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Lorenzo Siroli, Francesca Patrignani, Diana I. Serrazanetti, Fausto Gardini, Rosalba Lanciotti

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

3 Lorenzo Siroli<sup>(1)</sup>, Francesca Patrignani<sup>(1)</sup>, Diana I. Serrazanetti<sup>(2)</sup>, Fausto Gardini<sup>(1-2)</sup>, Rosalba  
4 Lanciotti<sup>(1-2)\*</sup>

5  
6 <sup>(1)</sup> Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna,  
7 Campus of Food Science, Piazza Goidanich 60, 47521 Cesena, Italy

8 <sup>(2)</sup> Interdepartmental Center for Industrial Agri-food Research, University of Bologna, Piazza  
9 Goidanich 60, 47521 Cesena (FC), Italy

10  
11 \*Corresponding Author: Rosalba Lanciotti, Department of Agricultural and Food Sciences, Alma  
12 Mater Studiorum, University of Bologna, Piazza Goidanich 60, 47521 Cesena, Italy.

13 E-mail: rosalba.lanciotti@unibo.it

14 Phone: +39 0547 338132

27 **Abstract**

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

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

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54 **1 Introduction**

55 Fresh fruits and vegetables are strongly recommended in the human diet because of their contents of  
56 vitamins, antioxidants, minerals and dietary fibres; additionally, a significant amount of  
57 epidemiological evidence has demonstrated that their consumption is beneficial to health (Boeing *et al.*,  
58 2012). They are generally consumed fresh, minimally processed, pasteurized or cooked by  
59 boiling in water or microwaving. Although heat treatments increase the safety and shelf-life of these  
60 products, heat treatments also decrease the nutritional properties and sensorial features of the raw  
61 materials. However, fresh produce and minimally processed products have a short shelf-life as a  
62 result of rapid microbial spoilage (Di Cagno *et al.*, 2008).

63 Minimally processed produce is more perishable than the original raw materials (Francis *et al.*,  
64 2012; Selma, Allende, López-Galvez, Conesa, & Gil, 2008). The increase in nutrient availability  
65 because of the presence of cut surfaces, the metabolism of tissues, the confinement of the products  
66 inside packages and the lack of treatments to ensure microbial stability favour the growth of both  
67 microorganisms deriving from raw materials and cross-contamination during handling and  
68 processing (peeling, cutting, etc.) (Francis *et al.*, 2012; Lanciotti, Gianotti, Patrignani, Belletti,  
69 Guerzoni, & Gardini, 2004). Although raw produce is expected to have a shelf-life of several weeks  
70 or months, minimally processed fruits and vegetables have only a very short storage life of 4 to 10  
71 days. Their shelf-life depends on various factors such as fruit and vegetable quality, production  
72 technology and the number and interactions among microbial groups (Selma *et al.*, 2008).

73 Mesophilic bacterial levels of  $10^3$  to  $10^6$  colony forming units (CFU)/g are common in minimally  
74 processed vegetables analysed immediately after packaging (Belletti, Lanciotti, Patrignani, &  
75 Gardini, 2008; Guerzoni, Gianotti, Corbo & Sinigaglia 1996; Ragaert, Devlieghere, & Debevere,  
76 2007; Siroli *et al.*, 2015). However, at the retail level, the counts are more variable, ranging between  
77  $10^3$  and  $10^9$  CFU/g (Belletti *et al.* 2008). Because of refrigerated storage, the dominating bacterial  
78 population mainly consists of species belonging to *Pseudomonadaceae* (particularly *Pseudomonas*

