Effect of *Pseudomonas graminis* strain CPA-7 on the ability of *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* to colonize Caco-2 cells after pre-incubation on fresh-cut pear

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ABSTRACT

In this work, we evaluated whether the prior interaction of the antagonist *Pseudomonas graminis* strain CPA-7 and *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* ser. Enteritidis on fresh-cut pear has an effect on the capacity of these pathogens to colonize human epithelial cells (Caco- 2 cell line) which is crucial in establishing infection. After 7 days of co-incubation with the antagonist at 10 °C, *L. monocytogenes* and *S. enterica* growth was reduced by 5.5 and 3.1 $\log_{10}$, respectively. CPA-7 attenuated the adherence of *S. enterica* to Caco-2 cells (by 0.8 $\log_{10}$) regardless of the pre-adaptation on the fruit. Conversely, adhesiveness of *L. monocytogenes* was

not influenced by the interaction with the antagonist but it was reduced by 0.5 $\log_{10}$ after incubation in the food matrix. The combination of the interaction pathogen-antagonist-food matrix and the pre-adaptation period was associated to a significant reduction of the relative invasiveness of both pathogens, by 1.3 $\log_{10}$ in the case of *L. monocytogenes* and to an undetectable level (below 5 CFU/g) for *S. enterica*. Increased relative adherence (by 0.6 $\log_{10}$) of CPA-7 to the monolayers positively correlated to the increase of multiplicity of infection of the antagonist with respect to Caco-2 cells. However, for the same level of inoculum, its internalization was only detected after seven days of pre-adaptation in the fruit (pH 4.5 - 5.0). Final populations of CPA-7 after gastrointestinal passage were reduced by 2 $\log_{10}$ more after the pre-incubation on the fruit than on inoculation day. Simulated digestion did not influence its adhesion index, but it reduced its invasiveness to an undetectable level, regardless of the habituation period on the fruit.

1. Introduction

Pseudomonas graminis strain CPA-7 is a Gram-negative bacteria with antagonistic effect on *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* and *Escherichia coli* O157:H7 on fresh-cut apples, peaches, pears and melons (Abadias et al., 2014; Alegre et al., 2013a, b). Our work group has been working on elucidating the mechanisms through which this strain controls populations of foodborne pathogens. Although a number of *in vitro* and *in vivo* experiments related to the production of antibiotics, biosurfactants and exo-proteases were performed, its mode of action could not be elucidated (Collazo et al., 2017). Hence the impairment of pathogenesis-related characteristics of foodborne pathogens was explored as another putative mechanism for antagonism.

To cause gastrointestinal infections, foodborne pathogenic bacteria must adhere to the epithelium, activating the release of enzymes and toxins that lead to the initiation of necrotic processes into the target cells facilitating the invasion process (Jankowska et

al., 2008; Tamburro et al., 2015). Among foodborne pathogens, *Salmonella* spp. and *Listeria* spp. are typical intracellular pathogens, capable of surviving and actively replicating inside epithelial cells (Götz and Goebelt, 2010).

Reduction of the colonization capacities of foodborne pathogens by interfering with their attachment to the human intestinal epithelium or by altering their pathways to penetrate into the enterocytes could be an effective mechanism to antagonize them which has been explored for probiotic bacteria (Feng et al., 2015; Tamburro et al., 2015). Anti-adherent abilities of probiotics can be mediated by the production of inhibitory substances and by exclusive competition because of their capacity to survive in low pH conditions, auto-aggregate or co-aggregate with pathogens and adhere to the human gastrointestinal tract (Pan et al., 2008; Vuotto et al., 2014). In such a way, *Lactobacillus acidophilus, L. casei and L. rhamnosus* were shown to actively reduce the adhesion of *S. enterica* and *L. monocytogenes* to human adenocarcinoma cell lines to less than 50% (Dutra et al., 2016; Tabasco et al., 2014). The ability of non-acidolactic probiotic bacteria such as *Clostridium butyricum* CB2 and *Enterococcus mundtii* ST4SA to impair foodborne pathogens adherence to intestinal epithelial cells have also been studied (Botes et al., 2008; Pan et al., 2008). However, to the best of knowledge, no information in the body of literature was found concerning the putative effect of non-probiotic antagonists, such as the one tested in the present study, on the behavior of the pathogens inhabiting contaminated food once they come into contact with the human gastrointestinal tract.

A great part of the studies that have focused on this matter have been carried out using Caco-2 cells, a human colon adenocarcinoma cell line, that reproduce morphological and functional properties of human intestinal epithelium. They differentiate in a similar manner to normal small intestinal cells, expressing characteristics of immature or mature enterocytes with functional brush border microvilli and apical hydrolases (Gaillard et al., 1996). After confluence (5 to 6 days of culture), the process of polarization of Caco-2 cells involves the whole monolayer while differentiation takes

another ten days to complete (Gaillard et al., 1996). Polarization and differentiation of this cell line has been shown to influence the invasion process of *Listeria* spp. and *Salmonella* spp. because surface molecules such as the receptors that mediate their internalization express themselves asymmetrically (Boumart et al., 2014; Jankowska et al., 2008).

The aim of this research was to study the effect of the interaction between the antagonist *P. graminis* CPA-7 and the enteropathogenic bacteria *L. monocytogenes* and *S. enterica* subsp. e*nterica* in a food matrix on the ability of these microorganisms to adhere and to invade differentiated colorectal human adenocarcinoma cells Caco-2. For this, the antagonist and each pathogen were pre-incubated aerobically for seven days on fresh-cut pear upon abused refrigerated conditions (10 ºC). Additionally, survival of CPA-7 during static simulated gastrointestinal digestion and its subsequent adhesion to and invasion into Caco-2 cells was evaluated.

2. Materials and methods.

2.1 Fruit

Pears (*Pyrus communis* L cv. 'Conference') were obtained from local packing-houses (Lleida, Spain). Prior to the experimental studies, they were washed in running tap water, disinfected with 700 mL/L ethanol and air-dried at room temperature. Pears were peeled and cut into eight wedges using a slicer/corer or into plugs (cylinders of 1.2 cm diameter, 1 cm thickness) using a cork borer. Quality parameters: pH, soluble solids, firmness, texture and titratable acidity were initially determined as described elsewhere (Colas-Meda et al., 2017).

