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Innovative strategies based on the use of bio-control agents to improve the safety, shelf-life and quality of minimally processed fruits and vegetables

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Abstract

The consumption of minimally processed fruits and vegetables has increased in recent years. Currently, the use chemical preservatives is unable to guarantee the safety of minimally processed fruits and vegetables. These conditions have stimulated research into alternative methods for increasing their safety and shelf-life. The use of protective cultures, particularly lactic acid bacteria, microorganisms from indigenous microflora and their antimicrobial products, has been proposed for minimally processed products. However, the application of bioprotective cultures has been limited at the industrial level. In this perspective, the aim of this review was to summarize the state-of-the-art application of biocontrol agents in minimally processed fruits and vegetables and their action mechanisms against spoilage and/or pathogenic microorganisms.

Keywords: minimally processed produce, biocontrol agents, lactic acid bacteria, safety and shelf-life

1 Introduction

Fresh fruits and vegetables are strongly recommended in the human diet because of their contents of vitamins, antioxidants, minerals and dietary fibres; additionally, a significant amount of epidemiological evidence has demonstrated that their consumption is beneficial to health (Boeing *et al.*, 2012). They are generally consumed fresh, minimally processed, pasteurized or cooked by boiling in water or microwaving. Although heat treatments increase the safety and shelf-life of these products, heat treatments also decrease the nutritional properties and sensorial features of the raw materials. However, fresh produce and minimally processed products have a short shelf-life as a result of rapid microbial spoilage (Di Cagno *et al.*, 2008).

Minimally processed produce is more perishable than the original raw materials (Francis *et al.*, 2012; Selma, Allende, López-Galvez, Conesa, & Gil, 2008). The increase in nutrient availability because of the presence of cut surfaces, the metabolism of tissues, the confinement of the products inside packages and the lack of treatments to ensure microbial stability favour the growth of both microorganisms deriving from raw materials and cross-contamination during handling and processing (peeling, cutting, etc.) (Francis *et al.*, 2012; Lanciotti, Gianotti, Patrignani, Belletti, Guerzoni, & Gardini, 2004). Although raw produce is expected to have a shelf-life of several weeks or months, minimally processed fruits and vegetables have only a very short storage life of 4 to 10 days. Their shelf-life depends on various factors such as fruit and vegetable quality, production technology and the number and interactions among microbial groups (Selma *et al.*, 2008).

Mesophilic bacterial levels of 10^3 to 10^6 colony forming units (CFU)/g are common in minimally processed vegetables analysed immediately after packaging (Belletti, Lanciotti, Patrignani, & Gardini, 2008; Guerzoni, Gianotti, Corbo & Sinigaglia 1996; Ragaert, Devlieghere, & Debevere, 2007; Siroli *et al.*, 2015). However, at the retail level, the counts are more variable, ranging between 10^3 and 10^9 CFU/g (Belletti *et al.* 2008). Because of refrigerated storage, the dominating bacterial population mainly consists of species belonging to *Pseudomonadaceae* (particularly *Pseudomonas* *fluorescens*) and *Enterobacteriaceae* (particularly *Erwinia herbicola* and *Rahnella aquatilis*), in addition to some species belonging to the lactic acid bacteria (LAB) (particularly *Leuconostoc mesenteroides*) (Bennik, Vorstman, Smid, & Gorris, 1998; de Azeredo, Stamford, Campos Nunes, Gomez Neto, de Oliveira, & de Souza, 2011; Nguyen-The & Carlin, 1994). Additionally, many different yeast species belonging to the genera *Candida, Cryptococcus, Rhodotorula*, *Trichosporon*, *Pichia* and *Torulaspora* have been identified during storage (Nguyen-The & Carlin, 1994, Ragaert *et al.*, 2007) whereas moulds are less important in these products because of the intrinsic properties of fruits and vegetables, such as a slightly acidic to neutral pH, which favours bacteria and yeasts (Barth, Hankinson, Zhuang, & Breidt, 2010). By contrast, the spoilage of minimally processed fruit primarily occurs because of the proliferation of its natural acid tolerant and osmophilic microflora (Belletti *et al.*, 2008; Lanciotti *et al.*, 2004). In fact, the microflora is mainly represented by yeasts, which are generally responsible for the fermented taste and carbon dioxide production; LAB, which can produce a buttermilk off-flavour; and moulds, which contribute to spoilage by their surface growth (Tournas, Heeres, & Burgess, 2006). However, yeasts are favoured compared to LAB because of the high sugar content and C/N ratio of the system (Patrignani, Tabanelli, Siroli, Gardini, & Lanciotti, 2013; Siroli *et al.*, 2014a). In addition to spoilage microorganisms, outbreaks of food-borne diseases associated with the consumption of fresh and minimally processed fruits and vegetables, which is primarily a result of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes*, have increased dramatically since the 1970s (Alegre, Abadias, Anguera, Oliveira, & Viñas, 2010; Ramos, Miller, Brandão, Teixeira, & Silva, 2013; Sant'Ana, Landgraf, Destro, & Franco, 2011). However, Ramos et al. (2013) also showed that *Clostridium botulinum*, *Shigella* spp., *Staphylococcus* spp., *Vibrio cholerae* and *Yersinia enterocolitica* are amongst the major fruit and vegetable pathogens associated with outbreaks. Moreover, numerous studies showed the presence of *Aeromonas hydrophila* and *Staphylococcus aureus* on fresh produce and related minimally processed products (Alegre *et al.*, 2010; Harris *et al.*, 2003; Nguyen-The & Carlin, 1994). Likewise, *Campylobacter jejuni* was isolated in minimally processed mushrooms from retail markets in the United States (EFSA 2013; FDA 2001). Castillo and Escartin (1994) showed that this pathogenic species could survive on sliced watermelon and papaya for a sufficient time to be a risk to the consumer. The contamination of vegetables and fruits with spores of *Bacillus cereus*, *Clostridium perfringens* and *C. botulinum* present in soil is common (FDA 2001). When fresh products are handled and processed in a manner that enables the germination of spores and the growth of vegetative cells, there is a threat to public health, particularly when the products are packaged in a modified atmosphere (FDA 2001). There were approximately 110 scientific papers and reports on outbreaks associated with the consumption of minimally processed fruits and vegetables according to the Food and Drug Administration (FDA), the Centres for Disease Control and Prevention (CDC) and the World Health Organization (WHO) (Ramos *et al.,* 2013).