79 *fluorescens*) and *Enterobacteriaceae* (particularly *Erwinia herbicola* and *Rahnella aquatilis*), in  
80 addition to some species belonging to the lactic acid bacteria (LAB) (particularly *Leuconostoc*  
81 *mesenteroides*) (Bennik, Vorstman, Smid, & Gorris, 1998; de Azeredo, Stamford, Campos Nunes,  
82 Gomez Neto, de Oliveira, & de Souza, 2011; Nguyen-The & Carlin, 1994). Additionally, many  
83 different yeast species belonging to the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*,  
84 *Pichia* and *Torulaspora* have been identified during storage (Nguyen-The & Carlin, 1994, Ragaert  
85 *et al.*, 2007) whereas moulds are less important in these products because of the intrinsic properties  
86 of fruits and vegetables, such as a slightly acidic to neutral pH, which favours bacteria and yeasts  
87 (Barth, Hankinson, Zhuang, & Breidt, 2010). By contrast, the spoilage of minimally processed fruit  
88 primarily occurs because of the proliferation of its natural acid tolerant and osmophilic microflora  
89 (Belletti *et al.*, 2008; Lanciotti *et al.*, 2004). In fact, the microflora is mainly represented by yeasts,  
90 which are generally responsible for the fermented taste and carbon dioxide production; LAB, which  
91 can produce a buttermilk off-flavour; and moulds, which contribute to spoilage by their surface  
92 growth (Tournas, Heeres, & Burgess, 2006). However, yeasts are favoured compared to LAB  
93 because of the high sugar content and C/N ratio of the system (Patrignani, Tabanelli, Siroli, Gardini,  
94 & Lanciotti, 2013; Siroli *et al.*, 2014a). In addition to spoilage microorganisms, outbreaks of food-  
95 borne diseases associated with the consumption of fresh and minimally processed fruits and  
96 vegetables, which is primarily a result of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria*  
97 *monocytogenes*, have increased dramatically since the 1970s (Alegre, Abadias, Anguera, Oliveira,  
98 & Viñas, 2010; Ramos, Miller, Brandão, Teixeira, & Silva, 2013; Sant'Ana, Landgraf, Destro, &  
99 Franco, 2011). However, Ramos *et al.* (2013) also showed that *Clostridium botulinum*, *Shigella*  
100 spp., *Staphylococcus* spp., *Vibrio cholerae* and *Yersinia enterocolitica* are amongst the major fruit  
101 and vegetable pathogens associated with outbreaks. Moreover, numerous studies showed the  
102 presence of *Aeromonas hydrophila* and *Staphylococcus aureus* on fresh produce and related  
103 minimally processed products (Alegre *et al.*, 2010; Harris *et al.*, 2003; Nguyen-The & Carlin,  
104 1994). Likewise, *Campylobacter jejuni* was isolated in minimally processed mushrooms from retail

105 markets in the United States (EFSA 2013; FDA 2001). Castillo and Escartin (1994) showed that  
106 this pathogenic species could survive on sliced watermelon and papaya for a sufficient time to be a  
107 risk to the consumer. The contamination of vegetables and fruits with spores of *Bacillus cereus*,  
108 *Clostridium perfringens* and *C. botulinum* present in soil is common (FDA 2001). When fresh  
109 products are handled and processed in a manner that enables the germination of spores and the  
110 growth of vegetative cells, there is a threat to public health, particularly when the products are  
111 packaged in a modified atmosphere (FDA 2001). There were approximately 110 scientific papers  
112 and reports on outbreaks associated with the consumption of minimally processed fruits and  
113 vegetables according to the Food and Drug Administration (FDA), the Centres for Disease Control  
114 and Prevention (CDC) and the World Health Organization (WHO) (Ramos *et al.*, 2013).

115 Because of the lack of processing steps to kill microbial contaminants, the use of high quality raw  
116 materials and efficient temperature control during manufacture, distribution and retailing are key  
117 factors for maintaining the microbiological quality and safety of minimally processed fruits and  
118 vegetables. However, the quality of raw materials and the maintenance of the cold chain are  
119 difficult to be implemented and controlled (Rediers, Claes, Peeters, & Willems, 2009; Siroli *et al.*,  
120 2014a). In fact, the quality of raw materials depends on several factors including agronomic  
121 practices, seasonal trends, storage conditions, etc. Moreover, an extensive amount of literature  
122 shows that thermal abuse is very frequent during product transport and selling (Lanciotti *et al.*,  
123 2004; Rediers *et al.*, 2009).

124 Decontamination methods are another tool for reducing the microbial cell loads of the raw materials  
125 and have been shown to have positive effects on product safety and shelf-life (Ramos *et al.*, 2013).

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

129 Disinfection processes incorporating chlorine are often applied to fresh vegetables to enhance safety  
130 and shelf-life profiles. However, numerous reports indicate that chlorine has limited antimicrobial

