 1). For treatments using spores, reductions of 0.64 log CFU mL^{-1} and 2.05 log CFU mL^{-1} were observed when 500 µg mL^{-1} and 1,000 µg mL^{-1} EO were used, respectively (Fig. 1).

Several studies have shown that the EO of *T. vulgaris* has several applications and can be used against the herpes virus (Nolkemper et al., 2006), fungi (Nguefack

et al., 2012; Prakash et al., 2015; Tajkarimi, Ibrahim and Cliver, 2010) and pathogenic bacteria (Abdollahzadeh, Rezaei and Hosseini, 2014; Rota et al., 2008; Tajkarimi, Ibrahim and Cliver, 2010). The EO of *T. vulgaris* is also effective as a food preservative, and it is used as a medicine and in the perfume industry (Mazzarrino et al., 2015; Nezhadali et al., 2014; Rota et al., 2008). However its application in orange juice against A. acidoterrestris was not mentioned in the literature.

According to Nezhadali et al. (2014), the EO of *T. vulgaris* showed better results against Gram-positive bacteria in antibacterial assays. The resistance of bacteria to the EO of *T. vulgaris* may be related to their membrane cell constituents (Gyawali and Ibrahim, 2014; Tajkarimi, Ibrahim and Cliver, 2010).

Studies performed by Nikolic et al. (2014) indicated that the EO of *T. vulgaris* showed good antimicrobial activity against several strains of *Streptococcus*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus* and *S. aureus* with MIC ranging from 80 µg mL^{-1} to 160 µg mL^{-1}. These results are inconsistent with those observed in the current study in which the MIC of *T. vulguris* EO ranged from 500 to 1,000 µg mL^{-1}. However, differences were observed between the major compounds isolated in the study by Nikolic et al. (2014), who found thymol (49.10%) and *p*-cymene (20.01%), and the compounds found in the current study, which were borneol (40.6%) and α-terpineol (19.9%). The concentration of EO constituents may vary according to the time of extraction, environmental conditions, harvest period, plant development stage and regions where the plants were collected (Burt, 2004; Nezhadali et al., 2014; Tajkarimi, Ibrahim and Cliver, 2010).

Nisin exhibited good bacteriostatic, bactericidal and sporicidal activity against *A. acidoterrestris*. The MIC and MBC for the vegetative forms were 15.60 µg mL^{-1} and 31.25 µg mL^{-1}, respectively (Tab. 1). The MSC with total elimination of the spores was reached at 62.50 µg mL^{-1} (Fig. 1).

The results observed in our assays were similar to the results of Ruiz et al. (2013). Rajendran, Nagappan and Ramamurthy (2011) observed good results in treatments evaluating the effect of nisin on *Bacillus cereus*, a spore-forming microorganism, in their study, nisin had an MIC of 70 µg mL^{-1} for the vegetative forms. In the study by Gyawali and Ibrahim (2014), nisin also showed better effects against Gram-positive bacteria, this may be related to the protection provided by the external membrane of these microorganisms.

3.2 Checkerboard method

The EO evaluations (*C. multijuga* and *T. vulgaris*) in combination with nisin were performed through the checkerboard method. For both treatments (*C. multijuga* + nisin or *T. vulgaris* + nisin), the FIC index was 0.75, and the combination of EOs + nisin produced an additive effect as shown in the isobologram (Fig. 2). Nisin and Eos *T. vulgaris* and *C. multijuga* has great biotechnological potential can be used as antibacterial agent of natural origin, and their combined effect provides a promising alternative for controlling several microorganisms and for use as preservatives in food matrices in addition to contributing to reductions in the cost of the product during the manufacturing process.

Nisin is a bacteriocin approved as a food preservative and used in over 50 countries. It is a good antibacterial agent and can be used alone or in combination with other antimicrobials. Nisin has broad spectrum effects and is active against several bacteria, including Gram-positive and Gram-negative bacteria, pathogenic or spoilage bacterial and spore-forming bacteria (Gyawali and Ibrahim, 2014; Huertas et al., 2014; Rajendran, Nagappan and Ramamurthy, 2011; Ruiz et al., 2013).

Nisin's antibacterial effect may be related to its ability to permeate the cell membrane and cause cell disruption and destruction (Lucera et al., 2012).

