Influence of fruit matrix and storage temperature on the survival of *Listeria monocytogenes* in a gastrointestinal simulation

Pilar Colás-Medà[1], Maribel Abadias[2]*, Marcia Oliveira[1], Josep Usall[2], Inmaculada Viñas[1]

*Corresponding author: Tel. 0034973003430 Fax. 0034973238301

E-mail address: isabel.abadias@irta.cat

[1] Food Technology Department, University of Lleida, XaRTA-Postharvest, Agrotecnio Center, Rovira Roure, 191, 25198-Lleida (Catalonia, Spain)

[2] IRTA, XaRTA-Postharvest, Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, Parc de Gardeny, 25003-Lleida (Catalonia, Spain)

Abstract

This study aimed to assess the effect of storage temperature and fruit matrix on the survival of *L. monocytogenes* after a gastrointestinal simulation. The growth of *L. monocytogenes* on different matrices (fresh-cut pear and melon and synthetic growth medium as a control) and storage temperature (1, 5, 10 and 20 °C) was evaluated. Subsequently, the ability of the pathogen on different fruit matrices to overcome the gastrointestinal simulation was evaluated. The highest reduction in the population of *L. monocytogenes* on fresh-cut pear and melon subjected to the gastrointestinal simulation was after 6 days of storage at 5 °C (0.84 and 2.12 log reduction on the pear and melon, respectively). Conversely, higher survival ratios of *L. monocytogenes* in both matrices were observed at 1 °C, even with logarithmic increases after the whole gastrointestinal simulation during the experiment. At 20 °C, the survival capacity of *L. monocytogenes* was higher than that under storage at 5 and 10 °C when grown on fresh-cut pear, whereas this was not observed on fresh-cut melon. In general, under the proper storage temperature of fresh-cut fruit (5 °C) and after 1 day of storage, the *L. monocytogenes* on the melon was more sensitive than that on the pear, and this behaviour was maintained for up to 9 days. The ability of *L. monocytogenes* to overcome the gastrointestinal tract was not enhanced when the pathogen grew on fresh-cut pear and melon under the proper storage temperature.

Keywords: foodborne pathogen; melon; pear; gastrointestinal simulation; acid-adaptation; cold

1 Introduction

The consumption of fruits and vegetables is associated with a healthy lifestyle. Thus, there has been an increasing market demand for minimally processed (MP) fruits and vegetables due to their fresh-like character, convenience, and human health benefits (Anon, 2014). Hurdles technology aims to improve the total quality of foods through the application of an intelligent mix of hurdles. 'Hurdles' are a popular analogy used to describe the concept that minimal food processing introduces sub-lethal stress that bacteria must overcome to survive or thrive in food systems. These cells, which have been 'prepared' or 'trained' to overcome hurdles, would have a significant advantage over wild cells. The most important hurdles used in food preservation are temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g., nitrate, sorbate, and sulphite), and competitive microorganisms (e.g., lactic acid bacteria) (Hill, Cotter, Sleator, & Gahan, 2002; Leistner, 2000). *Listeria (L.) monocytogenes* is capable of surviving and growing in environments where these factors are present, resulting in the long-term adaptation of this pathogen to sub-lethal environmental stress conditions (Farber & Peterkin, 1991). In minimally processed fruit, pathogenic bacteria must overcome different 'hurdles'. First, the intrinsic properties of the fruit are the pH of the tissue, the type of acidity, the sugar content or nutrient availability. Second are external properties linked to their processing (e.g., storage temperature and gas atmosphere) (Ragaert, Jacxsens, Vandekinderen, Baert, & Devlieghere, 2011). For successful foodborne infection, *L. monocytogenes* must survive through fresh-cut fruit storage (shelf-life) and the stress conditions encountered during gastrointestinal transit. Some studies have assessed the survival of *L. monocytogenes* inoculated on meat or cheese products after gastrointestinal simulation (Barbosa, et al., 2012; Barmpalia-Davis, Geornaras, Kendall, & Sofos, 2008; Dikici & Calicioglu, 2013; Formato, et al., 2007; Melo, Schrama, Hussey, Andrew, & Faleiro, 2013; Peterson, Faith, & Czuprynski, 2007; Ramalheira, et al., 2010; Stopforth, et al., 2005; Tompkins,

67 Mainville, & Arcand, 2011). However, little is known regarding the influence of fresh-cut fruit
68 and storage temperature on the ability of this microorganism to survive gastrointestinal
69 simulation. Thus, the aim of this study was to assess the effect of storage temperature and
70 fruit matrix on *L. monocytogenes* survival along with gastrointestinal simulation.

71 2 Material and Methods

72 2.1 Microbial strain

73 *L. monocytogenes* serovar 1/2a, which was isolated from commercial fresh-cut iceberg lettuce
74 (Abadias, Usall, Anguera, Solsona, & Viñas, 2008), was used in our study. It was grown for 20 -
75 22 h in 50 ml of tryptone soy broth (TSB, Oxoid, UK) supplemented with 6 g L^{-1} of yeast extract
76 (TSBYE) at 37 ± 1 °C and 120 rpm. The bacterial cells were harvested by centrifugation at 9800
77 × g for 10 min at 10 °C and then resuspended in 25 ml of saline peptone (SP; 8.5 g L^{-1} NaCl and
78 1 g L^{-1} peptone).

79 2.2 Fruit

80 'Conference' pears (*Pyrus communis* L. cv. Conference) were obtained from local
81 packinghouses in Lleida (Catalonia, Spain). 'Piel de sapo' melons (*Cucumis melo* L. var. Piel de
82 sapo) were purchased in a local supermarket. The pears were used when the firmness was
83 between 44 to 58 N, which is an optimum ripeness stage to obtain a better quality of fresh-cut
84 pear (Gorny, Cifuentes, Hess-Pierce, & Kader, 2000). Flesh firmness was measured on two
85 opposite sides of each fruit using a penetrometer (Effegi, Mila, Italy) equipped with a probe
86 that was 8 mm in diameter.

87 2.3 Sample preparation

88 Prior to cutting, the fruit was washed in running tap water and dried by hand with absorbent
89 paper. Then, the surface was disinfected with 70% ethanol. The pears were peeled and cut into
90 10 slices using a handheld apple slicer/corer. The melon was cut transversally in 14- to 16-mm
91 slices. The seeds and rind were removed, and each slice was then cut into trapezoidal pieces.

