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

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Abstract

The present work aimed to evaluate the antibacterial activity of the essential oils (EOs) of *Copaifera multijuga* and *Thymus vulgaris* against *Alicyclobacillus acidoterrestris*. The minimum inhibitory concentration (MIC) of the *C. multijuga* and *T. vulgaris* EOs were 300 and 500 µg/mL, respectively, and the minimum bactericidal concentration (MBC) of both EOs was >1,000 µg/mL. The best minimum sporicidal concentration (MSC) of *C. multijuga* was reached at a concentration of 500 µg/mL, with a reduction of 3.07 log CFU/mL. For the EO of *T. vulgaris*, better results were observed at concentrations higher than 500 µg/mL, and a reduction of 2.05 log CFU/mL was achieved at 1,000 µg/mL. The checkerboard method showed that the combination of EOs and nisin had an additive interaction. 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 with alterations in cell membrane integrity as compared to their spores. When EOs were added to reconstituted orange juice, antibacterial treatment was highly effective and when EOs were combined with nisin, there was a complete reduction of bacterial load. Thus, it is evident that the EOs of *C. multijuga* and *T. vulgaris* have potential for use as antibacterial agents against *A. acidoterrestris*.

Introduction

Alicyclobacillus acidoterrestris is a Gram-positive bacillus bacterium, which is thermoacidophilic, aerobic, food spoiling, non-pathogenic, catalase positive, with terminal or sub-terminal spores. 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 it can easily be introduced into industrial processes.

Spoilage caused by *A. acidoterrestris* occurs due to the production of guaiacol (2-methoxyphenol), 2,6-dibromophenol and 2,6-dichlorophenol, substances associated with an unpleasant taste and odour in contaminated food. This spoilage process is characterised by a lack of gas production, low turbidity and sedimentation, and thus is difficult to detect after filling (Bevilacqua *et al.*, 2008; Danyluk *et al.*, 2011; Witthuhn *et al.*, 2012; Sokolowska *et al.*, 2013).

Brazil is a major exporter of orange juice, exporting 98% of its entire production. The country accounts for 50% of the global production, and 85% of the global market of orange juice. Orange juice accounted for 56% of global production of agricultural products in 2009 (Neves *et al.*, 2014).

The demand for natural antimicrobial agents has increased because of the popularity of green consumerism and the concerns of consumers and

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regulatory agencies regarding the health and safety of food products. Several studies have indicated the potential of essential oils (EOs), their isolates, and nisin, either alone or in combination, as antibacterial agents due to their bacteriostatic, bactericidal and/or sporicidal properties (Bevilacqua *et al.*, 2008; Walker and Philips, 2008; Ruiz *et al.*, 2013). The combined use of such agents may have a synergistic effects, improving the efficiency of treatment which, when performed separately, is ineffective. In addition, this combination can reduce the cost of treatment and contributes to the quality of the juice, as EOs are considered natural products. However, no data was found in literature that reported the use of EOs of *T. vulgaris* and *C. multijuga*, either used alone or in combination with nisin, against *A. acidoterrestris*.

The genus *Copaifera* is found throughout Brazil. *Copaifera* multijuga is a large tree found in the states of Amazonas, Acre, Rondônia, Roraima and Mato Grosso (Mendonça and Onofre, 2009) belonging to the family Leguminosae. *Thymus vulgaris* is a medicinal aromatic plant native to the western Mediterranean, belonging 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 (Letchamo and Gosselin, 1996).

Several studies have shown that the EO of *T. vulgaris* has several applications and can be used for multiple treatments (Nolkemper *et al.*, 2006; Abdollahzadeh *et al.*, 2014; Prakash *et al.*, 2015). The EO of *T. vulgaris* is also effective as a food preservative, and is used as a medicine and in the perfume industry (Rota *et al.*, 2008; Nezhadali *et al.*, 2014; Mazzarrino *et al.*, 2015). However, its application against *A. acidoterrestris* in orange juice has not been mentioned in the literature.

Nisin is a bacteriocin produced by *Lactococcus lactis* subsp lactis, and 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 is generally recognized as safe (GRAS) (Stevens *et al.*, 1991; Yamazaki *et al.*, 2000; Walker and Philips, 2008).

The present work aimed to evaluate the antibacterial activity of the EOs of *C. multijuga* and *T. vulgaris* against the vegetative and sporulated forms of *A. acidoterrestris*, as well as evaluate the antibacterial activity in orange juice and assess the effects of these EOs when combined with nisin.