Because of the lack of processing steps to kill microbial contaminants, the use of high quality raw materials and efficient temperature control during manufacture, distribution and retailing are key factors for maintaining the microbiological quality and safety of minimally processed fruits and vegetables. However, the quality of raw materials and the maintenance of the cold chain are difficult to be implemented and controlled (Rediers, Claes, Peeters, & Willems, 2009; Siroli *et al.*, 2014a). In fact, the quality of raw materials depends on several factors including agronomic practices, seasonal trends, storage conditions, etc. Moreover, an extensive amount of literature shows that thermal abuse is very frequent during product transport and selling (Lanciotti *et al.*, 2004; Rediers *et al.*, 2009).

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 (Ramos *et al.*, 2013). However, the use of chemicals as disinfectants for raw materials is not sufficient to either eliminate or significantly delay microbial spoilage or to ensure product safety (Soliva-Fortuny & Martín-Belloso, 2003).

Disinfection processes incorporating chlorine are often applied to fresh vegetables to enhance safety and shelf-life profiles. 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 (Abadias, Usall, Anguera, Solsona, & Viñas, 2008). Its inefficacy to eliminate microbial cells was attributed to the inability of its aqueous solutions to wet the hydrophobic surface of the waxy cuticle of vegetables and to its inactivation by the organic matter (Carrasco, Pérez-Rodríguez, Valero, García-Gimeno, & Zurera, 2008; de Azeredo *et al.*, 2011). Additionally, the presence of biofilms on equipment has been reported to reduce the efficacy of chlorine against microorganisms that can cross-contaminate the products during processing (Carrasco *et al.*, 2008). Additional drawbacks of chlorine usage are the possible formation of carcinogenic chlorinated compounds, vapours having adverse health effects and the increase in microbial chlorine resistance (Abadias *et al.*, 2008; Gil, Selma, López-Gálvez, & Allende, 2009). For these reasons, the use of chlorine is prohibited or restricted in some European countries, such as the Netherlands, Sweden, Germany, Switzerland, Denmark and Belgium, for the disinfection of the raw materials used for the production of minimally processed vegetables (Gil *et al.*, 2009; Tirpanalan, Zunabovic, Domig, & Kneifel, 2011). Furthermore, disinfectants alternative to chlorine, such as ozone, H_2O_2, organic acids, calcium-based solutions and peroxiacetic acids, have demonstrated their inability to completely eradicate or kill microorganisms on fresh produce and their potential toxicity and side effects on the sensorial properties of the products (Ramos *et al.*, 2013; Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). In addition, the reduction of the naturally occurring population because of washing and sanitization can reduce the competition for space and nutrients against pathogenic species (Schuenzel & Harrison, 2002).

Consumer concern of 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, Oms-Oliu, Odriozola-Serrano, GonzálezAguilar, Álvarez-Parrilla, & Martín-Belloso, 2008). The use of generally recognized as safe (GRAS) microorganisms such as LAB and yeasts and/or their natural metabolites to inhibit the growth of pathogenic and spoilage microorganisms is a promising tool, and it is also perceived by the consumer as a natural food preservation method (Cosentino, Fadda, Deplano, Melis, Pomata, & Pisano, 2012; Ross, Morgan, & Hill, 2002). Bioprotective microorganisms have already shown their potential for practical application in various foods, such as meat (Vermeiren, Devlieghere, & Debevere, 2004) and plant derived products (Settanni & Corsetti, 2008; Trias, Baneras, Badosa, & Montesinos, 2008a; Trias, Baneras, Montesinos, & Badosa, 2008b).

In particular, LAB have shown a great potential as biocontrol agents of several non-fermented foods because they are widely used in fermented foods, have a long history of safe use, and have a GRAS status (Carr, Chill, & Maida, 2002). They have also been applied to increase the safety and shelf-life of minimally processed fruits and vegetables (Palmai, & Buchanan, 2002; Torriani, Orsi, Vescovo, 1997; Vescovo, Torriani, Orsi, Macchiarolo, & Scolari, 1996). However, several other bacteria and yeasts, often selected among the naturally occurring microbiota, including strains of *Pseudomonas syringae, Pseudomonas graminis, Gluconobacter asaii, Candida spp., Dicosphaerina fagi, Metschnikowia pulcherrima* and *Candida sake* have been proposed as biocontrol agents in these foods (Abadias, Usall, Alegre, Torres, & Vinas, 2009; Alegre, Vinas, Usall, Anguera, Altisent, & Abadias, 2013a; Trias *et al.*, 2008a).

This manuscript reviews the application of biocontrol agents belonging to LAB or to other microbial groups and their action mechanisms against spoilage and/or pathogenic microorganisms frequently associated with minimally processed fruits and vegetables.

2 Protective culture for minimally processed vegetables

LAB have been used to preserve meat and dairy products (Stiles & Holzapfel, 1997) and fermented vegetables or fruit juices (Ruiz-Barba, Cathcart, Warner, & Jimenez-Diaz, 1994).

LAB are also indubitably the most important bioprotective cultures for non-fermented foods including minimally processed vegetables. In fact, protective cultures of LAB have been developed over the last few decades to increase the safety and shelf-life of minimally processed vegetables. The potential of antagonistic LAB belonging to *Lactobacillus casei* or their culture filtrate to inhibit the growth of pathogenic bacteria in ready-to-eat vegetables was first demonstrated by Vescovo et al. (1996) and Torriani et al. (1997). In particular, Torriani et al. (1997) showed that the addition of 3% culture permeate of *Lb. casei* IMPC LC34 to mixed salads reduced the total mesophilic bacteria counts from 6 to 1 log CFU/g and suppressed coliforms, enterococci, and *A. hydrophila* after 6 days of storage at 8 ° C. *Lactobacillus plantarum* IMPC LP4 was able to prolong the very limited shelf-life of shredded carrots because of its ability in real systems to control the growth of *Leuconostoc* spp., which have been identified as the main spoilage agents of this minimally processed vegetable (Torriani, Scolari, Dellaglio, & Vescovo, 1999).