131 efficacy, allowing 1–2 logarithmic reductions in the bacterial population of raw materials at the  
132 permitted concentrations (Abadias, Usall, Anguera, Solsona, & Viñas, 2008). Its inefficacy to  
133 eliminate microbial cells was attributed to the inability of its aqueous solutions to wet the  
134 hydrophobic surface of the waxy cuticle of vegetables and to its inactivation by the organic matter  
135 (Carrasco, Pérez-Rodríguez, Valero, García-Gimeno, & Zurera, 2008; de Azeredo *et al.*, 2011).  
136 Additionally, the presence of biofilms on equipment has been reported to reduce the efficacy of  
137 chlorine against microorganisms that can cross-contaminate the products during processing  
138 (Carrasco *et al.*, 2008). Additional drawbacks of chlorine usage are the possible formation of  
139 carcinogenic chlorinated compounds, vapours having adverse health effects and the increase in  
140 microbial chlorine resistance (Abadias *et al.*, 2008; Gil, Selma, López-Gálvez, & Allende, 2009).  
141 For these reasons, the use of chlorine is prohibited or restricted in some European countries, such as  
142 the Netherlands, Sweden, Germany, Switzerland, Denmark and Belgium, for the disinfection of the  
143 raw materials used for the production of minimally processed vegetables (Gil *et al.*, 2009;  
144 Tirpanalan, Zunabovic, Domig, & Kneifel, 2011). Furthermore, disinfectants alternative to chlorine,  
145 such as ozone, H<sub>2</sub>O<sub>2</sub>, organic acids, calcium-based solutions and peroxiacetic acids, have  
146 demonstrated their inability to completely eradicate or kill microorganisms on fresh produce and  
147 their potential toxicity and side effects on the sensorial properties of the products (Ramos *et al.*,  
148 2013; Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). In addition, the reduction of the naturally  
149 occurring population because of washing and sanitization can reduce the competition for space and  
150 nutrients against pathogenic species (Schuenzel & Harrison, 2002).

151 Consumer concern of chemical synthetic additives has stimulated research into alternative methods  
152 for reducing the decay of minimally processed fruits and vegetables and improving product safety  
153 (Ayala-Zavala, Oms-Oliu, Odriozola-Serrano, GonzálezAguilar, Álvarez-Parrilla, & Martín-  
154 Belloso, 2008). The use of generally recognized as safe (GRAS) microorganisms such as LAB and  
155 yeasts and/or their natural metabolites to inhibit the growth of pathogenic and spoilage  
156 microorganisms is a promising tool, and it is also perceived by the consumer as a natural food



157 preservation method (Cosentino, Fadda, Deplano, Melis, Pomata, & Pisano, 2012; Ross, Morgan, &  
158 Hill, 2002). Bioprotective microorganisms have already shown their potential for practical  
159 application in various foods, such as meat (Vermeiren, Devlieghere, & Debevere, 2004) and plant  
160 derived products (Settanni & Corsetti, 2008; Trias, Baneras, Badosa, & Montesinos, 2008a; Trias,  
161 Baneras, Montesinos, & Badosa, 2008b).

162 In particular, LAB have shown a great potential as biocontrol agents of several non-fermented foods  
163 because they are widely used in fermented foods, have a long history of safe use, and have a GRAS  
164 status (Carr, Chill, & Maida, 2002). They have also been applied to increase the safety and shelf-life  
165 of minimally processed fruits and vegetables (Palmai, & Buchanan, 2002; Torriani, Orsi, Vescovo,  
166 1997; Vescovo, Torriani, Orsi, Macchiarolo, & Scolari, 1996). However, several other bacteria and  
167 yeasts, often selected among the naturally occurring microbiota, including strains of *Pseudomonas*  
168 *syringae*, *Pseudomonas graminis*, *Gluconobacter asaii*, *Candida spp.*, *Dicosphaerina fagi*,  
169 *Metschnikowia pulcherrima* and *Candida sake* have been proposed as biocontrol agents in these  
170 foods (Abadias, Usall, Alegre, Torres, & Vinas, 2009; Alegre, Vinas, Usall, Anguera, Altisent, &  
171 Abadias, 2013a; Trias *et al.*, 2008a).

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

175

## 176 ***2 Protective culture for minimally processed vegetables***

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

179 LAB are also indubitably the most important bioprotective cultures for non-fermented foods  
180 including minimally processed vegetables. In fact, protective cultures of LAB have been developed  
181 over the last few decades to increase the safety and shelf-life of minimally processed vegetables.

182 The potential of antagonistic LAB belonging to *Lactobacillus casei* or their culture filtrate to inhibit

183 the growth of pathogenic bacteria in ready-to-eat vegetables was first demonstrated by Vescovo et  
184 al. (1996) and Torriani et al. (1997). In particular, Torriani et al. (1997) showed that the addition of  
185 3% culture permeate of *Lb. casei* IMPC LC34 to mixed salads reduced the total mesophilic bacteria  
186 counts from 6 to 1 log CFU/g and suppressed coliforms, enterococci, and *A. hydrophila* after 6 days  
187 of storage at 8 °C. *Lactobacillus plantarum* IMPC LP4 was able to prolong the very limited shelf-  
188 life of shredded carrots because of its ability in real systems to control the growth of *Leuconostoc*  
189 spp., which have been identified as the main spoilage agents of this minimally processed vegetable  
190 (Torriani, Scolari, Dellaglio, & Vescovo, 1999).