Materials and methods

Bacterial strain and growth conditions

Strain of *A. acidoterrestris* (CBMAI 0244^T – source: soil) was provided by the CBMAI (Brazilian Collection of Environmental and Industrial 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 the assays. The strain was stored in cryovials containing BAT broth with 30% glycerol (v/v) (Thermo Fisher Scientific, Waltham, MA) at -20°C in the Laboratory of Water, Environment and Food Microbiology of Maringá State University (UEM), Paraná, Brazil. The bacterial stock used in the assays was maintained in Petri dishes (Inlab, Interlab, São Paulo, Brazil) with BAT agar at 4°C, and a subculture was maintained in BAT broth 45°C to assess the bacterial viability in every test performed.

Inoculum preparation and spore production

The vegetative cells of *A. acidoterrestris* were prepared as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012).

For the preparation of *A. acidoterrestris* spores, the inoculum was cultured in test tubes containing BAT broth (5 mL) and incubated at 45°C for 120 h for spore formation, until 80% sporulation was reached. The sporulation index was evaluated by direct counting using a phase contrast microscope. Subsequently, the spores were transferred to microtubes, centrifuged at 9,500 g for 3 min, and then rinsed in sterile deionised water three times. Serial dilution was then performed, followed by thermal shock in a hot bath (Nova Técnica, Piracicaba, Brazil) at 80°C for 10 min to count the viable cells of the spore suspension, using the spread plate method to determine the colony forming unit (CFU)/mL.

Essential oils

The *T. vulgaris* (thyme) EO was provided by the Laboratory of Toxicology of State University of Maringá (UEM), Brazil. The EO of *C. multijuga* was provided by the Laboratory of Microbiology of Natural and Synthetic Products, of the Department of Basic Health Sciences of UEM.

The EO of *T. vulgaris* was characterised by nuclear magnetic resonance (NMR) and gas chromatography coupled to a mass spectrometer (GC-MS) (Thermo Electron Corporation. Model DSQ II, San Jose, CA, EUA) in the Laboratory of Toxicology of UEM,

according to data already published in previous studies (Kohiyama *et al.*, 2015). The two major components in the EO of *T. vulgaris* were borneol (40.6%) and α-terpineol (19.9%).

The EO of *C. multijuga* was collected from the trunks of the copaiba tree, located in Manaus in the state of Amazonas. Specimens of the plant were deposited in the Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA-Manaus) under the specimen voucher number INPA 82.418. The EO was analysed by high-resolution chromatography (Hewlett-Packard model 5890) equipped with flame ionisation detectors, and characterised according to data already published (Santos *et al.*, 2008b). The major constituents of the *C. multijuga* EO were from the sesquiterpene (β-caryophyllene 57.5%) and diterpene (acid copalic 6.2%) groups. The EOs were stored in a sealed container under refrigeration at 4°C to retain their characteristics.

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 sterilised in a 0.22 µm membrane (Millipore, São Paulo, Brazil). Stock solutions of nisin ranged from 1,000 to 0.49 µg/mL.

Determination of the antibacterial activity of the essential oils and nisin

Antibacterial activity of the EOs and nisin was determined by serial dilution in 96-well microplates (TPP® – Techno Plastic Products, Trasadingen, Switzerland) containing 100 µL of sterile BAT broth in each well, as recommended by CLSI M7-A9, and adjusted inoculum was in a final concentration of 10^4 CFU/mL. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum sporicidal concentration (MSC) were then evaluated.

In MIC, MBC, and MSC assays, the stock EO solutions were prepared at concentrations of 1,000 to 0.49 µg/mL, and were solubilised in 0.05% (v/v) with Tween 80. To evaluate the toxicity of Tween 80, a positive control (free of EOs) was performed against *A. acidoterrestris* (CBMAI 0244T) under the same experimental conditions. After serial dilution, the microplates were incubated at 45°C for 24 h to evaluate the antibacterial activity of the EOs and nisin against the vegetative cells of *A. acidoterrestris*. A negative control was performed to control for the sterility of the culture medium and plate. A positive control was also conducted to evaluate cell growth, by adding the microorganism to the culture medium. The MIC

was defined as the lowest concentration capable of inhibiting visible bacterial growth in the microplate. Microcultures of suspensions (10 µL) from the wells with no visible bacterial growth were cultured in BAT agar to evaluate bacterial viability and determine the MBC. The MBC was defined as the lowest concentration capable of inhibiting *A. acidoterrestris* growth after inoculation and incubation in a specific medium free of the antibacterial agent at 45°C 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 sporicidal activity (MSC) of the EOs and nisin against *A. acidoterrestris* spores, the serial dilution technique was used, in accordance with previously described methodology for vegetative forms. As a negative control, only the BAT medium was used to control for the sterility of the culture medium and plaque. And as a positive control, the spores were added in the BAT medium to evaluate cell growth. However, after the incubation period of the spores at 45°C for 24 h, a heat shock was carried out at 80°C in a water bath (Nova Técnica, Piracicaba, SP, Brazil) for 10 min for spore activation. Then, 10 µL of the suspension was cultured in BAT agar to assess sporicidal concentration. The absence or reduction of the number of colonies as compared to the positive control indicated the treatment effectiveness against *A. acidoterrestris* spores. All of the assays were performed in triplicate, with independent batches.

