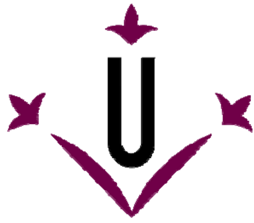

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1 **Studies on the biocontrol mechanisms of *Pseudomonas graminis* strain CPA-7**
2 **against food-borne pathogens *in vitro* and on fresh-cut melon.**

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11 ## Abstract
12 The present study was aimed at gaining insight into the mode of action of the
13 antagonistic bacteria *Pseudomonas graminis* CPA-7, which has been previously
14 identified as an effective biocontrol agent against *Listeria monocytogenes*, *Salmonella*
15 *enterica* and *Escherichia coli* O157:H7 on fresh-cut fruit. *In vitro* experiments did not
16 reveal any antimicrobial or proteolytic activity on solid media or any biosurfactant
17 activity on hydrophobic surfaces. Metabolites produced by CPA-7 in two different
18 culture media and on 'Galia' melon were unable to inhibit *L. monocytogenes*
19 populations on 'Galia' melon plugs at 25 °C or 5 °C. In contrast, at 25 °C the population
20 of this pathogen on 'Galia' plugs was reduced by 2.1 and 3.3 log-units when co-
21 inoculated with the antagonist in water, after 24 and 48 h, respectively. CPA-7 did not
22 form biofilms after 72 h at 25 °C (OD=0.03) or at 30 °C (OD=0.01) on polystyrene
23 plates and the production of alginate was close to the negative control. Studies of
24 nutritional profiles showed high overlap (NOI>0.9) between CPA-7 and *E. coli* O157:H7
25 regarding the use of carboxylic acids. This functional group could also contain putative

26 targets for competiveness between CPA-7 and *S. enterica*, although overlapping was
27 not restrictive enough (NOI=0.83).

28 **Keywords**
29 Biological control, action mechanism, antagonist, fresh-cut melon

30 ## Introduction

31 Biological control using native microbiota from fruits and vegetables surfaces is a
32 method that matches the ‘natural’ and ‘additive free’ criteria, which can aid in reducing
33 the risk of contracting food-borne diseases by pre-emptive exclusion of the pathogens
34 involved. As these microorganisms have the advantage of being in their natural
35 environment, they can easily colonise the food and inhibit pathogen growth when their
36 populations are high enough (Belak & Maraz, 2015). In particular, diverse
37 *Pseudomonas* spp. strains have the capacity to inhibit or suppress plant diseases and
38 foodborne illnesses usually by a combined strategy that includes the production of
39 antimicrobial compounds (Hernández-León *et al.*, 2015), siderophores (Solanki et al.,
40 2014) or hydrolytic enzymes, competition for nutrients (Saraoui et al., 2015), rapid
41 growth and formation of biofilms (Blumenstein et al., 2015) and elicitation of resistance
42 pathways in the host (Wang et al., 2015).

43 *Pseudomonas graminis* CPA-7 is a native strain from whole apple surfaces which
44 inhibits the growth of *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria*
45 *innocua* on fresh-cut fruits (Alegre, Viñas, Usall, Teixido, et al., 2013; Alegre, Viñas,
46 Usall, Anguera, et al., 2013; Abadias et al., 2014) . However, its effectiveness varies
47 depending on the pathogen, preservation conditions and food matrix. The present
48 study was aimed at gaining insight into the mode of action of *P. graminis* (CPA-7) for
49 controlling the growth of *Listeria monocytogenes, E. coli* O157:H7 and *S. enterica*
50 subsp. *enterica.*

51 ## 2. Materials and methods

52 ### *2.1 Fruits.*

53 Different batches of melon (*Cucumis melo* L.) of three cultivars: 'Piel de sapo', 54 'Cantaloupe' and 'Galia' were purchased from local markets (Lleida, Spain). Fruits were 55 washed with running tap water and surface-disinfected with 700 mL/L ethanol. Flesh 56 plugs of approximately 1.2 cm of diameter and 1 cm thickness were cut with a cork 57 borer. 'Piel de sapo' melon was also used to prepare fresh juice by crushing peeled 58 fruit pieces in a blender. Juice was filtered using gauze pads, bottled and autoclaved at 59 90 °C for 10 min. Fruit quality parameters were determined before each experiment as 60 described by Colas-Meda et al. (2017).

61 *2.2 Bacterial strains.*

62 Bacterial strains used in this study and their intended purpose in each section are listed 63 in Table 1. Culture conditions for each microorganism are described in each section.

64 *2.3 Biofilm formation.*

65 Biofilm formation was assessed by a microtiter plate assay (O'Toole & Kolter, 1998). 66 Briefly, microorganisms (Table 1) were cultured overnight in agitation as follows: CPA-7 67 and *P. fluorescens*, in tryptone soy broth (TSB, Biokar-Diagnostics, Beauvais, France) 68 at 25 °C, *S. enterica* and *E. coli* in TSB at 37 °C and *L. monocytogenes*, in TSBYE 69 (TSB supplemented with 6 g/L yeast extract) at 37 °C. One microlitre of culture of each 70 microorganism was added to 500 µL of M63 minimal medium (Pardee, Jacob, & 71 Monod, 1959) (supplemented with 2 g/L glucose; 5 g/L casaminoacids) in order to 72 obtain inocula that were transferred (100 µL/well) to 96-well, polystyrene microplates 73 (Grainier Bio-one, NC, USA). Two plates were inoculated: one with CPA-7 and *P.* 74 *fluorescens* which was incubated at 25 °C and the other with *L. monocytogenes, S.* 75 *enterica, E. coli* and CPA-7, which was incubated at 30 °C. Fresh M63 medium was 76 included as negative control in both plates, which were stored statically during 72 h in a 77 closed container with a moistened filter paper to maintain humid atmosphere. Optical 78 density (OD) was measured at 570 and 750 nm (Epoch Microplate Spectrophotometer,

79 Biotek-Instruments, Winooski, USA). The experiment was performed twice and
80 included six replicates per microorganism.

