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Efficacy of *Pseudomonas graminis* CPA-7 against *Salmonella* spp. and *Listeria monocytogenes* on fresh-cut pear and setting up of the conditions for its commercial application

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ABSTRACT

Pseudomonas graminis CPA-7 has been reported to control foodborne pathogens on fresh-cut apple, peach and melon. The first aim of this study was to assess its antagonistic activity against *Salmonella* spp. and *L. monocytogenes* on fresh-cut pear. CPA-7 was able to control both pathogens on fresh-cut pear stored in air conditions at 5, 10 and 20 °C. However, when CPA-7 antagonistic effect was tested by simulating commercial application (with antioxidant solution and passive modified atmosphere packaging), its effect decreased and no reductions of foodborne pathogens were reported at 10 °C. Therefore, the second aim was to optimise the antioxidant solution and the packaging in order to retain its antagonistic capacity. The selected antioxidant solution was 2% ascorbic acid +2% sodium citrate +1% $CaCl_2$ according to growth and effect of CPA-7. Film permeability, which affects gas composition inside fruit packages, influenced CPA-7 efficacy. If the biopreservative strain is used, film has to be sufficiently gas permeable to allow CPA-7 function and at the same time to maintain product quality.

1. Introduction

Fresh fruits and vegetables are an important part of a healthy lifestyle due to their high levels of vitamins and minerals. It is known that a diet rich in fruits and vegetables is protective against coronary heart disease and many cancers if they are ingested in an adequate quantity. The WHO (2003) reported that a daily intake of 400 g of fruit and vegetables contribute to a good health.

Because of the on-going consumer interest and another positive factors, the consumption of fresh-cut fruit is increasing. Processing of fresh-cut fruit and storage conditions could determine produce shelf life. At global scale, 'Conference' pear is the second most important pear cultivar after 'Williams' pear. In Europe, this variety is the most important of the total production (around 20%) (Deckers and Schoofs, 2001). Moreover, 'Conference' is the main pear variety that is produced in Lleida, Spain and this variety is one of the most suitable for minimal processing (Arias et al., 2008; Colás-Medà et al., 2016). Soliva-Fortuny et al. (2004) reported that the ripeness state of 'Conference' pear influences commercial shelf life of the fresh-cut product. Moreover, in order to extend shelf life of fresh-cut fruit, different antioxidant solutions and modified atmospheres packaging (MAP) are used (Arias et al., 2008). Low O_2 concentrations inside packaging reduce respiration rate and, consequently, delay the loss of produce quality. MAP can be obtained actively by the replacement or displacemet of gas inside the packaging, or passively by using films with different permeability. Francis and O'Beirne (1998) reported that antimicrobial dipping or MAP can affect growth and composition of natural microbiota in fresh-cut vegetables, and also survival and growth of pathogens. Soliva-Fortuny and Martin-Belloso (2003) demonstrated that MAP controlled the proliferation of spoilage microorganisms and avoided nutritional losses. Similarly, not only spoilage and pathogenic microorganisms could be affected, but also biopreservation cultures that have been intentionally added to obtain a beneficial effect.

Whole fruit is supposed to be microbiologically safe; however, fresh-cut fruits have been vehicles for the transmission of human pathogens and have been associated with foodborne outbreaks. Most of them have been attributed to *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* (CDC, 2016a), being the pathogen most frequently associated with consumption of fruit and

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vegetables (Sivapalasingam et al., 2004). In recent years, *Salmonella* caused 18% of outbreaks in the USA and 20% in the European Union (EU) that were related to consumption of fruit and vegetables (Callejon et al., 2015). *L. monocytogenes* is a psychrotrophic bacteria, that is able to grow at refrigeration temperatures, and although it has been responsible for outbreaks related to fresh fruit and vegetables, its incidence level is lower than for *Salmonella* (Callejon et al., 2015). However, it is a relevant pathogen due to its high mortality rate (15–23%) (CDC, 2015, 2016b; EFSA, 2015, 2016).

The processing of fruit could enhance the contamination of edible tissue. Moreover, the high quantity of nutrients available in these products in combination with bad storage conditions could promote the growth of pathogen and spoilage microorganisms. Therefore, other control measures are necessary to reduce the risks such as chemical products, which are usually used to control pathogenic and spoilage bacteria in fresh-cut products or refrigeration temperatures. Nevertheless, consumers have a negative perception of chemical products and for this reason, researchers are looking for safer alternatives to control pathogens and improve food safety, such as the use of antagonistic microorganisms (Jordan et al., 2014). Non-pathogenic microorganisms could compete with pathogens for physical space and nutrients, and they are able to produce antagonistic compounds that negatively affect viability of pathogens (Parish et al., 2003). It is known that native microbiota of fresh fruits and vegetables is able to inhibit or enhance the growth of pathogen microorganisms (Nguyen-the and Carlin, 1994). Results obtained by Schuenzel and Harrison (2002) suggest that native microbiota isolated from a produce could not be present in other fresh-cut produce, therefore the native microflora is specific for each product. Several authors have investigated indigenous microflora as being antagonist against foodborne pathogens in fresh vegetables as well as fruits. Ukuku et al. (2004) showed the antagonistic effect of indigenous microbiota against *L. monocytogenes* on fresh-cut melon. Leverentz et al. (2006) observed the antagonistic effect of native microbiota against *L. monocytogenes* on fresh-cut apples. Liao (2007) proved that naturally occurring microbiota control the growth of pathogens on baby carrots. Alegre et al. (2012) demonstrated an *Enterobacteriaceae* species, isolated from fresh-cut apples, controlled the growth of foodborne pathogens on minimally processed (MP) apples and peaches.