Antibacterial combinations

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

For this assay, a 1:2 serial dilution, 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 antibacterial agents were based on their respective MIC results. The concentrations of nisin, EO of *C. multijuga*, or *T. vulgaris*, ranged from 500 - 0.25, 2,400 - 18.75, and 4,000 - 31.25 µg/mL, respectively, were evaluated. At the end of the dilutions, 5 µL of the inoculum at 10^4 CFU/mL was added to each well, and the plates were incubated at 45°C 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 FIC_A = MIC_A in combination, divided by MICA alone and FIC_B = MIC_B in combination, divided by MIC_B alone. The tests detected whether the concentrations of antibacterial agents exerted any of the following effects: synergistic (FIC_{total} ≤ 0.5), additive (0.5 ≤ FIC_{total} ≤1), indifferent (1 < FIC_{total} ≤ 4), or antagonistic (FIC > 4) (Gutierrez *et al.*, 2008). All of the assays were performed in triplicate.

Application of essential oils in reconstituted orange juice

Commercially obtained, concentrated orange juice (approximately 65 °Brix) was used in these tests. Prior to testing, the juice was checked to ensure the absence of *Alicyclobacillus* spp. Following this evaluation, the juice was reconstituted with sterilised water, resulting in a concentration of approximately 11 °Brix. The juice (5 mL) was then placed in 24 wells (TPP® – Techno Plastic Products, Trasadingen, Switzerland) and the EOs of *T. vulgaris* and *C. multijuga*, diluted to 0,05% (v/v) with Tween 80 were added. The tests were performed at concentrations of 1×, 4×, 8×, and 16× MIC. Following the addition of the EOs, the inoculum of *A. acidoterrestris*, at a final concentration of 5×10^4 CFU/mL, was added. Next, the plates were incubated at 45°C for 24 h. Following incubation, the orange juice was diluted, plated in BAT agar, and incubated at 45°C for 24 h. All the tests were performed in triplicate.

An evaluation of the action of EOs (*T. vulgaris* and *C. multijuga*) was done in combination with nisin. The orange juice was aseptically reconstituted at approximately 11 °Brix, pH 4.0 in 24 well plates (TPP® – Techno Plastic Products, Trasadingen, Switzerland), in a volume of 5 mL. The combined concentrations of EOs (*T. vulgaris* or *C. multijuga*) plus nisin used in this assay were 1×, 0.5×, 0.25×, and 0.125× MIC. The EOs of *T. vulgaris* and *C. multijuga* were solubilised in Tween 80 at 0.05% (v/v). After the dilutions, the inoculum of *A. acidoterrestris* at a concentration of 5×10^4 CFU/mL was incubated at 45°C for 24 h. Following incubation, 10 μL (triplicate) microculture was performed on BAT agar to evaluate the cell viability, followed by incubation at 45°C for 24 h.

Death time curve

This test was performed in accordance with Ruiz *et al.* (2013), with modifications. To construct 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, and diluted in BAT media at final concentrations adjusted to 8×, 4×, 2×, 1×, 0.5×, and 0.25× MIC, at 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 10^4 CFU/mL, was added and incubated at 45°C for 48 h. The bacterial growth evaluations were conducted at 0, 3, 6, 9, 12, 24, and 48 h. This assay was performed in triplicate.

Dose response effect

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

Cytotoxicity assay

The cytotoxicity 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 metabolise the tetrazolium salt of formazan, thereby providing information on the cytotoxicity of the tested compound.

The Vero cells were cultivated in 96-well plates (TPP®) with 10% foetal 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°C with 5% CO_2 until a confluent layer was formed.

The evaluated concentrations of the EOs of *C. multijuga* and *T. vulgaris* ranged from 1,000 - 31.25 μg/mL. A positive (free of EOs) and blank control were prepared. The plates were incubated for 72 h under the same conditions described above. Following incubation, the culture medium was removed and 50 μL of MTT solution (2.0 mg/mL in distilled water) was added to each microplate well. Then, the plates were incubated at 37°C for 4 h. Subsequently, the MTT solution was discarded and 150 μL of DMSO was added to solubilise 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 cytotoxicity of the EOs against the Vero cells was compared using the selectivity index (SI), which was determined by dividing the 50% cytotoxic concentration of the Vero cells (CC_{50}) by the 50% inhibitory concentration of the bacteria (IC_{50}) (Santos *et al.*, 2008a). The experiment was performed in triplicate.

