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Lactic acid bacteria and natural antimicrobials to improve the safety and shelf-life of minimally processed sliced apples and lamb's lettuce

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ABSTRACT

Outbreaks of food-borne disease associated with the consumption of fresh and minimally processed fruits and vegetables have increased dramatically over the last few years. Traditional chemical sanitizers are unable to completely eradicate or kill the microorganisms on fresh produce. These conditions have stimulated research to alternative methods for increasing food safety. The use of protective cultures, particularly lactic acid bacteria (LAB), has been proposed for minimally processed products. However, the application of bioprotective cultures has been limited at the industrial level. From this perspective, the main aims of this study were to select LAB from minimally processed fruits and vegetables to be used as biocontrol agents and then to evaluate the effects of the selected strains, alone or in combination with natural antimicrobials (2-(E)-hexenal/hexanal, 2-(E)-hexenal/citral for apples and thyme for lamb's lettuce), on the shelf-life and safety characteristics of minimally processed apples and lamb's lettuce. The results indicated that applying the *Lactobacillus plantarum* strains CIT3 and V7B3 to apples and lettuce, respectively, increased both the safety and shelf-life. Moreover, combining the selected strains with natural antimicrobials produced a further increase in the shelf-life of these products without detrimental effects on the organoleptic qualities.

1. Introduction

Fruits and vegetables are strongly recommended in the human diet due to their vitamins, antioxidants, mineral and dietary fiber content. They are generally consumed fresh, minimally processed, pasteurized or cooked by boiling in water or microwaving. Although treating food with heat increases the product safety and shelf-life, it also decreases the nutritional properties and sensorial features of the raw materials, whereas fresh produce and minimally processed products are characterized by a short shelf-life due to rapid microbial spoilage (Di Cagno et al., 2008). In addition, outbreaks of food-borne disease associated with the consumption of fresh and minimally processed fruits and vegetables, primarily due to *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes*, have increased dramatically since the 1970s (Abadias et al., 2011). In fact, numerous studies have reported the presence of pathogenic species, including *L. monocytogenes*, *Salmonella* spp., *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Staphylococcus aureus* (Alegre et al., 2010), on fresh produce and related minimally processed products. Currently, modified atmosphere packaging and refrigeration are the most utilized tools for improving the shelf-life of these foods to delay microbial growth and the physiological degradation of vegetable tissue (Alegre et al., 2010). Decontamination methods are another tool for reducing the microbial cell loads of the raw materials and have been shown to have positive effects on product safety and shelf-life (Manzocco et al., 2011; Ramos et al., 2013). Presently, chlorine is the most widely used washing and sanitizing agent among those available for fresh produce. However, numerous reports indicate that chlorine has limited antimicrobial efficacy, allowing 1–2 logarithmic reductions in the bacterial population of raw materials at the permitted concentrations, which have been

