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Control of foodborne pathogens on fresh-cut fruit by a novel strain of *Pseudomonas graminis*

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ABSTRACT

The consumption of fresh-cut fruit has substantially risen over the last few years, leading to an increase in the number of outbreaks associated with fruit. Moreover, consumers are currently demanding wholesome, fresh-like, safe foods without added chemicals. As a response, the aim of this study was to determine if the naturally occurring microorganisms on fruit are "competitive with" or "antagonistic to" potentially encountered pathogens. Of the 97 and 107 isolates tested by co-inoculation with *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on fresh-cut apple and peach, respectively, and stored at 20 °C, seven showed a strong antagonistic capacity (more than 1-log unit reduction). One of the isolates, CPA-7, achieved the best reduction values (from 2.8 to 5.9-log units) and was the only isolate able to inhibit *E. coli* O157:H7 at refrigeration temperatures on both fruits. Therefore, CPA-7 was selected for further assays. Dose-response assays showed that CPA-7 should be present in at least the same amount as the pathogen to adequately reduce the numbers of the pathogen. From the results obtained in *in vitro* assays, competition seemed to be CPA-7's mode of action against *E. coli* O157:H7. The CPA-7 strain was identified as *Pseudomonas graminis*. Thus, the results support the potential use of CPA-7 as a bio-protective agent against foodborne pathogens in minimally processed fruit.

1. Introduction

The consumption of minimally processed fruits and vegetables has increased continuously during the last decades due to a change in consumer tendencies, such as the lack of time to buy and cook food, the public consciousness of the health benefits associated with the consumption of produce, the year-round availability of vegetable products, and an increase in the variety of the commodities offered.

Spoilage bacteria, yeast and moulds dominate the microflora on raw fruits and vegetables; however, the occasional presence of pathogenic bacteria, parasites, and viruses capable of causing human infections, and therefore, outbreaks of foodborne diseases linked to fresh fruits and vegetables consumption, have been reported (Beuchat, 2002). For example, cantaloupes, honeydew melons, tomatoes, pears, watermelons, strawberries, mangoes and grapes have been implicated in outbreaks caused by *Salmonella* and *Escherichia coli* O157:H7 (CDC, 2007; Harris et al., 2003). *Listeria monocytogenes* has been shown to contaminate vegetables such as lettuce, broad-leaved endive, broccoli, radishes, cabbages, potatoes and cucumbers (Beuchat, 1996; Carlin and Nguyenthe, 1994; Little and Gillespie, 2008).

During the processing of fresh-cut produce, cutting, slicing, skinning and shredding remove or damage the protective surfaces of the plant or fruit so that nutrients become more available, and pathogens can be spread from the contaminated to the uncontaminated parts (EU Scientific Committee on Food, 2002). Moreover, a treatment to guarantee the total elimination of microorganisms from fresh-cut fruits and vegetables does not exist, so fresh-cut produce is particularly susceptible to the growth of spoilage bacteria and pathogens. Previous studies have demonstrated the capability of *Salmonella* Michigan, *E. coli* O157:H7 and *Listeria innocua* to grow on fresh-cut apples (Abadias et al., 2009; Alegre et al., 2010a; Conway et al., 2000; Dingman, 2000; Gunes and Hotchkiss, 2002; Janisiewicz et al., 1999b; Leverentz et al., 2006) and peaches (Alegre et al., 2010b).

In the fresh-cut industry, chlorine is commonly used to disinfect produce. However, chlorine does not ensure elimination or even an efficient reduction in the pathogen levels (Beuchat, 1998). A prolonged exposure to chlorine vapour may cause irritation to the skin

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and respiratory tract of the handlers. In addition, chlorinated organic compounds, such as trihalomethanes, can be produced when the chlorine contacts the organic matter. In addition, there is an increasing demand for "natural" and "additive-free" products. Therefore, it is desirable to preserve foods by natural means (Kim, 1993). Biological control fits well with this new trend, and several bacteria and yeast have been identified as bioprotective agents (Vermeiren et al., 2004). The native microflora established on food may have inhibitory properties against contaminating foodborne pathogens and therefore, via competition or antibiosis, function as a hurdle to pathogen growth and survival (Leistner and Gorris, 1995; Schuenzel and Harrison, 2002). Therefore, it should be possible to find specific organisms among the natural microflora that are responsible for exhibiting these pathogenic features. For example, the strain *Pseudomonas syringae* L-59-66, which was commercialised as BioSave 110 (EcoScience Corp. Orlando, FL) for controlling the postharvest decay of apples and pears, prevented the growth of *E. coli* O157:H7 on wounded apple tissue (Janisiewicz et al., 1999a). The growth of *L. monocytogenes* and *Salmonella enterica* in fresh-cut apples has been prevented using fungal antagonists (Leverentz et al., 2006). Trias et al. (2008) found five strains of lactic acid bacteria that were able to inhibit *L. monocytogenes* and *Salmonella typhimurium* in apple wounds but were not effective in reducing the amount of *E. coli*. Recently, Abadias et al. (2009) found that the application of the fungal postharvest antagonist *Candida sake* CPA-1 reduced the growth of a mixture of *E. coli* strains in apple wounds at 25 °C.

The objective of this study was to evaluate the native microorganisms from fresh and fresh-cut fruit that showed an inhibitory potential against the foodborne pathogens (FBP) *E. coli* O157:H7, *Salmonella* and *L. innocua* on minimally processed apples and peaches. The best antagonist was tested for phytopathogenicity, antimicrobial substances production, and minimum inhibitory concentration. Finally, this antagonist was identified.