Scanning electron microscopy

In this assay, the morphological alterations of the vegetative cells and spores of *A. acidoterrestris*, in comparison with a negative control (no treatment), was determined. The vegetative cells and spores were treated with the EOs of *C. multijuga* and *T. vulgaris* at concentrations of 1× and 0.5× MIC for vegetative cells and 500 µg/mL for spores. After incubation at 45°C 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 before 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 graded ethanol, 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).

Flow cytometry

A. acidoterrestris spores, standardised to a concentration of 10^4 CFU/mL, were treated with 500 or 1,000 µg/mL, *C. multijuga* or *T. vulgaris* EOs, whereas the vegetative cells (10^4 CFU/mL) were treated with the MICs of the EOs. Then, the vegetative cells and spores were incubated at 45°C for 24 h. For the spores of *A. acidoterrestris*, a thermal shock at 80°C 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 neutralised, centrifuged, washed, and stained with propidium iodide (PI; Invitrogen) in PBS (2 µg/mL). 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 (dos Anjos *et al.*, 2013). CellQuest Software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA) was used for data analysis.

Results and discussion

Determination of the antibacterial activity of the essential oils and nisin

The MIC and MBC of the EOs of *C. multijuga* and *T. vulgaris* were determined for the vegetative cells and spores of *A. acidoterrestris*. MIC values below 100 µg/mL indicated good antibacterial activity of the EOs; values ranging from 100 to 500 µg/mL indicated moderate activity; values ranging from 500 to 1,000 µg/mL indicated weak activity; and values above 1000 µg/mL indicated no activity (Holetz *et al.*, 2002).

The EOs displayed weak antibacterial activity against *A. acidoterrestris* spores, since the highest concentration (1,000 µg/mL) was not sufficient to completely eliminate the spores, therefore only the reduction in spore concentration was evaluated (Figure 1). The resistance of spores to treatment with EOs may be related, among other factors, to the presence of dipicolinic acid found in the endospores. This characteristic implies high resistance of the spores to thermal and chemical treatments (Setlow, 2006; Paredes-Sabja *et al.*, 2011; Bevilacqua *et al.*, 2015). However there are few studies on the mechanism of resistance of *A. acidoterrestris*. Both EOs were most effective against the vegetative cells, resulting in moderate antibacterial activity.

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

According to Santos *et al.* (2008a), the EO extracted from copaiba showed broad spectrum antimicrobial activity, obtaining better results against *Bacillus subtilis* (MIC/MBC of 125 µg/mL). Such results may be related to the constituents of the sesquiterpene and diterpene groups.

The major constituents present in the EO of *C. multijuga*, according to Santos *et al.* (2008b), and used in our experiments, were β-caryophyllene (57.5%) and acid copalic (6.2%), belonging to the sesquiterpene and diterpene groups, respectively. These lipophilic constituents can cross and alter the cell wall and cytoplasmic membrane of the bacterium (de Alencar Filho *et al.*, 2017). The antibacterial activity of β-caryophyllene may be linked to its high antioxidant capacity. Copalic acid has a high biological activity of broad spectrum, acting as a good antibacterial agent (Souza *et al.*, 2011; Dahham *et al.*, 2015).

The MIC and MBC of the EO of *T. vulgaris*,

against the vegetative forms of *A. acidoterrestris*, were 500 and > 1,000 µg/mL, respectively. For treatments using spores, reductions of 0.64 log and 2.05 log CFU/mL were observed when 500 and 1,000 µg/mL EO were used, respectively (Figure 1).
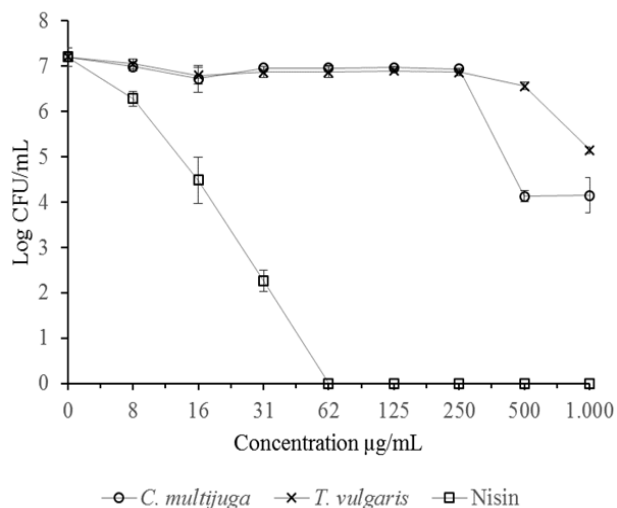

Figure 1. *A. acidoterrestris* spore load (log CFU/mL) after treatment with the essential oils of *Copaifera multijuga* and *Thymus vulgaris* and bacteriocin nisin.