The application of a central composite design (CCD) to modulate the carbon dioxide concentration in the packaging atmosphere, the *Lb. casei* inoculum size and the storage temperature allowed the obtaining of models that emphasized the role of the biocontrol agent initial level in controlling *A. hydrophila* and permitted the identification of combinations of the selected variables to reduce the survival of the pathogenic species (Vescovo, Scolari, Orsi, Sinigaglia, & Torriani, 1997).

Bennik, van Overbeek, Smid and Gorris (1999) studied the potential of two *Pediococcus parvulus* strains and one *Enterococcus mundtii* strain to control the growth of *L. monocytogenes* on refrigerated, modified atmosphere stored mung-bean sprouts. These bacteriocinogenic biocontrol agents, previously isolated from minimally processed vegetables, were shown to grow in culture broth at 4, 8, 15 and 30 °C. However, only *E. mundtii* was capable of bacteriocin production at 4–8 °C and was subsequently evaluated for its ability to control the growth of *L. monocytogenes* on vegetable agar and fresh mung-bean sprouts under a modified atmosphere at 8 °C. The growth of *L. monocytogenes* was inhibited by bacteriocinogenic *E. mundtii* on sterile vegetable-medium but not on fresh produce. Otherwise, bacterial cultures that were isolated from the same type of vegetable or product in which they were used as biocontrol agents were reported to have the greatest chance of success in controlling pathogens (Bennik *et al.*, 1999; Siroli *et al.*, 2015).

Palmai and Bouchanan (2002) assessed the inhibitory activity of *Lactococcus lactis* against *L. monocytogenes* inoculated in model systems and sprouts at levels of approximately 2 log CFU/g, thus demonstrating that their inhibitory activity was substantially reduced on alfalfa compared to that observed in a model system. The apparent decrease in effectiveness of the biocontrol agents in real systems compared to model systems was attributed to the inhibitory activity of naturally occurring microflora (Bennik *et al.*, 1999; Palmai & Bouchanan 2002). Otherwise, there were more variables affecting the success of the biocontrol agents in a real system than in a model system, and they were often unpredictable in a real system. Additionally, the interference of naturally occurring microbiota cannot be exactly identified because it varies according to the raw material and process conditions.

The effectiveness of the strain used by Palmai and Bouchanan (2002) was not a result of the production of bacteriocin but of its ability to produce high amounts of lactic acid. However, *L. monocytogenes* was able to proliferate in the control samples in sprouts (without the biocontrol agent), reaching levels of approximately 6 log CFU/g within 48 hours. When *L. lactis* was co-inoculated onto the seeds, the maximum levels of *L. monocytogenes* were approximately 1 log lower than those observed in the control samples. The reduction of *L. monocytogenes* observed by Palmai and Bouchanan (2002) was similar to that observed by Cai, Ng, & Farber (1997) using a bacteriocin-producing *L. lactis* strain at a lower inoculation level (5 log CFU/g).

Scolari and Vescovo (2004) performed several challenge experiments on scarola salad leaves by simultaneously inoculating *Lb. casei* and pathogenic species such as *S. aureus*, *A. hydrophila*, *E. coli* and *L. monocytogenes*. These authors showed a remarkable inhibitory effect by the LAB towards all the pathogenic strains. Scolari, Vescovo, Zacconi and Bonadé (2004) studied the influence of *Lb. plantarum* on the growth of *S. aureus* through an impedometric method and by varying the inoculum size of the single strain and the growth temperature according to a CCD. These authors showed that temperature affected the growth of both *S. aureus* and *Lb. plantarum* strains. The pathogenic strain, independent of its inoculum size, was inhibited by *Lb. plantarum* at all the tested temperatures. The authors outlined that a proper combination of specific LAB and storage temperature should improve the safety of the minimally processed vegetables. Trias, Badosa, Montesinos and Baneras (2008c) characterized ten *L. mesenteroides* strains and one *Leuconostoc citreum* strain isolated from fresh fruits and vegetables for their antagonistic capacity against *L. monocytogenes*; they identified organic acids, hydrogen peroxide and bacteriocins as the main inhibition mechanisms. In a successive study, Trias et al. (2008a) studied the ability of the selected biocontrol agents to inhibit the growth of foodborne human pathogens when inoculated in iceberg lettuce leaf cuts. The selected strains grew on the substrates and did not cause negative effects on the general aspect of the lettuce tissues. In addition, the treatment of the lettuce cuts with the antagonistic strains reduced the cell count of *Salmonella typhimurium* and *E. coli* by 1 to 2 log CFU/wound or g, whereas the growth of *L. monocytogenes* was completely inhibited.

Although the importance of the biocontrol agent inoculum size had been previously reported by others authors, Trias et al. (2008c) used a dose response assay to determine the efficacy of *Leuconostoc* strains as bioprotective agents against *L. monocytogenes* inoculated in minimally processed lettuce, thus demonstrating that the efficacy of biocontrol agents was affected by the cell loads of both the pathogenic and biocontrol agents.