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

196 Bennik, van Overbeek, Smid and Gorris (1999) studied the potential of two *Pediococcus parvulus*  
197 strains and one *Enterococcus mundtii* strain to control the growth of *L. monocytogenes* on  
198 refrigerated, modified atmosphere stored mung-bean sprouts. These bacteriocinogenic biocontrol  
199 agents, previously isolated from minimally processed vegetables, were shown to grow in culture  
200 broth at 4, 8, 15 and 30 °C. However, only *E. mundtii* was capable of bacteriocin production at 4–8  
201 °C and was subsequently evaluated for its ability to control the growth of *L. monocytogenes* on  
202 vegetable agar and fresh mung-bean sprouts under a modified atmosphere at 8 °C. The growth of *L.*  
203 *monocytogenes* was inhibited by bacteriocinogenic *E. mundtii* on sterile vegetable-medium but not  
204 on fresh produce. Otherwise, bacterial cultures that were isolated from the same type of vegetable  
205 or product in which they were used as biocontrol agents were reported to have the greatest chance  
206 of success in controlling pathogens (Bennik *et al.*, 1999; Siroli *et al.*, 2015).

207 Palmi and Bouchanan (2002) assessed the inhibitory activity of *Lactococcus lactis* against *L.*  
208 *monocytogenes* inoculated in model systems and sprouts at levels of approximately 2 log CFU/g,

209 thus demonstrating that their inhibitory activity was substantially reduced on alfalfa compared to  
210 that observed in a model system. The apparent decrease in effectiveness of the biocontrol agents in  
211 real systems compared to model systems was attributed to the inhibitory activity of naturally  
212 occurring microflora (Bennik *et al.*, 1999; Palmai & Bouchanan 2002). Otherwise, there were more  
213 variables affecting the success of the biocontrol agents in a real system than in a model system, and  
214 they were often unpredictable in a real system. Additionally, the interference of naturally occurring  
215 microbiota cannot be exactly identified because it varies according to the raw material and process  
216 conditions.

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

225 Scolari and Vescovo (2004) performed several challenge experiments on scarola salad leaves by  
226 simultaneously inoculating *Lb. casei* and pathogenic species such as *S. aureus*, *A. hydrophila*, *E.*  
227 *coli* and *L. monocytogenes*. These authors showed a remarkable inhibitory effect by the LAB  
228 towards all the pathogenic strains. Scolari, Vescovo, Zacconi and Bonadé (2004) studied the  
229 influence of *Lb. plantarum* on the growth of *S. aureus* through an impedometric method and by  
230 varying the inoculum size of the single strain and the growth temperature according to a CCD.  
231 These authors showed that temperature affected the growth of both *S. aureus* and *Lb. plantarum*  
232 strains. The pathogenic strain, independent of its inoculum size, was inhibited by *Lb. plantarum* at  
233 all the tested temperatures. The authors outlined that a proper combination of specific LAB and  
234 storage temperature should improve the safety of the minimally processed vegetables. Trias,

235 Badosa, Montesinos and Baneras (2008c) characterized ten *L. mesenteroides* strains and one  
236 *Leuconostoc citreum* strain isolated from fresh fruits and vegetables for their antagonistic capacity  
237 against *L. monocytogenes*; they identified organic acids, hydrogen peroxide and bacteriocins as the  
238 main inhibition mechanisms. In a successive study, Trias et al. (2008a) studied the ability of the  
239 selected biocontrol agents to inhibit the growth of foodborne human pathogens when inoculated in  
240 iceberg lettuce leaf cuts. The selected strains grew on the substrates and did not cause negative  
241 effects on the general aspect of the lettuce tissues. In addition, the treatment of the lettuce cuts with  
242 the antagonistic strains reduced the cell count of *Salmonella typhimurium* and *E. coli* by 1 to 2 log  
243 CFU/wound or g, whereas the growth of *L. monocytogenes* was completely inhibited.

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

249 Siroli et al. (2015) characterized several LAB strains that were previously isolated from commercial  
250 minimally processed fruits and vegetables for their ability to grow at low temperature and low pH  
251 values and to antagonize the pathogenic species frequently associated with these food products. In  
252 addition, these authors studied the effect of the biocontrol agents to prolong the shelf-life of the  
253 product. In fact, most of the literature available studied the effects of biocontrol cultures on  
254 minimally processed vegetable safety without considering the effects on product shelf-life and  
255 quality. On the basis of the results obtained, these authors selected *Lb. plantarum* V7B3 and *Lb.*  
256 *casei* V4B4 to be used as biocontrol agents alone or in combination with thyme essential oil (EO) in  
257 lamb's lettuce. The results obtained indicated that applying the *Lb. plantarum* V7B3 strain to lettuce  
258 during the washing phase at a level of 6 log CFU/ml instead of chlorine increased product shelf-life  
259 and safety. In fact, *Lb. plantarum* V7B3 showed an interesting potential for controlling *L.*  
260 *monocytogenes* and *E. coli* when deliberately inoculated in washing solution at levels ranging