Studies performed by Nikolić *et al.* (2014) indicated that the EO of *T. vulgaris* displayed good antimicrobial activity against several strains of *Streptococcus*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus* and *S. aureus* with MICs ranging from 80 - 160 µg/mL. These results are inconsistent with those observed in the present work, in which the MIC of *T. vulgaris* EO ranged from 500 - 1,000 µg/mL. However, differences were observed between the major compounds isolated in the study by Nikolić *et al.* (2014), who found thymol (49.10%), and *p*-cymene (20.01%), and the compounds found in the present work were borneol (40.6%) and α-terpineol (19.9%).

Borneol and α-terpineol belong to the group of monoterpene alcohols. These compounds exhibit antibacterial activity against Gram-negative strains, with major action against Gram-positive bacteria. The antibacterial activity of monoterpenes may be associated with their lipophilic nature and their action on membrane flexibility and permeability, and interfering in metabolic functions (Tiwari *et al.*, 2009; Luo *et al.*, 2014).

EOs are formed by a mixture of compounds, and can exert a synergistic action against bacterial cells with a varied mechanism of action. The composition of the EO can be differentiated by several factors, among them: the methodology used in its extraction, the time of year, the age and the part of the plant used, and the location, among other factors (Burt, 2004;

Bakkali *et al.*, 2008; Kohiyama *et al.*, 2015).

Nisin exhibited good bacteriostatic, bactericidal and sporicidal activities against *A. acidoterrestris*. The MIC and MBC for the vegetative forms were 15.60 and 31.25 µg/mL, respectively. The MSC with total spore inactivation was reached at 62.50 µg/mL (Figure 1).

The results observed in the present work are similar to the results of Ruiz *et al.* (2013). Rajendran *et al.* (2011) observed good outcomes in treatments evaluating the effect of nisin on *Bacillus cereus*, a spore-forming bacteriuk, with nisin having an MIC of 70 µg/mL for the vegetative forms. In the study by Gyawali and Ibrahim (2014), nisin was also more effective against Gram-positive bacteria, which may be related to the protection provided by the cell wall of these microorganisms.

Antibacterial combinations

The evaluations of EO (*C. multijuga* and *T. vulgaris*) in combination with nisin were performed via the checkerboard method. For both treatments (nisin plus *C. multijuga* or *T. vulgaris*), the FIC index was 0.75, and the combination of EOs plus nisin produced an additive effect. The use of nisin and the *T. vulgaris* and *C. multijuga* EOs has great biotechnological potential as an 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 manufacturing cost of the product.

The antibacterial effect of nisin may be related to its ability to permeate the cell membrane and cause cell disruption and destruction (Lucera *et al.*, 2012). 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 acid efflux, and pH gradient of the bacteria (Turgis *et al.*, 2012).

Application in reconstituted orange juice

Figure 2 shows the results of the evaluation of the antibacterial activity of the EOs of *C. multijuga* and *T. vulgaris*, applied to reconstituted orange juice, against *A. acidoterrestris*. This assay found that both EOs were effective in the reduction and elimination of *A. acidoterrestris* after 24h of treatment. At the concentration of 8× MIC of EO of *C. multijuga*, it was possible to eliminate the entire bacterial load present in the orange juice, acting as a bactericidal substance in the reconstituted orange juice. For the EO of *T. vulgaris*, at a concentration of 16× MIC, there was a reduction of 2.68 log CFU/mL after 24 h of treatment.
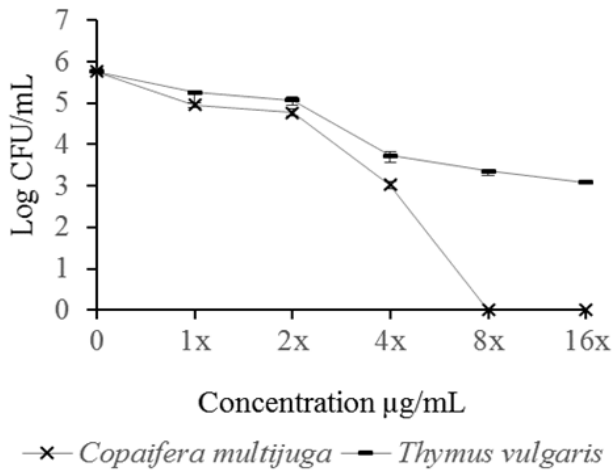
Figure 2. Antibacterial activity of the essential oils of *Copaifera multijuga* (MIC: 300 µg/mL) and Thymus vulgaris (MIC: 500 µg/mL) in orange juice reconstituted to 11 °Brix, and contaminated with *A. acidoterrestris* incubated at 45°C for 24 h. X - Amount of multiplication by the concentration of MIC.

