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Short communication

Synergistic inhibition of bacteria associated with minimally processed vegetables in mixed culture by carvacrol and 1,8-cineole

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ABSTRACT

This study assessed the effect of the combined application of carvacrol (CAR) and 1,8-cineole (CIN) against bacteria associated with minimally processed vegetables (MPV), namely *Listeria monocytogenes*, *Aeromonas hydrophila* and *Pseudomonas fluorescens*, in mixed culture. For this, Fractional Inhibitory Concentration (FIC) index, time-kill in vegetable-based broth, vegetable matrices and confocal and scanning electron microscopy analysis were carried out. CAR and CIN displayed Minimum Inhibitory Concentrations (MIC) of 1.25 and 40 μL/mL, respectively. FIC index of the combined compounds was 0.25 against the mixed inoculum, suggesting a synergic interaction. The application of CAR and CIN alone (MIC) or combined (1/8 MIC + 1/8 MIC) in vegetable-based broth caused a decrease ($p < 0.05$) in viable cell counts over 24 h. CAR and CIN in combination reduced ($p < 0.05$) the mixed inoculum in vegetable broth and in experimentally inoculated fresh vegetables. The exposure to these compounds changed the membrane permeability and caused ultrastructural changes in surfaces of bacterial cells. The enhancing inhibitory effects observed for subinhibitory concentrations of CAR and CIN in combination against a mixed culture of bacteria associated with MPV suggest that the combination of these compounds is effective for controlling bacterial growth and survival in these foods.

1. Introduction

Minimally processed vegetables (MPV) undergo processes involving physical modification, including selection, washing, peeling, cutting, sanitization, rinsing, drying and packaging, to extend their shelf life and preserve their nutritive properties but still retain characteristics of fresh food (Food and Drug Administration [FDA], 2008). MPV are consumed primarily raw, representing a potential microbiological safety hazard, especially when processing occurs under unsatisfactory sanitary conditions (Gleeson & Beirne, 2005).

The emergence of pathogenic microorganisms not previously associated with raw vegetable products has enhanced the potential for foodborne outbreaks related to MPV (Martin-Belloso, 2007). Among the emerging food-related bacteria, psychrotrophic pathogens, such as *Aeromonas hydrophila* (Uyttendaele, Neyts, Vanderswalmen, Notebaert, & Debevere, 2004) and *Listeria monocytogenes* (Carrasco, Pérez-Rodríguez, Valero, García-Gimeno, & Zurera, 2008), and spoilage bacteria of the genus *Pseudomonas*, such as *Pseudomonas fluorescens* (Ragaert, Devlieghere, & Debevere, 2007), have been associated with MPV, usually products stored at low temperatures.

Therefore, research on novel and effective practices to control bacterial survival and growth in MPV are needed. In this context, essential oils and their constituents have been considered potential candidates to be applied to MPV to ensure their microbiological safety and stability (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). The essential oils from *Origanum vulgare* L. (oregano) and

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Rosmarinus officinalis L. (rosemary), whose main constituents are carvacrol (CAR) (66.9%) and 1,8-cineole (CIN) (32.2%), respectively (Azerêdo et al., 2011), have shown broad-spectrum antimicrobial activity that includes the inhibition of known bacterial contaminants of MPV (Sousa, Azerêdo, et al., 2012; Sousa, Torres, et al., 2012).

Although some studies have demonstrated the antimicrobial activity of CAR and CIN against bacteria in individual culture, studies regarding the efficacy of these compounds when applied alone or in combination in inhibiting bacterial mixed cultures are scarce or non-existent. Since the use of mixed inoculum has been recommended in studies using specifically defined conditions that limit the growth of bacterial species (Buchanan, Bagi, Goins, & Phillips, 1993; Romero, Fernández Pinto, Patriarca, & Vaamonde, 2010), the response of bacteria in mixed cultures when challenged with CAR and/or CIN could provide more realistic information regarding the antimicrobial efficacy of these compounds in food matrices.

This study assessed the antibacterial effects of CAR and CIN alone or in combination against a mixed culture of bacteria associated with the contamination of MPV using the Fractional Inhibitory Concentration (FIC) index, time-kill assays in vegetable broth, vegetable matrices and confocal and scanning electron microscopy analysis.

2. Materials and methods

2.1. Materials

CAR and CIN were supplied by Sigma Aldrich (Sigma, France). Solutions of these compounds were prepared within a range of 160–0.075 μL/mL using bacteriological agar (0.15 g/100 mL) as a stabilizing agent (Bennis, Chami, Chami, Bouchikhi, & Remmal, 2004).

L. monocytogenes ATCC 7644, *A. hydrophila* ATCC 7966 and *P. fluorescens* ATCC 11253, obtained from the Microorganism Collection, Laboratory of Food Microbiology, Federal University of Paraíba (João Pessoa, Brazil), were used as test microorganisms. Inocula of each strain (7 log cfu/mL) were obtained as previously described (Sousa, Azerêdo, et al., 2012). The mixed inoculum was obtained by mixing the different bacterial suspensions at a ratio of 1:1:1.

