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Biopreservative methods to control the growth of foodborne pathogens on fresh-cut lettuce

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ABSTRACT

Fruits and vegetables can become contaminated by foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes*, and it has been demonstrated that current industrial sanitizing treatments do not eliminate the pathogens when present. Chemical control is widely used, but biological control appears to be a better solution, mainly using the native microbiota present on fresh produce. The first objective of this study was to isolate native microbiota from whole and fresh-cut produce and to determine whether these bacteria were antagonistic toward foodborne pathogens. A total of 112 putative antagonist isolates were screened for their ability to inhibit the growth of *Salmonella enterica* on lettuce disks. Five different genera reduced *S. enterica* growth more than 1-log unit at 20 °C at the end of 3 days. When tested against *L. monocytogenes* 230/3, only *Pseudomonas* sp. strain M309 (M309) was able to reduce pathogen counts by more than 1-log unit. Therefore, M309 strain was selected to be tested on lettuce disks at 10 °C against *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes*. M309 strain was only able to reduce *S. enterica* and *E. coli* O157:H7 populations. The second objective was to test different biopreservative methods including M309 strain, *Pseudomonas graminis* CPA-7 (CPA-7), bacteriophages (Listex P100 and Salmonelex) and nisin at conditions simulating commercial applications against *Salmonella* and *L. monocytogenes* on fresh-cut lettuce. The addition of the biopreservative agents did not result in a significant reduction of *Salmonella* population. However, CPA-7 strain together with nisin reduced *L. monocytogenes* numbers after 6 days of storage at 10 °C. The cocktail of *Salmonella* and *L. monocytogenes* was not markedly inactivated by their respective bacteriophage solutions. This study highlighted the potential of biocontrol, but the combination with other technologies may be required to improve their application on fresh-cut lettuce.

1. Introduction

The increase in fresh fruit and vegetable consumption has led to an increase in foodborne outbreaks associated with their consumption. A number of outbreaks associated with consumption of lettuce contaminated with *Listeria monocytogenes* (Francis et al., 1999; Sagoo et al., 2003), *Salmonella* spp. (Crook et al., 2003; Horby et al., 2003; Takkinen et al., 2005) and *Escherichia coli* O157:H7 (Ethelberg et al., 2010; Friesema et al., 2007, 2008) have been reported.

Minimally processed vegetables can be contaminated by pathogens and there is no kill step involved in the processing of these vegetables. Therefore the need for intervention methods to maintain the safety of minimally processed produce is very important (Abadias et al., 2008, 2011; Beuchat, 1996). A variety of disinfectants (including chlorine, hydrogen peroxide, organic acids and ozone) have been used to reduce initial bacterial populations on minimally processed produce (Beuchat, 1998). Chlorine is the most widely used sanitizer in the fresh produce industry. However, studies indicate that chlorine concentrations traditionally used (50–200 ppm) are not effective in reducing pathogen loads on fresh-cut produce (Behrsing et al., 2000; Delaquis et al., 2002; Lee and Baek, 2008). Moreover, a prolonged exposure to chlorine vapor may cause adverse effects to the workers, may affect the quality of foods and also adversely affect the environment (Beuchat, 1998). Thus, there is a need for better, safer and more environmentally friendly methods to reduce the contamination. Therefore, it is desirable to preserve foods by natural means.

Biological control fits well with this new tendency, and several bacteria and yeasts have been identified as bioprotective agents (Vermeiren et al., 2004). The native microbiota present on the surface of fresh produce can play an important role as they compete with the pathogens for physical space and nutrients and/or producing antagonistic compounds that negatively affect the viability of pathogens (Liao and Fett, 2001; Parish et al., 2003). Several antagonistic microorganisms have been used to inhibit the growth of foodborne pathogens (FBP) (Janisiewicz et al., 1999; Leverentz et al., 2006). Recently, a strain of

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Pseudomonas graminis CPA-7 has been used to prevent the growth of FBP in fresh-cut apples (Alegre et al., 2013a,b) and melon (Abadias et al., 2014).