92 Erlenmeyer flasks containing 100 ml of TSBYE medium were used as a control for the growth
93 experiment.
94 Before inoculation, the pH, soluble solids content (SSC) and titratable acidity (TA) of the fresh-
95 cut pears and melons were determined. The pH of the flesh of the fruits was determined using
96 a pH-meter (Model GLP22, Crison, Instruments S.A., Barcelona, Spain) equipped with a
97 penetration electrode (5231 Crison, Instruments S.A., Barcelona, Spain). Before inoculation,
98 nine determinations were performed per lot of fruit (n=9). After the pH reading, the pears and
99 melons were squeezed separately, and the soluble solids content (SCC) of the extracted juice
100 was determined in triplicate at 20 °C using a hand-held refractometer (Atago CO., Ltd., Tokyo,
101 Japan). The data were expressed in °Brix. To measure the titratable acidity (TA), triplicate
102 samples of 10 ml of the pear or melon juice were diluted with 10 ml of deionized water, and 2
103 drops of a phenolphthalein solution 1% (Panreac, Barcelona, Spain) were added. The samples
104 were then titrated with a sodium hydroxide solution (NaOH, 0.1 mol l^{-1}) until a specific colour
105 change of the pH indicator was achieved. The results were calculated as g L^{-1} of malic acid for
106 pears and g L^{-1} of citric acid for melons.
107 2.4 Inoculation
108 For the inoculum preparation, a volume of the *L. monocytogenes* suspension was added to
109 deionized water to obtain approximately 10^5 CFU ml^{-1}. Fresh-cut pears and melons were
110 inoculated separately by immersion in the *L. monocytogenes* suspension (1:2 w/v) and shaken
111 at 150 rpm for 2 min. Afterwards, the liquid was drained off and the fruits were left to air-dry
112 in a biosafety cabinet. As a growth control, Erlenmeyer flasks containing 100 ml of TSBYE were
113 inoculated with a specific volume of the *L. monocytogenes* suspension and then homogenized.
114 Previous experiments were performed to establish the correct volume of the pathogen
115 suspension for flask inoculation to obtain similar initial populations for fresh-cut fruit.
116 2.5 Storage and sampling points

Inoculated fresh-cut fruit samples (100 ± 5 g) were placed into polypropylene trays of a 375 ml volume and sealed with a polypropylene plastic film. To obtain the air conditions, nine 400-µm holes were made in the sealed film using a needle. Once packed, the fruit trays were stored at 20 ± 1 °C, 10 ± 1 °C, 5 ± 1 °C and 1 ± 1 °C. TSBYE flasks were stored at the same temperatures but under shaking conditions (120 rpm). The samples stored at 1 and 5 °C were examined on the day of inoculation and after 1, 2, 6 and 9 days of storage. The samples stored at 10 °C were examined on the day of inoculation and after 1, 2 and 6 days of storage, and the samples stored at 20 °C were examined on the day of inoculation and after 1 and 2 days of storage. The headspace of the gas composition (carbon dioxide and oxygen) on the trays was measured using a handheld gas analyser (CheckPoint O_2/CO_2, PBI Dansensor, Denmark) at each sampling point for all of the evaluated temperatures to confirm that the packages remained under aerobic conditions throughout storage. Once the trays were opened, the fruit pH values were measured in two fruit pieces from each tray (n=6) using a pH-meter equipped with a penetration electrode Model GLP22 (Crison Instruments S.A., Barcelona, Spain), and the same trays were used to evaluate the survival of *L. monocytogenes* throughout the gastrointestinal simulation as described below (part 2.6). There were three trays/Erlenmeyer flasks per temperature and the sampling time and experiment was repeated twice. The quality data from each trial were combined after testing to determine if they showed any significant differences.

2.6 Gastrointestinal solutions

Our *in vitro* digestion model procedure was performed according to Oomen et al. (2003) and Oliveira et al. (2011) with some modifications. These models describe a three-step procedure simulating digestive progress in the mouth, stomach and small intestine. Synthetic saliva fluid (SSF) was prepared with the following composition per litre: 0.90 g potassium chloride (KCl), 0.20 g potassium thiocyanate (KSCN), 1.15 g sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), 0.57 g sodium sulphate (Na_2SO_4), 0.30 g sodium chloride (NaCl), 0.07 g sodium hydroxide (NaOH), 0.20 g urea ($CO(NH_2)_2$), 145 mg α-amylase (Sigma), 15 mg uric acid

143 ($C_5H_4N_4O_3$) and 50 mg mucin (Sigma). The pH was adjusted to 6.5 with hydrochloric acid (HCl,
144 0.1 N). The synthetic gastric fluid (SGF) was prepared with the following composition per litre:
145 0.82 g KCl, 0.35 g $NaH_2PO_4 \cdot 2H_2O$, 2.75 g NaCl, 0.40 g calcium chloride dihydrate ($CaCl_2 \cdot 2H2O$),
146 0.31 g ammonium chloride (NH_4Cl), 0.09 g $CO(NH_2)_2$, 0.65 g glucose ($C_6H_{12}O_6$), 0.02 g glucuronic
147 acid ($C_6H_{10}O_7$), 0.33 g glucosamine hydrochloride ($C_6H_{14}ClNO_5$), 1.00 g bovine serum albumin
148 fraction V (BSA, Sigma), 1.00 g pepsin (Sigma) and 3.00 g mucin (Sigma), and adjusted to pH 2.0
149 with HCl (6 mol l^{-1}). Two solutions of synthetic intestinal fluid (SIF) were prepared to simulate a
150 duodenal and bile solution. The duodenal solution (DS) was prepared with the following
151 composition per litre: 0.56 g KCl, 7.00 g NaCl, 3.39 g sodium bicarbonate ($NaHCO_3$), 0.08 g
152 potassium dihydrogen phosphate (KH_2PO_4), 0.05 g magnesium chloride ($MgCl_2$), 0.20 g
153 $CaCl_2 \cdot 2H_2O$, 0.10 g $CO(NH_2)_2$, 1.00 g BSA (Sigma), 3.00 g pancreatin (Sigma) and 0.50 g lipase
154 (Sigma). The bile solution (BS) was prepared with the following composition per litre: 0.38 g
155 KCl, 5.26 g NaCl, 5.79 g sodium bicarbonate ($NaHCO_3$), 0.22 g $CaCl_2 \cdot 2H_2O$, 0.25 g $CO(NH_2)_2$, 1.80
156 g BSA (Sigma) and 6.00 g bile (Sigma). The pH was adjusted to 7.8 and 8.0 in the duodenal and
157 bile solutions, respectively, with NaOH (2 mol l^{-1}).