Siroli et al. (2015) characterized several LAB strains that were previously isolated from commercial minimally processed fruits and vegetables for their ability to grow at low temperature and low pH values and to antagonize the pathogenic species frequently associated with these food products. In addition, these authors studied the effect of the biocontrol agents to prolong the shelf-life of the product. In fact, most of the literature available studied the effects of biocontrol cultures on minimally processed vegetable safety without considering the effects on product shelf-life and quality. On the basis of the results obtained, these authors selected *Lb. plantarum* V7B3 and *Lb. casei* V4B4 to be used as biocontrol agents alone or in combination with thyme essential oil (EO) in lamb's lettuce. The results obtained indicated that applying the *Lb. plantarum* V7B3 strain to lettuce during the washing phase at a level of 6 log CFU/ml instead of chlorine increased product shelf-life and safety. In fact, *Lb. plantarum* V7B3 showed an interesting potential for controlling *L. monocytogenes* and *E. coli* when deliberately inoculated in washing solution at levels ranging between 3 and 4 log CFU/ml. The presence of the *Lb. plantarum* V7B3 strain increased the *E. coli* death kinetics and reduced the viability of *L. monocytogenes* over the 9 days of refrigerated storage of lamb's lettuce. Moreover, combining the selected strains with natural antimicrobials produced a further increase in the shelf-life (12 days) of the product without detrimental effects on the organoleptic quality compared to the traditional products washed with chlorine (120 ppm), thus contributing to the substitution of this chemical raw material sanitizer. Moreover, Siroli, Patrignani, Salvetti, Torriani, Gardini and Lanciotti (2014b) showed the good performance of a nisin producing strain, *L. lactis* CBM21, which was inoculated at a level of 7 log CFU/ml in the washing solution of minimally processed lamb's lettuce and combined or not with thyme EO, to inhibit both the inoculated *L. monocytogenes* and *E. coli* and the total mesophilic species, significantly increasing the product shelf-life. In fact, the addition of the biocontrol agent did not affect the quality parameters (*i.e.*, colour parameters and sensory attributes) of lamb's lettuce. The use of *L. lactis* CBM21 and/or thyme EO added in the tap water used for lamb's lettuce washing was also experienced at the industrial level, confirming their potential as an alternative to chlorine (Siroli et al. unpublished results). In fact, the products obtained with the innovative washing solutions showed the same safety and shelf-life of the controls but with improved sensorial properties. Moreover, the products added with the biocontrol agent maintained a good appearance for up to 12 days (Figure 1).

In addition to LAB, some authors studied the competitive, inhibitory, or antagonistic activity of biocontrol agents selected among the naturally occurring microbiota of fresh or minimally processed vegetables. Several studies showed that fresh-cut produce are sources of competitive microorganisms (Francis & O'Beirne 1998; Janisiewicz, Conway, & Leverentz, 1999; Liao & Fett 2001; Schuenzel & Harrison, 2002). Liao and Sapers (1999) also demonstrated that potential soft rot microorganisms belonging to the natural resident microflora, such as *P. fluorescens* and *P. viridiflava*, can have great potential as biocontrol agents, inhibiting the growth of *L. monocytogenes* inoculated on potato tuber slices. Additionally, Carlin, Nguyen-The and Morris (1996) found that *P.*

fluorescens was able to inhibit the growth of *L. monocytogenes* on endive leaves maintained at 10 °C by approximately 1 log compared to controls when the endive leaves were inoculated with the *Pseudomonas* at levels ranging between 6 and 7 log CFU/g. Using a model system, Buchanan and Bagi (1999) reported that the inhibition of *L. monocytogenes* by *P. fluorescens* was limited to a repression in the maximum levels attained and that the extent of inhibition was dependent on the water activity and pH of the environment.

Liao and Fett (2001) demonstrated the inhibitory action against *Salmonella chester*, *L. monocytogenes*, and *E. coli* from *Pseudomonas* species on green pepper, romaine lettuce, baby carrots, alfalfa and clover. The six isolates that inhibited at least one pathogen were *Bacillus* spp. (3 isolates), *Pseudomonas aeruginosa* (1 isolate), *P. fluorescens* (1 isolate), and a yeast (1 isolate). On green pepper disks inoculated with *P. fluorescens* and the yeast isolates, the growth of *S. chester* and *L. monocytogenes* was reduced by 1 and 2 logs, respectively, over a period of 3 days.

Schuenzel and Harrison (2002) screened isolates from fresh-cut produce for antimicrobial activity against *S. aureus*, *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella montevideo*. Of the 180 isolates screened, 37 were found to have various degrees of inhibitory activity against at least one pathogen.

Johnston, Harrison and Morrow (2009) evaluated the competitive, inhibitory, or antagonistic activity of native microflora obtained from fresh-cut iceberg lettuce and bagged baby spinach against *E. coli* O157:H7. These authors isolated 495 inhibitors of *E. coli* O157:H7, demonstrating that naturally occurring microorganisms on foods can have inhibitory activities towards foodborne pathogens. A summary of the biocontrol agents used for vegetables and minimally processed vegetables are reported in Table 1.

3 Protective culture for minimally processed fruits

The use of protective cultures and biocontrol agents has also been reported in minimally processed fruits because they can be an alternative to chemical treatments to increase the product safety, shelf-life and quality (Abadias *et al.*, 2009). Biocontrol agents have been utilized alone or in combination with modified-atmosphere packaging, natural antimicrobials (Siroli *et al.*, 2015), gamma radiation (Mostafavi, Mirmajlessi, Fathollahi, Shahbazi, & Mirjaliliet, 2013), reducing agents (Alegre *et al.*, 2013a; Alegre, Viñas, Usall, Teixidó, Figge, & Abadias, 2013b) and heat treatments (Leverentz, Janisiewicz, Conway, Saftner, & Camp, 2003) to obtain a synergic effect on both the safety and quality of fruit, postharvest fruit and minimally processed produce.