The mechanisms of action of EOs have not been totally elucidated because EOs are composed of several chemical groups, and each group can act differently on bacterial cells.

EOs and nisin used in combination contribute to the formation of pores in the membrane, thus changing its permeability and altering the proton motive force, amino acids efflux and pH gradient of the bacteria (Turgis et al., 2012).

Turgis et al. (2012) observed an additive effect of the EO of *T. vulgaris* combined with nisin against *Bacillus cereus* and *Listeria monocytogenes*. Solomakos et al. (2008) also observed good results when testing the combination EO of *T. vulgaris* and nisin against *L. monocytogenes*. The combination had a synergistic effect when 0.6% *T. vulgaris* EO and 1,000 UI/g nisin were added, and the treatment reduced the bacteria below the limit established by the European Union (2 Log CFU mL^{-1}). In our studies, an additive effect of EOs combined with nisin against *A. acidoterrestris* was observed when 500 µg mL^{-1} of *T. vulgaris* EO was combined with

15.7 µg mL^{-1} nisin and 300 µg mL^{-1} *C. multijuga* EO was combined with 15.7 µg mL^{-1} nisin.

3.3 Death time curve

The death time curve method was conducted to assess the antibacterial activity of the EOs. At a concentration of 8x MIC *C. multijuga* EO, there was complete reduction of the bacterial load of *A. acidoterrestris* in the first three hours after treatment. With concentrations of 4x MIC and 2x MIC, a longer treatment was necessary, with 48 h required to eliminate the vegetative forms of *A. acidoterrestris*. With 1x MIC for 24 h, a reduction of approximately 4.65 log CFU mL^{-1} was obtained (Fig. 3 - A).

The treatment with *T. vulgaris* EO exhibited a smaller reduction compared with the treatment using the *C. multijuga* EO. Total bacterial elimination was reached after 48 h of treatment using the concentration of 8x MIC. In the treatment using 1x MIC for 24 h and 48 h, there was a reduction of 3.81 log CFU mL^{-1} and approximately 4.11 Log CFU $mL^{-1,}$ respectively (Fig. 3 - B).

The results observed in this assay were similar to the results of MBC, with values from 2,000 µg mL^{-1} to *C. multijuga* and 10,000 µg mL^{-1} to *T. vulgaris* in 24 h treatments (data not shouwn).

3.4 Dose-response effect and cell viability

The cytotoxic effect of the EOs on Vero cells was indicative of cell survival, wherein dead cells cannot metabolize MTT. The results obtained in this assay revealed that the EOs of *C. multijuga* and *T. vulgaris* had CC_{50} values of 54.2 µg mL^{-1} and 142.3 µg mL^{-1}, respectively, causing a reduction of 50% of the viable Vero cells at these concentrations. The IC_{50} results of *A. acidoterrestris* using the EOs of *C. multijuga* and *T. vulgaris* were 500 µg mL^{-1} and 1,000 µg mL^{-1}, respectively, and there was 50% inhibition of bacterial growth with these concentrations. Comparing the activity of the EOs against Vero cells (CC_{50}) with the toxic effect against *A. acidoterrestris* (IC_{50}), we obtained the selectivity index (SI). Values less than 1.0 are regarded as the SI being less selective for the microorganisms, indicating that the tested sample is more toxic (Santos et al., 2008a). This comparison showed that the

EOs were less selective to *A. acidoterrestris* and more toxic to Vero cells, showing SI of the EO of *C. multijuga* and *T. vulgaris* of 0.11 and 0.14, respectively.

3.5 Scanning electron microscopy

After treatment (45 ºC for 24 h) using the *C. multijuga* and *T. vulgaris* Eos at concentrations of 500 µg mL^{-1} for spores and 1x MIC and 0.5x MIC for the vegetative cells of *A. acidoterrestris*, external morphological alterations were observed in both treatments and cell forms compared with the control group using scanning electron microscopy (Fig. 4).

Santos et al. (2008b) showed the effectiveness of the EO of *Copaifera martii* against *S. aureus,* which caused morphological and structural alterations in the bacteria, indicating that the EO of the *Copaifera* genus can affect the cell wall, which is consistent with the results of our study.