2.2 Bacterial strains and culture conditions

The strain *P. graminis* CPA-7 (Alegre et al., 2013b) was used as antagonist. Five different strains of *S. enterica* subsp. *enterica* [(ex. Kauffmann and Edwards) Le Minor and Popoff]: ATCC BAA-707, ATCC BAA-709, ATCC BAA-710, ATCC BAA-711 and

106 CECT 4300 belonging to serovars Agona, Michigan, Montevideo, Gaminara and
107 Enteritidis , respectively, were used. Five strains of *L. monocytogenes* [(Murray *et al.*
108 1926) Pirie 1940]: CECT 4031, CECT 933, CECT 940, CECT 4032 and Lm 230/3,
109 (Abadias et al., 2008) belonging to serotypes 1a, 3a, 4d, 4b and 1/2a, respectively,
110 were tested.

111 Strains of *S. enterica* and *L. monocytogenes* were grown individually as described by
112 Abadias et al. (2014) in order to obtain concentrated suspensions. CPA-7 was grown in
113 tryptone soy broth, TSB and a concentrated suspension was obtained following the
114 above mentioned procedure. Concentrations were checked by plating appropriate ten-
115 fold dilutions in saline peptone (8.5 g/L NaCl and 1 g/L peptone) onto Xylose Lysine
116 Deoxycholate Agar, XLD-agar for *S. enterica*, onto Palcam Agar Base with selective
117 supplement, for *L. monocytogenes* and onto tryptone soy agar, TSA for CPA-7. *S.*
118 *enterica* and *L. monocytogenes* plates were incubated at 37 °C for 24 and 48 h,
119 respectively, and CPA-7 was grown at 30 °C for 48 h. All synthetic culture media were
120 purchased from Biokar Diagnostics, Beauveais, France.

121 ### 2.3 Caco-2 cells culture conditions

122 Human intestinal Caco-2 cells (ECACC 86012202) were grown in 24-wells cell culture
123 polystyrene plates (Falcon, USA) in Dulbecco's Modified Eagle Medium (DMEM 1X,
124 Gibco, Waltham, MA, USA) supplemented with 200 mL/L inactivated fetal bovine
125 serum (Gibco, BRL) and 10 mL/L non-essential aminoacids solution as described by
126 Jankowska et al. (2008). Plates were maintained at 37 °C in a humidified incubator at
127 5% CO_2. For the experiments, cells were seeded at 5 x 10^4 cells per well and grown
128 until differentiation (12-13 days), refreshing the culture medium every two days.
129 Penicillin (20.000 U/mL) and streptomycin (20 mg/mL) were added to the culture
130 medium except for 24 h prior to virulence assays. Antibiotics and aminoacids were
131 purchased from Sigma-Aldrich, St Louis, USA.

2.4 Selection of pathogenic strains

General experimental design is shown in figure 1. Preliminary trials were performed in order to select one strain of *S. enterica* subsp. *enterica* and one strain of *L. monocytogenes* out of five strains from different serovars of each species from our laboratory collection. Susceptibility of each strain to be effectively controlled by CPA-7 on pear plugs at 10 °C and high virulence, evaluated by their ability to adhere and invade Caco-2 cells were used as selective criteria.

For the antagonist effectiveness test, bacterial suspensions with 10^5 CFU/mL of each pathogenic strain inoculated alone or in combination with the antagonist, this latter inoculated at 10^7 CFU/mL, were prepared with deionized water. In the same way, a CPA-7 control treatment was included. Pear plugs were dip-inoculated at a ratio of 1:2 (pear weight: volume of bacterial suspension) for 2 min in agitation in an orbital tabletop shaker and subsequently drained and air-dried. Samples were analyzed just after inoculation and after six days of incubation in sterile glass tubes at 10 °C, allowing air exchange. Bacterial populations were evaluated in triplicated by plate count. For this, 1 g of sample was homogenized in 9 mL of buffered peptone water (BPW, Oxoid LTD, UK) at 7 strokes/s during 2 min in a Bagmixer 100 (Minimix, Interscience, Saint Nom, France).

For virulence screening, each strain was inoculated individually in DMEM medium to achieve a final concentration of 10^5 CFU/mL. Afterwards, two rows of 24-well plates containing post-confluent differentiated Caco-2 cells were inoculated with 500 µL of each treatment. The plates were incubated for 1 h in a humidified incubator at 37 °C and 5% CO_2. Non-adhered bacterial cells were removed by two washes with phosphate buffered saline (PBS, 0.137 mol/L NaCl, 0.0027 mol/L KCl, 0.01 mol/L Na_2HPO_4, 0.0018 mol/L KH_2PO_4, pH 7.4) and Caco-2 cells from six wells/replicate were lysed with cold 1 mL/L Triton X-100 (Sigma, UK) and collected in sterile glass analysis tubes. The other six wells were incubated for 2 h in the same conditions with DMEM

159 supplemented with Gentamicin (150 µg/mL) to kill extracellular bacteria. Viable
160 adhered and internalized cells were estimated by plate count, as previously described.

161 ## 2.5 Antagonist-pathogen interaction on fresh-cut pear.

162 Prior to *in vitro* virulence assays, the selected strains *L. monocytogenes* CECT 4032
163 and *S. enterica* subsp. *enterica* CECT 4300 were submitted to the interaction with
164 CPA-7 in separate trials which included three treatments that were prepared using
165 deionized water: **i**) CECT 4032 or CECT 4300 control, **ii**) CPA-7 + CECT 4032 or
166 CECT 4300 and **iii**) CPA-7 control. In all cases, concentrations of the pathogens (10^7
167 CFU/mL) and the antagonist (10^9 CFU/mL) were two $\log_{10}$ above the amount used in
168 the selection phase, in order to detect bacterial cells at the end of the invasion process,
169 considering the expected population reduction due to antagonism. Pear slices were
170 dip-inoculated in each treatment at a ratio of 1:2 (pear weight: volume of bacterial
171 suspension) as previously described. Samples were examined on the day of
172 inoculation and after 7 days of incubation at 10 °C in 500 mL lidded-polyethylene trays,
173 not hermetically closed to allow air exchange. Populations of *S. enterica, L.*
174 *monocytogenes* and CPA-7 were determined at each sampling time following the same
175 procedure explained in section 2.2. For this, 10 g of pear of each sample was
176 previously homogenized in 90 mL BPW within a 400 mL sterile full-page filter bag
177 (Bagpage, Interscience, Saint Nom, France) in a Masticator (IUL, Barcelona, Spain) set
178 at 4.2 strokes/s for 90 s. The experiments were performed two independent times for
179 each pathogen including three replicates per treatment and sampling time. Each of
180 these replicates was analyzed in duplicate in the subsequent adhesion and invasion
181 assays.