158 2.7. *In vitro* gastrointestinal simulation

159 At each sampling point, 10 g of inoculated fruit sample (pear or melon) were placed into a
160 sterile plastic bag (80 ml, BagPage®, Interscience BagSystem, Saint Nom, France). Three
161 different trays of inoculated pear or melon were used for each sampling point and
162 temperature. Prior to the experiment, the digestive solutions (SSF, SGF, DS and BS) were kept
163 in a water bath (TectronBio-100, J.P. Selecta, Barcelona, Spain) at 37 °C.

164 All of the samples underwent the same digestive process (Fig. 1). Nine millilitres of SSF was
165 added to 10 g of sample and homogenized in a blender for 2 min at high speed (MiniMix,
166 Interscience, Saint Nom, France). The samples were then incubated for 5 min at 37 °C.
167 Afterwards, the pH of the mixture was measured, and duplicate 0.5 ml samples were collected
168 for microbial analysis ('post-saliva count'). Previous experiments showed that the counts of

169 *L. monocytogenes* (CFU g^{-1} fruit) obtained from a 10 g sample plus 90 ml SP or a 10 g sample
170 plus 9 ml of synthetic saliva fluid (SSF) after 5 min of contact were the same. Thus, the 'post-
171 saliva count' was used as the 'initial count'. The same method was used to recover the
172 population of *L. monocytogenes* in TSBYE medium along with the storage at different
173 temperatures to compare with the initial pathogen values on fresh-cut fruit. Then, 13.5 ml of
174 SGF were added to the remaining sample and the mixture was homogenized and the pH
175 measured again. The pH could be altered by the buffering effect of each type of matrix (pear or
176 melon). Thus, to begin the gastric step with the same values, the pH was adjusted to 3.5 with
177 hydrochloric acid (HCl, 0.1 mol l^{-1}). The samples were incubated for 1 h at 37 °C. After
178 incubation, the pH was measured and duplicate 0.5 ml samples were collected for microbial
179 analysis ('post-SGF' count). The remaining sample (32.5 ml) was mixed with 27 ml of DS and 9
180 ml of BS, homogenized and the pH of the mixture measured. The sample was incubated for 2 h
181 at 37 °C. Then, duplicate 0.5 ml samples were collected for microbial analysis ('post-SIF' count),
182 and the pH of the final mixture was measured. For microbial analysis, the decimal dilutions
183 were prepared using SP. Enumeration was performed by plating in duplicate on Palcam agar
184 media. The plates were incubated at 37 °C for 48 h.
185 At each sampling point, all of the cell counts were calculated considering the dilution factors
186 due to the continuous addition of gastrointestinal fluids. To represent the growth of
187 *L. monocytogenes* on minimally processed pear and melon and in TSBYE medium during the
188 storage, all CFU g^{-1} or ml^{-1} data were transformed to log CFU g^{-1} or ml^{-1}. In addition, the data
189 were expressed as initial (population before the gastric step; log CFU g^{-1} of fruit), post-SGF
190 population (after exposure to the gastric step; log CFU g^{-1} of fruit) and post-SIF population
191 (after exposure to the intestinal step; log CFU g^{-1} of fruit). To compare between the fruit
192 matrices, the logarithmic variation of the pathogen population after whole gastrointestinal
193 simulation was calculated as the log N_{SIF}/N_0, where N_{SIF} is the pathogen population count at the
194 end of the gastrointestinal simulation and N_0 is the initial cell density.

195 2.8 Statistical analysis

196 *L. monocytogenes* populations were statistically compared between the gastrointestinal steps
197 at each sampling day for each matrix. Data on the *L. monocytogenes* population and quality
198 parameters were analysed using a general linear model analysis with the JMP8 software (SAS
199 Institute, Cary, NC, USA). The statistical significance was judged at the level of $P < 0.05$. When
200 the analysis was statistically significant, the Tukey's test for the separation of the means was
201 used.

202 3 Results

203 3.1 Quality parameters of fresh-cut fruits

204 The initial pH of the fresh-cut pear was between 4.39 and 5.87 (mean 5.08), and the initial pH
205 of fresh-cut melon was between 5.16 and 6.72 (mean 5.85). The fresh-cut pear had SSC values
206 from 12.5 to 15.7 °Brix (mean 14.5 °Brix) and TA values between 1.27 and 2.14 g malic acid L^{-1}
207 (mean 1.62 g malic acid L^{-1}). The fresh-cut melon had SSC values between 8.4 and 12.4 °Brix
208 (mean 10.5 °Brix) and TA values between 1.07 and 2.87 g citric acid L^{-1} (mean 1.70 g citric acid
209 L^{-1}). The initial pH of the TSBYE medium was between 7.05 and 7.20.

210 No significant differences in the pH value were observed between the untreated and
211 *L. monocytogenes*-treated fresh-cut pear and melon throughout the study at each storage
212 temperature and sampling day (data not shown). Furthermore, the pH of the treated fresh-cut
213 pear and melon did not change throughout the study under any of the storage conditions (data
214 not shown). In contrast, the pH of the inoculated TSBYE medium exhibited a significant
215 decrease at all of the temperatures throughout storage. At 1, 5, 10 and 20 °C, the inoculated
216 TSBYE medium reached pH values of 6.99, 6.30, 5.94 and 5.61, respectively, at the end of the
217 experiment (data not shown).

218 The gas composition of the headspace of the trays was measured at every sampling point, and
219 the results demonstrated that air conditions were maintained throughout the experiment in all
220 of the trays (data not shown).