81 *2.4 Alginate production.*

82 Production of alginate by CPA-7 was evaluated using supernatants from overnight
83 cultures in TSB at 25 °C. Alginate detection was carried out using the Periodic acid
84 Schiffs (PAS) procedure adapted to 96-well microplate format (Houghton et al., 2014).
85 Cell-free supernatants, cell pellets and fresh medium were subjected to hydrolysis with
86 alginate-lyase (0.05 mg/mL) (Sigma-Aldrich, St Louis, USA) for 2 h at 30 °C.
87 Quantification of the exopolysaccharides that were present in the samples before and
88 after hydrolysis was carried out by interpolating OD_{550nm} values in a sodium alginate
89 (PubChem CID: 5102882) (Sigma-Aldrich, St Louis, USA) standard curve. Three
90 independent assays including three replicates of each sample were performed.

91 *2.5 Proteolytic, hemolytic and biosurfactant activities.*

92 Casein proteolysis was evaluated on plate count agar (PCA, Biokar-Diagnostics,
93 Beauvais, France) containing 0.1 g/mL skim milk powder (Vanderzant & Splittstoesser,
94 1992). After 72 h at 25 °C, the plates were flooded with 1 mol/L HCl. Hemolysis was
95 tested on Columbia blood agar plates (Bioser, Barcelona, Spain) during 48 h. CPA-7
96 and the negative control (Table 1) were incubated at 30 °C and the positive control was
97 incubated at 37 °C. Biosurfactants production was assessed by checking the collapse
98 of a supernatant droplet previously stained with methylene blue (PubChem CID: 6099)
99 on parafilm (Sigma-Aldrich, St Louis, USA) (Kuiper et al., 2004). Supernatants were
100 obtained after centrifugation (10,976 × *g* for 15 min at 10 °C) of 72 h cultures of each
101 microorganism (Table 1) grown in King´s B medium. All experiments were performed
102 three times and included three replicates of each microorganism.

103 *2.6 Antimicrobial activity on solid media.*

104 The production of soluble antimicrobial substances by CPA-7 in two different culture
105 media at different stages of growth was evaluated by the agar spot inoculation method

106 (Alegre et al., 2013b) with some modifications. To obtain cell-free supernatants (CFS),
107 CPA-7 was grown in TSB and in M63 minimal medium (pH=6.5) at 25 °C.
108 Supernatants were collected by centrifugation (10,976 × *g* for 15 min at 10 °C) at 24,
109 48 and 72 h and filtered through sterile, 0.2 µm-pore, nitrocellulose membranes
110 (Millipore, County Cork, Ireland). Agar plates were overlaid with soft agar media (7 g/L
111 agar) that were inoculated with the correspondent pathogen strain (10^5 CFU/mL) (Table
112 1). Plates were marked into quadrants and 10 µL of **i**) CFS, **ii**) antagonist cell
113 suspension (10^8 CFU/mL), **iii**) fresh culture medium and **iv**) streptomycin (100 µg/mL)
114 were spotted onto the sections and allowed to soak into the agar for 2 h at 5 °C. Then,
115 the plates were incubated for 72 h at 30 °C and checked for hyaline zones. Two
116 independent assays to test the supernatants of each culture media at each sampling
117 time were conducted, including three replicates per each pathogen.

118 *2.7 Inhibitory effect in melon juice.*

119 Cultivar (cv) 'Piel de sapo' melon juice was used to test the effect of any inhibitory
120 soluble substance produced by CPA-7 in TSB medium until the stationary growth
121 phase on *L. monocytogenes* (*Lm*) and *S. enterica* (*Sal*) growth in a liquid matrix
122 containing fruit components. Aliquots of a mixture containing 4 mL of melon juice and 1
123 mL of either CPA-7 CFS (prepared as described in section 2.6) or water were
124 transferred to sterile glass tubes. Inocula of the antagonist and the pathogens were
125 obtained as described by Abadias et al. (2014) and added to the water-juice or the
126 CFS-juice mixtures as follows: **i)** *Lm* + *Sal* (10^5 CFU/mL each), **ii)** *Lm* + *Sal* (10^5
127 CFU/mL each) + CPA-7 (10^7 CFU/mL), **iii)** CPA-7 (10^7 CFU/mL). Bacterial populations
128 were estimated initially and after five days of incubation at 5 °C or 10 °C by plate count
129 onto the appropriate media as described by Abadias et al. (2014) except that CPA-7
130 was grown in TSA. The experiment was carried out twice and included three replicates
131 per treatment and sampling time.

132 *2.8 Inhibitory effect on melon plugs.*

133 The antagonistic effect of CPA-7 CFS from overnight TSB-cultures against a cocktail of
134 *L. monocytogenes* (*Lm*) (Table 1) was tested on melon-flesh plugs of three different
135 cultivars. Twelve melon plugs were dipped for 2 min at 185 rpm in 25 mL of water or
136 CFS inoculated with *Lm* (10^5 CFU/mL) or with both *Lm* (10^5 CFU/mL) and CPA-7 (10^7
137 CFU/mL). A control of CPA-7 (10^7 CFU/mL) in water was also included. Samples were
138 placed in 10 ml sterile glass tubes and analysed just after inoculation and after
139 incubation for 24 or 48 h at 25 °C or for five days at 5 °C. Microbial populations were
140 estimated by plate count in three replicate fruit plugs of each treatment at each
141 sampling time as explained in the previous section. The experiment was carried out
142 two independent times.