The application of lactic acid bacteria (LAB) as biocontrol agents in fresh and minimally processed fruits has not yet been fully developed (Settanni & Corsetti, 2008; Trias *et al.*, 2008a) because the high sugar content associated with the low pH of these food matrices favours yeast growth compared to bacterial growth. The use of LAB as bioprotective agents in fruit was proposed as an optional method to circumvent the limitations found with other antagonists such as *Candida* and *Gluconobacter* species by Trias et al. (2008a). These authors isolated *L. mesenteroides* and *L. citreum* from fruits and vegetables in a survey from commercial products in Spain and tested them against *L. monocytogenes* inoculated in the wounds of Golden Delicious apples. The use of *Leuconostoc* strains as bioprotective agents provided encouraging results in inhibiting *L. monocytogenes* growth. Promising results of LAB biocontrol cultures were also obtained by Siroli et al. (2015). These authors selected some interesting LAB from apples and lamb's lettuce and used these strains as biocontrol agents in minimally processed Golden Delicious apples packaged in a modified atmosphere alone or in combination with natural antimicrobials such as 2-(E)-hexenal/hexanal and 2-(E)-hexenal/citral. The most promising strain resulted from *Lb. plantarum* CIT3, which, when inoculated at levels of 6-7 log CFU/g in the dipping solution of sliced apples, both alone or in combination with natural antimicrobials, increased the safety features of the products. This strain was able to significantly inhibit the growth of yeast but negatively affected the sensory characteristics of the product, which is an important consumer factor choice. However, the colour of the samples inoculated with LAB remained acceptable for up to 9 days of storage at 6 °C.

Combining the selected strains with the natural antimicrobials prolonged the shelf-life quality for up to 16 days without detrimental effects on the organoleptic.

Moreover, Siroli et al. (unpublished results) showed that the nisin-producer *L. lactis* CBM21, inoculated at a level of 7 log CFU/ml in the dipping solution of sliced apples in combination or not with 2-(E)-hexenal/hexanal and/or 2-(E)-hexenal/citral, limited the growth of yeasts below 5 log CFU/g during 28 days of storage. This strain also inhibited the growth of *L. monocytogenes* during 28 days of storage, particularly when used in combination with the proposed natural antimicrobials. Negative effects on colour parameters were observed but only after 16 days of storage in the presence of natural antimicrobials. Similar results were obtained by Siroli et al. (unpublished results) on the shelf-life of sliced apples that were produced on an industrial scale by adding the mixture of hexanal/2-(E)-hexenal and/or *L. lactis* CBM21 to the dipping solution. The products obtained at the industrial level with the innovative dipping solutions maintained good microbiological, organoleptic and textural characteristics for up to 20 days. These results are promising because one of the most important selection criteria of a biocontrol agent is the maintenance of their performance in real production conditions.

Biocontrol agents, different from LAB, have also been selected for their application in minimally processed fruits. *P. syringae* L-59-66 prevented the growth of *E. coli* on apple wounds (Janisiewicz *et al.*, 1999). The growth of *L. monocytogenes* and *Salmonella* on fresh-cut apples was reduced by *G. asaii*, *Candida* spp., *D. fagi* and *M. pulcherrima* (Leverentz, Conway, Janisiewicz, Abadias, Kurtzman, & Camp, 2006). These antagonists reduced the *L. monocytogenes* populations and, except for the *Candida* spp., the *S. enterica* serovar Poona populations. This reduction was higher at 25 °C than at 10 °C, and the growth of the antagonists and pathogens increased at higher temperatures (Leverentz *et al.*, 2006).

The postharvest biocontrol agent *C. sake* CPA-1 reduced *E. coli* growth on apple wounds but not in minimally processed apples (Abadias *et al.*, 2009). In particular, this yeast was effective at colonizing apple wounds and tissues, and the competition for nutrients could play the main role in the biocontrol of *C. sake* CPA-1 on pome fruits. Trials were conducted with a mixture of five strains of *E. coli* isolated from apples. The results provided evidence that *E. coli* was unable to grow in apple juice at 5, 15 and 25 °C, but it was able to survive. At 10 °C and above, *E. coli* thrived in fresh-cut apples and wounds. When *E. coli* was inoculated in apple wounds with the yeast antagonist *C. sake*, its growth was reduced by approximately 1 log CFU/wound at 25 °C. At 5 °C, no effect of the biocontrol agent was observed. The biocontrol agent *C. sake*, which was developed to prevent fruit decay during storage, also reduced *E. coli* growth in wounded apples at abusive temperatures.

However, none of these studies were performed under realistic conditions for minimally processed apples. Beyond microbiological contamination, the development of fresh cut apple slices has been hampered by the rapid oxidative browning of apple flesh. Alegre et al. (2013a) tested the combination of antioxidant treatment and packaging atmosphere conditions to improve the efficacy of the biocontrol agent *P. graminis* CPA-7 in reducing the viability of a cocktail of four *Salmonella* and five *L. monocytogenes* strains deliberately inoculated on minimally processed apples under simulated commercial processing.

The antagonistic strain increased the activity of NatureSeal AS1 (6%, w/v) (a commercial anti-browning agent) on apple wedges stored at 10 °C with or without modified atmosphere packaging (Rößle, Gormley, & Butler, 2009). Moreover, in a semi-commercial assay, the efficacy of *P. graminis* CPA-7 inoculated at 5 and 7 log CFU/ml against *Salmonella* and *L. monocytogenes* was evaluated on minimally processed apples with NatureSeal and modified atmosphere packaging and stored at 5 and 10 °C. Although high CPA-7 concentrations avoided *Salmonella* growth at 10 °C and lowered the *L. monocytogenes* population increases, the effect was not instantaneous. No effect on apple sensory properties was detected. Therefore, CPA-7 could avoid pathogen growth on minimally processed apples during storage when used as part of a hurdle technology in combination with disinfection techniques, low storage temperature and modified atmosphere packaging. Recently, the ability of *P. graminis* CPA-7 to reduce *E. coli* O157:H7, *Salmonella* and *Listeria* *innocua* on minimally processed apples and peaches was demonstrated (Alegre et al., 2013b). The results support the potential use of CPA-7 as a bioprotective agent against foodborne pathogens in minimally processed fruit.

Alegre et al. (2012) showed the efficacy of the CPA-6 strain, an unidentified species of *Enterobacteriaceae* that was isolated from minimally processed apples, to control non-pathogenic strains of *Escherichia coli* O157:H7, *Salmonella* and *Listeria innocua* on minimally processed apples and peaches. In fact, CPA-6 inoculated at a level of 6 log CFU/plug inhibited the growth, or in some cases reduced the growth, of pathogen populations (inoculated at a level of 5 log CFU/plug) to below the limit of detection compared to the pathogen inoculated alone. A summary of the biocontrol agents used for fruits and minimally processed fruits are reported in Table 2.