221 3.2 Population of *L. monocytogenes* on fresh-cut pear and melon during a storage time of 1, 5,
222 10 and 20 °C
223 The initial populations of *L. monocytogenes* after inoculation were 3.63 log CFU g^{-1} on pear and
224 3.62 log CFU g^{-1} on melon (Fig. 2a and 2b). When the inoculated fresh-cut pear samples were
225 stored at 1 °C, *L. monocytogenes* reached 3.72, 3.73, 4.03 and 3.81 log CFU g^{-1} and after 1, 2, 6
226 and 9 days of storage, respectively. For the inoculated fresh-cut melon, *L. monocytogenes*
227 reached 3.70, 3.79, 4.22 and 4.63 log CFU g^{-1} after 1, 2, 6 and 9 days of storage at 1 °C,
228 respectively. Under the proper storage conditions (5 °C), *L. monocytogenes* on fresh-cut pears
229 showed populations of 3.87, 4.28, 5.78 and 6.30 log CFU g^{-1}, whereas the pathogen on fresh-
230 cut melon reached 3.61, 4.11, 6.11 and 7.46 log CFU g^{-1} after 1, 2, 6 and 9 days of storage,
231 respectively. The higher pathogen population increases were observed under storage at 10
232 and 20 °C. When inoculated fresh-cut pears were stored at 10 °C, the *L. monocytogenes*
233 population reached 4.49, 5.77 and 6.80 log CFU g^{-1} after 1, 2 and 6 days of storage,
234 respectively. On the fresh-cut melon, *L. monocytogenes* reached populations of 4.81, 5.79 and
235 8.61 log CFU g^{-1} after 1, 2 and 6 days of storage, respectively. In both food matrices, the
236 storage of samples at 20 °C caused the highest pathogen increase. On fresh-cut pears after 1
237 day, the *L. monocytogenes* population was 6.87 and increased until it reached 7.71 log CFU g^{-1}
238 after 2 days of storage. On the fresh-cut melon, the *L. monocytogenes* population was 8.07
239 after 1 day and increased until it reached 9.25 log CFU g^{-1} after 2 days.
240 The growth of *L. monocytogenes* in an optimum growth media (TSBYE) under storage at 1, 5,
241 10 and 20 °C is shown in Figure 2c. After 1 day of storage at 1 and 5 °C, slight reductions of the
242 pathogen were observed in TSBYE, whereas that pathogen on fresh-cut pear and melon always
243 showed a population increase under the same storage conditions. After 2 days under these
244 cold conditions, the pathogen in TSBYE increased exponentially until 9 days and reached 5.99
245 and 9.06 log CFU ml^{-1} at 1 °C and 5 °C, respectively. In the experiment at 10 °C, the
246 *L. monocytogenes* count in the TSBYE medium was 6.19 log CFU ml^{-1} after 2 days of storage,

247 whereas after 6 days of storage it was 9.37 log CFU ml^{-1}. After 1 and 2 days of storage at 20 °C,
248 the *L. monocytogenes* population in the TSBYE medium was 7.86 and 9.36 log CFU ml^{-1},
249 respectively.
250 3.3 Survival of *L. monocytogenes* throughout the gastrointestinal simulation after different
251 storage temperatures
252 In matrices that underwent the digestive process immediately after inoculation (0 d), there
253 were no significant reductions in the pathogen throughout the gastric step in both matrices
254 (Fig. 3); nevertheless, after the intestinal step, population increases were observed on fresh-
255 cut pears and melon. Overall, the logarithmic reduction after the whole gastrointestinal
256 simulation did not cause any effect on *L. monocytogenes* upon the fresh-cut pear, whereas on
257 fresh-cut melon, the growth of the pathogen throughout the gastrointestinal simulation was
258 observed.
259 Considering the logarithmic variation value throughout the entire gastrointestinal simulation,
260 no variation of the *L. monocytogenes* population occurred when the matrix was pear, whereas
261 a 0.43 logarithmic increase was noticed in melon (Table 1). After 1 day of storage at 1 °C and
262 10 °C (Fig. 4), *L. monocytogenes* inoculated on fresh-cut pear (Fig. 4a) showed a reduction
263 throughout gastric step, whereas on fresh-cut melon, the reduction was observed in samples
264 stored at 5 and 10 °C (Fig. 4b). *L. monocytogenes* on fresh-cut pears showed a significant
265 logarithmic reduction after the entire process of gastrointestinal simulation only in samples
266 stored at 10 °C (0.91 log reduction), whereas on fresh-cut melon, *L. monocytogenes* showed a
267 significant logarithmic reduction after gastrointestinal simulation on samples stored at 5, 10
268 and 20 °C.
269 After 2 days of storage at different temperatures, the population of *L. monocytogenes* on
270 fresh-cut pears throughout the gastric step was reduced when the samples were stored at 5 °C
271 (Fig. 5a), and the same behaviour was observed in the pathogen on fresh-cut melon in samples
272 stored during 2 days at 5 and 10 °C (Fig. 5b). Regarding to pathogen logarithmic variation after

the whole gastrointestinal simulation, *L. monocytogenes* on both fresh-cut fruits was more sensible when the samples were stored at 10 °C for 2 days with 0.77 and 1.16 log reduction. After 6 days, *L. monocytogenes* on fresh-cut melon showed high population reductions after the gastric step in samples stored at 5 and 10 °C (Fig. 6b). However, at 10 °C, there was an increase of the population during the intestinal step. Thus, after 6 days of storage, a significant logarithmic reduction was observed only in fresh-cut melon stored at 5 °C. For the long storage period (9 days), the population of *L. monocytogenes* on both matrices was reduced throughout the gastric step when the samples were stored at 5 °C (Fig. 7). Conversely, any reduction of the *L. monocytogenes* population in both matrices was observed after the gastric step and when the samples were stored at 1 °C.

Considering the logarithmic variation of *L. monocytogenes* during the whole gastrointestinal simulation (Table 1) and during the storage at 1 °C, similar logarithmic variation values after gastrointestinal simulation were observed for *L. monocytogenes* on fresh-cut pears until 9 days, when it increases up to 0.31 log, and the pathogen on fresh-cut melon had the same logarithmic variations after the gastrointestinal simulation during the study. At 5 °C, a significantly higher logarithmic reduction after the gastrointestinal simulation was observed in pathogen populations on both matrices after 6 days of storage (0.84 and 2.12 log reductions on pear and melon, respectively). At 10 °C, *L. monocytogenes* showed noteworthy reduction throughout the gastrointestinal simulation regardless of the support matrix and after 1 and 2 days of storage (0.91 and 0.72 log reductions after 1 day of storage and 0.77 and 1.16 log reductions after 2 days of storage, on pear and melon, respectively). Finally, at 20 °C, the survival of *L. monocytogenes* on pear remained constant throughout the gastrointestinal simulation for all of the storage times evaluated, whereas the pathogen grown on melon showed a weak reduction after 1 or 2 days of storage (0.38 and 0.31 log reductions after 1 and 2 days of storage, respectively).