143 Moreover, the effect of the metabolites produced by CPA-7 in 'Galia' melon during 24
144 and 48 h at 25 °C in air, was also tested against *L. monocytogenes (Lm)* on 'Galia'
145 melon plugs. For this, 25 g of the plugs was dip-inoculated in a suspension of CPA-7
146 (10^7 CFU/mL) in deionised water, drained, air-dried and homogenised with 75 mL of
147 deionised water to obtain CFS as previously described. The resultant 24 and 48 h
148 CPA-7 CFS, along with a filtered supernatant obtained from non-inoculated 'Galia'
149 plugs, were inoculated with *Lm* as described above and used to immerse 'Galia' plugs.
150 Water treatments inoculated with **i)** *Lm,* **ii)** *Lm* + CPA-7 and **iii)** CPA-7 at the same
151 concentrations used in the previous experiment were included to corroborate the
152 antagonistic activity of CPA-7. Samples were stored and analysed by plate count at the
153 same sampling times and incubation temperatures of the previous experiment. The
154 experiment was carried out twice and it included three replicate fruit plugs per
155 treatment and sampling time.

156 *2.9 Nutritional similarity.*

157 Individual nutritional profiles based upon single substrate utilisation were defined for
158 Gram-negative and Gram-positive bacteria (Table 1) using GN2 and GP2 MicroLog

159 System, respectively, according to the manufacturer's instructions (Biolog, Hayward,
160 USA). Briefly, colonies of each microorganism, grown in BUG-B (5% sheep blood)-
161 AGAR (Biolog, Hayward, USA) at 30 °C, were re-suspended in GN/GP inoculation fluid
162 (10^7 CFU/mL) and transferred to 96-well microplates (150 µL/well) which were
163 incubated statically for 96 h at 30 °C. Transformation of tetrazolium chloride to purple-
164 coloured Formazan was visually recorded and analysed as presence/absence data
165 every 24 h for four days. Each strain was tested in duplicate in independent assays.

166 Nutritional similarity between CPA-7 and the pathogens was estimated by the niche
167 overlap index (NOI) = number of compounds used by both the antagonist and the
168 pathogen/number of compounds used by the pathogen (Ji & Wilson, 2002). NOIs were
169 calculated for the 95 sources that were tested and for specific groups: carbohydrates,
170 carboxylic acids, amino acids, polymers, amines/amides and miscellaneous (Dobranic
171 & Zak, 1999). Niche size (NS) was calculated for each microorganism: NS = number of
172 compounds used/95 and NS = number of compounds used/subtotals of each functional
173 group that has been previously mentioned.

174 *2.10 Statistical analysis*

175 Microbiological results measured as CFU/mL were transformed to log_{10}CFU/g fruit.
176 Population increase of the pathogen was calculated for each treatment: log_{10}CFU/g
177 fruit at a specific sampling time - log_{10}CFU/g fruit at initial time, and expressed as mean
178 ± standard error of the mean. In the biofilm formation experiment the mean values of
179 OD_{570nm} - OD_{750nm} were calculated. OD results are expressed as mean ± standard error
180 of the mean. Statistical analyses were performed using the Statistical software JMP
181 (version 8.0.1 SAS Institute Inc., NC, USA). All data were verified for normal
182 distribution and homoscedasticity and accordingly, compared by using analysis of
183 variance (ANOVA) or by Welch's test and separated by Tukey's test (P < 0.05).

184 **3. Results and discussion**

185 *3.1 Biofilm formation.*

186 Production of biofilms by CPA-7 after 72 h at 25 °C (0.03 ± 0.01) or at 30 °C (0.012 ±
187 0.004) was close to the negative control (0.012 ± 0.003). These results differed
188 significantly ($P < 0.05$) from the positive control *P. fluorescens* (0.48 ± 0.04) and *L.*
189 *monocytogenes* (0.14 ± 0.03). Adhesiveness of *S. enterica* var. *enterica* CECT 4300
190 was similar (0.03 ± 0.01) to the negative control (0.012 ± 0.004) while *E. coli* O157:H7
191 was significantly higher (0.048 ± 0.004). Minimal medium M63 supplemented with
192 glucose and casaminoacids was selected for this test based on previous results, when
193 it promoted the formation of biofilms by Pseudomonads such as *P. fluorescens*
194 WCS365 on plastic surfaces (O'Toole & Kolter, 1998). However, it was not able to
195 induce the production of significant amounts of biofilm by CPA-7 as it did for *L.*
196 *monocytogenes*. The adaptive advantage of CPA-7, with respect to the pathogens
197 tested, cannot be explained by its adherence capacity under the conditions examined
198 although under different conditions, biofilm formation could be possible (Ude, Arnold,
199 Moon, Timms-Wilson, & Spiers, 2006).

200 *3.2 Alginate production.*

201 To assess the potential capacity of CPA-7 to adhere other surfaces, the production of
202 alginate, reported as a matrix biofilm component of Pseudomonads such as *P.*
203 *aeruginosa, P. fluorescens, P. putida, P. syringae P. mendocina* and *P. extremaustralis*
204 (Govan, Fyfe, & Jarman, 1981; Raiger Iustman et al., 2014), was also quantified.
205 However, the amounts of exopolysaccharides present in fresh culture medium (0.3 ±
206 0.2 mg/mL), cell-free supernatants (0.2 ± 0.2 mg/mL) and cell pellets (0.2 ± 0.1 mg/mL)
207 were not significantly different. Likewise, they were similar to those obtained after
208 hydrolysis with alginate lyase (0.4 ± 0.2 mg/mL, 0.2 ± 0.2 mg/ml, 0.3 ± 0.3 mg/mL,
209 respectively) suggesting that any of the polysaccharides detected was alginate.

210 *3.3 Proteolytic, hemolytic and biosurfactant activities.*

211 Proteolytic activity was observed as translucent haloes of 5 mm width around the
212 bacterial growth of *P. fluorescens* in PCA medium supplemented with skim milk, but no

213 halo was obtained for CPA-7 (Fig. 1A). Translucent haloes, that are characteristic of
214 complete hemolysis, were not produced in Columbia blood agar plates by any of the
215 microorganisms tested. A greenish-coloured halo indicating incomplete hemolysis was
216 observed surrounding the positive control growth and no halo was produced by CPA-7
217 or *L. innocua* (data not shown).