182 ## 2.6 Adhesion to and invasion into Caco-2 cells

183 Fifty milliliters of each pear homogenate were collected at each sampling time,
184 individually centrifuged at 15777 × *g* for 20 min at 4 °C in a Sorvall Legen XTR
185 centrifuge (Thermo Scientific, USA) and re-suspended in 13 mL of DMEM medium to

be used as inocula for the adhesion and invasion assays. Afterwards, 1 mL of bacterial suspension per replicate per treatment was added to each of 12 wells containing 2 x 10^6 differentiated Caco-2 cells/well. Multiplicity of infection (MOI) was set at 0.1:1 (bacteria: Caco-2 cells) for both pathogens and at 1:1 for the antagonist control on the initial day of the experiments. The plates were incubated and analyzed following the same experimental scheme that is described in section 2.4. Inoculated, adhered and internalized bacteria were estimated by plate count as previously described.

Concomitantly, the viability of CPA-7 in the conditions assayed (37 °C in humid atmosphere with 5% CO_2), was assessed. For this, 1 mL of the CPA-7 suspension in DMEM, as mentioned above, was added in triplicate to six wells of a 24-wells plate and analyzed by plate count after 1, 2 and 3 h as described in section 2.2.

The impact of MOI in *S. enterica* and *L. monocytogenes* adhesion and invasion was evaluated using bacterial suspensions in DMEM medium of concentrations ranging from 10^2 to 10^7 CFU/mL. Then, 1 mL of each suspension was inoculated in triplicate on Caco-2 plates and adhered and internalized cells were estimated following the procedure previously described. All experiments were performed twice.

2.7 Survival and colonization abilities of CPA-7 in a simulated gastrointestinal tract

Additionally, to assess the possibility for competition between CPA-7 and the evaluated pathogens once in contact with the intestinal epithelium in a more realistic scenario, its survival under *in vitro* static gastrointestinal passage and the subsequent adhesion to and invasion into Caco-2 cells was tested. For this, pear wedges were inoculated as previously described and then submitted to simulated digestion at initial time or after seven days of storage at 10 °C in air. Gastrointestinal digestion protocol was adapted from that described by Zudaire et al. (2017) based on the standardized method of Minekus et al. (2014). Briefly, 5 g of inoculated pear wedges was placed into a sterile filter plastic bag (BagPage 80 mL, Interscience BagSystem, Saint Nom, France) with

3.5 mL of synthetic salival fluid tempered at 37 °C. The mixture was then homogenized 7 strokes/s during 2 min in a Bagmixer 100 (Minimix, Interscience, Saint Nom, France). Then, 1.5 mL of salivary enzymatic solution was added to the sample and incubated statically at 37 °C for 5 min. Afterwards, the sample was mixed with 7.5 mL of synthetic gastric fluid and 2.4 mL of gastric enzymatic solution, adjusted to pH 3 with 1 mol/L NaOH and incubated statically for 2 h at 37 °C. Then, 11 mL of synthetic intestinal fluid and 9 mL of intestinal enzymatic solution was added to each sample and incubated statically at 37 °C for 2 h. After every phase, pH was measured and 1 mL of each sample was used to estimate CPA-7 populations by plate count as previously described. All enzymes were purchased from Sigma-Aldrich, St Louis, USA.

Subsequently, adhesiveness and invasiveness of CPA-7 before and after habituation on pear wedges followed by *in vitro* gastrointestinal simulation was tested in the same experimental conditions as previously described and following the same scheme. For this, resulting cells from the gastrointestinal passage were harvested by centrifugation at 15777 × *g* for 20 min at 4 °C, were diluted in 1 mL of DMEM medium at a concentration of 10^6 CFU/g fruit and used as the inoculum that was added to the Caco-2 plates. Adhered and internalized cells were estimated by plate count on TSA as previously described. These assays were performed in triplicate, two independent times.

2.8 Expression of results and statistical analysis

Microbiological data was calculated as colony forming units per milliliter (CFU/mL) and transformed to $\log_{10}$ CFU/g fruit. The adhesion and invasion efficiencies of each pathogen were expressed as logarithmic reductions calculated as follows: $\log_{10} N_1/N_0$; where N_1 refers to the bacterial count per gram of fruit at the end of the adhesion or invasion step and N_0 refers to the initial bacterial count per gram of fruit inoculated onto the Caco-2 cells. Survival capacity of CPA-7 after each phase of gastrointestinal simulation was calculated as logarithmic reductions: $\log_{10} N_1/N_0$, where N_1 is the

bacterial count per gram of fruit at the end of the analyzed digestion phase and N_0 is the initial count per gram of fruit. All data were tested for agreement to normal distribution and homoscedasticity. The significance of the differences between factors were determined by one-way analysis of variance (ANOVA) ($P < 0.05$) and separated by using Tukey's test. All statistical analyses were performed using Statistical software JMP (version 8.0.1 SAS Institute Inc., NC, USA).

3. Results and discussion

3.1 Selection of pathogenic strains

Initial screening of the pathogenic strains of *L. monocytogenes* and *S. enterica* usually used in our laboratory was carried out to select one strain of each species for the subsequent evaluation of the effect of pathogen-antagonist interaction on pathogen´s virulence. In biological control assays, the initial populations of CPA-7 on the fruit were around 6.3 $\log_{10}$ CFU/g fruit which represents at least 2 $\log_{10}$ above each pathogen's population. Populations of *L. monocytogenes* strains were initially 3.9 $\log_{10}$ CFU/g fruit. After six days of incubation at 10 °C strains CECT 4032, CECT 940 and Lm 230/3 were reduced by more than 3 $\log_{10}$ when co-inoculated with the antagonist with respect to the control (Fig. 2A). Compared to the mentioned strains, CECT 4031 was significantly less inhibited (reduction by 2.6 $\log_{10}$) while no inhibitory effect was observed on strain CECT 933. On the other hand, initial populations of *S. enterica* isolates on fruit plugs were around 4.1 $\log_{10}$ CFU/g fruit. As observed at day six, strains BAA-711 and CECT 4300 were the most susceptible to biological control (reduced by more than 4 $\log_{10}$) (Fig. 2A).