3.6 Flow cytometry

To determine whether the bacterial cell membrane was disrupted, the cells were stained with PI, a DNA marker that binds to the genetic material and transmits fluorescence that can be read by a cytometer. In this assay, the vegetative cells of *A. acidoterrestris* treated with the EOs of *C. multijuga* and *T. vulgaris* at their respective MICs had a higher percentage of cells with alterations in the cell membrane integrity compared with cells of the negative control (upper left and right quadrant) (Fig. 5). However, the spores treated with the EOs of *C. multijuga* and *T. vulgaris* at concentrations of 500 µg mL^{-1} and 1,000 mL^{-1}, respectively, showed no changes relative to the negative control (upper left and right quadrant) (Fig. 5). Therefore, we cannot conclude that the main mechanism of action of the EOs is changes in the cell membrane because the percentage of cells with membrane alterations was low in all treatments, with values ranging from 4.24% to 29.94%.

4. Conclusion

Our results indicate that the EOs of *C. multijuga* and *T. vulgaris* showed good antibacterial activity against the vegetative cells and spores of *A. acidoterrestris* in in

vitro assays. Although the combination of EOs with nisin exhibited additive effects, the results suggest that the use of these antibacterial in orange juice should be performed with caution, not exceeding concentrations indicated as safe. However, other studies should be conducted EOs should be performed because the SI indicated that the concentrations used in the current study had greater cytotoxic effects on Vero cells. Nevertheless, the EOs showed great biotechonological potential having antibacterial effect against *A. acidoterrestris*.

Therefore, further studies should evaluate the effects of *C. multijuga* and *T. vulgaris* EOs as well as of their isolates in combination with other chemicals and physical mechanisms to determine whether they can reduce or eliminate *A. acidoterrestris* in orange juice. Such results would increase the treatment efficacy and contribute to the production and distribution of orange juice with increased quality and shelf life, and enabling cost reductions.

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Table 1- Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the essential oils of *Copaifera multijuga* and *Thymus vulgaris* against vegetative cells *Alicyclobacillus acidoterrestris*.

Treatment	**MIC (µg mL^{-1})**	**MBC (µg mL^{-1})**
Copaifera multijuga	300	>1,000
Thymus vulgaris	500	>1,000
Nisin	15.60	31.25

FIGURE 1

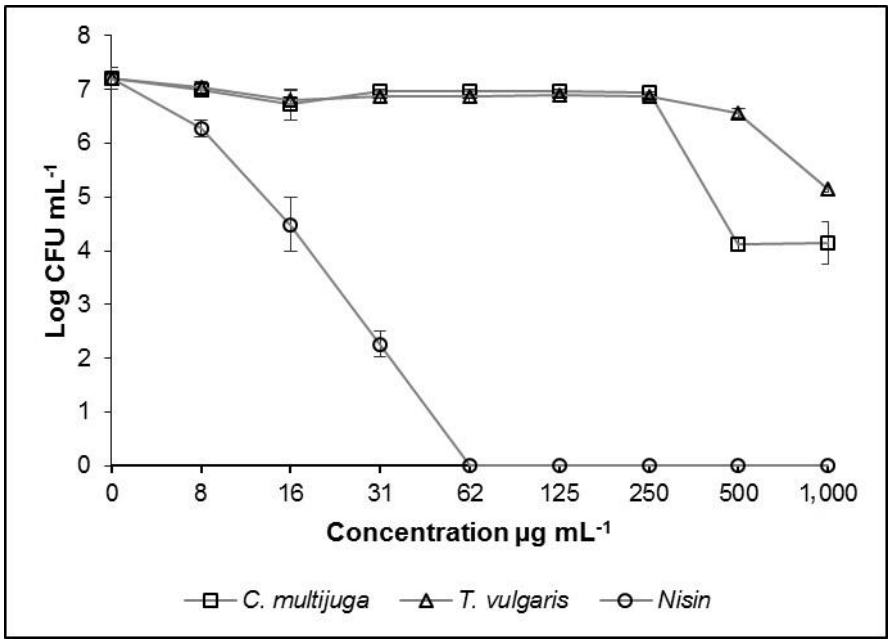
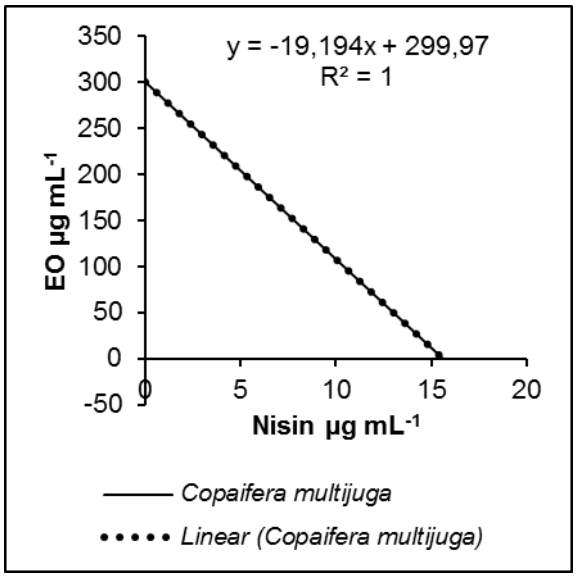