Although research on the use of biocontrol agents in minimally processed fruits and vegetables has increased in recent decades, a critical analysis of the literature available clearly indicates that the efficacy of biocontrol agents, independent of the species and strains used, is affected by the inoculation level, the presence of background microflora, the physic-chemical and compositional features of the products and the storage conditions. These aspects make it difficult to standardize the bio-preservative approaches based on the use of live cells and, consequently, their scaling up at the industrial level in which process conditions can also interfere with maintaining their effectiveness.

4 Action mechanisms of biocontrol agents

Numerous studies have shown the potential of several microorganisms to inhibit the growth of foodborne pathogens in minimally processed fruits and vegetables (Alegre *et al.* 2012; Alegre *et al.* 2013b; Leverentz, *et al.*, 2006; Scolari & Vescovo, 2004; Torriani *et al.*, 1997; Trias *et al.*, 2008a; Vescovo *et al.*, 1996). In particular, LAB have shown great potential as biocontrol agents in these types of products. The preservation abilities of LAB are a result of several mechanisms of action and are mainly related to the production of antimicrobial compounds, organic acids, hydrogen peroxide, bacteriocins and diacetyl (Cleveland, Montville, Nes, & Chikindas, 2001; Trias *et al.*, 2008c). Moreover, they compete with pathogens and spoilage microorganisms for nutrients (vitamins, minerals, trace elements and peptides). The decreased pH values and antibacterial activities of organic acids produced by LAB represent the main mechanisms for the biopreservation of fermented foods (Galvez, Abriouel, Benomar, & Lucas, 2010).

Several bacteriocin-producing LAB have been shown to be effective against spoilage and pathogenic microorganisms in minimally processed fruits and vegetables (Allende, Martınez, Selma, Gil, Suarez, & Rodrıguez, 2007; Bennik *et al.,* 1999; Randazzo, Pitino, Scifo, & Caggia, 2009). In fact, many LAB are able to produce bacteriocins and bacteriocin-like molecules. Bacteriocins are antimicrobial peptides produced by bacteria to compete against bacteria of the same species or even other genera (Cotter, Hill, & Ross, 2005). Both gram-positive and gram-negative bacteria are able to produce bacteriocins. However, bacteriocins produced by LAB appear to be more promising for potential use in the food industry as natural preservatives (Settanni & Corsetti, 2008) because they are normally designed as GRAS by the U.S. Food and Drug Administration (FDA), in particular when they are familiar with the selected food product.

Bacteriocins are ribosomally synthesized peptides and proteinaceous inhibitors that act through the depolarization of the target cell membrane or through the inhibition of cell wall synthesis (Heng & Tagg, 2006). They have a wide or limited spectrum of action. For example, lactococcins can inhibit only lactococci; however, the lantibiotic nisin has a broad range of antimicrobial activity (Ross *et al.,* 2002). Moreover, bacteriocins are secondary metabolites, and consequently the physiological status of the protective culture is a key factor affecting its effectiveness when inoculated in food. Bacteriocins can be divided according to Heng & Tagg (2006) into four classes: Class I includes the lantibiotics family, Class II includes peptide bacteriocins and small, heat-stable, non-lanthionine-containing bacteriocins; Class III includes bacteriolytic and non-lytic large proteins; and Class IV includes cyclic peptides. Furthermore, some strains are able to produce more than one bacteriocin; additionally, this aspect can play a determinant role in the inhibition mechanism and spectrum of the antimicrobial actions of biocontrol cultures.

It appears that the mechanisms of action of bacteriocins are related to the permeabilization of the cell membrane. They are cationic and amphiphilic or hydrophobic (Hasper *et al.*, 2006). However, it is demonstrated that each bacteriocin possesses more than one mode of action on the target microorganism (Hasper *et al.*, 2006).

Although the number of known bacteriocins is very large, nisin is the most characterized bacteriocin and the only one to have realized widespread commercial use (Ross *et al.*, 2002).

The direct application of bacteriocins on fresh-cut products has been tested in recent years. In particular, bacteriocins such as nisin, pediocin PA-1/AcH and enterocin AS-48 have been tested in tinned vegetables, fruit juices, and salads against pathogens such as *E. coli* O157:H7, *S. aureus*, and the spoilage bacterium *Alicyclobacillus acidoterrestris* (Cleveland *et al.*, 2001; Cobo-Molinos *et al.*, 2005). Randazzo et al. (2009) showed a reduction in *L. monocytogenes* cell loads of 1.9 log unit and of 2.7 log units in iceberg lettuce that was washed with commercial nisin and RUC9 bacteriocin, respectively, compared to samples without bacteriocin after the 7th day of refrigerated storage. Additionally, Allende et al. (2007) reported that washing fresh-cut lettuce with solutions containing a mix of nisin, plantaricin, lacticin, coagulin and pediocin PA-1 reduced the viability of *L. monocytogenes* by 1.2–1.6 log units immediately after treatment. Cai et al. (1997) showed that the addition of nisin in ready-to-eat Caesar salad caused a reduction of 1.4 log in *Listeria* cell loads. Cobo-Molinos et al. (2005) found a reduction of *L. monocytogenes* of 2.0–2.4 log CFU/g on fresh alfalfa sprouts, soybean sprouts and green asparagus added with enterocin AS-48.

The direct use of bacteriocins on fresh products may not be completely satisfactory, which is mainly a result of the adsorption or deactivation of the added antimicrobials (Allende *et al.*, 2007; Settanni & Corsetti, 2008; Trias *et al.*, 2008c). For this reason, the application of the bacteriocin-producer strains on the product can avoid these problems and provide other advantages, including the production of other antimicrobial compounds and competition for space and nutrients with spoilage and pathogenic microorganisms (Settanni & Corsetti, 2007; Trias *et al.*, 2008c). However, Bennik et al. (1999) showed that bacteriocin production is dependent on temperature.