Regarding their virulence, *L. monocytogenes* CECT 4032, CECT 933, CECT 940 and Lm 230/3 and were the most adhesive to Caco-2 cells showing less reduction of adhered cells in respect of the initial inoculum (Fig. 2B). In the same way, CECT 4032 was the most efficient in terms of invasion into epithelial cells. Among *S. enterica*

265 strains, BAA-707, BAA-710 and CECT 4300 had similar attachment efficiencies that
266 were higher than the observed for the strains BAA-709 and BAA-711 (Fig. 2B). BAA-
267 707 and CECT 4300 were also the most invasive strains. According to the obtained
268 results, *L. monocytogenes* CECT 4032 and *S. enterica* CECT 4300 isolates, which
269 showed a combination of high virulence (adherence and invasion capacities) and
270 marked susceptibility to CPA-7, were selected to investigate the antagonistic effect of
271 the CPA-7 on their colonization properties.

272 ### 3.2 Antagonist-pathogen interaction on fresh-cut pear

273 *S. enterica* CECT 4300 and *L. monocytogenes* CECT 4032 were submitted to
274 interaction with CPA-7 on pear wedges during 7 days at 10 °C in air. We selected 10
275 ºC as it has shown to be a chilling temperature which reproduces better real conditions
276 on open refrigerated exhibitors where processed fruit and vegetable are usually stored
277 in supermarkets. It is also a suitable refrigerated temperature for tracking changes in
278 the populations of *S. enterica* and *L. monocytogenes* during biological control assays
279 as it fits into the growth temperature range for both microorganisms (8 - 45 ºC and 0 -
280 45 ºC, respectively) (Khaleque and Bari, 2015).

281 At initial time, CPA-7 populations on the fruit were 7.5 $\log_{10}$ CFU/g fruit on average,
282 which represents approximately 1.8 $\log_{10}$ above the populations of the pathogens (Fig.
283 3). After seven days of co-incubation with CPA-7, *S. enterica* populations were reduced
284 by 5.5 $\log_{10}$ while *L. monocytogenes* populations were reduced by 3.1 $\log_{10}$ with
285 respect to the control. When compared altogether, control populations of both
286 pathogens reached similar numbers after the evaluated incubation period but CPA-7
287 showed significantly more effectiveness ($P < 0.0001$) at inhibiting the growth of *S.*
288 *enterica* than of *L. monocytogenes* in co-inoculated samples. Similar storage conditions
289 has been previously used in experiments that have shown the effectiveness of CPA-7
290 at reducing the populations of cocktails of *L. monocytogenes* and *S. enterica* by 3 and

291 4 $\log_{10}$ respectively, on fresh-cut melon and apple (Abadias et al., 2014; Alegre et al.,
292 2013a).

293 ### 3.3 Adhesion to and invasion into Caco-2 cells

294 We evaluated the effect of the antagonist-foodborne pathogen previous interaction in a
295 food matrix on the virulence of the pathogens, focusing on adhesion to and invasion of
296 human gastrointestinal epithelial cells because these are preliminary steps in infection.
297 Interference with the adherence and invasion of pathogens into epithelial cells has
298 previously shown to be an effective mechanism for antagonistic activity (Burkholder &
299 Bhunia, 2010; Cells, Coconnier, Lie, Lorrot, & Servin, 2000).

300 Fresh-cut pear homogenates resulting from samples inoculated individually with CPA-
301 7, *S. enterica* CECT 4300 and *L. monocytogenes* CECT 4032 or with a combination of
302 each pathogen and the antagonist were obtained at initial day and after seven days of
303 storage at 10 ºC and used as inocula for adhesion and invasion assays. The relative
304 adhesion to Caco-2 cells of *L. monocytogenes* co-inoculated with CPA-7 was similar to
305 that of the control (Fig. 4A) at both times of analysis. Adhesion of *L. monocytogenes*
306 was significantly reduced (by 0.5 $\log_{10}$) ($P < 0.001$) after habituation on the fruit matrix
307 regardless of the interaction with CPA-7. Similar storage conditions, period of
308 incubation and fruit matrix that the used in the present study have previously shown to
309 reduce the adhesion to Caco-2 cells of other strain of *L. monocytogenes* (Lm 230/3,
310 serotype 1/2a) (Colas-Meda et al., 2017b).

311 In contrast, the relative adherence of *S. enterica* was significantly reduced in presence
312 of the antagonist (by 0.8 $\log_{10}$) compared to the control treatment regardless of the
313 habituation in the fruit matrix (Fig. 4A). Similarly, the relative adhesiveness of the CPA-
314 7 control did not show to be influenced by the incubation period and it was similar to
315 that of *S. enterica*. Reduction of adhesiveness of *S. enterica* in the presence of CPA-7,
316 could be due to competition for sites on Caco-2 surfaces as CPA-7 cells were two

317 orders of magnitude more abundant than *S. enterica* cells and both microorganisms
318 had a similar efficiency of adhesion which was lower to that of *L. monocytogenes*.

319 Regarding the capacity of invasion, no significant differences could be observed for any
320 of the analyzed pathogens when exposed to CPA-7 compared to the control treatments
321 on inoculation day (Fig. 4B). Nevertheless, after 7 days, the number of non-internalized
322 cells of *L. monocytogenes* increased by 1.3 $\log_{10}$, thus the invasion efficiency of this
323 strain was significantly reduced when co-inoculated with CPA-7 compared to the
324 control. The combination of the incubation period and the interaction with the
325 antagonist resulted in the reduction of invasion of *S. enterica* since no viable
326 internalized cells could be detected after the incubation period for inocula with
327 concentrations ranging from 3 to 6 $\log_{10}$ CFU g/ fruit. However, they could be detected
328 in the control treatment using the inocula within the mentioned range of concentration
329 (about 7 $\log_{10}$) after and before the preadaptation period. On the other hand,
330 habituation on the fruit resulted in an increase of relative invasion of CPA-7 from an
331 undetectable level (detection limit: 5 CFU/g) to about 2 $\log_{10}$ CFU/g for an inoculum of
332 7.4 $\log_{10}$ CFU/g.