4 Discussion

299 The first objective of our study was to assess the growth of *L. monocytogenes* (serotype 1/2a)
300 isolated from ready-to-eat lettuce on minimally processed fruits (pear and melon) for their
301 different pH and during storage at 1, 5, 10 and 20 °C. Our study confirms that temperature is
302 not a limiting factor for *L. monocytogenes* growth on fresh-cut mild acid fruit as weak growth
303 was observed in melon even at 1 °C. Under extreme cold conditions (1 and 5 °C), the fresh-cut
304 fruit matrix helped *L. monocytogenes* overcome the cold stress after 1 day of storage but
305 growth in liquid medium was reduced, although a population increase was observed after 2
306 days under both temperatures.
307 Second, our hypothesis was that growing *L. monocytogenes* on fresh-cut pears and melon
308 stored at refrigeration temperatures could enhance *L. monocytogenes* survival to subsequent
309 exposure in the gastrointestinal simulation. Several authors confirmed through *in vitro* assays
310 that the optimum range of pH, in which habituation resulted in increased acid resistance, was
311 5.0-6.0 (Davis, Coote, & Obyrne, 1996; Koutsoumanis & Sofos, 2004; O'Driscoll, Gahan, & Hill,
312 1996; Shen, Soni, & Nannapaneni, 2014). The pH of our evaluated fruit matrices was
313 approximately within this range; nevertheless, it is known that several other factors play
314 critical roles in controlling the induction of acid-stress adaptation in *L. monocytogenes*.
315 Extensive studies determined the influence of sub-lethal acid concentrations, exposure time,
316 the type of acidulant, temperature and bacterial growth stage on acid-stress adaptation in
317 *L. monocytogenes* (Shen, et al., 2014). In addition, in the presence of a mild concentration of
318 weak acid preservatives, organisms have been shown to adapt by making changes in their cell
319 membrane permeability and fluidity (Diakogiannis, et al., 2013).
320 Our study evaluated whether the surrounding food environment present during the minimally
321 processed fruit shelf-life (matrix, time and temperature) could affect the survival of
322 *L. monocytogenes* to subsequent exposure to acid stress. Some studies focused on the
323 production chain of minimally processed products because *L. monocytogenes* may encounter
324 various hurdles (stresses) in food processing environments (e.g., acidity, salinity, sanitizers,

etc.) (Chorianopoulos, Giaouris, Grigoraki, Skandamis, & Nychas, 2011). Disinfection is one of the most critical processing steps in fresh-cut vegetable production and affects the quality, safety and shelf-life of the end product (Gil, Selma, Lopez-Galvez, & Allende, 2009). Pathogens from contaminated produce can be dislodged from the plant surface by the cleaning action of the wash process, and the sanitising agent eliminates them in suspension (Gil, et al., 2009; Zhou, Luo, Nou, Lyu, & Wang, 2015). Chlorine is the most widely used sanitizer but other alternatives exist such as the use of peroxyacetic acid, chlorine dioxide, hydrogen peroxide, organic acids, electrolyzed water or physical methods such as ultrasound, high pressure, high-intensity electric field pulses, ultraviolet radiation and radio frequency and ionizing radiation (Artes, Gomez, Aguayo, Escalona, & Artes-Hernandez, 2009; Gil, et al., 2009). Potential acid habituation in a sublethal pH environment of an acid decontaminated food may enhance the survival of pathogens during transit through the stomach and increase the likelihood of intestinal colonization and thus their virulence potential (Samara & Koutsoumanis, 2009). Samara et al. (2009) studied the potential adaptation of the phenomena induced by acid decontamination (lactic acid, acetic acid, propionic acid and citric acid at concentrations 0.5 and 1.0%) by studying the behaviour of *L. monocytogenes* during exposure to simulated gastric fluid following storage (48 h at 5 °C and 20 °C) on decontaminated lettuce. The results showed that the tested decontamination treatments did not increase the acid tolerance of *L. monocytogenes*. Moorman et al. (2008) observed changes in membrane lipids after exposure of *Listeria innocua* to acid conditions and found that adaptation to acid conditions decreased the pathogen membrane fluidity. The same result was observed as a response to prolonged exposure of *L. monocytogenes* to sublethal levels of benzalkonium chloride (2.5 mg L^{-1}) (Bisbiroulas, et al., 2001). This physiological modification may enhance the survival of the pathogen during transit through the stomach. Nevertheless, the survival of *L. monocytogenes* after exposure to simulated gastric fluid was not evaluated after those stresses.

After the disinfection step, Allende et al. (2004) observed that shredding, rinsing and centrifugation affect the microbial and sensory quality of fresh processed lettuce and show increased bacterial counts. Thus, every step from production through consumption will influence the microbiology of fresh produce and the proper use of a good cleaning and disinfection programme should be a major priority of the fresh processed industry (Allende, et al., 2004).

In the current study, when the pathogen behaviour was evaluated at 20 °C (without cold stress), we observed that *L. monocytogenes* on fresh-cut pears was more resistant to the gastrointestinal simulation after 1 and 2 days of contact than the pathogen on melon. The mild-low pH of pear flesh could have switched on the acid tolerance response (ATR) of *L. monocytogenes* in this stressful environment and subsequently enhanced the pathogen survival in the gastrointestinal simulation (acid shock, pH 3.5).

For the temperature effect on acid adaptation, Shen et al. (2014) showed that the acid-stress adaptation that typically occurs when *L. monocytogenes* cells are pre-exposed to sub-lethal acid at 20 °C or 37 °C was not induced when the cells were pre-exposed to sub-lethal acid at 4 °C. It is likely that between the acid and cold adaptation, cold adaptation becomes the priority task for the bacterium. A cold stress environment may block the penetration of acid into the cytoplasm and therefore fail to trigger the intracellular response to acid stress (Shen, et al., 2014). This result might explain our findings at 1 °C, where the lowest pathogen reductions were observed in both matrices and probably due to the hydrochloric acid internalization from the SGF solution to the cytoplasm, which was more difficult due to the cold pre-adaptation of the cells. Al-Nabulsi et al. (2015) found that cold (4 °C), acid (5.0) and osmotic (2, 4, 6 and 12% NaCl) stresses increased the resistance of *L. monocytogenes* to nine currently used antibiotics. The increase of the survival of *L. monocytogenes* after the entire gastrointestinal simulation observed after 9 days of storage at 5 °C and 6 days at 10 °C in both fresh-cut matrices could be caused by the pathogen entering into a stationary-phase. After entering into the stationary-

phase, cells become naturally resistant because of the activation of a stringent response (mediated by ppGpp) and general stress response (mediated by σ^B) (Shen, et al., 2014).