Time-kill assays, fluorescent cell assays and assays to observe changes in utrastructural aspects of cells were performed using a vegetable-based broth composed of iceberg lettuce (*Lactuca sativa* L.), chard (*Beta vulgaris* L. var. *cicla*) and rocket (*Eruca sativa* L.) as substrate for bacterial cultivation, which was prepared as previously described (Azerêdo et al., 2011).

2.2. Determination of the Minimum Inhibitory Concentration (MIC), FIC index and time-kill assays

The MIC values of CAR and CIN were determined using macrodilution in broth (Nostro et al., 2001). The checkerboard method was performed using macrodilution in broth to obtain the FIC index for the combined application of CAR and CIN. The FIC index was calculated as follows: (MIC of CAR in combination with CIN/MIC of CAR alone) + (MIC of CIN in combination with CAR/MIC of CIN alone). CAR and CIN were assayed at the MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC, 1/16 MIC and 1/32 MIC in different combinations of each of the different concentrations of each compound. Synergy was defined as FIC $\leq$ 0.5; indifference (no interaction) was defined as FIC > 0.5–4; and antagonism was defined as FIC > 4 (Hemaiswarya, Kruthiventi, & Doble, 2008; Mackay, Milne, & Gould, 2000).

The effect of the compounds alone (MIC) and in combination (1/8 MIC CAR + 1/8 MIC CIN) on the cell viability of bacterial strains in vegetable-based broth over 24 h at 7 °C was evaluated by the viable cell count procedure, using selective medium for each bacteria: *Listeria* selective agar (Himedia, India), *Aeromonas* selective isolation medium (Himedia, India) and *Pseudomonas* selective agar (Himedia, India) (Azerêdo et al., 2011; Sousa, Azerêdo, et al., 2012) for 48 h at 35 °C. Control flasks without CAR or CIN were similarly tested. The results were expressed as the log of cfu/mL.

2.3. The effect of CAR and CIN on the survival of bacteria in fresh vegetables

Portions (90 g) of a pool of iceberg lettuce, chard or rocket (in a rate of 1:1:1) that were previously washed with sterile distilled water were shredded aseptically and inoculated with the bacteria according to the following procedure: the portion of vegetables was submerged in 900 mL of the mixed inoculum, softly rotated with a sterile glass stem for 5 min to ensure effective inoculation and air-dried for 1 h in a bio-safety cabinet. Subsequently, the vegetables were submerged in 250 mL of solutions of CAR or CIN alone (MIC) or in a mixture (1/8 MIC CAR + 1/8 MIC CIN) for 5 min at 25 °C. Then, a 25-g sample of the vegetables was aseptically obtained, transferred into a sterile stomacher bag containing 225 mL of sterile peptone water (1 g/L) and homogenized for 60 s. Subsequently, a decimal dilution was made in the same diluent, and bacterial enumeration was performed by spread-plating 0.1 mL of the appropriate sample dilution on sterile selective agar (Xu et al., 2007). Control flasks containing sterile distilled water were tested in the same way. The results were expressed as the log of cfu/mL.

2.4. Fluorescent cell assay

Mixed inocula were exposed for 15 and 30 min (7 °C) to CAR or CIN alone (MIC) or combined (1/8 MIC CAR + 1/8 MIC CIN) in vegetable broth and stained using a LIVE/DEAD BacLight bacterial viability kit for microscopy (Molecular Probes, Eugene, OR, USA) as described by the manufacturer. This assay uses mixtures of SYTO® 9 green fluorescent nucleic acid stain, which stains all cells green, and the red fluorescent nucleic acid stain propidium iodide (PI), which only penetrates those cells whose cell membranes have been damaged. Double staining with PI and SYTO 9 was performed by incubating the samples with 1.50 μM PI and 250.5 nM SYTO 9 for 15 min at room temperature. Aliquots of the mixed bacterial inoculum exposed to isopropyl alcohol (PI) (700 mL/L) for 1 h were used as positive controls for membrane permeabilization, and mixed bacterial cells incubated in the absence of CAR and CIN were used as a negative control. The cells were observed using a TCS SP2 AOBS confocal microscope (Leica Microsystems, Wetzlar, Germany) at excitation/emission wavelengths of 480/500 nm for the SYTO 9 stain and 490/635 nm for the PI stain (Leonard, Virijevic, Reginier, & Combrinck, 2010). The collected images were analyzed using the Lite 2.0 software.

2.5. Scanning electron microscopy (SEM)

Mixed inoculum exposed to CAR and CIN alone (MIC) or in combination (1/8 MIC CAR + 1/8 MIC CIN) in vegetable broth for 1 h (7 °C) were harvested by centrifugation at 10,000 × *g* for 12 min at 4 °C, washed in PBS and fixed for 24 h at 4 °C with 2.5% glutaraldehyde in 0.1 M PBS. After washing in the same buffer, the cells were post-fixed for 30 min with osmium tetroxide (0.01 g/L) in 0.1 M cacodylate buffer (pH 7.2) and allowed to adhere to poly-lysine-coated coverslips. The samples were dehydrated in ethanol, critical-point-dried with CO_2, coated with a 20-nm-thick gold layer and observed using a Quanta 200 FEG (FEI, Hillsboro, OR, USA) scanning electron microscope. The bacterial cells not exposed