The bacteriocin nisin actually has GRAS (generally recognized as safe) status by FAO (Food and Agriculture Organization of the United Nations), WHO (World Health Organization) as well as the FDA (USA Food and Drug Administration). In the European Union (EU) it is an approved preservative additive for use in certain foods (E-234) (Randazzo et al., 2009). Nisin and other bacteriocins produced by Lactic Acid Bacteria (LAB) have received a great deal of attention because they are produced by bacteria largely considered beneficial to human health and to food production. The use of nisin as a biopreservative has been widely investigated in a large variety of fresh and processed foods. Concerning vegetables, Allende et al. (2007) and Randazzo et al. (2009) evaluated the effect of bacteriocin-containing washing solutions on survival of *L. monocytogenes* in fresh-cut lettuce at refrigerated temperatures. Both authors observed a decrease on *L. monocytogenes* populations after treatment with nisin.

Bacteriophage (phage) prophylaxis is also a possible natural method to be used as a biopreservative. Phages are bacterial viruses that invade specific bacterial cells, disrupt bacterial metabolism, and cause the bacterium to lyse without compromising the viability of other flora in the habitat. They are the most abundant microorganisms in our environment (Brussow and Hendrix, 2002) and are present in high numbers in water and foods (Hsu et al., 2002; Kennedy et al., 1986). Promising results using phage biocontrol have been reported for several pathogens, including *Salmonella* spp. (Guenther et al., 2012; Kocharunchitt et al., 2009; Leverentz et al., 2001), *L. monocytogenes* (Carlton et al., 2005; Dykes and Moorhead, 2002; Guenther et al., 2009; Leverentz et al., 2003; Oliveira et al., 2014) and *E. coli* O157:H7 (Abuladze et al., 2008; Sharma et al., 2009; Viazis et al., 2011). There are several commercialized phage preparations, such as ListShield™ and EcoShield™ (Intralytix, Inc., USA), Agriphage™ (Omnilytics, Inc., USA), Listex™ P100 and Salmonellex™ (Micreos Food Safety, The Netherlands).

The aim of this study was to evaluate native microorganisms from fresh and fresh-cut fruits and vegetables for potential inhibitory effect against FBP on lettuce. The best antagonist together with *P. graminis* CPA-7, nisin and two commercial phage preparations (Listex P100 and Salmonellex) were tested as potential biopreservative agents on minimally processed lettuce under simulated commercial conditions.

2. Material and methods

2.1. Samples

A total of 300 samples of whole vegetables, fresh-cut fruit and vegetables and sprouts were used. Samples of whole vegetables, fresh-cut vegetables and sprouts were purchased from different supermarkets in Lleida (Spain). Fresh-cut fruit samples were obtained from vending machines. The samples analyzed included: 104 single-ingredient salad samples (5 of arugula, 18 of grated carrot, 21 of corn salad, 21 of endive, 29 of lettuce (Iceberg, Batavia and Romaine) and 10 of spinach); 132 ready-to-eat salads, containing from three to six ingredients such as lettuce (different varieties), endive, carrots, corn salad, spinach, red beet and soybean sprouts; 21 single-ingredient ready-to-eat fruits (apple, pineapple, orange, mango and peach); 15 sprout samples (soybean and alfalfa) and 28 samples of whole fresh vegetables (Iceberg, Oakleaf, Romaine and Trocadero lettuces, endive and lettuce hearts).

2.2. Isolation of putative antagonists

Putative antagonists were isolated from whole vegetables, fresh-cut fruit and vegetables and sprouts. For whole products, the 3–4 outer leaves were discarded and the inner portion was cut up in the laboratory.