Log CFU mL-1
8
7
6
5
4
3
2
1
0
0
8
16
31
62
125
250
500
1,000
Concentration µg mL-1
C. multijuga
T. vulgaris
Nisin

FIGURE 2

A


y = -19,194x + 299,97
R² = 1
EO µg mL-1
Nisin µg mL-1
Copaifera multijuga
Linear (Copaifera multijuga)

B

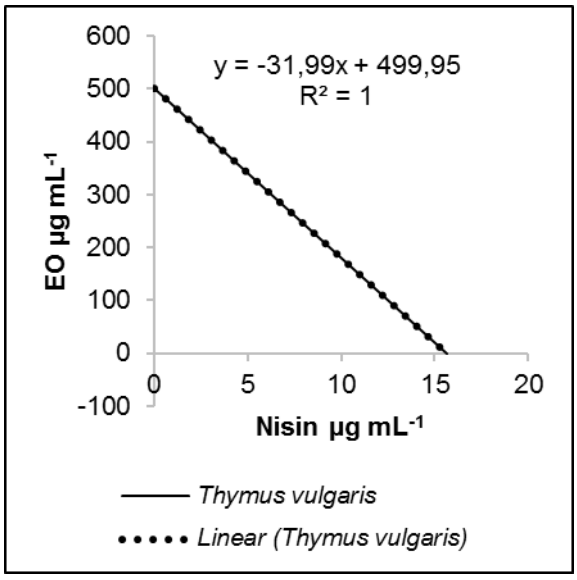
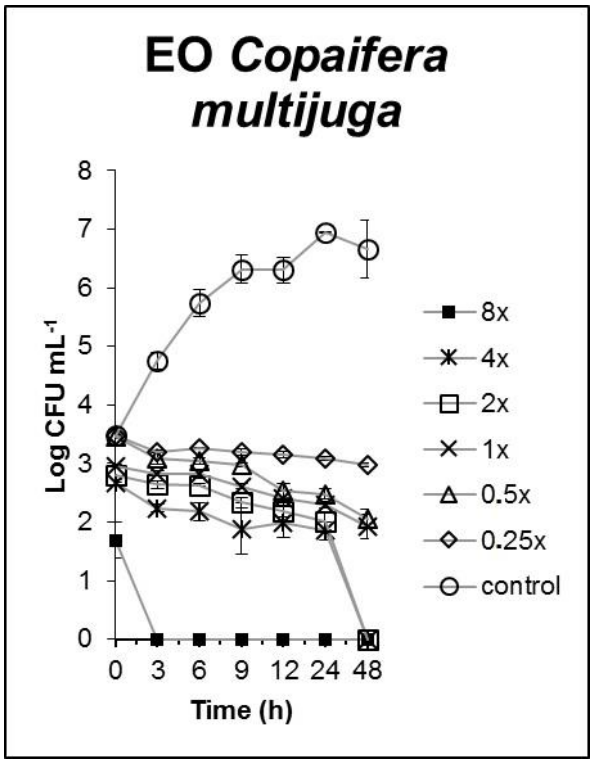

y = -31,99x + 499,95
R² = 1
EO µg mL-1
Nisin µg mL-1
Thymus vulgaris
Linear (Thymus vulgaris)