In conclusion, when the samples had not been stored, *L. monocytogenes* on melon was more resistant to the gastrointestinal simulation than the pathogen on the pear. In general, under the proper storage temperature of fresh-cut fruit (5 °C) and in general after 1 day of storage, *L. monocytogenes* on melon was more sensible than on pear and this behaviour was maintained until 9 days when the microorganism in both matrices showed less sensitivity to the gastrointestinal simulation. However, *L. monocytogenes* on pears at 5 °C could seem more hazardous for consumers and it is necessary to consider that the population of *L. monocytogenes* on melon was always higher than on pear due to its physicochemical properties. Some authors confirm that the ability of *L. monocytogenes* to survive the acidic conditions of the stomach could contribute to increasing its virulence and thus the likelihood of intestinal colonization. Therefore, more studies should be conducted to determine whether *L. monocytogenes* on fresh-cut fruits during their shelf-life might increase its ability to infect.

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507
508

Highlights

- The ability of *Listeria* to overcome the gastrointestinal simulation was evaluated on fruit
- After 1 day of storage at 5 °C, the *Listeria* on melon was more sensitive than that on pear
- At 20 °C, the survival capacity of *L. monocytogenes* on pear was higher than that at 5 °C
- High levels of pathogen after gastrointestinal simulation were observed at 1 °C

Table 1

The logarithmic variation of the population of *L. monocytogenes* inoculated onto fresh-cut pear and melon and exposed to the whole gastrointestinal simulation.

Logarithmic variation of the *L. monocytogenes* population after gastrointestinal simulation																								
Storage time	1 °C						5 °C						10 °C						20 °C					
	pear				melon		pear				melon		pear				melon		pear				melon	
0 day		0.01	b	*	a 0.43			0.01	a	*	a 0.43			0.01	a	*	a 0.43			0.01	a	*	a 0.43	
1 day	AB	-0.16	b	*	a 0.31	X	A	0.02	a	*	b -0.60	Y	B	-0.91	b		b -0.72	Y	A	0.01	a	*	b -0.38	Y
2 days	A	0.09	ab		a 0.36	X	AB	-0.16	ab	*	b -0.59	Y	B	-0.77	b		b -1.16	Z	AB	-0.10	a		b -0.31	Y
6 days	A	-0.08	b	*	a 0.23	X	A	-0.84	c	*	c -2.12	Y	A	-0.25	ab		a 0.10	X						
9 days	A	0.31	a		a -0.05	X	B	-0.56	bc		b -0.55	X												

The values are the mean of gastrointestinal survival, log N_{SIF}/N_0 with N_0 being the pathogen population count at the beginning of the gastrointestinal simulation ('initial count', CFU g^{-1}) and N_{SIF} the pathogen population count at the end of the gastrointestinal simulation ('post-SIF' count, CFU g^{-1}). Within each storage temperature (columns) and within each matrix, the values with different lowercase letters are significantly different ($P < 0.05$) among the storage times. Within each storage time (rows) and within each matrix, the values with different uppercase letters (A, B and C for pear and X, Y and Z for melon) are significantly different ($P < 0.05$) among the storage temperatures. Within each storage temperature (columns) and within each storage day, the values with an asterisk are significantly different among the matrices.

538 **Figure captions**

539 **Fig 1.** Schematic overview of the gastrointestinal simulation (* post-saliva *L. monocytogenes*
540 enumeration, ** post-SGF *L. monocytogenes* enumeration and *** post-SIF *L. monocytogenes*
541 enumeration).

542 **Fig. 2** Population (log CFU g^{-1} or ml^{-1}) of *L. monocytogenes* inoculated onto fresh-cut pear (A),
543 melon (B) and TSBYE medium (C) during storage at 1 °C (diamonds), 5 °C (squares), 10 °C
544 (triangles) and 20 °C (cross). The results are the means of two biological replicates each with
545 three technical replicates (n=6), and the vertical bars indicate the standard deviation of the
546 mean.

547 **Fig. 3** Survival of *L. monocytogenes* after inoculation onto fresh-cut pear and melon and
548 subsequent exposure to gastric and intestinal steps. The values are the means of two biological
549 replicates and each with three technical replicates (n=6), and the bars represent the standard
550 error. Within each fruit matrix, the values with different letters are significantly different ($P < 0.05$)
551 among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF)
552 and post-simulated intestinal fluid (post-SIF)).

553 **Fig. 4** Survival of *L. monocytogenes* inoculated onto fresh-cut pears (a) and melon (b) after 1
554 day of storage at 1, 5, 10 and 20 °C and exposed to gastric and intestinal steps. The values are
555 the means of two biological replicates each with three technical replicates (n=6), and the bars
556 represent the standard error. Within each storage temperature, values with different letters
557 are significantly different ($P < 0.05$) among the evaluated gastrointestinal steps (initial, post-
558 simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF)).

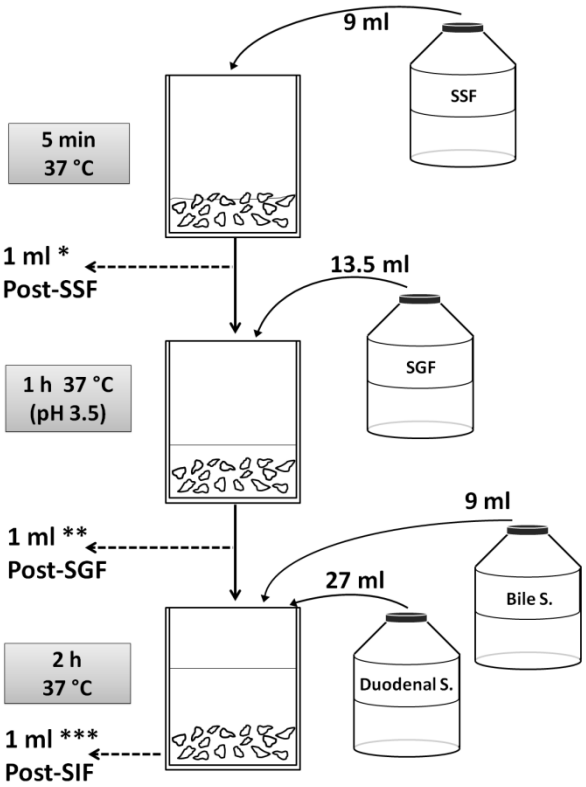
559 **Fig.5** Survival of *L. monocytogenes* inoculated onto fresh-cut pears (a) and melon (b) after 2
560 days of storage at 1, 5, 10 and 20 °C and exposed to gastric and intestinal steps. The values are
561 the means of two biological replicates and each with three technical replicates (n=6), and the

bars represent the standard error. Within each storage temperature, values with different letters are significantly different ($P < 0.05$) among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF)).