261 between 3 and 4 log CFU/ml. The presence of the *Lb. plantarum* V7B3 strain increased the *E. coli*  
262 death kinetics and reduced the viability of *L. monocytogenes* over the 9 days of refrigerated storage  
263 of lamb's lettuce. Moreover, combining the selected strains with natural antimicrobials produced a  
264 further increase in the shelf-life (12 days) of the product without detrimental effects on the  
265 organoleptic quality compared to the traditional products washed with chlorine (120 ppm), thus  
266 contributing to the substitution of this chemical raw material sanitizer. Moreover, Siroli, Patrignani,  
267 Salvetti, Torriani, Gardini and Lanciotti (2014b) showed the good performance of a nisin producing  
268 strain, *L. lactis* CBM21, which was inoculated at a level of 7 log CFU/ml in the washing solution of  
269 minimally processed lamb's lettuce and combined or not with thyme EO, to inhibit both the  
270 inoculated *L. monocytogenes* and *E. coli* and the total mesophilic species, significantly increasing  
271 the product shelf-life. In fact, the addition of the biocontrol agent did not affect the quality  
272 parameters (*i.e.*, colour parameters and sensory attributes) of lamb's lettuce. The use of *L. lactis*  
273 CBM21 and/or thyme EO added in the tap water used for lamb's lettuce washing was also  
274 experienced at the industrial level, confirming their potential as an alternative to chlorine (Siroli et  
275 al. unpublished results). In fact, the products obtained with the innovative washing solutions  
276 showed the same safety and shelf-life of the controls but with improved sensorial properties.  
277 Moreover, the products added with the biocontrol agent maintained a good appearance for up to 12  
278 days (Figure 1).

279 In addition to LAB, some authors studied the competitive, inhibitory, or antagonistic activity of  
280 biocontrol agents selected among the naturally occurring microbiota of fresh or minimally  
281 processed vegetables. Several studies showed that fresh-cut produce are sources of competitive  
282 microorganisms (Francis & O'Beirne 1998; Janisiewicz, Conway, & Leverentz, 1999; Liao & Fett  
283 2001; Schuenzel & Harrison, 2002). Liao and Sapers (1999) also demonstrated that potential soft  
284 rot microorganisms belonging to the natural resident microflora, such as *P. fluorescens* and *P.*  
285 *viridiflava*, can have great potential as biocontrol agents, inhibiting the growth of *L. monocytogenes*  
286 inoculated on potato tuber slices. Additionally, Carlin, Nguyen-The and Morris (1996) found that *P.*

287 *fluorescens* was able to inhibit the growth of *L. monocytogenes* on endive leaves maintained at 10  
288 °C by approximately 1 log compared to controls when the endive leaves were inoculated with the  
289 *Pseudomonas* at levels ranging between 6 and 7 log CFU/g. Using a model system, Buchanan and  
290 Bagi (1999) reported that the inhibition of *L. monocytogenes* by *P. fluorescens* was limited to a  
291 repression in the maximum levels attained and that the extent of inhibition was dependent on the  
292 water activity and pH of the environment.

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

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

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

309

### 310 ***3 Protective culture for minimally processed fruits***

311 The use of protective cultures and biocontrol agents has also been reported in minimally processed  
312 fruits because they can be an alternative to chemical treatments to increase the product safety, shelf-

313 life and quality (Abadias *et al.*, 2009). Biocontrol agents have been utilized alone or in combination  
314 with modified-atmosphere packaging, natural antimicrobials (Siroli *et al.*, 2015), gamma radiation  
315 (Mostafavi, Mirmajlessi, Fathollahi, Shahbazi, & Mirjaliliet, 2013), reducing agents (Alegre *et al.*,  
316 2013a; Alegre, Viñas, Usall, Teixidó, Figge, & Abadias, 2013b) and heat treatments (Leverentz,  
317 Janisiewicz, Conway, Saftner, & Camp, 2003) to obtain a synergic effect on both the safety and  
318 quality of fruit, postharvest fruit and minimally processed produce.