218 Additionally, production of biosurfactants was explored as they have shown microbial
219 inhibitory activity and they can influence biofilm formation (Kuiper et al., 2004). No
220 reduction of the surface tension of King's B medium, suggesting biosurfactant activity,
221 was detected for CPA-7, whereas supernatant of *P. aeruginosa* resulted in a collapsed
222 droplet (Fig. 1B).

223 *3.4 Antimicrobial activity on solid media.*

224 No inhibition zone indicating antimicrobial activity on any of the pathogens tested was
225 observed for either the CFS or CPA-7 cells, irrespective of the medium (TSB or M63)
226 or the time of incubation tested (24, 48, 72 h) (data not shown). This contrasted with
227 the hyaline zone observed around the antibiotic spot (Fig. 2). Overnight growth of CPA-
228 7 in M63 medium was too low, thus it was not included in the study. In previous studies
229 of overnight cultures of CPA-7 in TSB medium, no antimicrobial activity against *E. coli*,
230 *S. enterica or L. monocytogenes* was detected (Alegre, et al., 2013b). In the present
231 study, we extended the analysis to different stages of growth in the same rich medium
232 and in a minimal medium because it has been reported that production of antibiotics by
233 *Pseudomonas* spp. can be regulated by the presence or limitation of certain carbon
234 and nitrogen sources and may differ according to the growth stage (He, Xu, & Zhang,
235 2008; Kremmydas, Tampakaki, & Georgakopoulos, 2013). Growth conditions may
236 influence the production by *Pseudomonas* spp. of certain groups of antibiotics, such as
237 phenazines, pyoluteorin, pyrrolnitrin and 2,4 diacetylphloroglucinol, as well as cyclic
238 lipopeptides and rhamnolipids (Hernández-León et al., 2015; Tupe et al., 2015;
239 Ramette et al., 2011; Raaijmakers, de Bruijn, & de Kock, 2006). For instance, glucose

240 repressed pyrrolnitrin synthesis and induced the production of phenazines in *P.*
241 *chlororaphis* at higher levels during the stationary phase (Park et al., 2011). As the
242 CPA-7 CFS obtained from any of the assayed liquid media did not show lethal effects
243 on the pathogens on solid media, a more sensitive method was used which entailed
244 monitoring the effect of the CFS in the progression of the pathogen´s populations in
245 fruit matrices.

246 ### *3.5 Inhibitory effect in melon juice.*

247 The effect of the secondary metabolites produced by CPA-7 in TSB medium on the
248 growth of *L. monocytogenes* or *S. enterica* in cv 'Piel de sapo' melon juice was
249 compared with the direct pathogen-antagonist interaction in this matrix, at 5 °C and 10
250 °C. Initial populations of CPA-7 and *S. enterica* were 8.0 ± 0.3 log_{10}CFU/mL and 5.8 ±
251 0.3 log_{10}CFU/mL, respectively. After five days of incubation at 5°C, the counts of *S.*
252 *enterica* remained invariable in the four treatments tested. When incubated at 10 °C, *S.*
253 *enterica* populations increased by an average of 2.4 ± 0.6 log-units in all treatments.
254 No significant differences were observed between pathogen´s populations in the
255 supernatant-treated and in the water control treatments.

256 With respect to *L. monocytogenes*, the initial count was about 5.4 ± 0.2 log_{10}CFU/mL in
257 all treatments. After 5 days of incubation, it increased by 1.2 and 2.3 log-units in the
258 treatments stored at 5 °C and at 10 °C, respectively. *L. monocytogenes* populations
259 were not reduced when inoculated in the supernatant-juice mix compared to the control
260 juice. In the same way, no inhibition of *L. monocytogenes* growth was observed when
261 co-inoculated with the antagonist cells in the supernatant-juice or in the water control at
262 any of the incubation temperatures assayed.

263 These results showed that CPA-7 was not effective for controlling the populations of *S.*
264 *enterica* or *L. monocytogenes* in melon juice, regardless of the temperature of
265 incubation analysed, even when inoculated two orders of magnitude above the
266 pathogens, which has been previously observed as an effective proportion for

267 biocontrol on apples slices at 5 °C and 20 °C and on melon plugs at 10 °C and 20 °C
268 (Alegre et al., 2013a; Abadias et al., 2014). Furthermore, metabolites produced by
269 CPA-7 in cv ‘Piel de sapo’ melon juice did not show any reduction of *L. monocytogenes*
270 or *S. enterica* populations in the conditions that were tested (this including the effect of
271 the antagonist cells) indicating that an alternative mechanism must be responsible for
272 the inhibitory effect observed in the previous experiments performed at 10 °C (Abadias
273 et al., 2014). The fluidity of this matrix, although its composition is similar to that of fruit
274 pieces, implies that local changes in the concentration of microbial growth and the
275 accumulation of final metabolic products cannot occur, avoiding competition between
276 bacterial species resulting from a near spatial distribution (Wilson et al., 2002). The
277 antagonistic activity of Pseudomonads such as *P. syringe* and *P. fluorescens* against *L.*
278 *monocytogenes* and *S. enterica* has been previously reported to be hampered in fruit
279 juices (Pacetto, Bella, Catara, La Rosa, & Cirvilleri, 2003).

280 *3.6 Inhibitory effect on melon plugs.*

281 The effect of CPA-7 CFS obtained from cultures in TSB to control *L. monocytogenes*
282 populations was tested at the optimal growth temperature for CPA-7 (25 °C) and under
283 refrigerated conditions (5 °C) in three different melon cultivars and the results were
284 compared to the effectiveness of the antagonist cells.