FIGURE 3

A


EO Copaifera multijuga
Log CFU mL-1
Time (h)
0 3 6 9 12 24 48
0 1 2 3 4 5 6 7 8
8x
4x
2x
1x
0.5x
0.25x
control

B

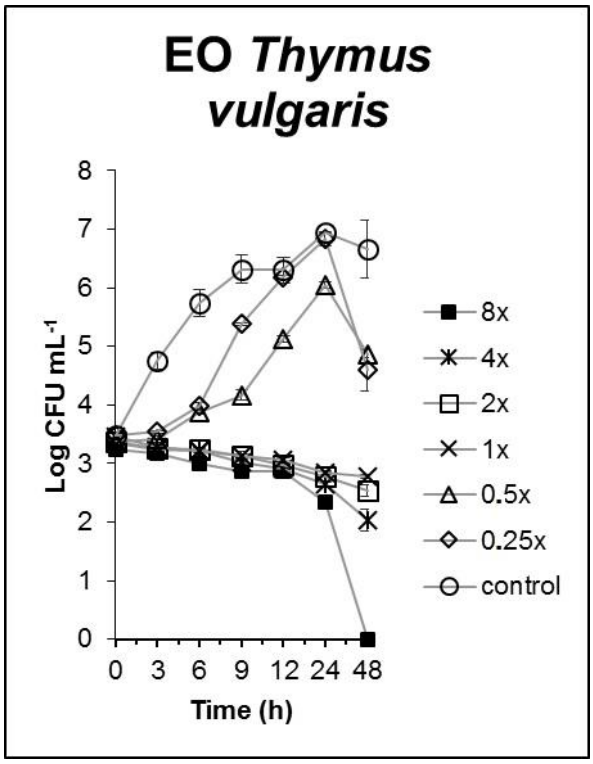
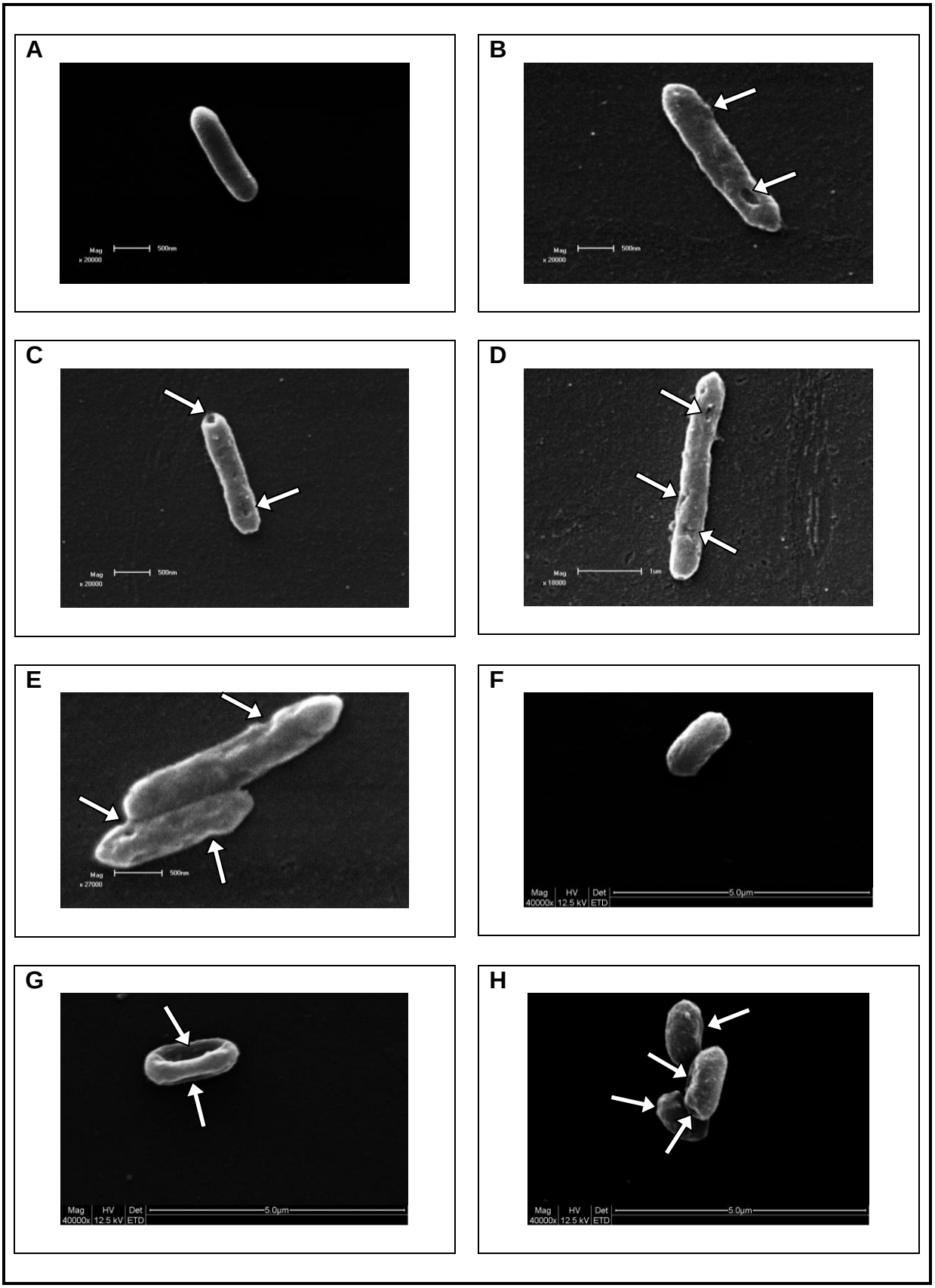