333 Furthermore, in the present study, the pre-incubation period in the food matrix led to a
334 variation of the population sizes of the pathogens, i.e. an increase of the control
335 populations of *S. enterica*, *L. monocytogenes* and CPA-7 and a concomitant reduction
336 of pathogen's populations (by 3 and 4 $\log_{10}$ respectively) when co-inoculated with CPA-
337 7. Thus, the population dynamics of both pathogens was related to a variation of the
338 multiplicity of infection (MOI) in the subsequent adhesion and invasion assays.

339 Analysis of MOI using dilutions of each pathogens in DMEM medium showed that
340 relative adhesion of *S. enterica* CECT 4300 was the same in a range of MOI of
341 0.0001:1 to 0.01 but it increased at an MOI of 1:1 while for *L. monocytogenes* CECT
342 4032 the adhesion increased in a different MOI range (0.001:1 < 10:1 < 0.1:1) (data not

343 shown). Increased relative adherence (by 0.6 $\log_{10}$) of CPA-7 to the monolayers
344 positively correlated to the increase of multiplicity of infection of the antagonist with
345 respect to Caco-2 cells (0.1:1 < 1:1 = 100:1).

346 As for invasive capacities, we could not observe an effect of MOI on *S. enterica*
347 internalized cells in a range of 0.0001:1 to 1:1 which correspond to the levels obtained
348 after interaction with the antagonist. An increase of MOI from 0.001:1 to 0.1:1 shown to
349 be associated to an increase of invasion of *L. monocytogenes* while it remained stable
350 at higher levels (10:1). These results agree with previous reports regarding the
351 adhesion and invasion capacities of *S. typhymurium* strain C52 to monolayers of
352 intestine cells (Int-407), which varied between certain MOI ranges (0.1:1 = 1:1 < 100:1)
353 (Kusters et al., 1993). MOIs have previously shown to influence the efficiencies of
354 infection of both *Salmonella* spp. and *Listeria* spp. at different levels according to the
355 strain and to the experimental conditions tested (Kushwaha & Muriana, 2010; Kusters,
356 Mulders-Kremers, Van Doornik, & Van der Zeijst, 1993).

357 When analyzing this factor along with the habituation in the food matrix, we observed
358 that after seven days of incubation the relative adhesiveness of the analyzed
359 pathogens when they were inoculated alone either remained stable or it was reduced,
360 in the case of *S. enterica* and *L. monocytogenes,* respectively, even when their
361 populations increased and consequently, the multiplicity of infection augmented.
362 However, the combination of these two factors do not show to have an influence in the
363 invasiveness of any of the analyzed pathogens.

364 Mechanisms for adherence of antagonists have shown to be either nonspecific, which
365 are mediated by electrostatic interactions and hydrophobic or steric forces; or specific,
366 which involve the adhesin/receptor interaction and/or the secretion of extracellular
367 proteinaceous adherence-promoting factors, as previously demonstrated in
368 experiments performed with probiotics (Muñoz-Provencio et al., 2009; Neeser et al.,

2000; Wang et al., 2014; Sribuathong et al., 2014). However, the antagonistic bacterium evaluated in the present study is not a probiotic. Although a number of experiments have been performed, we have not been able to demonstrate any production of antimicrobial metabolites in synthetic media or in fresh-cut fruit which could suggest a putative ability to exclude or displace foodborne pathogens *in situ* (Collazo et al., 2017). Furthermore, it was reported that it fails to grow at temperatures higher than 33 ºC on TSA plates (Alegre et al., 2013a). However, its viability in the conditions tested in the present study had not previously been assessed. We observed that CPA-7 was able to survive and maintain its populations in DMEM medium at 37 ºC in 5% CO_2 in humidified atmosphere for at least 3 h (data not shown). We also demonstrated that it can adhere to Caco-2 cells with higher adherence capacity according to increasing MOIs 1:1 > 0.1:1 reaching a saturation point at 100:1. This would enable it for steric disruption or competition for adhesion in the conditions assayed in this study. These results agree with previous experiments performed with isolates of *P. fluorescens* of different origins: MF37 (crude milk isolate) and MFN1032 (clinical isolate). These isolates had also been considered to be psychrotrophic and unable to grow at temperatures above 32 ºC, but showed to be able to survive and even adapt to growth at 37 ºC or above and subsequently adhere to and translocate across the cytoplasm to the basal membrane of differentiated intestinal epithelial cells (Caco-2/TC7) (Madi et al., 2010). Antagonistic strains of *Lactobacillus (L. plantarum* PD110 and *L. cellobiosus* RE 33) have also shown increasing adherence ability at inocula ranging from 10^6 to a saturation point of 2 x 10^8 CFU/mL (MOI 1:1 and 1:100, respectively) when bacterial clusters disrupted adhesion (Sribuathong et al., 2014).

The combined effect of cell-to-cell contact, food matrix microenvironment and storage conditions could have led to the modulation of the production or the functionality of the molecules involved in adhesion, invasion and multiplication of the pathogens evaluated in the intestinal epithelium. Several reports have stated the role of environmental

396 factors such as growth temperatures, osmotic stress, pH, anaerobiosis, and cell-to-cell
397 signaling prior to infection, in the regulation of virulence genes of intracellular
398 foodborne pathogens (Ivy, Chan, Bowen, Boor, & Wiedmann, 2010; Pricope-Ciolacu,
399 Nicolau, Wagner, & Rychli, 2013; Zilelidou et al., 2015). For instance, cell-to-cell
400 contact between several combinations of *L. monocytogenes* strains (e.g. Scott A
401 serovar 4b and PL25 serovar 1/2b) co-cultured in a nutrient-rich broth resembling a
402 food matrix, has previously been associated to an inhibitory effect in their growth as
403 well as in their capacity to invade and multiply within Caco-2 cells (Zilelidou et al.,
404 2015).