Pseudomonas graminis strain CPA-7, which was isolated from apple surface, has been reported to control the growth of foodborne pathogens in fresh-cut apples and peaches (Alegre et al., 2013a, 2013b) and fresh-cut melon (Abadias et al., 2014). Moreover, there were no significant differences on physical quality parameters and visual appearance between fruit treated with CPA-7 and non-treated fruit in the tested conditions for MP apples (Alegre et al., 2013a) and fresh-cut melon (Plaza et al., 2016). The first objective of this work was to ascertain the efficacy of CPA-7 against *Salmonella* spp. and *L. monocytogenes* on fresh-cut pear. In order to broaden its spectrum of action, the second objective was to find the most appropriate antioxidant treatment and packaging conditions to preserve quality parameters of fruit and to improve the survival of CPA-7 and with that, its antagonistic effect against foodborne pathogens during produce shelf life.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

The five *Salmonella enterica* subsp. *enterica* serovars were: Agona (ATCC BAA-707), Michigan (ATCC BAA-709), Montevideo (ATCC BAA-710), Gaminara (ATCC BAA-711) and Enteritidis (CECT-4300). *Salmonella* strains were grown individually in tryptone soy broth (TSB, Biokar Diagnostic) medium for 20–24 h at 37 ± 1 °C.

To prepare the cocktail of *Listeria monocytogenes*, the serovars used were: serovar 1a (CECT 4031), serovar 3a (CECT 933); serovar 4d (CECT 940), serovar 4b (CECT 4032) and serovar 1/2a, this latter being previously isolated in our laboratory from a fresh-cut lettuce sample (Abadias et al., 2008). *L. monocytogenes* strains were grown individually in TSB supplemented with 6 g/L of yeast extract (TSBYE) for 20–24 h at 37 ± 1 °C.

Strain *Pseudomonas graminis* CPA-7, which was isolated in our laboratory from an apple surface, (Alegre et al., 2013b) was used as antagonist. It was grown in TSB for 20–24 h at 25 ± 1 °C.

Bacterial cells were harvested by centrifugation at 9800×g, 10 min at 10 °C. The broth was decanted and the pathogen cells were resuspended in saline solution in proportion 2:1 (v/v) (SS; 8.5 g/L NaCl) and CPA-7 cells were resuspended in sterile distilled water. Equal volumes of the five *Salmonella* concentrated suspensions were mixed to produce a single suspension. Also, equal volumes of the five *L. monocytogenes* concentrated suspensions were mixed to produce a single suspension.

For the inoculum preparation, an aliquot of each of the bacterial concentrated suspensions was added to deionised water or antioxidant solution (depending on the assay conditions) to obtain approximately 10^5 CFU/mL in the case of *Salmonella* and *L. monocytogenes* and 10^7 CFU/mL for CPA-7. Inoculum concentration was checked by plating appropriate dilutions onto XLD (Xylose-Lysine-Desoxycholate Agar, Biokar Diagnostics, France) for *Salmonella*, onto Palcam agar (Palcam Agar Base with selective supplement, Biokar Diagnostics, France) for *L. monocytogenes*, and onto trypto-casein soy agar (TSA, Biokar Diagnostics, France) for CPA-7. The plates were incubated at 37 ± 1 °C for 24 for *Salmonella* and 48 h for *L. monocytogenes*, and CPA-7 plates at 30 ± 1 °C for 48 h.

2.2. Fruit processing

'Conference' pears (*Pyrus communis* L. cv. Conference) were used as matrix. Fruit was stored at 0 °C until use. The pears were ripened by storage at 20 °C until the optimum ripeness stage for processing (44 ± 3.2 N) was reached (Soliva-Fortuny et al., 2004). The firmness of pear was measured in two opposing sites of the pear using a penetrometer (Effegi, Mila, Italy) that was equipped with an 8 mm probe.

For each experiment, fruit was processed as follows: pears were washed in running tap water, dried with paper and disinfected with ethanol 70% by spraying them. After that, pears were let dry at room temperature and were peeled and cut into 10 wedges using a handheld apple corer and slicer.

2.3. Experiment 1: Evaluation of CPA-7 efficacy against Salmonella and L. monocytogenes on minimally processed pears

Once the fruit was processed, pear wedges were inoculated with the microorganisms. Pears were dipped (1:2, w/v) for 2 min at 150 rpm in deionised water inoculated with bacteria according to the following treatments: (a) Sal + Lm: deionised water with 10^5 CFU/mL *Salmonella* and *L. monocytogenes*, (b) CPA-7: deionised water inoculated with CPA-7 (10^7 CFU/mL) and (c) Sal + Lm + CPA-7: deionised water containing *Salmonella* and *L. monocytogenes* at 10^5 CFU/mL and CPA-7 at 10^7 CFU/mL. Pear wedges (50 ± 5 g) were placed in polyethylene terephthalate trays (PET, 500 mL) in ambient air (21% O_2 and 0% CO_2) and stored at 20 °C during 2 days and at 5 and 10 °C for 10 days. Each tray was a replicate and there were three replicates for each treatment and each sample date. For the analysis, 10 g of pear from each tray were mixed with 90 mL of buffered peptone water (BPW, Oxoid, LTD, Basingstoke, Hampshire, England)