285 In the experiment carried out at 25 °C, the initial counts of CPA-7 on melon plugs of
286 cultivars ‘Cantaloupe’, ‘Galia’ and ‘Piel de sapo’ were 5.8 ± 0.1, 5.8 ± 0.2 and 6.0 ± 0.3
287 log_{10}CFU/g fruit, respectively (data not shown). After 24h of incubation, control
288 populations of CPA-7 increased by approximately 4 orders of magnitude in all cultivars.
289 CPA-7 TSB-CFS had no inhibitory effect ($P < 0.05$) on *L. monocytogenes* populations
290 in any of the melon cultivars that were tested, after 24 or 48 h of incubation (Table 2).

291 In cv ‘Galia’ melon, co-inoculation of *L. monocytogenes* with CPA-7 in water resulted in
292 a reduction of the pathogen’s growth by 2.4 and 3.8 log-units with respect to the water
293 control, after 24 and 48 h of incubation, respectively. In cv ‘Piel de sapo’ melon, co-

294 inoculation with CPA-7 in water or in CFS, did not result in a significant reduction of *L.*
295 *monocytogenes* populations, which differs from previous results obtained in similar
296 experiments performed at 20 °C (Abadias et al., 2014). This could be related with the
297 higher incubation temperature used in the present study which can also favour an
298 increase of the growth rate of the pathogen and other mesophyles present on the
299 samples. On cv 'Cantaloupe' melon, *L. monocytogenes* population growth declined by
300 1.8 log-units when co-inoculated with CPA-7 compared to the pathogen inoculated
301 alone, in the water-treated samples at 48 h post-inoculation. However, no significant
302 reduction was observed at 24 h. Furthermore, when the 'Cantaloupe' plugs were
303 treated with TSB-CFS, in the presence of the antagonist cells *L. monocytogenes*
304 growth was reduced by 1.1 log-units at 24 and at 48 h, compared to the control.

305 At 5 °C, *L. monocytogenes* populations were not significantly reduced when inoculated
306 in CPA-7 TSB-CFS compared to the control inoculated in water in any of the three
307 melon cultivars (Table 3). On plugs of cv 'Cantaloupe', *L. monocytogenes* populations
308 increased more on the fruit treated with the CFS than on the control treatment. A
309 favourable combination of nutrients of the culture medium and the matrix could
310 promote the pathogen's growth, but on the other hand it also enhanced the
311 antagonistic activity of CPA-7. A general trend of reduction of *L. monocytogenes*
312 populations was observed in the samples treated with CPA-7 cells in water compared
313 with the treatments where the pathogen was inoculated alone in water or in TSB-CFS.
314 However, no significant reduction could be determined on cv 'Piel de sapo' or cv
315 'Galia'. The loss of efficacy of the biocontrol exerted by the antagonist could be related
316 to the variability of the composition and the structure of the matrix with the ripening
317 stage. During this process, the breakdown of the cell wall changes its texture,
318 increasing the fluidisation and modifying the compounds that accumulate in the
319 microenvironment (Wilson et al., 2002). Furthermore, the proportion of sugars also
320 changes (i.e. increase of sucrose and reduction of glucose and fructose) which could

321 also diminish the possibility of competition, probably as a result of a lesser efficiency in
322 using the available Carbon sources (Villanueva, Tenorio, Esteban & Mendoza, 2004).

323 As CPA-7 reduced *L. monocytogenes* growth in cv 'Galia', the CFS obtained from
324 cultures of the antagonist that was grown for 24 and 48 h in this matrix stored at 25 °C,
325 were used to dip-inoculate 'Galia' plugs with this pathogen to determine whether the
326 inhibitory effect was associated with the production of antimicrobial metabolites. The
327 population increase of *L. monocytogenes* on 'Galia' plugs treated with the CPA-7-24 h
328 or 48 h-CFS was similar ($P < 0.05$) to plugs treated with the supernatant obtained from
329 non-inoculated melon, regardless of the temperature of incubation and the sampling
330 time analysed (Table 4). These results indicate that the compounds produced as a
331 result of the metabolic activity of CPA-7 in the 'Galia' melon were not responsible for
332 the reduction of growth of *L. monocytogenes* observed in this matrix in the conditions
333 tested. This suggests that it could be associated with the competition for nutrients
334 and/or rapid replication of the antagonist during the logarithmic phase of growth.

335 *3.7 Nutritional similarity.*

336 GN2/GP2 microplates, initially developed for identification of Gram-negative bacteria,
337 have shown to be useful for exploring interactions and functional diversity of microbial
338 communities (Horemans, Smolders, & Springael, 2013) and for approaching to niche
339 overlapping in biological control interactions (Janisiewicz, 1996). In this study,
340 nutritional niche size that was calculated including the 95 carbon/nitrogen sources, was
341 similar for all of the Gram-negative microorganisms tested (Table 5). However, the
342 analysis of specific functional groups showed differences among them. For instance, *E.*
343 *coli* used 31% more carbohydrates than CPA-7 of the 26 tested. Similar results were
344 obtained when nutritional overlap indexes (NOI) were calculated for all sources,
345 showing a high level of coexistence between CPA-7 and the pathogens tested.
346 However, NOIs calculated for specific functional groups allowed a better approach to

347 competitive relations at an ecologically-significant resource dimension. Carbohydrates
348 utilisation (adonitol, L-arabinose, D-arabitol, L-fucose, D-galactose, α-D-glucose, m-
349 inositol, D-mannitol, D-mannose, D-sorbitol) showed the lowest overlapping between
350 the antagonist and the pathogens of all groups of compounds analysed (Table 5). From
351 the major soluble sugars (sucrose, glucose and fructose) present in melon, pear and
352 apple, which are some of the fruits where CPA-7 has shown to be effective against the
353 pathogens tested, this antagonist was able to use only glucose (Wu, Gao, Zhao, Liao,
354 & Chen, 2007; Beaulieu, Lea, & Eggleston, 2003). This could be due to a high level of
355 specialisation and it could represent an advantage to effectively use this compound in
356 the fruit matrix as *E. coli* and *S. enterica* also oxidised it and were not able to
357 metabolise sucrose.