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

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

340 Moreover, Siroli et al. (unpublished results) showed that the nisin-producer *L. lactis* CBM21,  
341 inoculated at a level of 7 log CFU/ml in the dipping solution of sliced apples in combination or not  
342 with 2-(E)-hexenal/hexanal and/or 2-(E)-hexenal/citral, limited the growth of yeasts below 5 log  
343 CFU/g during 28 days of storage. This strain also inhibited the growth of *L. monocytogenes* during  
344 28 days of storage, particularly when used in combination with the proposed natural antimicrobials.  
345 Negative effects on colour parameters were observed but only after 16 days of storage in the  
346 presence of natural antimicrobials. Similar results were obtained by Siroli et al. (unpublished  
347 results) on the shelf-life of sliced apples that were produced on an industrial scale by adding the  
348 mixture of hexanal/2-(E)-hexenal and/or *L. lactis* CBM21 to the dipping solution. The products  
349 obtained at the industrial level with the innovative dipping solutions maintained good  
350 microbiological, organoleptic and textural characteristics for up to 20 days. These results are  
351 promising because one of the most important selection criteria of a biocontrol agent is the  
352 maintenance of their performance in real production conditions.

353 Biocontrol agents, different from LAB, have also been selected for their application in minimally  
354 processed fruits. *P. syringae* L-59-66 prevented the growth of *E. coli* on apple wounds (Janisiewicz  
355 *et al.*, 1999). The growth of *L. monocytogenes* and *Salmonella* on fresh-cut apples was reduced by  
356 *G. asaii*, *Candida* spp., *D. fagi* and *M. pulcherrima* (Leverentz, Conway, Janisiewicz, Abadias,  
357 Kurtzman, & Camp, 2006). These antagonists reduced the *L. monocytogenes* populations and,  
358 except for the *Candida* spp., the *S. enterica* serovar Poona populations. This reduction was higher at  
359 25 °C than at 10 °C, and the growth of the antagonists and pathogens increased at higher  
360 temperatures (Leverentz *et al.*, 2006).

361 The postharvest biocontrol agent *C. sake* CPA-1 reduced *E. coli* growth on apple wounds but not in  
362 minimally processed apples (Abadias *et al.*, 2009). In particular, this yeast was effective at  
363 colonizing apple wounds and tissues, and the competition for nutrients could play the main role in



364 the biocontrol of *C. sake* CPA-1 on pome fruits. Trials were conducted with a mixture of five  
365 strains of *E. coli* isolated from apples. The results provided evidence that *E. coli* was unable to grow  
366 in apple juice at 5, 15 and 25 °C, but it was able to survive. At 10 °C and above, *E. coli* thrived in  
367 fresh-cut apples and wounds. When *E. coli* was inoculated in apple wounds with the yeast  
368 antagonist *C. sake*, its growth was reduced by approximately 1 log CFU/wound at 25 °C. At 5 °C,  
369 no effect of the biocontrol agent was observed. The biocontrol agent *C. sake*, which was developed  
370 to prevent fruit decay during storage, also reduced *E. coli* growth in wounded apples at abusive  
371 temperatures.

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

379 The antagonistic strain increased the activity of NatureSeal AS1 (6%, w/v) (a commercial anti-  
380 browning agent) on apple wedges stored at 10 °C with or without modified atmosphere packaging  
381 (Röbke, Gormley, & Butler, 2009). Moreover, in a semi-commercial assay, the efficacy of *P.*  
382 *graminis* CPA-7 inoculated at 5 and 7 log CFU/ml against *Salmonella* and *L. monocytogenes* was  
383 evaluated on minimally processed apples with NatureSeal and modified atmosphere packaging and  
384 stored at 5 and 10 °C. Although high CPA-7 concentrations avoided *Salmonella* growth at 10 °C  
385 and lowered the *L. monocytogenes* population increases, the effect was not instantaneous. No effect  
386 on apple sensory properties was detected. Therefore, CPA-7 could avoid pathogen growth on  
387 minimally processed apples during storage when used as part of a hurdle technology in combination  
388 with disinfection techniques, low storage temperature and modified atmosphere packaging.  
389 Recently, the ability of *P. graminis* CPA-7 to reduce *E. coli* O157:H7, *Salmonella* and *Listeria*

390 *innocua* on minimally processed apples and peaches was demonstrated (Alegre et al., 2013b). The  
391 results support the potential use of CPA-7 as a bioprotective agent against foodborne pathogens in  
392 minimally processed fruit.

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

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

407

#### 408 ***4 Action mechanisms of biocontrol agents***

409 Numerous studies have shown the potential of several microorganisms to inhibit the growth of  
410 foodborne pathogens in minimally processed fruits and vegetables (Alegre *et al.* 2012; Alegre *et al.*  
411 2013b; Leverentz, *et al.*, 2006; Scolari & Vescovo, 2004; Torriani *et al.*, 1997; Trias *et al.*, 2008a;  
412 Vescovo *et al.*, 1996). In particular, LAB have shown great potential as biocontrol agents in these  
413 types of products. The preservation abilities of LAB are a result of several mechanisms of action  
414 and are mainly related to the production of antimicrobial compounds, organic acids, hydrogen  
415 peroxide, bacteriocins and diacetyl (Cleveland, Montville, Nes, & Chikindas, 2001; Trias *et al.*,

416 2008c). Moreover, they compete with pathogens and spoilage microorganisms for nutrients  
417 (vitamins, minerals, trace elements and peptides). The decreased pH values and antibacterial  
418 activities of organic acids produced by LAB represent the main mechanisms for the biopreservation  
419 of fermented foods (Galvez, Abriouel, Benomar, & Lucas, 2010).