EO Thymus vulgaris
Log CFU mL-1
Time (h)
0 3 6 9 12 24 48
0 1 2 3 4 5 6 7 8
8x
4x
2x
1x
0.5x
0.25x
control

FIGURE 4


A
Mag
x 20000
500nm
B
Mag
x 20000
500nm
C
Mag
x 20000
500nm
D
Mag
x 18000
1um
E
Mag
x 27000
500nm
F
Mag
40000x
HV
12.5 kV
Det
ETD
5.0µm
G
Mag
40000x
HV
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H
Mag
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HV
12.5 kV
Det
ETD
5.0µm

FIGURE 5

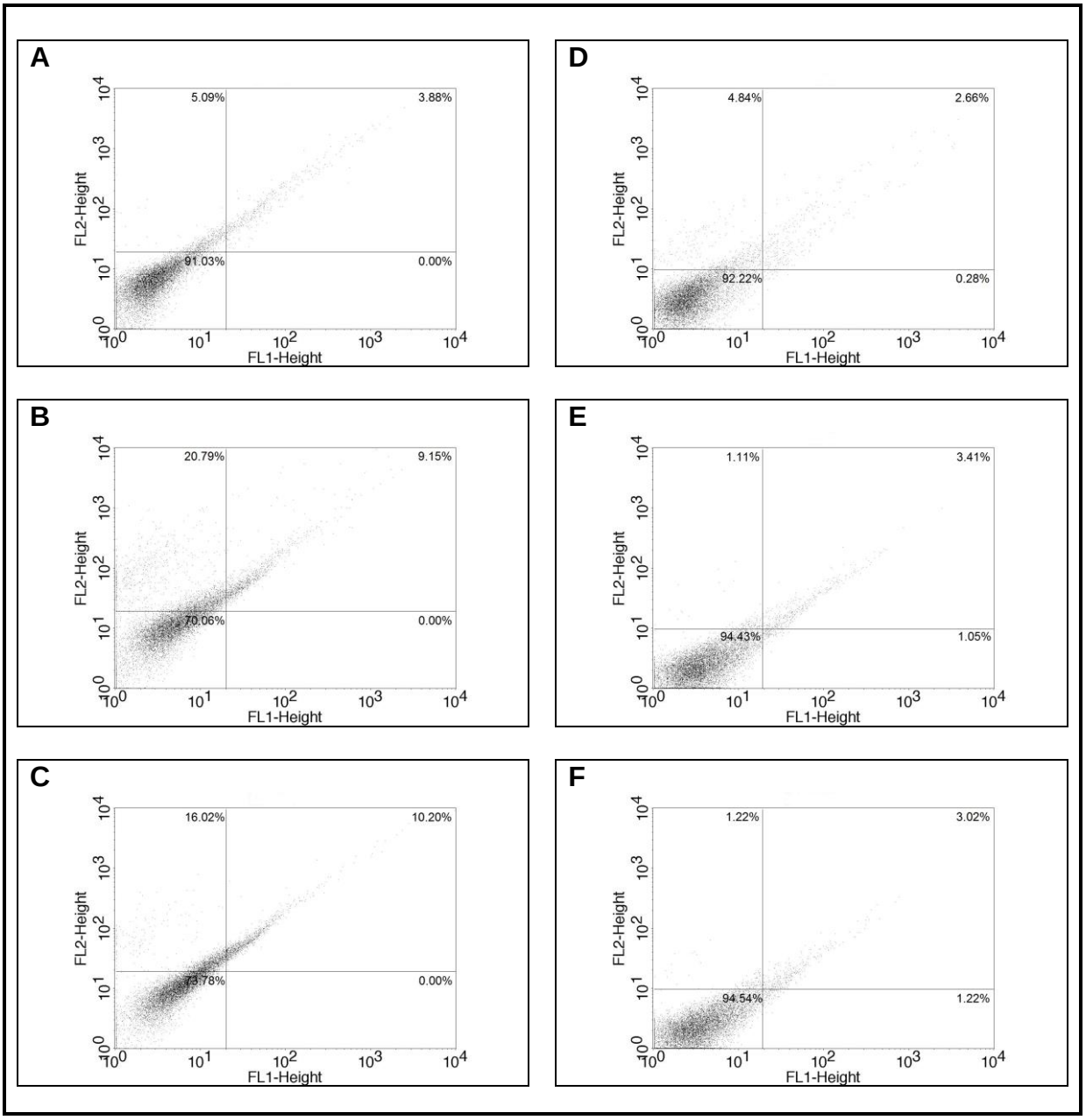
A
5.09%
3.88%
91.03%
0.00%
B
20.79%
9.15%
70.06%
0.00%
C
16.02%
10.20%
73.78%
0.00%
D
4.84%
2.66%
92.22%
0.28%
E
1.11%
3.41%
94.43%
1.05%
F
1.22%
3.02%
94.54%
1.22%
FL2-Height
FL1-Height