The results of the present work indicated that both EOs performed satisfactorily as antibacterial agents in reconstituted orange juice. According to FDA (2018a; 2018b), essential oils, oleoresins (without solvents) and natural extracts (including distillates) are generally recognized as safe (GRAS).

When the combined treatment (EO plus nisin) was applied to the reconstituted orange juice, there was a considerable improvement for both treatments (nisin plus *T. vulgaris* or *C. multijuga*). At the concentration of 1× MIC for both combined treatments, it was possible to reduce the entire bacterial load of *A. acidoterrestris*, thereby establishing the combination as bactericidal. The combination of 250 µg/mL of EO of *T. vulgaris* with 7.81 µg/mL nisin (0.5× MIC) led to a reduction of 2.58 log CFU/mL. For the treatment with 150 µg/mL of *C. multijuga* EO with 7.81 µg/mL of nisin, the reduction was 3.58 log CFU/mL.

Death time curve

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

Treatment with *T. vulgaris* EO exhibited a smaller reduction than treatment using the *C. multijuga* EO. Total bacterial elimination was reached after 48 h of treatment using a concentration of 8× MIC. In

treatment using 1× MIC for 24 h and 48 h, there was a reduction of 3.81 log CFU/mL and, approximately, 4.11 log CFU/mL, respectively (Figure 3B). The results observed in this assay were similar to the results for MBC, with values from 2,000 µg/mL for *C. multijuga* and 10,000 µg/mL for *T. vulgaris* in 24 h treatments.
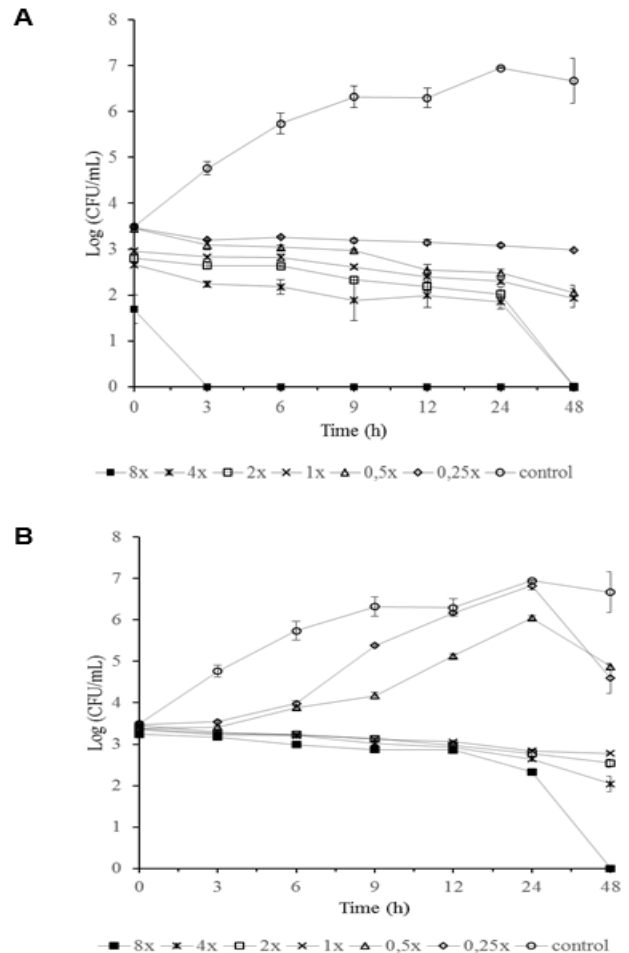

Figure 3. Death time curve for the essential oils of (A) *Copaifera multijuga* (MIC: 300 µg/mL) and (B) *Thymus vulgaris* (MIC: 500 µg/mL) against the vegetative cell of *A. acidoterrestris*. Bacteria grown in medium BAT, temperature of 45°C. X - Amount of multiplication by the concentration of MIC.