The best effects of bacteriocins and bacteriocin-producing LAB on food products have been achieved when the use of bacteriocins was combined with other preservation methods (Ananou, Maqueda, Martinez-Bueno, & Valdivia, 2007). Their use combined with chemical additives, natural antimicrobials, physical treatments, or new physical methods (HHP, pulsed electric field, *vacuum*, or modified atmosphere packaging) was reported mainly for meat products (Ananou *et al.*, 2010). The use of physical or chemical treatments increases the permeability of the outer-membrane, thus improving the effectiveness of some LAB bacteriocins against gram-negative cells, which are generally resistant. Siroli et al. (unpublished results) used a nisin-producer *L. lactis* strain CBM21, in combination with the mixture of natural antimicrobials hexanal/2-(E)-hexenal, during the washing of minimally processed sliced apples and obtained a significant increase in product safety and shelf-life.

The key role of the native microbial community that is naturally present on the surfaces of fresh produce in maintaining the health-supporting status of minimally processed produce (Nguyen-The & Carlin, 1994) is attributed to out-competing the pathogens for physical space and nutrients and/or producing antagonistic compounds that reduce the viability of pathogens (Leverentz *et al.*, 2006; Liao & Fett, 2001). Therefore, there is potential for the use of native microflora to reduce pathogen growth and survival on fruits and vegetables (Siroli *et al.*, 2015). These organisms have the advantage of being part of the natural microbial community that is already established on the target produce, which may facilitate their colonization and survival when applied in appropriate numbers (Leverentz et al., 2006). Amongst biocontrol agents, yeasts have been successfully used in minimally processed fruits because of their ability to rapidly overcome the naturally occurring bacterial population. However, there are only a few reports about their use to control human pathogens on fresh and minimally processed fruits (Janisiewicz *et al.* 1999; Liao & Fett, 2001).

5 Conclusion

The results reported in this review provide encouraging information concerning the effects of biocontrol agents on the safety and shelf-life of minimally processed fruits and vegetables. The results also highlight the importance of the isolation and selection of appropriate biocontrol agents from the products themselves. In fact, the superior performance of the strains used was not only against deliberately inoculated pathogens but also against spoilage microorganisms that are naturally present in fruits and vegetables. These abilities have been attributed to the capability of the strains to colonize the product and survive under the stringent conditions of refrigerated storage. Moreover, the ability of biocontrol agents to not adversely affect the quality of the product is important. Several authors reported negative effects of added biocontrol agents on the colour and texture parameters of the products (Leverentz *et al.,* 2006; Siroli *et al.* 2015 Trias *et al.,* 2008a). The combination of biocontrol agents with anti-browning solutions reduced these negative effects. Therefore, some of the proposed biocontrol agents, particularly in combination with other preservative methods, may represent a good strategy to increase the safety and shelf-life of minimally processed fruits and vegetables. However, the introduction of biocontrol agents can be further optimized by focusing on the level and mode of inoculation and by limiting the negative effects observed on the colour parameters.

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Figure Captions

Figure 1. Lamb's lettuce, produced at industrial levels by using different washing solutions, immediately after washing and after 12 days of storage. The controls were washed with 120 mg/l of

chlorine. The samples added to the biocontrol agent were washed in solution containing 6 log CFU/ml of *L. lactis* CBM21. The samples washed with the biocontrol agent and thyme essential oil were washed in a solution containing 6 log CFU/ml of *L. lactis* CBM21 and 250 mg/l of thyme essential oil.

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Biocontrol Agent	Target Organism	Vegetable	Reference
Bacillus spp. and *Pseudomonas* spp.	*Salmonella chester*, *Listeria monocytogenes, Escherichia coli*	green pepper, romaine lettuce, baby carrots, alfalfa, and clover	Liao and Fett, 2001
Enterococcus mundtii, Pediococcus parvulus	*Listeria monocytogenes*	mung bean sprouts	Bennik et al., 1999
Gram negative bacteria	*Staphylococcus aureus*, *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella montevideo*	model system	Schuenzel and Harrison, 2002
Lactobacillus casei	*Staphylococcus aureus*, *Aeromonas hydrophila*, *Escherichia coli*, *Listeria monocytogenes*	scarola salad leaves	Scolari and Vescovo, 2004
Lacobacillus casei	coliforms, enterococci and *Aeromonas hydrophila*	mixed salads	Torriani et al., 1997
Lactobacillus casei, Lactobacillus plantarum, Pediococcus spp.	*Aeromonas hydrophila, Salmonella typhimurium and Staphylococcus aureus, Listeria monocytogenes*	salads and juice prepared from vegetable salads	Vescovo et al., 1996
Lactobacillus plantarum	*Leuconostoc* spp.	shredded carrots	Torriani et al., 1999
Lactobacillus plantarum	*Staphylococcus aureus*	minimally processed vegetables	Scolari et al., 2004
Lactobacillus plantarum and *Lactobacillus casei*	*Listeria monocytogenes* and *Escherichia coli*	lamb's lettuce	Siroli et al., 2015
Lactococcus lactis	*Listeria monocytogenes*	alfalfa sprouts	Palmai et al., 2002
Lactococcus lactis	*Listeria monocytogenes*	ready to eat Caisar salad	Cai et al., 1999
Leuconostoc mesenteroides and *Leuconostoc citreum*	*Listeria monocytogenes*	iceberg lettuce	Trias et al., 2008c
Leuconostoc spp.	*Salmonella typhimurium, Escherichia coli, Listeria monocytogenes*	iceberg lettuce leaf cuts	Trias et al., 2008a
Pseudomonas fluorescens	*Listeria monocytogenes*	endive leaves	Carlin et al., 1996
Pseudomonas fluorescens	*Listeria monocytogenes*	model system	Buchanan and Bagi, 1999

Pseudomonas fluorescens and *Pseudomonas viridiflava*	*Listeria monocytogenes*	potato tuber slices	Liao and Sapers, 1999
Weissella cibaria and lactic acid bacteria	*Xanthomonas campestris, Erwinia carotovora, Penicillium expansum, Monilinia laxa, Botrytis cinerea*	model system	Trias et al., 2008b

Table 1. Summary of the biocontrol agents isolated and used for vegetable and minimally processed vegetable.