358 Carboxylic acids were the carbon sources that were most used by CPA-7 (64%) in
359 relation to the total tested while for both strains of *S. enterica* and for *E. coli* its use was
360 close to 50%. Although the highest level of coincidence among the microorganisms
361 analysed was observed for the polymers (NOI=1.00), only dextrine from the five tested
362 is present in food matrices and it is not naturally present in fruits. Thus, at a biologically
363 significant level, carboxylic acids are probable targets for competiveness between *E.*
364 *coli* O157:H7 and the antagonist, as NOI was above 0.9 (Blumenstein *et al.*, 2015).
365 CPA-7 and both strains of *S. enterica* also matched in the use of this group of
366 compounds although the level of overlapping was not restrictive enough (NOI=0.83).
367 From the 25 organic acids tested, acetic acid, cis-aconitic acid, citric acid, D-
368 galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, D,L-lactic
369 acid, propionic acid, D-saccharic acid, succinic acid and bromosuccinic acid, could be
370 used by the four microorganisms, while α-keto glutaric acid was only used by CPA-7
371 and *E. coli*. It is noteworthy that the shared use of citric and succinic acids, which are
372 predominant organic acids in melons, suggests suitable targets for competition
373 between the antagonist and the three pathogens (Beaulieu et al., 2003).

374 The use of amino acids by all the microorganisms analysed was around 55% of the 20
375 that were tested. However, overlapping between the antagonist and the three
376 pathogens evaluated was close to 0.7. L-proline, D-alanine, L-alanine, L-alanyl-glycine,
377 L-asparagine, L-aspartic acid and L- glutamic acid were used by all of the
378 microorganisms tested. L-pyroglutamic acid was metabolised by both CPA-7 and *S.*
379 *enterica* CECT 711 while L-histidine was used by CPA-7 and both strains of *S.*
380 *enterica*. All of these aminoacids except for L-pyroglutamic acid, have been identified in
381 both cv 'Cantaloupe' and cv 'Galia', from which glutamic acid, aspartic acid and alanine
382 are the most predominant in both of them, but they are almost to times more abundant
383 in cv 'Cantaloupe' than in cv 'Galia' (Lamikanra, Chen, Banks, & Hunter, 2000).

384 The GP2 MicroLog System was used to evaluate the use of 95 carbon and nitrogen
385 sources by two strains of *L. monocytogenes* (Table 1). After 96 h of incubation, a
386 change of colour was only detected for carbohydrates, 10 of which (arbutin, D-
387 cellobiose, D-frutose, D-glucose, N-acetyl-D-glucosamine, D-mannose, D-ribose, D-
388 trehalose, turanose, D-xylose) were transformed by strain 230 and 8 of them (the
389 above-mentioned, except for N-acetyl-D-glucosamine and D-fructose) by strain
390 CECT940. From these compounds, only α-D-glucose and L-mannose were oxidized by
391 both *L. monocytogenes* strains as well as by CPA-7 and could represent target for
392 competiveness in fresh-cut fruit. This narrow use of Carbon and Nitrogen sources by
393 both strains of *L. monocytogenes* could indicate that the technology used is not
394 suitable for the analysis of these strains, although it has been used in comparative
395 studies including several variants of other strains of *L. monocytogenes* (Kastbjerg,
396 Hein-Kristensen, & Gram, 2014). Similar technologies with a broader spectrum of
397 carbon compounds (i. e. Phenotype microarrays, Biolog MicroArray^{TM}), has shown to
398 be useful for selecting highly competitive potential biocontrol agents (Blumenstein et
399 al., 2015; Fox, Leonard, & Jordan, 2011) and could be suitable to further analyse the *L.*
400 *monocytogenes/*CPA-7 niche overlap.

401 ## 4. Final remarks

402 Although the number of putative mechanisms involved in the antagonistic activity of
403 CPA-7 that have been explored in this study, we were not be able to clarify its mode of
404 action. We could not demonstrate inhibition of growth of the foodborne pathogens
405 tested in agar plates, fruit juices or fruit pieces, using the supernatants from CPA-7
406 monocultures or the homogenates of 'Galia' melon plugs. These results suggest that
407 the concentration of bactericidal compounds, if any, was insufficient to be detected
408 using the methods that were applied in our assay, or that the inhibition of growth could
409 be mediated by a contact- or proximity-dependent mechanism involving bactericidal
410 compounds or quorum-sensing molecules which can achieve inhibition at very low
411 concentrations. Furthermore, as the production of antimicrobial molecules can be
412 medium dependent, the specific conditions tested do not completely exclude the
413 possibility of this occurring in the presence of different Carbon/Nitrogen sources or in
414 specific restrictive or stressful conditions.

415 On the other hand, niche differentiation that was estimated from *in vitro*
416 Carbon/Nitrogen utilisation profiles that were performed using GN2/GP2 plates did not
417 provide enough information to establish a suitable number of targets for competiveness
418 allowing to explain the antagonistic effect observed in fruit matrices. Therefore, further
419 studies of niche overlapping including a wider range of Carbon/Nitrogen sources that
420 could be targeted for competition between this antagonist and foodborne pathogens as
421 well as the induction of the host defence mechanisms, need to be carried out in order
422 to ascertain its mode of action.