405 Molecular and physiological basis of virulence reduction have been associated to
406 changes in expression of stress-response genes such as *sigB* as well as of virulence
407 genes (*plcA, iap,* and *hly*) in *L. monocytogenes* of different serovars, during habituation
408 in different food matrices at refrigerated temperatures (4 ºC and 12 ºC) (Rantsiou et al.,
409 2012). Down-regulation of genes involved in the invasion process (including *actA*, *hly,*
410 *inlA,* and *plcA*) leading to differential protein expression, has also resulted in reduced
411 invasiveness in antagonist-pathogen interactions (*L. monocytogenes* CMCC54001 and
412 *Bifidobacterium longum* NCC2705) (Tan et al., 2012).

413 ### 3.4 Survival and colonization abilities of CPA-7 in a simulated gastrointestinal tract

414 In view of the positive results obtained in the adhesion and invasion assays, the
415 abilities of CPA to survive simulated human gastrointestinal passage and to
416 subsequently colonize the intestinal epithelium were evaluated. On the first day of the
417 experiments, the populations of CPA-7 inoculated onto 'Conference' pear wedges
418 remained unaltered after the oral phase but they were drastically reduced after the
419 gastric phase (by 6.5 $\log_{10}$) (Fig. 5). However, a significant population increase (by 2.7
420 $\log_{10}$) was recorded after the intestinal phase. At this sampling point, CPA-7 was able
421 to survive in acidic conditions, bile salts and corporal temperature of the host, but its
422 populations were drastically reduced after the gastric phase, concomitantly with the

423 reduction in pH from 6.4 to 2.0. Then, they recovered during the intestinal phase in
424 correlation to the rise of pH to 7.5.
425 After seven days of incubation in the fruit matrix CPA-7 initial populations were 1.8
426 log_{10} above the recorded at inoculation day. Although, this number was reduced by 0.7
427 log_{10} during the oral phase. At this sampling time, the means of the pH measured after
428 the oral, gastric and intestinal phases were 6.0, 2.9 and 8.2, respectively.
429 Concomitantly with this pH variation, populations dropped significantly (by 6.0 log_{10})
430 during the gastric phase. However, they remained stable during the intestinal phase
431 showing that even after habituation in the food matrix, surviving cells were unable to
432 grow during the subsequent phase. Regardless of the pre-incubation period,
433 populations reached similar numbers at the end of the gastrointestinal passage,
434 although logarithmic reduction after digestion with respect of the initial population was 2
435 log_{10} higher after seven days on the fruit than at inoculation day. In spite of the low
436 number of viable cells after gastrointestinal passage, the adhesion capacity of CPA-7
437 was the same regardless of the stress experienced during digestion, suggesting that
438 the cells that reach the intestinal epithelium could stablish competition for adhesion
439 sites. In previous experiments no viable cells of this antagonist could be detected after
440 simulated gastric digestion but assayed conditions (pH 2; 2 mmol/L HCl, 0.3 g/L
441 pepsin) and food matrix (fresh-cut 'Golden delicious' apple) differed from the evaluated
442 in the present work (Alegre et al., 2013b).
443 We expected the population size of *L. monocytogenes* to remain invariable after the
444 whole gastrointestinal passage and evaluated directly its ability to attach and to
445 penetrate intestinal cells, based on the observations in a similar model of static
446 gastrointestinal simulation, where bacterial counts of the strain CECT 4032 inoculated
447 on fresh-cut 'Conference' pear were maintained at the end of gastrointestinal passage,
448 after seven days of storage at 10 ºC (Iglesias et al., 2017). Similarly, populations of *L.*
449 *monocytogenes* strain 230/3 inoculated on 'Conference' pear did not change along the

passage through a simulated static gastrointestinal tract after 6 days of storage at 10 ºC in air (Colas-Meda et al., 2017a).

4. Conclusions

The data presented herein show that the interaction of the antagonist with both of the analyzed pathogens during the pre-incubation period was associated to the reduction of their virulence features. Invasiveness of *S. enterica* subsp. *enterica* ser. Enteritidis CECT 4300 and *L. monocytogenes* CECT 4032 to Caco-2 cells were reduced after seven days of co-incubation with *P. graminis* CPA-7 on fresh-cut pear at 10 ºC. This could be due to a combined effect of several processes, i) cell-contact-dependent competition established in the food matrix which could modulate the expression of genes involved in adhesion and invasion leading to the impairment by the antagonist of the pathogen's virulence traits associated with host colonization; ii) physical competition for adhesion to the epithelial membranes which is linked to the proportion of antagonist-pathogen cells and; to some extent, to the multiplicity of infection.

Further investigation should be carried out to determine the effect of the CPA-7 in the regulation of specific virulence-related genes of *L. monocytogenes* and *S. enterica* during co-incubation at different temperatures and storage conditions. To the best of our knowledge, this is the first study on the influence of the interaction of a non-probiotic antagonistic bacterium on fresh-cut fruit in the colonization abilities of human enteropathogenic bacteria.

