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Effectiveness of polymeric coated films containing bacteriocin-producer living bacteria for *Listeria monocytogenes* control under simulated cold chain break

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Abstract

Nisin, enterocin 416K1 and living bacteriocin-producer *Enterococcus casseliflavus* IM 416K1 have been entrapped in polyvinyl alcohol (PVOH) based coatings applied to poly(ethylene terephthalate) (PET) films, and their effectiveness in the control of the growth of *Listeria monocytogenes* ATCC 19117 has been tested**.** The anti-listerial activity of the doped coated films was evaluated by both a modified agar diffusion assay and a direct contact with artificially contaminated precooked chicken fillets stored at 4°C, 22°C and under simulated cold chain break conditions (one day at 30°C).

The live-*Enterococcus*-doped film showed a more remarkable activity than nisin- and enterocin-doped films over long times both at 4°C and 22°C. The use of this film at 22°C resulted in full inactivation of *L. monocytogenes* from the seventh day of the test. Live-*Enterococcus*-doped film displayed a much better antilisterial activity in comparison to nisin- and enterocin-doped films also in samples incubated at 4°C, and submitted at one

27 day (3rd or 7th day) of storage at 30°C, to simulate cold chain break conditions. All results
28 suggest that the live-*Enterococcus*-doped film can behave as a smart active food
29 packaging, very effective in cold chain break conditions when the Listeria growth is fast.

30

31 **Keywords**

32 Antimicrobial coatings; entrapped living bacteria; food preservation; cold chain break;
33 bacteriocins; *L. monocytogenes*

34

35 ## 1. Introduction

36

37 Diseases caused by the consumption of contaminated food represent a significant health
38 problem and economic damage. It has been estimated that about 30% of people in
39 industrialized countries suffer from a foodborne disease each year and at least two million
40 people die from diarrhoeal disease worldwide (WHO, 2007). The economic damage
41 caused by illness due to contaminated meat (poultry, pork, beef, deli and other meats) and
42 produce is $6.65 billion, and $1.44 billion, respectively (Batz et al., 2012). Recent changes
43 in processing technologies and food production seem to have increased the occurrence of
44 food borne infections. In particular, the trend toward consumption of mildly processed
45 refrigerated foods, most attractive for the consumers, arouses concern. Preferences in
46 food consumption are increasingly geared towards fresh-like foods that are ready-to-eat
47 (RTE) or easy to prepare, making minimal processed refrigerated foods the most rapidly
48 growing segments of the food processing industry. On the other hand, this new kind of
49 refrigerated fresh foods presents many safety and quality complications, especially those
50 with extended shelf-life, more susceptible to microbial contamination for the absence of
51 chemical preservatives (Jol et al., 2005; Coulomb, 2008). Food processing that increases

52 the shelf-life of refrigerated foods without including effective barriers to pathogenic and
53 spoilage bacteria, greatly enhances the risk of unsafe or poor quality products, with a
54 consequent commercial damage for loss of food products and possible consequences for
55 the health of consumers. Actually, the most critical microbial problem due to the trend
56 towards the consumption of minimally processed RTE and refrigerated foods is the
57 increase of infectious diseases, caused by psychrotrophic microorganisms, such as
58 *Listeria monocytogenes* (Rocourt and Bille, 1997; Goulet et al., 2008; Jeddi et al., 2014).
59 Outbreaks and sporadic cases of listeriosis have been associated with the contamination
60 of various food items, including milk, soft cheese, meat and meat products, vegetables,
61 seafood products, RTE foods (CAC, 2007), and cantaloupes (Lomonaco et al., 2013), as
62 the ubiquitous nature of the pathogen allows easy access to food products during various
63 phases of production, such as processing, manufacturing and distribution (White et al.,
64 2002). Since refrigeration is one of the most common ways to increase the shelf-life of
65 foods, the ubiquity and the psychrotrophy of *L. monocytogenes* make its control extremely
66 difficult (Gandhi and Chikindas, 2007). As chemical additives are less accepted by the
67 consumers and limited by more restrictive laws, a widely used alternative approach to
68 conventional food preservation methods is the use of natural antimicrobials such as
69 bacteriocins from lactic acid bacteria (LAB). Nisin, the most popular one (Muriana, 1996),
70 is a small heat–stable bacteriocin classified as a lantibiotic (Holzapfel et al., 1995),
71 produced by some strains of *Lactococcus lactis*, active against Gram-positive bacteria,
72 including listeria, and recognized as safe (GRAS) for use as biopreservative in food
73 systems. The anti-listerial activity of nisin has been well studied and applied in a variety of
74 foods, including vegetable, meat and dairy products (Irkin and Esmer, 2015). Other
75 bacteriocins could be of interest as natural anti-listerial compounds. Many enterocins
76 (bacteriocins produced by enterococci), have already demonstrated considerable