423 Nonetheless, the inability of CPA-7 to produce biosurfactants, to form biofilms or to
424 secrete alginate, and the lack of proteolytic or hemolytic activities in the conditions
425 tested, could represent additional advantages to evaluate this strain for the Qualified
426 Presumption of Safety (QPS) of microorganisms, which is required in the European

427 Union (EU) for market authorisation of biological agents that are intentionally added to
428 foods.

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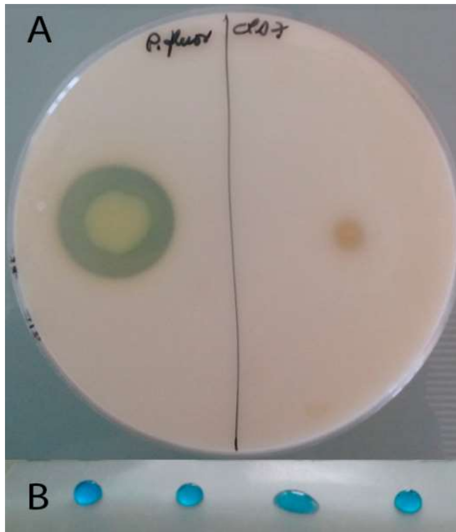

Figure 1. **A.** Protease production assay on PCA medium supplemented with 0.1 g/mL skim milk. Left section: positive control *Pseudomonas fluorescens*, right section: CPA-7. **B.** Biosurfactant activity of 20 µl droplets of 72h King's B medium - supernatants stained with methylene blue on a hydrophobic surface (parafilm). From left to right: water, fresh King's B medium, positive control (*Pseudomonas aeruginosa*) and *Pseudomonas graminis* (CPA-7). Each assay was performed three times and included three replicates.

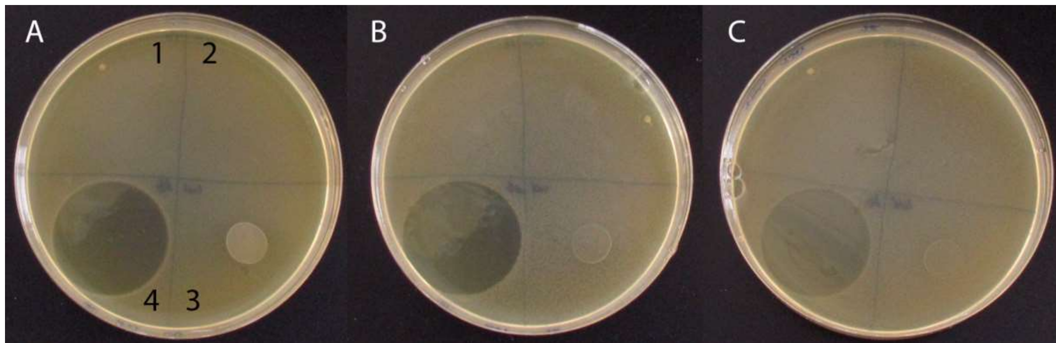

Figure 2. Antibiosis diffusion assay on agar medium inoculated with: **A.** *L. monocytogenes* CECT 4032, **B.** *Salmonella enterica* var. *enterica* CECT 4300 or **C.** *Escherichia coli* O157:H7. On each plate: 1. Fresh M63 medium, 2. CPA-7 cell-free supernatant obtained from 72 h culture in M63 medium, 3. CPA-7 cells (10^8 CFU/mL), 4. Streptomycin (100 µg/mL). The assay was repeated two independent times including three replicates per pathogen.

575 Table 1. Bacterial strains used in this study.

Microorganism	Serovar (identification code)	Purpose of use (section of this article)
Salmonella enterica subsp. *enterica* (Smith) Weldin	Agona (ATCC BAA-707)	Pathogen cocktail (2.7) Nutrients profiling (2.9)
Salmonella enterica subsp. *enterica* (Smith) Weldin	Michigan (ATCC BAA-709)	Pathogen cocktail (2.7)
Salmonella enterica subsp. *enterica* (Smith) Weldin	Montevideo (ATCC BAA-710)	Pathogen cocktail (2.7)
Salmonella enterica subsp. *enterica* (Smith) Weldin	Gaminara (ATCC BAA-711)	Pathogen cocktail (2.7) Nutrients profiling (2.9)
Salmonella enterica subsp. *enterica* (Smith) Weldin	Enteritidis (CECT 4300)	Pathogen (2.3, 2.6)
Listeria monocytogenes (Murray) Pirie	1a (CECT 4031)	Pathogen cocktail (2.7, 2.8)
Listeria monocytogenes (Murray) Pirie	3a (CECT 933)	Pathogen cocktail (2.7, 2.8)
Listeria monocytogenes (Murray) Pirie	4d (CECT 940)	Pathogen cocktail (2.7, 2.8) Nutrients profiling (2.9)
Listeria monocytogenes (Murray) Pirie	1/2a (Lm 230), Microbiology laboratory collection (UdL- IRTA, Lleida)	Pathogen cocktail (2.7, 2.8) Nutrients profiling (2.9)
Listeria monocytogenes (Murray) Pirie	4b (CECT 4032)	Pathogen cocktail (2.7, 2.8) Pathogen (2.3, 2.6)
Streptococcus gordonii (Kiliam)	(CECT 804)	Positive control in hemolysis assay (2.5)
Listeria innocua (Seeliger)	(CECT 910)	Negative control in hemolysis assay (2.5)
Pseudomonas graminis	(CPA-7), Microbiology laboratory collection (UdL - IRTA, Lleida)	Antagonist (2.1-2.9)
Pseudomonas aeruginosa (Schroeter) Migula	(NCTC 12903)	Positive control in biosurfactant activity assay (2.5)
Pseudomonas fluorescens	(1.62) Food Technology department collection, UdL, Lleida)	Positive control (2.3) Positive control in proteolysis assay (2.5)
Escherichia coli	O157:H7, shigatoxin negative (NCTC 12900)	Pathogen (2.3, 2.6) Nutrients profiling (2.9)

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578 Table 2. *Listeria monocytogenes* population increase, calculated as $\log_{10}$ CFU/g fruit at a
579 specific sampling time − $\log_{10}$ CFU/g fruit at initial time, on plugs of cv 'Galia', cv 'Piel de
580 sapo' and cv 'Cantaloupe' melons, after incubation at 25 ºC in air, when inoculated alone or
581 co-inoculated with *Pseudomonas graminis* (CPA-7) in water or in cell-free supernatants
582 (CFS) obtained from 24 h cultures of CPA-7 in tryptone soy broth medium (TSB).