Biocontrol Agent	Target Organism	Fruit	Reference
Candida sake	*Escherichia coli*	apple wounds	Abadias et al., 2009
Candida sp.; Gluconobactera saii, Candida spp., Dicosphaerina fagi and *Metschnikowia pulcherrima*	*Listeria monocytogenes* and *Salmonella enterica*	minimally processed apples	Leverentz et al., 2006
Enterobacteriaceae	*Escherichia coli, Salmonella, Listeria innocua*	Minimally processed apples and peaches	Alegre et al., 2012
Lactobacillus plantarum	*Listeria monocytogenes* and *Escherichia coli*	sliced apples	Siroli et al., 2015
Lactococcus lactis	*Listeria monocytogenes*	sliced apples	Siroli et al., 2014
Lactococcus lactis	spoliage microrganisms	industrial sliced apples	Siroli et al., unpublished results
Leuconostoc mesenteroides and *Leuconostoc citreum*	*Listeria monocytogenes*	apple Golden delicious	Trias et al., 2008a
Pseudomonas fluorescens	*Penicillium expansum*	apple, apple wounds	Mostafavi et al., 2013
Pseudomonas graminis	*Salmonella* and five *Listeria monocytogenes*	minimally processed apples	Alegre et al., 2013a
Pseudomonas graminis	*Escherichia coli*, *Salmonella* and *Listeria innocua*	minimally processed apples	Alegre et al., 2013b
Pseudomonas syringae	*Escherichia coli*	apple wounds	Janisiewicz et al., 1999

Table 2. Summary of the biocontrol agents isolated and used for fruits and minimally processed fruits.

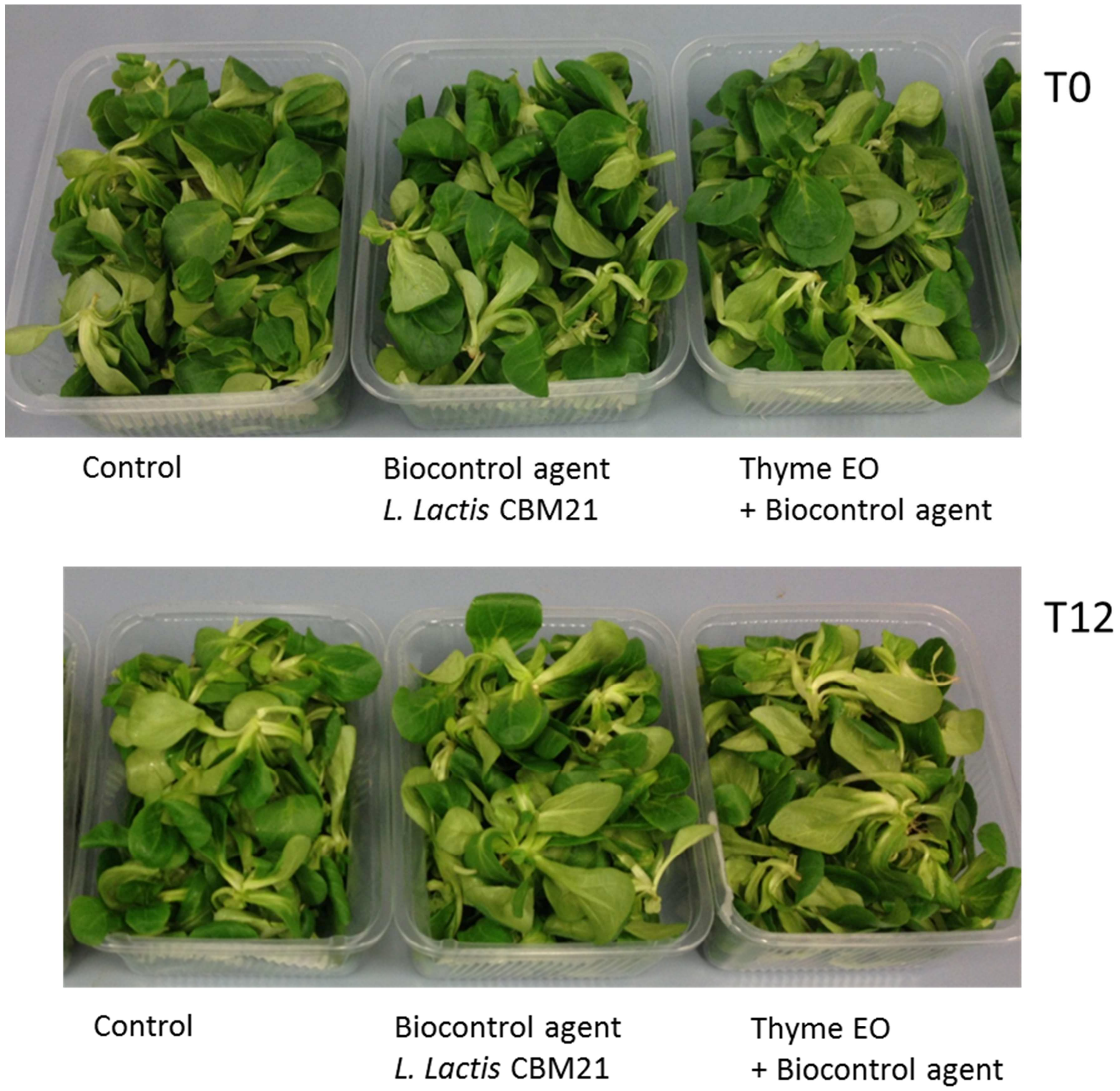
T0
Control
Biocontrol agent
L. Lactis CBM21
Thyme EO
+ Biocontrol agent
T12
Control
Biocontrol agent
L. Lactis CBM21
Thyme EO
+ Biocontrol agent

Figure 1.

- Biocontrol agents are able to prolong shelf-life and safety of minimally processed fruits

- Bioprotection of minimally processed vegetables

- LAB to increase safety and shelf-life of minimally processed products

- Mechanisms of action of biocontrol agents