420 Several bacteriocin-producing LAB have been shown to be effective against spoilage and  
421 pathogenic microorganisms in minimally processed fruits and vegetables (Allende, Martinez,  
422 Selma, Gil, Suarez, & Rodriguez, 2007; Bennik *et al.*, 1999; Randazzo, Pitino, Scifo, & Caggia,  
423 2009). In fact, many LAB are able to produce bacteriocins and bacteriocin-like molecules.  
424 Bacteriocins are antimicrobial peptides produced by bacteria to compete against bacteria of the  
425 same species or even other genera (Cotter, Hill, & Ross, 2005). Both gram-positive and gram-  
426 negative bacteria are able to produce bacteriocins. However, bacteriocins produced by LAB appear  
427 to be more promising for potential use in the food industry as natural preservatives (Settanni &  
428 Corsetti, 2008) because they are normally designed as GRAS by the U.S. Food and Drug  
429 Administration (FDA), in particular when they are familiar with the selected food product.

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

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

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

448 The direct application of bacteriocins on fresh-cut products has been tested in recent years. In  
449 particular, bacteriocins such as nisin, pediocin PA-1/AcH and enterocin AS-48 have been tested in  
450 tinned vegetables, fruit juices, and salads against pathogens such as *E. coli* O157:H7, *S. aureus*, and  
451 the spoilage bacterium *Alicyclobacillus acidoterrestris* (Cleveland *et al.*, 2001; Cobo-Molinos *et al.*,  
452 2005). Randazzo *et al.* (2009) showed a reduction in *L. monocytogenes* cell loads of 1.9 log unit and  
453 of 2.7 log units in iceberg lettuce that was washed with commercial nisin and RUC9 bacteriocin,  
454 respectively, compared to samples without bacteriocin after the 7th day of refrigerated storage.  
455 Additionally, Allende *et al.* (2007) reported that washing fresh-cut lettuce with solutions containing  
456 a mix of nisin, plantaricin, lacticin, coagulin and pediocin PA-1 reduced the viability of *L.*  
457 *monocytogenes* by 1.2–1.6 log units immediately after treatment. Cai *et al.* (1997) showed that the  
458 addition of nisin in ready-to-eat Caesar salad caused a reduction of 1.4 log in *Listeria* cell loads.  
459 Cobo-Molinos *et al.* (2005) found a reduction of *L. monocytogenes* of 2.0–2.4 log CFU/g on fresh  
460 alfalfa sprouts, soybean sprouts and green asparagus added with enterocin AS-48.

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

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

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

491

## 492 **5 Conclusion**

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

509

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513

#### 514 *Figure Captions*

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

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

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

<i>Pseudomonas fluorescens</i> and <i>Pseudomonas viridiflava</i>	<i>Listeria monocytogenes</i>	potato tuber slices	Liao and Sapers, 1999
<i>Weissella cibaria</i> and lactic acid bacteria	<i>Xanthomonas campestris</i> , <i>Erwinia carotovora</i> , <i>Penicillium expansum</i> , <i>Monilinia laxa</i> , <i>Botrytis cinerea</i>	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
<i>Candida sake</i>	<i>Escherichia coli</i>	apple wounds	Abadias et al., 2009
<i>Candida sp.</i> ; <i>Gluconobacteria saii</i> , <i>Candida spp.</i> , <i>Dicosphaerina fagi</i> and <i>Metschnikowia pulcherrima</i>	<i>Listeria monocytogenes</i> and <i>Salmonella enterica</i>	minimally processed apples	Leverentz et al., 2006
<i>Enterobacteriaceae</i>	<i>Escherichia coli</i> , <i>Salmonella</i> , <i>Listeria innocua</i>	Minimally processed apples and peaches	Alegre et al., 2012
<i>Lactobacillus plantarum</i>	<i>Listeria monocytogenes</i> and <i>Escherichia coli</i>	sliced apples	Siroli et al., 2015
<i>Lactococcus lactis</i>	<i>Listeria monocytogenes</i>	sliced apples	Siroli et al., 2014
<i>Lactococcus lactis</i>	spoliage microorganisms	industrial sliced apples	Siroli et al., unpublished results
<i>Leuconostoc mesenteroides</i> and <i>Leuconostoc citreum</i>	<i>Listeria monocytogenes</i>	apple Golden delicious	Trias et al., 2008a Mostafavi et al., 2013
<i>Pseudomonas fluorescens</i>	<i>Penicillium expansum</i>	apple, apple wounds	
<i>Pseudomonas graminis</i>	<i>Salmonella</i> and five <i>Listeria monocytogenes</i>	minimally processed apples	Alegre et al., 2013a
<i>Pseudomonas graminis</i>	<i>Escherichia coli</i> , <i>Salmonella</i> and <i>Listeria innocua</i>	minimally processed apples	Alegre et al., 2013b
<i>Pseudomonas syringae</i>	<i>Escherichia coli</i>	apple wounds	Janisiewicz et al., 1999