77 potentiality for food preservation applied to food products in numerous ways in form of
78 purified or semi-purified extracts (Aymerich et al., 2000; Giraffa, 2003; Ananou et al., 2005;
79 Marcos et al., 2008; Iseppi et al., 2008; Gálvez et al., 2009). In several studies enterococci
80 were tested *in situ* as protective cultures (Sabia et al., 2003; Cocolin et al., 2007; Pingitore
81 et al., 2012; Coelho et al., 2014; Devi et al., 2014; Hassanzadazar et al., 2014; Vandera et
82 al. 2017). In this context food-packaging industries are developing packaging concepts for
83 maintaining food safety and quality, in particular in minimally processed foods. Given that
84 food contamination usually starts at the food surface, a variety of barriers to microbial
85 growth, as the incorporation of additives into packaging systems, has been developed
86 (Suppakul et al., 2003; Kerry e al., 2006; Joerger, 2007; Iseppi et al., 2008; Neetoo et al.,
87 2008; Irkin and Esmer, 2015; Malhotra et al., 2015; Damania et al., 2016). One drawback
88 of this approach is that the antibacterial activity decreases with time due to the progressive
89 depletion of antibacterial additive in the packaging film. A possible way to overcome this
90 problem is the inclusion of living microorganisms able to produce bacteriocins in the film.
91 This approach has been proposed very rarely in the literature (Altieri et al., 2004; Iseppi et
92 al., 2011).
93 The aim of this paper is to extend the previous study on the antibacterial effectiveness of
94 film packaging entrapping living bacteria (Iseppi et al., 2011). In particular, this study
95 compares, *in vitro* and directly on food, the anti-listerial activity of living *Enterococcus*
96 *casseliflavus* IM 416K1 entrapped in PVOH-based coatings applied to PET films to that of
97 commercial nisin and enterocin 416K1 included in similar PVOH-based coatings. In
98 addition, the effect of the break of the cold chain conditions on antibacterial activity of
99 these packaging films has been investigated. For this purpose, food samples (precooked
100 chicken fillets) stored at refrigeration temperatures (4°C) were put at 30°C for 24 h in order
101 to simulate cold chain break conditions.

102

103 **2. Materials and methods**

104

105 2.1. *Bacterial strains*

106 The following microorganisms were used: (i) *Enterococcus casseliflavus* IM 416K1, a
107 bacteriocin (enterocin 416K1) producer isolated from naturally fermented Italian sausages
108 (Sabia et al., 2002), and identified by biochemical (API 50 CHL system, bioMérieux, Marcy
109 l'Etoile, France) and PCR analyses; (ii) *Listeria monocytogenes* ATCC 19117 purchased
110 from American Type Culture Collection (Manassas, VA, USA), used as an artificial
111 contaminant in precooked chicken fillets.

112 *E. casseliflavus* IM 416K1 was cultured in de Man, Rogosa, Sharpe medium (MRS, Oxoid,
113 Milan, Italy) and incubated at 30 °C for 24h. *L. monocytogenes* ATCC 19117 was grown in
114 Tryptic Soy broth or Tryptic Soy agar (TSB or TSA, Difco Laboratories, Detroit, MI), under
115 the same incubation conditions. All strains were maintained at -80°C in the appropriate
116 cultivation broth containing 20% (v/v) glycerol (Merck, Darmstadt, Germany).

117

118 2.2 *Enterocin 416K1 biosynthesis at different temperatures*

119 Sterile flasks containing 250 ml of MRS broth were inoculated with 10 µl of an overnight
120 culture of *E. casseliflavus* IM 416K1, resulting in an initial cell density of about 10^4 CFU/ml,
121 and were incubated at 4°C, 22°C and 30°C. At appropriate intervals (4h, 8h, 12h, 16h, 24h
122 and 48h) samples were removed for the measurement of bacteriocin activity assaying
123 serial twofold dilutions of the purified cell-free supernatant (CFS) by an agar well diffusion
124 assay (Rogers and Montville, 1991) against *L. monocytogenes* ATCC 19117.