Dose-response effect and cell viability

The cytotoxic effect of the EOs on Vero cells was indicative of cell survival, as dead cells cannot metabolise MTT. The results obtained in this assay revealed that the EOs of *C. multijuga* and *T. vulgaris* had CC_{50} values of 54.2 and 142.3 µg/mL, respectively, causing a reduction of 50% of viable Vero cells at these concentrations. The IC_{50} results of *A. acidoterrestris*, using the EOs of *C. multijuga* and *T. vulgaris*, were 500 and 1,000 µg/mL, respectively, and there was a 50% inhibition of bacterial growth with these concentrations. The selectivity index (SI)

was used to compare the activity of the EOs against Vero cells (CC_{50}) with the toxic effect against *A. acidoterrestris* (IC_{50}). Values < 1.0 indicate that the SI is less selective for the microorganisms, revealing that the tested sample is more toxic (Santos *et al.*, 2008b). This comparison showed that the EOs were less selective for *A. acidoterrestris* and more toxic to Vero cells, as the SIs of the EOs of *C. multijuga* and *T. vulgaris* were 0.11 and 0.14, respectively.

Scanning electron microscopy

After treatment (45°C for 24 h) with *C. multijuga* and *T. vulgaris* EOs at concentrations of 500 µg/mL for spores and 1× MIC and 0.5× MIC for the vegetative cells of *A. acidoterrestris*, external morphological alterations were observed in cell forms with both treatments, as compared with the control group, using scanning electron microscopy (Figure 4).

The vegetative cells of *A. acidoterrestris* were in rod form, and in the control, the bacterial membrane was smooth and integrated (Figure 4A). When comparing with the cells in which EO treatment was applied, we could verify the changes that occurred due to the treatment (Figure 4A, 4B, 4D and 4E). The same occurred with the sporulated cells of *A. acidoterrestris*, where the control for the spores presented a smooth and integrated membrane (Figure 4F) but the treated spores showed clear deformations (Figure 4G and 4H).

According to data found in the literature, the constituents present in the essential oils include hydrophobic compounds that have the capacity to alter the chemical and physical structures of the cell wall and membrane, consistent with results found in this assay (Sikkema *et al.*, 1995). In addition, the lipophilic characteristic of the EOs means they can more easily penetrate the cytoplasmic membrane of the bacterium, a factor that may also contribute to the alterations found in the vegetative cells and in the spores of *A. acidoterrestris* (de Alencar Filho *et al.*, 2017).

Santos *et al.* (2008a) 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 obtained in the present work.

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
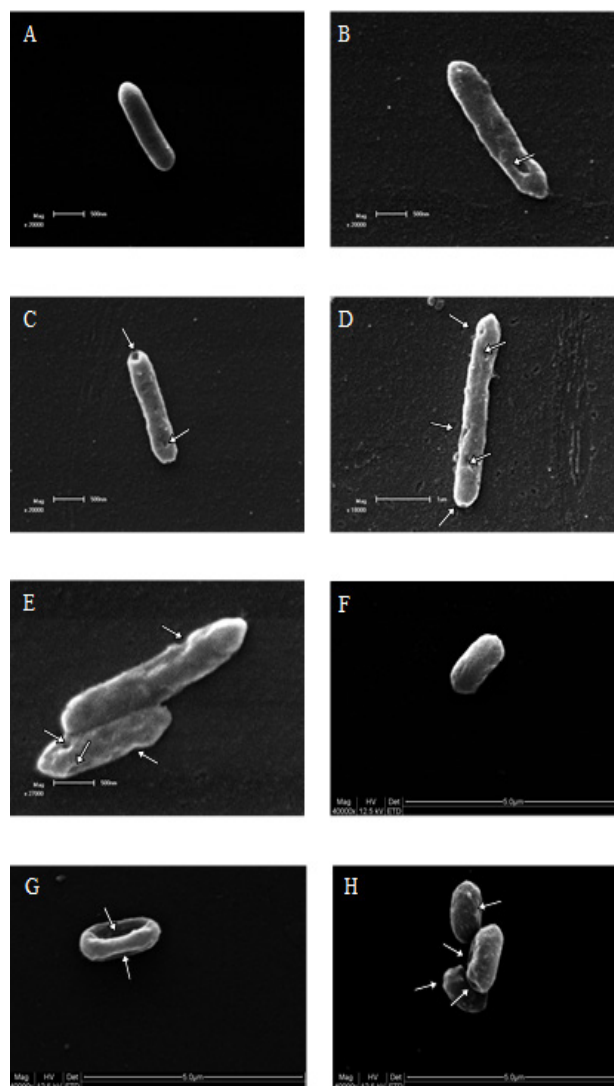