Figure 1- Quantification of *A. acidoterrestris* spores (Log CFU mL^{-1}) after treatment with the EOs of *C. multijuga* and *T. vulgaris* and bacteriocin nisin.

Figure 2- Isobologram showing the interactivity between nisin and the EOs of *Copaifera multijuga* (A) and *Thymus vulgaris* (B) in *Alicyclobacillus acidoterrestris*. The solid line over the dashed line indicates that the combined effect of both treatments represents an additive effect (FIC index = $0.5 \leq FIC_{total} \leq 1$).

Figure 3- Death time curve for the essential oils of *Copaifera multijuga* (MIC: 300 µg mL^{-1}) (A) and *Thymus vulgaris* (MIC: 500 µg mL^{-1}) (B) against the vegetative cell of *A. acidoterrestris*.

Figure 4- Scanning electron microscopy of *A. acidoterrestris* vegetative cells and spores after 24 h of treatment using the *C. multijuga* and *T. vulgaris* EOs: (A) Control – vegetative cell. Vegetative cell treated with (B) *C. multijuga* EO at 150 µg mL^{-1}; (C) *C. multijuga* EO at 300 µg mL^{-1}; (D) *T. vulgaris* EO at 250 µg mL^{-1}; (E) *T. vulgaris* EO at 500 µg mL^{-1}. (F) Control - spores. Spores treated with (G) *C. multijuga* EO at 500 µg mL^{-1} and (H) *T. vulgaris* EO at 500 µg mL^{-1}. The arrows point the external morphological changes.

Figure 5- Flow cytometry plot showing the PI staining of *A. acidoterrestris* vegetative cells and spores after 24 h treatment using the EOs of *C. multijuga* and *T. vulgaris*: (A) Control – vegetative cell. Vegetative cell treated with (B) *C. multijuga* EO at 300 µg mL^{-1} and (C) *T. vulgaris* EO at 500 µg mL^{-1}. (D) Control – spores. Spores treated with (E) *C. multijuga* EO at 500 µg mL^{-1} and (F) *T. vulgaris* EO at 1,000 µg mL^{-1}.