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

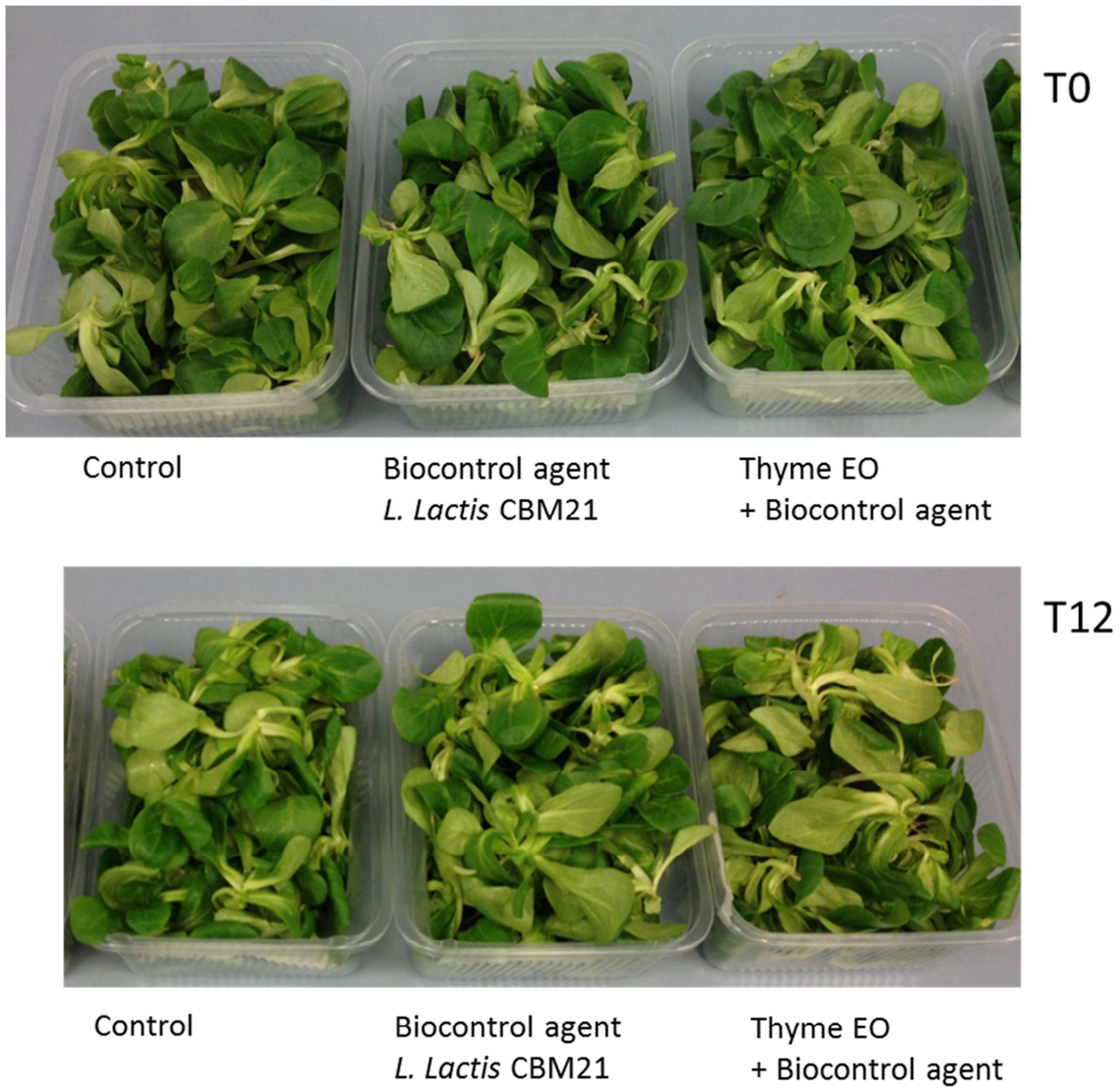


Figure 1.

- 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

ACCEPTED MANUSCRIPT