Treatment	cv 'Galia'		cv 'Piel de sapo'		cv 'Cantaloupe'	
	24 h	48 h	24 h	48 h	24 h	48 h
Lm (in water)	5.0 ± 0.1 a	5.1 ± 0.1 a	4.9 ± 0.2 a	4.9 ± 0.1 a	4.5 ± 0.3 ab	5.1 ± 0.2 ab
Lm + CPA-7 (in water)	2.6 ± 0.6 b	1.3 ± 0.3 b	5.0 ± 0.4 a	4.5 ± 0.3 a	3.9 ± 0.1 b	3.3 ± 0.4 c
Lm (in TSB-CFS)	5.1 ± 0.1 a	5.1 ± 0.1 a	5.3 ± 0.1 a	4.7 ± 0.4 a	4.7 ± 0.2 a	5.3 ± 0.1 a
Lm + CPA-7 (in TSB-CFS)	4.2 ± 0.6 ab	4.2 ± 0.7 a	4.4 ± 0.4 a	4.2 ± 0.3 a	3.7 ± 0.3 b	4.2 ± 0.2 bc

Results are expressed as mean ± standard error of the mean. Within columns, different letters represent significantly different means corresponding to different treatments analysed separately for each melon cultivar and sampling time, according to Welch's test ($P < 0.05$) and Tukey's test for n=6.

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586 Table 3. *Listeria monocytogenes* population increase, calculated as $\log_{10}$ CFU/g fruit at 120 h
587 post-inoculation − $\log_{10}$ CFU/g fruit at initial time, on plugs of cv 'Galia', cv 'Piel de sapo' and cv
588 'Cantaloupe' melons, after incubation at 5 ºC in air, when inoculated alone or co-inoculated with
589 *P. graminis* (CPA-7) in water or in cell-free supernatants (CFS) obtained from 24 h cultures of
590 CPA-7 in tryptone soy broth medium (TSB).

Treatment	**cv 'Galia'**	**cv 'Piel de sapo'**	**cv 'Cantaloupe'**
Lm (in water)	1.4 ± 0.3 ab	1.1 ± 0.3 ab	1.5 ± 0.1 a
Lm + CPA7 (in water)	0.4 ± 0.2 b	0.6 ± 0.2 b	1.7 ± 0.1 ab
Lm (in TSB-CFS)	1.6 ± 0.2 a	1.7 ± 0.1 a	2.1 ± 0.2 b
Lm + CPA7 (in TSB-CFS)	1.1 ± 0.4 ab	1.4 ± 0.1a	1.6 ± 0.1 a

Results are expressed as mean ± standard error of the mean. Within columns, different letters represent significantly different means corresponding to different treatments analyzed separately for each melon cultivar, according to Welch's test ($P < 0.05$) and Tukey's test for n=6.

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596 Table 4. *Listeria monocytogenes* population increase, calculated as $\log_{10}$ CFU per gram of fruit
597 at a specific sampling time − $\log_{10}$ CFU per gram of fruit at initial time, on plugs of cv 'Galia'
598 melon after incubation at 25 ºC or 5 ºC in air, when inoculated alone or co-inoculated with
599 *Pseudomonas graminis* (CPA-7) in water or in cell-free supernatants (CFS) obtained from 24 or
600 48 h cultures of CPA-7 on 'Galia' melon plugs.

601

Treatment	**cv 'Galia'**		
	24 h at 25 °C	**48 h at 25 °C**	**120 h at 5°C**
Lm (in water)	5.1 ± 0.1 a	4.7 ± 0.1 a	2.3 ± 0.1 a
Lm + CPA-7 (in water)	2.4 ± 0.5 b	1.0 ± 0.2 c	1.4 ± 0.1 c
Lm (in non-inoculated Galia supernatant)	4.8 ± 0.1 a	4.0 ± 0.1 b	3.0 ± 0.1 b
Lm (in 24h-Galia-CFS)	5.0 ± 0.2 a	4.1 ± 0.1 ab	2.5 ± 0.2 ab
Lm (in 48h-Galia-CFS)	5.3 ± 0.1 a	4.0 ± 0.2 b	2.5 ± 0.1 ab

Results are expressed as mean ± standard error of the mean. Within columns, different letters represent significantly different means corresponding to different treatments, analysed separately for each sampling time, according to a Welch's test ($P < 0.05$) and Tukey's test for n=6.

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607 Table 5. Nutritional profile analysis of *Pseudomonas graminis* (CPA-7) compared to *E. coli*
608 O157:H7 and *S. enterica* var. *enterica*, strains CECT 707 (Sal 707) and CECT 711 (Sal 711).
609 Niche size represents the proportion of single carbon/nitrogen sources utilization from the total
610 of compounds analysed or from the subtotals of each functional group used by the pathogen.
611 NOI (niche overlap index) represents the proportion of substrates able to support the growth of
612 both the antagonist and the pathogen from the total of compounds used by the pathogen or
613 from the subtotals of each functional group used by the pathogen. Each strain was analysed
614 twice.

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		Niche size				**NOI**		
Functional group	Number of compounds analysed	*E. coli*	Sal 707	Sal 711	CPA-7	CPA-7/*E. coli*	CPA-7/Sal 707	CPA-7/Sal 711
Carbohydrates	26	0.69	0.50	0.58	0.38	0.39	0.50	0.50
Aminoacids	20	0.50	0.55	0.60	0.55	0.70	0.73	0.75
Carboxylic	25	0.52	0.48	0.48	0.64	0.92	0.83	0.83
Amines/Amides	8	0.25	0.25	0.25	0.63	0.00	0.50	0.50
Polymers	5	0.20	0.60	0.60	0.60	1.00	1.00	1.00
Miscellaneous	11	0.73	0.73	0.73	0.73	0.88	0.88	0.88
Total	95	0.55	0.52	0.55	0.56	0.63	0.73	0.71