Twenty-five grams of each sample were diluted in 225 mL of saline peptone solution (SP, 8.5 g/L NaCl and 1.0 g/L peptone) and homogenized for 2 min at normal speed in a Stomacher (Model 400 Circulator, Seward). Serial dilutions of the suspension were made in SP and plated on the following different media: de Man, Rogosa and Sharpe medium (MRS, Biokar Diagnostics, Beauvais, France) for Lactic Acid Bacteria (LAB) isolation and Nutrient Agar (NA, Biokar Diagnostics) to isolate psychrotrophic microorganisms. Plates were incubated at 30 °C for 3 days and at 7 °C for 10 days, respectively. Based on different morphologies and color, colonies were selected and further isolated. Eighteen postharvest fungal antagonists: CPA-1 (*Candida sake*, Viñas et al., 1998), CPA-2 (*Pantoea agglomerans*, Nunes et al., 2001), CPA-3 (*Pantoea ananatis*, Torres et al., 2005), CPA-5 (*Pseudomonas syringae*, Nunes et al., 2007), CPA-7 (*Pseudomonas graminis*, Alegre et al., 2013b) and EL 8, PN5, PN6, 128-M, C-9P-21, F-13, F-10, PO, C-9 17, P-12, C-5 10, C-10 5, and RG4 strains (unpublished results); belonging to the Pathology Laboratory collection (IRTA, Lleida), was also tested.

2.3. In vivo assay of antagonistic activity

2.3.1. Lettuce preparation

'Romaine' lettuce (*Lactuca sativa* var. *longifolia*) was obtained from a local supermarket. The outer or damaged leaves of lettuce were removed and discarded. Leaf disks (2.3 cm in diameter) were cut using an aseptic cork borer and placed in commercial 500 mL food plastic bowls. The covers on the plastic bowls allowed sufficient air exchange to prevent modified atmosphere creation.

2.3.2. Microorganisms and inoculum preparation

Salmonella enterica subsp. *enterica* (Smith) Weldin serotype Michigan (BAA-709, ATCC) was adapted to grow on Tryptone Soy Agar (TSA, Oxoid, UK) supplemented with 100 μg/mL of streptomycin sulfate salt (TSA-St), thereby enabling detection on selective medium in the presence of the antagonists and the native microbiota associated with lettuce. *S. enterica* was grown in Tryptone Soy Broth (TSB, Oxoid, UK) supplemented with streptomycin (TSB-St) for 20–24 h at 37 °C. Five strains of *L. monocytogenes*: serovar 1a (CECT 4031), serovar 3a (CECT 933), serovar 4a (CECT 940), serovar 4b (CECT 4032) and serovar 1/2a (LM230/3, isolated from Abadias et al., 2008); and a non-pathogenic strain of green fluorescent protein (GFP)-expressing and ampicillin resistant *E. coli* O157:H7 (B6-914 GFP-91) (Fratamico et al., 1997) were also used. *L. monocytogenes* strains were grown individually on TSA supplemented with 6.0 g/L yeast extract, 2.5 g/L glucose and 2.5 g/L dipotassium hydrogen phosphate (TYSEA) at 37 °C for 20–24 h. Each strain was transferred to TSB supplemented with 6.0 g/L yeast extract (TYSEB) at 37 °C for 20–24 h. *E. coli* O157:H7 was grown on Sorbitol MacConkey agar supplemented with Cefixime and Tellurite (CT-Smac, Biokar Diagnostic) and 50 mg/mL Ampicillin (ampicillin sodium salt, Sigma, St. Louis, USA) (CT-Smac + Amp) at 37 °C for 20–24 h. A single colony was transferred into a flask with TSB supplemented with 50 mg/mL Ampicillin (TSB + Amp) at 37 °C for 20–24 h.

The cultures were harvested individually by centrifugation at 9820 ×*g* for 10 min at 10 °C and resuspended in a sterile 8.5 g/L NaCl solution (SS) and equal volumes of each concentrate were mixed to obtain the five strain cocktail.

A total of one hundred and twelve microorganisms were tested for their potential antagonistic activity. The antagonists were grown in plates as previously described in the isolation of putative antagonists section. The colonies were rubbed from the medium and a suspension of 30 ± 5% transmittance ($\lambda = 420$ nm) was prepared in 5 mL of sterile deionized water. 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.