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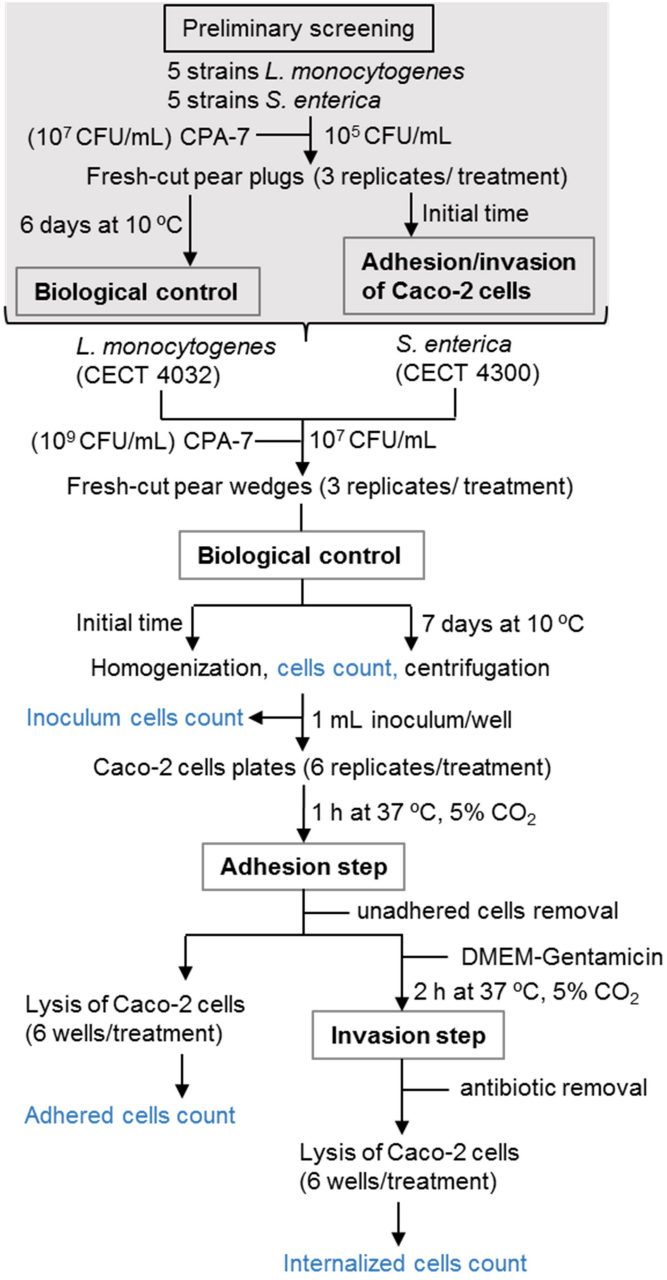
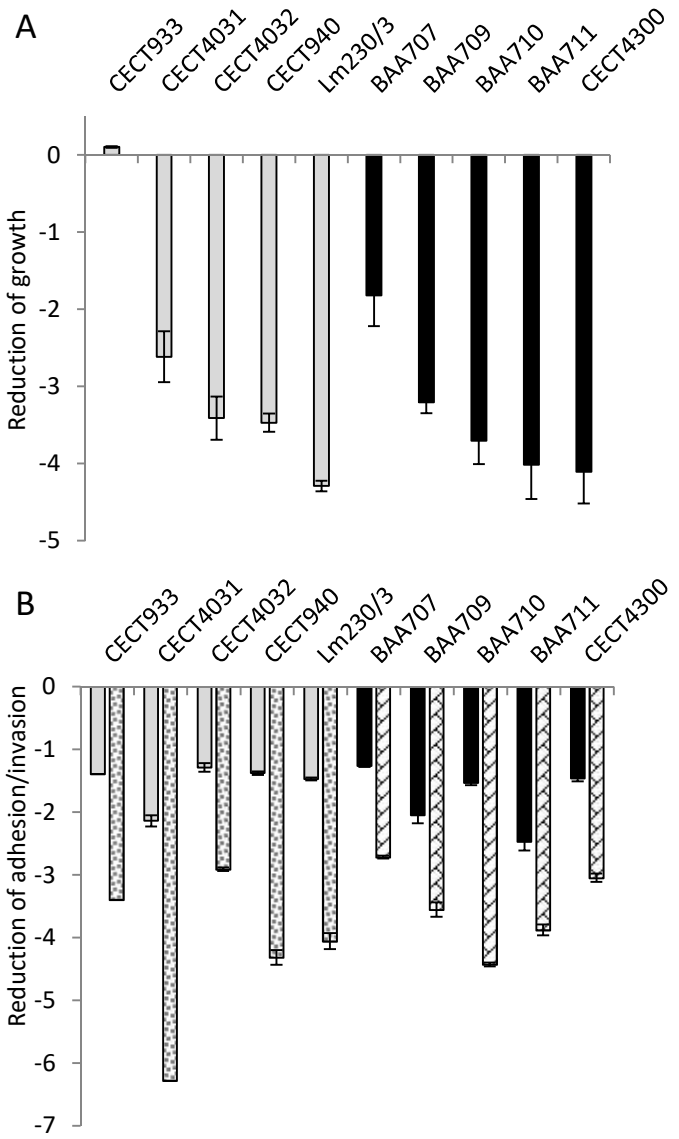
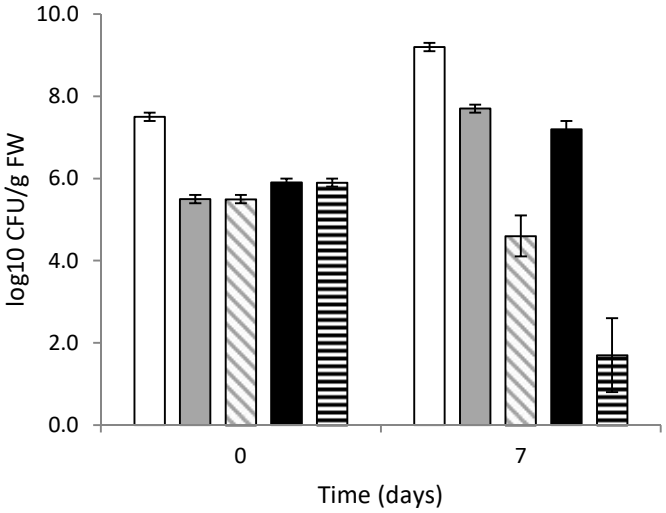


Figure 1. Schematic experimental design of biological control and adhesion/invasion assays.



615 Figure 2. Preliminary screening including several strains of L. monocytogenes and S.
616 enterica subsp. enterica. (A) Inhibition of growth of *L. monocytogenes* (□) and *S. enterica*
617 (■) when co-inoculated with *P. graminis* (CPA-7) on pear plugs after 6 d of aerobic
618 incubation at 10 °C. (B) Non-adhered L. monocytogenes cells (□) and *S. enterica* cells (■)
619 and non-internalized *L. monocytogenes* cells (⊡) and S. enterica cells (⊠) when
620 inoculated individually in DMEM 1×medium onto Caco-2 cells. Columns represent the
621 means of $\log_{10}(N_1/N_0)$, where (A) N1 is the bacterial count of the pathogen-CPA-7 co-
622 inoculated treatment at day six and N0 is the bacterial count of the pathogen control
623 treatment at this sampling point, (B) N1 is the bacterial count after the adhesion or the
624 invasion step and N0 is the bacterial count of the inoculum added to the Caco-2

monolayer. Each experiment was performed twice including three replicates per each strain. Error bars represent standard errors of the mean.