2. Materials and methods

2.1. Fruit

"Golden Delicious" apples and "Royal Glory," "Elegant Lady," "Merry O'Henry," "Tardibelle," "Placido" and "Roig d'Albesa" peaches were used in these experiments. The different varieties of peaches were used due to the high seasonality and low storage capability of these fruits. The fruit, which had not received any postharvest treatment, was obtained from the IRTA Experimental Station and from the packinghouses in Lleida (Catalonia, Spain).

The fruit was washed in running tap water and surface-disinfected with 70% ethanol. The fruit was cut in half, and 1-cm long plugs of 1.2 cm of diameter were taken out using a cork borer. The plugs were placed into sterile glass test tubes.

On the day of the assay, the fruit was evaluated by particular quality parameters. A sample of each of the apples and the peaches used was tested for pH with a penetration electrode (5231 Crison, and pH-meter Model GLP22, Crison Instruments S.A., Barcelona, Spain). After the pH determination, the fruit was crushed, and the soluble solids content was determined at 20 °C using a handheld refractometer (Atago Co., LTD., Japan). To measure the titratable acidity, 10 mL of fruit juice was diluted with 10 mL of distilled water, and this solution was then titrated with 0.1 N NaOH up to pH 8.1. The results were calculated as g of malic acid L^{-1}.

2.2. Antagonists

The bacteria and yeast to be tested as putative antagonists were isolated from fresh-cut apples, peaches and pineapples and from the surface of fresh apples, peaches and nectarines. The whole fruit was rinsed with sterile deionised water, submerged in saline peptone (SP, 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) and sonicated for 10 min. To isolate microorganisms from the fresh-cut fruit, 25 g of the products was mixed with 225 mL of SP in a stomacher blender for 2 min with 150 rpm (Stomacher 400 Circulator, Seward). Several dilutions of either whole or fresh-cut fruit were plated on the following different media: nutrient yeast dextrose agar (NYDA, 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 15 g L^{-1} agar), NYDA supplemented with imazalil (20 ppm, Sigma, Madrid, Spain) for bacteria isolation or with streptomycin sulphate salt (500 ppm, St, Sigma, St. Louis, MO) for yeast and mould isolation and de Man, Rogosa and Sharpe medium (MRS, Biokar Diagnostics, Beauvais, France) for lactic acid bacteria isolation. The plates were incubated at 25 ± 1 °C for 3 days. Colonies of different morphologies were selected and isolated.

A collection of fungal antagonists belonging to the Pathology Laboratory collection, which have demonstrated efficacy in reducing fungal postharvest diseases, was also tested.

The antagonists were grown on NYDA plates at 25 ± 1 °C for 2–3 days. The colonies were scraped from the medium, and a suspension of 30 ± 5% transmittance ($\lambda = 420$ nm), which corresponded to a concentration between 10^6 and 10^8 cfu mL^{-1}, was prepared in 5 mL of sterile, deionised water.

2.3. Biological control in in vivo tests on fresh-cut apples and peaches

A non-pathogenic strain of *E. coli*, O157:H7 (NCTC 12900), and a pathogenic strain of *S. enterica* subsp. *enterica* (Smith) Weldin serotype Michigan (BAA-709, ATCC) were used. Both of these strains were adapted to grow on tryptone soy agar (TSA, Oxoid, UK) supplemented with 100 μg mL^{-1} of streptomycin sulphate salt, thereby enabling their detection on selective medium (TSA-St) in the presence of the antagonists and the natural microbial flora associated with apples and peaches. The strains were grown in tryptone soy broth (TSB, Oxoid, UK) supplemented with streptomycin (TSB-St) for 20–24 h at 37 °C. The CECT-910 strain of *L. innocua* was used as a microbial surrogate for *L. monocytogenes* because previous studies have demonstrated that it is a valid model for *L. monocytogenes* (Francis and O'Beirne, 1997). *L. innocua* was grown overnight in TSB supplemented with 6 g L^{-1} of yeast extract (tryptone yeast extract soy broth, TYSEB) at 37 °C.

E. coli O157:H7, *Salmonella* and *L. innocua* cells were harvested by centrifugation at 9820 × *g* for 10 min at 10 °C and then resuspended in a sterile 8.5 g L^{-1} NaCl solution (SS) to obtain a concentrated suspension. The concentration was estimated using a spectrophotometer set at $\lambda = 420$ nm according to previously determined standard curves.

For the inoculum preparation, a volume of the FBP concentrated suspension was added to the 30%-transmittance antagonist suspension to obtain a pathogen concentration of approximately 1×10^7 cfu mL^{-1}. The antagonist and pathogen suspension was pipetted (15 μL) onto fruit tissue plugs, and the fruit plugs were stored at 20 ± 1 °C for 2 days. The control treatments consisted of a pathogen suspension without an antagonist. To recover the pathogen, each fruit plug was placed into a sterile plastic bag (Bagpage 80 mL, Interscience BagSystem, St Nom La Breteche, France), and 9 mL of SP was added. The sample was homogenised in a stomacher blended for 120 s at a high speed (Bagmixer 100 Minimix, Interscience). Aliquots of this mixture were serially diluted and spread onto TSA-St plates for *E. coli* O157:H7 and *Salmonella* or on Palcam agar plates (Palcam Agar Base with Palcam selective supplement, Biokar Diagnostics) for *L. innocua*. The agar plates were incubated overnight at 37 ± 1 °C. The size of the initial pathogen