Figure 4. Scanning electron microscopy of *A. acidoterrestris* vegetative cells and spores after 24 h of treatment using the *Copaifera multijuga* and *Thymus vulgaris* essential oils (EOs): (A) control – vegetative cell, bar: 50 nm, magnification: 20,000×; (B) vegetative cell treated with *C. multijuga* EO at 150 µg/mL, bar: 50 nm, magnification: 20,000×; (C) *C. multijuga* EO at 300 µg/mL, bar: 50 nm, magnification: 20,000×; (D) *T. vulgaris* EO at 250 µg/mL, bar: 1 µm, magnification: 18,000×; (E) *T. vulgaris* EO at 500 µg/mL, bar: 500 nm, magnification: 27,000×; (F) control – spore, bar: 5 µm, magnification: 40,000×; (G) spores treated with *C. multijuga* EO at 500 µg/mL, bar: 5 µm, magnification: 40,000×, and (H) *T. vulgaris* EO at 500 µg/mL, bar: 5 µm, magnification: 40,000×. The arrows point the external morphological changes.

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 than cells from the negative control (Figure 5). However, spores treated with the EOs of *C. multijuga* and *T. vulgaris*, at concentrations of 500 and 1,000 µg/mL, respectively, showed no changes as compared to the negative control (Figure 5).
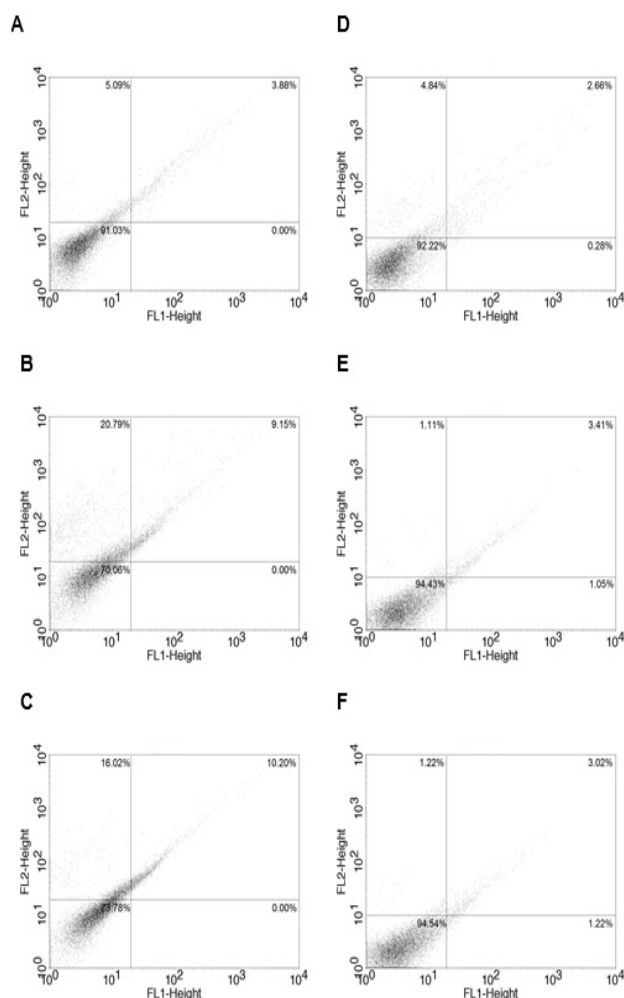
Figure 5. Flow cytometry plot showing the PI staining of A. acidoterrestris vegetative cells and spores after 24 h treatment using the EOs of *Copaifera multijuga* and *Thymus vulgaris*: (A) control – vegetative cell. Vegetative cell treated with (B) *C. multijuga* EO at 300 μg/mL and (C) *T. vulgaris* EO at 500 μg/mL. (D) control – spores. Spores treated with (E) *C. multijuga* EO at 500 μg/mL and (F) *T. vulgaris* EO at 1,000 μg/mL.

Conclusion

In conclusion, the results of the present work indicate that the EOs of *C. multijuga* and *T. vulgaris* displayed antibacterial activity against the vegetative cells and spores of *A. acidoterrestris* during in vitro assays. When applied to reconstituted orange juice, both EOs proved to be effective antibacterial agents against *A. acidoterrestris*. The combination of EOs with nisin exhibited additive effects. The SI results indicated that concentrations used in the present work had cytotoxic effects on Vero cells. However, due to the additive effect found, the combination of these antimicrobials could be utilised in orange juice at lower concentrations, considered safe. Apart from toxicity, other studies should be conducted regarding the sensory changes that EOs is likely to cause in

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

Acknowledgement

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