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Novel antimicrobial agents as alternative to chlorine with potential applications in the fruit and vegetable processing industry

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ABSTRACT

There has been an increasing demand for fresh fruit and vegetables in recent years. Along the processing line in fresh-cut vegetable production, disinfection is one of the most important processing steps affecting the quality and safety, and the shelf-life of the end produce. Although a range of antimicrobial compounds commonly termed biocides or disinfectants are available, chlorine has long been used to disinfect washing waters of fresh-cut vegetables. However, since chlorine reactions with organic matter lead to the production of by-products, alternative disinfectants to chlorine must be evaluated. A synthetic washing water formula has been developed to determine the antimicrobial efficiency of different families of potential disinfectants: quaternary ammonium compounds (QACs) as benzalkonium chloride (BZK), and didecyldimethylammonium chloride (DDAC); isothiazolinones (mixture of methylchloroisothiazolinone and methylisothiazolinone, CMIT:MIT 3:1 and 1:1); and essential oils (carvacrol, CAR). The twin configuration and higher length of the chains of alkyl groups of DDAC compared to BZK have led to a higher antimicrobial efficiency. In both cases, Gram-positive bacteria seemed to be much more sensitive to the QAC attack than Gram-negative. The opposite happened for CMIT:MIT. The chloro-substituted isothiazolinone (CMIT) has been proven to be much more effective than its unsubstituted form (MIT). In addition, in contrast to chlorine, its antimicrobial activity together with that of DDAC was not decreased when increasing the organic matter content of the water. Synergetic antimicrobial effects have been confirmed when combining BZK and CAR. MBC values were determined in SWW, during 90 s of contact time and *Salmonella* concentration of 10^3 CFU/mL, corresponding to: 100 (BZK), 30 (DDAC), 50 (CMIT:MIT 3:1), 100 (CMIT:MIT 1:1), 300 (CAR), 75 (BZK)-200 (CAR), and 9 (free chlorine) mg/L. MBC values for inactivating similar concentration of *E. faecalis* corresponded to: 50 (BZK), and 10 (DDAC) mg/L. Increasing contact times up to 5 min did not lead to higher antimicrobial efficiencies. CMIT:MIT 3:1 together with DDAC, and combinations of BZK-CAR seem to be a plausible alternative to chlorine.

1. Introduction

In recent years, there has been an increase in consumption of fresh vegetables and fruit due to their convenience of use and health benefits. As many fresh vegetables and fruit are consumed raw, they have been catalogued as high risk food products. In fact, number of documented outbreaks of human toxiinfections associated with the consumption of raw and minimally processed fruit and vegetables has increased considerably over the past decades (Oms-Oliu et al., 2010). *Salmonella* has been responsible for numerous outbreaks (Oms-Oliu et al., 2010). Hence, it is considered one of major bacterial challenges of food safety.

In fresh produce processing, wash water retains soil, juice from the cut produce, viruses and bacteria. Therefore, reusing processing water may result in the build-up of microbial loads, including undesirable pathogens from the produce. Thus, wash water of inadequate quality has the potential to be a direct source of contamination and a vehicle for spreading bacterial contamination (Gil et al., 2009). The vegetable wash water may increase bacterial counts. Thus, the most widely applied approach to reduce the microbial contamination and maintain the water quality is to use sanitizing agents in vegetable wash water.

Disinfection of water is a critical step to minimize the potential transmission of pathogens from water to produce, among produce

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within a lot, and between lots over time (FAO/WHO, 2008). NaOCl (50–200 mg/L), with a contact time of 1–2 min (Beuchat and Ryu, 1997), is often used to reduce microbial populations in the water used during washing operations. Its antimicrobial effect is due to the attack of amino groups in nucleic acids (Denyer and Stewart, 1998). Since chlorine reacts with organic matter, its use may lead to the formation of some potentially hazardous by-products such as trihalomethanes and chloramines, which are potential carcinogens. Its use only yields bacterial concentration reductions around 1–2 log units (90–99%) in both, water and produce (Sapers, 2001). Natural cracks, crevices, and waxes in fruits and vegetables have been reported to provide protection to the product, hindering the effectiveness of chlorine. Although both FAO and U.K. has stated that washing foods with chlorine does not pose a health risk to consumers (COT, 2007), there is a tendency in removing chlorine from the disinfection processes (Ölmez and Kretzschmar, 2009).

Extensive research is being developed to explore the potential of different antimicrobial agents in preserving food safety and food quality (Sun, 2014) with less environmental and health impact.

Quaternary ammonium compounds (QACs) are cationic antibacterial agents widely used as disinfectants even to prevent cross-contamination from equipment/machines to food in processing industries (Gilbert and Moore, 2005; Møretrø et al., 2017; Shirron et al., 2009; Yoshimat and Hiyama, 2007). It is known that the antibacterial effect of QACs depends on the chain lengths of alkyl groups which affect their hydrophobicity (Gilbert and Moore, 2005). Benzalkonium chloride (BZK) is a commonly used type of QAC, which contains one long-chain alkyl group of C12-C16. At low concentrations, these positively charged molecules bind strongly to the anionic sites found on the membrane surface and cell walls of bacteria because of their opposite, negative charge (Gilbert and Moore, 2005). Antimicrobial activity of didecyldimethylammonium chloride (DDAC) is based on the typical structure of twin long-chain of alkyl groups of C22, which leads to a stronger antimicrobial effect (Yoshimat and Hiyama, 2007).