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associated with the production of potentially toxic substances (Abadias et al., 2008). Also disinfectants alternative to chlorine, such as hydrogen peroxide, organic acids and ozone, demonstrated their inability to completely eradicate or kill microorganisms on fresh produce, as well as their potential toxicity (Alegre et al., 2013). The washing procedures are able to remove only a part of spoilage or pathogenic microbial cells from raw fruits and vegetables; the remaining part can survive the sanitizing agents that attach to the surfaces of the raw material (Allende et al., 2008). Peeling, slicing, and shredding fresh produce stimulate growth among the surviving microorganisms and transfer them to both the inner tissues and the released nutrients (Lanciotti et al., 2003). In addition, the reduction of the naturally occurring population, due to washing and sanitization, can reduce the competition for space and nutrients among human–pathogenic species (Schuenzel and Harrison, 2002). Consumer concern to chemical synthetic additives has stimulated research into alternative methods for reducing the decay of minimally processed fruits and vegetables and improving product safety (Ayala-Zavala et al., 2008). The use of protective cultures has been proposed due to their potential benefits for minimally processed fruits and vegetables (Rodgers, 2001). Protective cultures of lactic acid bacteria (LAB) to increase safety and shelf-life have been developed over the last few decades (Vescovo et al., 1996; Leroy et al., 2003; Palmai and Buchanan, 2002). For example, Torriani et al. (1997) and Scolari and Vescovo (2004) demonstrated that a strain of *Lactobacillus casei* could be used to increase the safety of minimally processed vegetables due to the inhibition of *A. hydrophila*, *Staph. aureus*, *E. coli* and *L. monocytogenes*. Selected strains of *Pseudomonas syringae*, *Pseudomonas graminis*, *Gluconobacter asaii*, *Candida* spp., *Dicosphaerina fagi* and *Metschnikowia pulcherrima* have shown great potential as biocontrol agents for minimally processed fruits due to their ability to antagonize several foodborne pathogens under laboratory conditions (Abadias et al., 2009; Trias et al., 2008; Alegre et al., 2013). However, the use of bioprotective cultures has been limited for commercial products at the industrial level because satisfactory outcomes under laboratory settings are unable to guarantee success under real processing and distribution conditions (Trias et al., 2008; Abadias et al., 2009). Even if, some authors have shown that microorganisms isolated from the same commercial type of product were able to successfully control spoilage and pathogenic microorganisms (Vescovo et al., 1996; Reina et al., 2006; Rodgers, 2008). However, in our knowledge no previous reports regard the selection and use of lactic acid bacteria as biocontrol agents in minimally processed lamb's lettuce and apples. In addition, natural antimicrobials, such as essential oils (EOs) and some of their components, have been proposed as a means to increase the quality and safety of minimally processed fruit and vegetables (Allende et al., 2008; Gutierrez et al., 2009; Vandekinderen et al., 2009; De Azeredo et al., 2011; Siroli et al., 2014). Based on numerous literature reports, citral, hexanal and 2-(E)-hexenal were shown to be the most effective for increasing the safety and shelf-life of ready-to-eat fruit (Lanciotti et al., 1999, 2003; Corbo et al., 2000; Belletti et al., 2008; Siroli et al., 2014). Moreover, these molecules mediated an enhancement of the sensorial properties and a retention of the original color of sliced fresh apples packaged under a modified atmosphere (Lanciotti et al., 1999; Corbo et al., 2000). In addition, *Thymus vulgaris* EO has been proposed as an interesting alternative to chlorine for minimally processed vegetables that is able to increase the safety and shelf-life of products without detrimental effects on their sensorial properties (Gutierrez et al., 2009). However, in our knowledge no previous reports regard the use of lactic acid bacteria as biocontrol agents in combination with natural antimicrobials in minimally processed lamb's lettuce and apples. From this perspective, the main aims of this research were as follows: i) to isolate, identify and characterize LAB from minimally processed fruits and vegetables and select some of those strains for use as biocontrol agents in the same products; ii) to evaluate the effects of selected strains on the shelf-life and safety characteristics of minimally processed apples and lamb's lettuce; iii) to evaluate the combined effects of the most effective biocontrol agents and natural antimicrobials on minimally processed lamb's lettuce and apple in terms of safety, shelf-life and sensorial characteristics.

2. Materials and methods

2.1. Natural antimicrobials

Hexanal, 2-(E)-hexenal and citral were purchased from Sigma–Aldrich (Milano, Italy). Thyme EO was obtained from Flora s.r.l. (Pisa, Italy).

2.2. Isolation and identification of LAB from minimally processed apples and lamb's lettuce

Samples of commercially sliced apples and minimally processed lamb's lettuce were obtained from a local market. A 10-g portion of each vegetable was suspended in 90 ml of sterile saline solution (0.9% NaCl, w/v) and homogenized using a Stomacher for 2 min at room temperature. Serial dilutions were made, plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid Ltd., Basingstoke, United Kingdom), and incubated at 30 °C for 48–72 h under anaerobic conditions, to isolate presumptive mesophilic LAB. For lamb's lettuce, a 24 h enrichment in MRS broth at 30 °C was necessary. Serial dilutions of the cultures were then plated on MRS agar. Multiple colonies, possibly with distinct morphologies, were isolated from the MRS plates. Gram-positive, catalase-negative, non-motile rods and cocci were cultivated in MRS broth at 30 °C for 24 h and then re-streaked onto MRS agar. Stock cultures were stored at −20 °C in MRS broth with 10% (v/v) glycerol.

Genomic DNA was extracted from each strain of presumptive LAB using the InstaGene Matrix kit (Bio-Rad Laboratories, Milano, Italy). A total of 39 representative isolates were identified using RAPD-PCR (primer M13) and by sequencing the 16S rRNA region according to the protocol described by De Angelis et al. (2006).

2.3. Phenotypic characterization and evaluation of antagonistic activity of identified LAB

The identified strains were characterized based on their ability to grow under various environmental conditions, such as various temperatures (4, 8, 15 and 30 °C), various levels of sodium chloride (2, 4 and 6%), high concentrations of sucrose (20%) and low pH values (3.5, 4.0 and 4.5). The strains were grown overnight after were inoculated at a level of approximately 5 log CFU/ml in tubes with 10 ml of MRS broth to evaluate growth at various temperatures or in MRS broth supplemented with the selected concentrations of NaCl or sucrose. Regarding the conditions at low pH values, glacial acetic acid was used to create the selected pH conditions. The inoculated tubes (5 repetitions for each condition) were stored at 30 °C with the exception of the tubes used to evaluate the minimum growth temperatures. The growth of the strains was evaluated based on the optical density at 600 nm (OD_{600}) using a UV-1204 spectrophotometer (Shimadzu, Kyoto, Japan). If growth was not observed, the viability of the strains was verified by counting on MRS agar plates.

To evaluate the ability of the identified LAB to antagonize the *L. monocytogenes* Scott A, *E. coli* 555 and *Salmonella enteritidis* E5