Fig. 6 Survival of *L. monocytogenes* inoculated onto fresh-cut pears (a) and melon (b) after 6 days of storage at 1, 5, 10 and 20 °C and exposed to gastric and intestinal steps. The values are the means of two biological replicates each with three technical replicates (n=6), and the bars represent the standard error. Within each storage temperature, the values with different letters are significantly different ($P < 0.05$) among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF)).

Fig. 7 Survival of *L. monocytogenes* inoculated onto fresh-cut pears (a) and melon (b) after 9 days of storage at 1, 5, 10 and 20 °C and exposed to gastric and intestinal steps. The values are the means of two biological replicates each with three technical replicates (n=6), and the bars represent the standard error. Within each storage temperature, the values with different letters are significantly different ($P < 0.05$) among the evaluated gastrointestinal steps (initial, post-simulated gastric fluid (post-SGF) and post-simulated intestinal fluid (post-SIF)).

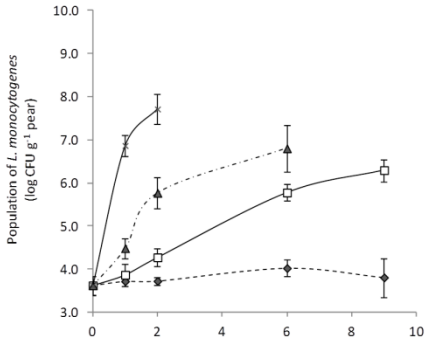
580 **Fig. 1**


9 ml
SSF
5 min
37 °C
1 ml *
Post-SSF
13.5 ml
SGF
1 h 37 °C
(pH 3.5)
1 ml **
Post-SGF
9 ml
27 ml
Bile S.
Duodenal S.
2 h
37 °C
1 ml ***
Post-SIF

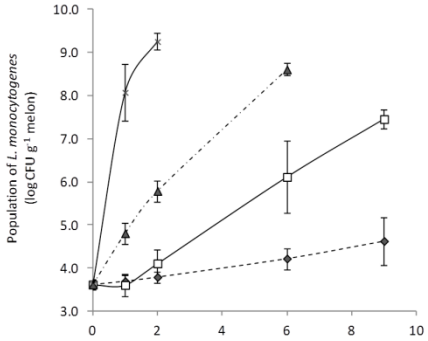
581

Fig. 2

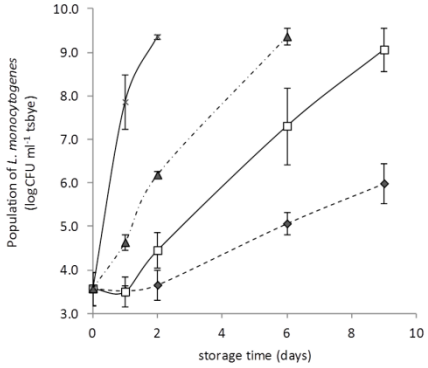
a)


Population of L. monocytogenes (log CFU g-1 pear)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
2
4
6
8
10

b)

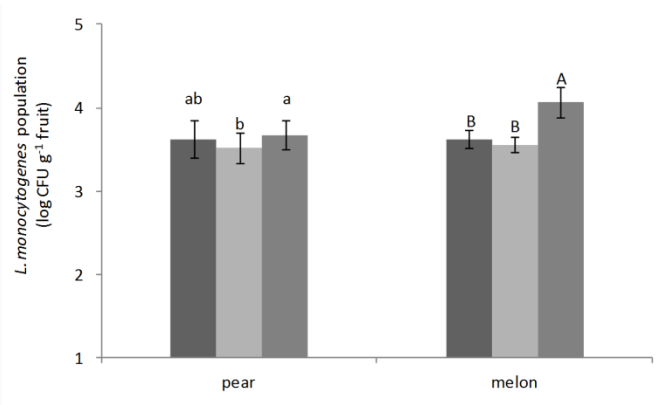

Population of L. monocytogenes (log CFU g-1 melon)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
2
4
6
8
10

c)


Population of L. monocytogenes (logCFU ml-1 tsbye)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
2
4
6
8
10
storage time (days)

582

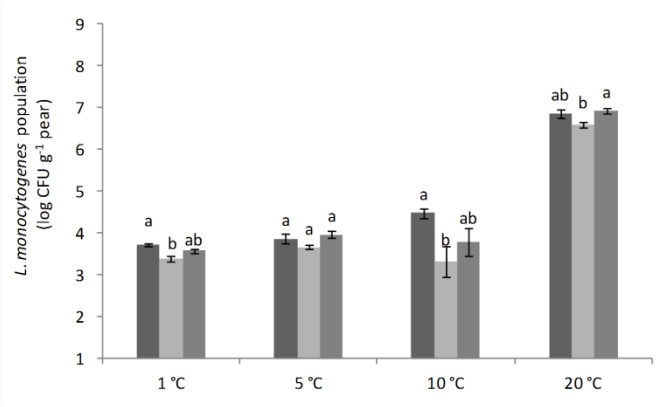
Fig. 3


L. monocytogenes population
(log CFU g-1 fruit)
5
4
3
2
1
ab
b
a
B
B
A
pear
melon

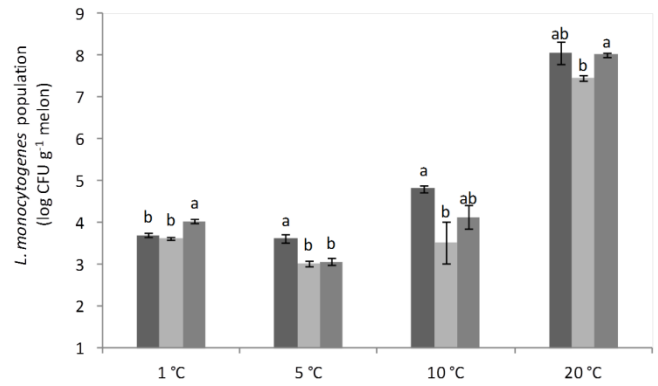
583

Fig. 4

a)