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630 Figure 3. Populations of *Pseudomonas graminis* strain CPA-7 control (□), *L.*
631 *monocytogenes* CECT 4032 (Lm) control (▩), Lm co-inoculated with CPA-7 (▨),
632 *S. enterica* subsp. *enterica* CECT 4300 (Sal) control (■) and Sal co-inoculated with CPA-7
633 (▤) in pear wedges at initial time and after seven days at 10 °C in air. Values above
634 columns are the means bacterial counts transformed to log10 CFU per gram of fresh
635 weight of fruit (FW) of three replicates from two independent assays per each pathogen.
636 Error bars represent the standard errors of the means. Different letters above columns
637 indicate significant differences among treatments analyzed separately by sampling time
638 ($P < 0.0001$) according to analysis of variances (ANOVA) and Tukey's test.

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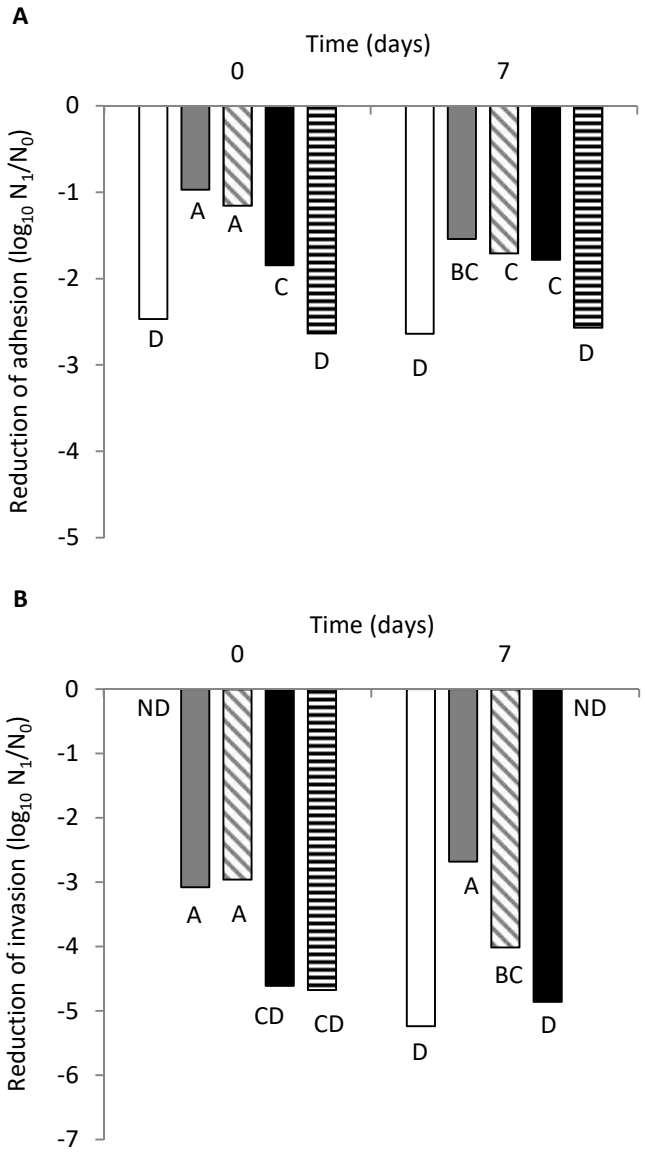

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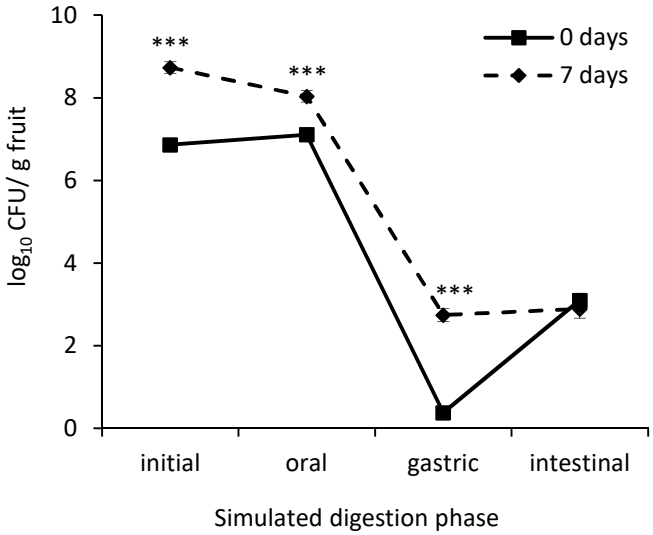
Figure 4. (A) Non—adhered and (B) non— internalized bacteria into Caco-2 cells after the invasion step performed at initial time and after seven days of aerobic incubation on pear slices at 10 °C. Columns represent (□) *P. graminis* CPA-7, (■) *L. monocytogenes* CECT 4032 control, (▨) CECT 4032 co-inoculated with CPA-7, (■) *S. enterica* subsp. *enterica* CECT 4300 control and (▤) CECT 4300 co-inoculated with CPA-7. Results are represented as means of logarithmic reductions of viable counts of internalized cells (N_1) per gram of fresh fruit in relation to the inoculum added to Caco-2 cells monolayers (N_0), from two independent trials per each microorganism. Error bars represent standard error of the mean (n = 12). ND, below detection limit of 5 CFU/g fruit. Asterisks indicate means which are significantly different compared by analysis of variance (ANOVA) and separated by Tukey's test ($P < 0.05$).



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643 Fig. 5. *Pseudomonas graminis* strain CPA-7 population dynamics during *in vitro* static
644 gastrointestinal simulation at inoculation day (continuous line) and after seven days
645 (dotted line) of storage on fresh-cut 'Conference' pear at 10 °C in air. Symbols
646 represent means and error bars represent standard errors of the mean (n = 6).
647 Asterisks represent significant differences among different phases at each sampling
648 time according to analysis of variances ANOVA and Tukey's test ($P < 0.0001$).

649