Generally, natural antimicrobials as plant essential oils (EOs) such as carvacrol, eugenol, and thymol play a vital role as flavoring agents in food products. However, it is proven that many also possess a wide range of antimicrobial properties. Although, they can impart aromatic compounds to the product, they are Generally Recognized As Safe (GRAS) (Sun, 2014). The mechanism of action of carvacrol and EOs in general against microorganisms involves the interaction of phenolic compounds with the proteins (porins) in the cytoplasmic membrane altering microbial cell permeability (Roller, 2000; Sun, 2014). The application of EOs and carvacrol-containing washing solutions to control the growth of foodborne pathogens and food spoilage has been reported in different fruits and vegetables (Abadias et al., 2011; Martínez-Hernández et al., 2017; Millan-Sango et al., 2016).

Isothiazolinone-derived biocides are used for controlling microbial growth and biofouling in industrial water treatment applications. They are also authorized to be used as antimicrobials in food contact materials (EC 10/2011). Two of the most widely used isothiazolinone biocides are 5-chloro-2-methyl-4-isothiazolin-3-one (chloromethylisothiazolinone or CMIT) and 2-methyl-4-isothiazolin-3-one (methylisothiazolinone or MIT), which are the active ingredients in a 3:1 mixture (CMIT:MIT) sold commercially as Kathon® or Predator 8000®. Isothiazolinones are responsible for the inhibition of key enzymes, in particular thiol-containing cytoplasmatic and membrane-bound enzymes. This leads to the inhibition of bacterial metabolism and growth, requiring a certain contact time to produce cell death (Denyer and Stewart, 1998; Williams, 2007).

As generally, antimicrobial activity is depending on the chemical structure of disinfectant as well as concentration, this study aims at assessing the effect of antimicrobials from different families at different concentrations as alternatives to NaOCl. Antimicrobial properties of quaternary ammonium compounds (QACs), isothiazolinones (CMIT:MIT), EOs, and their combinations have been studied in comparison with control treatments with NaClO in simulated wash water against Gram-negative and Gram-positive bacteria.

2. Materials & methods

2.1. Bacterial strain and inoculum preparation

Gram-negative gentamicin-resistant and chromosomally green fluorescent protein (GFP) labeled *Salmonella enterica* sv Thompson pGT-Kan mB156 provided by Dr. F. Pérez-Rodríguez, Department of Food Science and Technology, University of Córdoba; and Gram-positive *Enterococcus faecalis* (ATCC 11700), provided by the CECT (Colección Española de Cultivos Tipo, Valencia, Spain) were studied at levels of concentration of 6 and 3 $\log_{10}$ colony forming units (CFU)/mL in independent experiments. *E. faecalis* was grown in tryptic soy broth (TSB, Scharlau, Barcelona, Spain), whereas *Salmonella* was grown in TSB supplemented with gentamicin 15 μg/mL (Sigma-Aldrich, Darmstadt, Germany). Both were kept at 37 °C under rotary shaking for 24 h. Fresh bacterial cultures were obtained at stationary phase with around 9 $\log_{10}$ CFU/mL. A volume of 5 mL of viable cells was harvested by centrifugation (3500 rpm, for 25 min), washed, and resuspended in 5 mL of sterile saline solution (NaCl, 0.9%). Experiments for *E. faecalis* and *Salmonella* were carried out independently unless otherwise specified.

2.2. Simulated vegetable wash water preparation

Simulated wash water (SWW) was prepared based on physico-chemical characterization of real wash waters of fresh-cut lettuce processing lines from a Spanish processor. The main chemical properties of the prepared simulated wash water can be summarized as: Total organic carbon (TOC) (150 mg/L), turbidity (100 NTU), conductivity (1000 μS/cm), pH (6.2), F^- (0.14 mg/L), Cl^- (282 mg/L), NO_2^- (0.030 mg/L), Br^- (10.2 mg/L), NO_3^- (51.6 mg/L), SO_4^{2-} (51.0 mg/L), Na^+ (87.7), NH_4^+ (1.24 mg/L), K^+ (108.0 mg/L), Mg^{2+} (9.55), Ca^{2+} (47.1 mg/L). These compounds were supplied by Scharlau, Barcelona, Spain. TOC was provided by adding malt extract for microbiology (Appli. Chem. Panreac, Castellar del Vallés, Spain) (350 mg/L); and turbidity was obtained by supplemented kaolin powder (Merck, Madrid, Spain) (100 mg/L) dissolved 24 h prior to use.

2.3. Antimicrobial suspension preparation

Disinfectant agents of different families of chemical compounds were purchased: QACs as Benzalkonium chloride (BZK) (CAS 63449-41-2) and didecyldimethylammonium chloride (DDAC) (CAS 7173-51-5) (Sigma-Aldrich, Darmstadt, Germany); isothiazolones as Kathon® (Dow®, Houston, USA) and Predator 8000® (Innospec Limited, Barcelona, Spain) containing CMIT/MIT composed of a 3:1 ratio at a final concentration of 1.5% total active ingredient; and CMIT/MIT 1:1 (CAS 55965-84-9) (Sigma-Aldrich, Darmstadt, Germany); EOs as carvacrol (CAR) (CAS 499-75-2) (Sigma-Aldrich, Darmstadt, Germany); and sodium hypochlorite solution (NaOCl) (containing 4.00–4.99% active chlorine) (CAS 7681-52-9) (Sigma-Aldrich, Darmstadt, Germany). A colorimetric determination of free chlorine was measured in NaOCl suspensions by a reagent kit (0–5 ppm) (HI93701-01, Hanna Instruments) (Scharlau, Barcelona, Spain).

Fresh stock solutions were prepared in deionized water of 2000 mg/L for DDAC; of 1000 mg/L for CMIT/MIT (3:1), CMIT/MIT (1:1), and BZK; of 500 mg/L for CAR, and free chlorine. All the stock solutions were stored in dark under 4 °C. Fresh stock suspensions were prepared every 7 days.

2.4. Determination of antimicrobial activity

Antibacterial dose–response assays were performed in the presence