L. monocytogenes population (log CFU g-1 pear)
9
8
7
6
5
4
3
2
1
a
b
ab
a
a
a
a
b
ab
ab
b
a
1 °C
5 °C
10 °C
20 °C

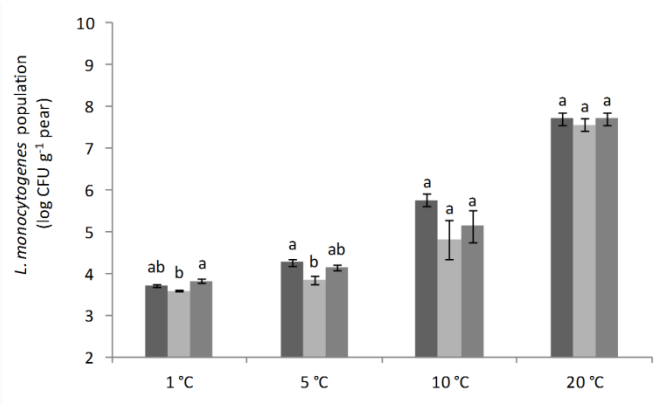
b)


L. monocytogenes population (log CFU g-1 melon)
9
8
7
6
5
4
3
2
1
b
b
a
a
b
b
a
b
ab
ab
b
a
1 °C
5 °C
10 °C
20 °C

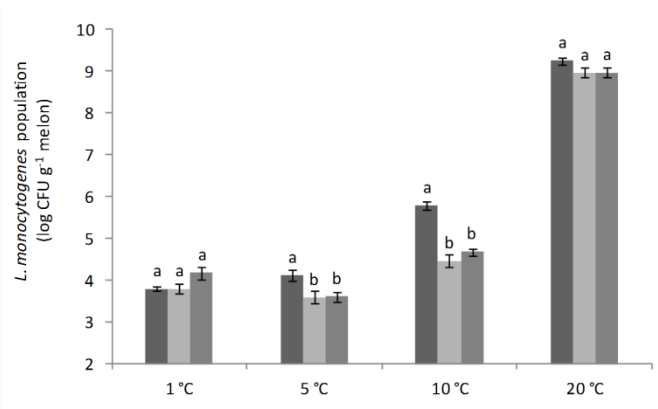
584

Fig. 5

a)


L. monocytogenes population (log CFU g-1 pear)
10
9
8
7
6
5
4
3
2
ab b a
a b ab
a a a
a a a
1 °C
5 °C
10 °C
20 °C

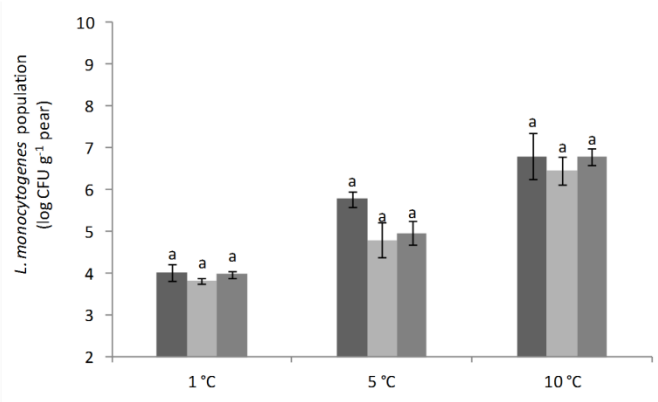
b)


L. monocytogenes population (log CFU g-1 melon)
10
9
8
7
6
5
4
3
2
a a a
a b b
a b b
a a a
1 °C
5 °C
10 °C
20 °C

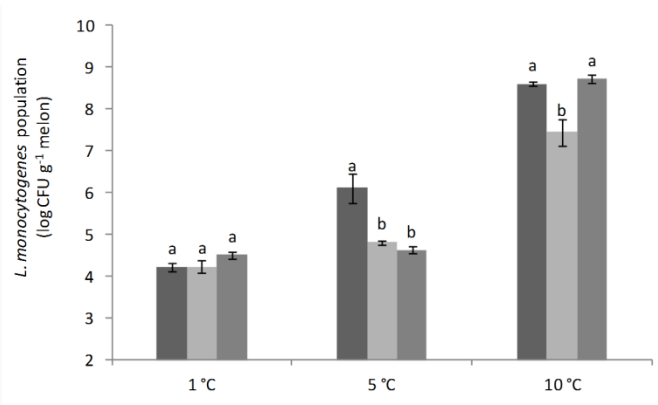
585

Fig. 6

a)


L. monocytogenes population (log CFU g-1 pear)
10
9
8
7
6
5
4
3
2
a
a
a
a
a
a
a
a
a
1 °C
5 °C
10 °C

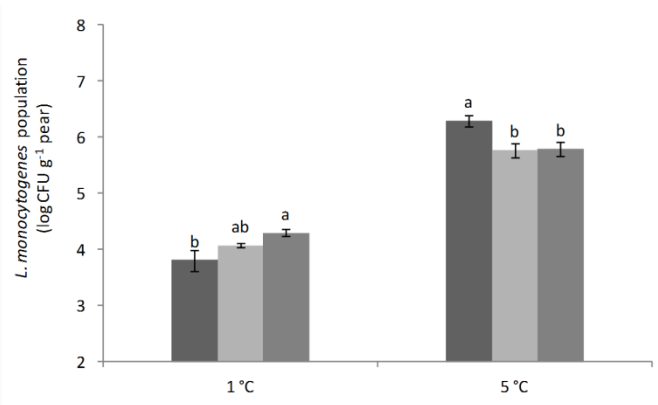
b)


L. monocytogenes population (log CFU g-1 melon)
10
9
8
7
6
5
4
3
2
a
a
a
a
b
b
a
b
a
1 °C
5 °C
10 °C

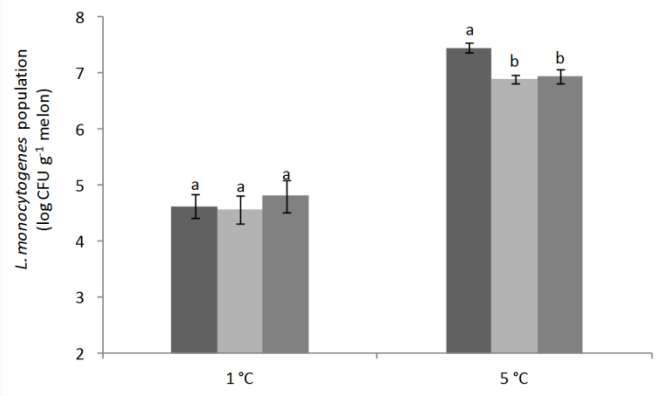
586

Fig. 7

a)


L. monocytogenes population (log CFU g-1 pear)
8
7
6
5
4
3
2
b
ab
a
a
b
b
1 °C
5 °C

b)


L. monocytogenes population (log CFU g-1 melon)
8
7
6
5
4
3
2
a
a
a
a
b
b
1 °C
5 °C

587