125 Enterocin IM 416K1 shows a bactericidal activity against *L. monocytogenes*, as already
126 demonstrated in our previous study (Sabia et al., 2002). CFS was collected by

127 centrifugation (10.000 rpm, for 10 min at 4°C), separated from the cellular pellet, dialyzed
128 against 30 mmol/l sodium acetate buffer (pH 5.3) and filter sterilized (0.45 µm pore-size
129 filter; Millipore Corp., Bedfort, Mass.). The antimicrobial titer of enterocin 416K1 was
130 defined as the reciprocal of the highest dilution producing a distinct inhibition of the
131 indicator lawn and expressed in terms of arbitrary units per millilitre (AU/ml) according to
132 Mayr-Harting et al., (1972).

133 2.3. *Preparation of* E. casseliflavus *IM 416K1, enterocin 416K1 and nisin to be entrapped*
134 *in the coating applied to the PET films*

135 The antibacterial products to be entrapped in the coating applied to the PET films were
136 prepared as described below:

137 i) Enterocin 416K1 from an overnight culture at 30°C in MRS broth of *E. casseliflavus* IM
138 416K1 was collected and treated as previously described.

139 ii) The pellet of *E. casseliflavus* IM 416K1, washed twice with sterile Ringer's solution, was
140 maintained at refrigeration temperature and added to 5 ml of fresh MRS broth just before
141 the coating preparation.

142 iii) 6,6 mg of nisin powder (kindly supplied by Handary; Nisin Ap, > 38000 IU/mg, Handary,
143 Bruxelles) was placed in a graduated cylinder and 0.02 M hydrochloric acid was added to
144 the 100 ml mark obtaining a nisin concentration of 2500 IU/ml (Neetoo et al., 2008). The
145 antibacterial activity of the nisin solution was evaluated by an agar well diffusion assay as
146 previously reported for enterocin 416K1.

147

148 2.4. *Preparation and application of coatings to PET substrate*

149 Poly(ethylene terephthalate) thin films (PET, 80 µm thick; Enhance 80 Laminating
150 Pouches, Fellowes Leonardi Spa, Italy) were used as polymer substrate for coatings. In
151 order to avoid any surface contamination, PET films were washed with methanol and

152 accurately dried just before coating application. Partially hydrolyzed polyvinyl alcohol
153 (PVOH, Mowiol 4-88, Mw ≈ 31000 g mol^{-1}, 86.7–88.7 mol% hydrolysis), 3-
154 (triethoxysilyl)propyl isocyanate (ICPTES, 95%), glacial acetic acid, potassium acetate and
155 diethyl ether were supplied by Sigma-Aldrich (Milano, Italy) and used as received without
156 further purification.

157 In order to allow crosslinking of polyvinyl alcohol (PVOH) under mild conditions (after
158 application of the coating to the PET substrate), commercial PVOH was chemically
159 modified by replacing a limited fraction (about 5%) of –OH groups with trialkoxysilane
160 groups (PVOH-Si). For this purpose PVOH was dissolved in N,N-dimethylformamide and
161 reacted for 1 hour at 50°C with ICPTES in a molar ratio of about 1:20 with respect to
162 monomeric units of the polymer. The resulting triethoxysilane functionalized polymer
163 (PVOH-Si) was recovered by precipitation in diethyl ether and then dried at 80°C. The
164 details of the synthesis and the characterizations of the functionalized polymer are
165 reported in a previous paper (Iseppi et al. 2011).

166 The preparation of the aqueous coating solutions was carried out as follows: PVOH-Si (2
167 g) was dissolved in bidistilled water (24 ml) and then a CH_3COOH/CH_3COOK buffer
168 solution (6 ml, pH 4.5) was added as catalyst. The mixture was then added with 2 ml of *E.*
169 *casseliflavus* IM 416K1 in MRS broth (5 x 10^9 CFU ml^{-1}) under stirring just before
170 application of the solution to the PET substrate. The same procedure was used to prepare
171 films coated with PVOH-Si alone (undoped, used as negative control) and PVOH-Si
172 entrapping enterocin 416K1 or nisin (by adding 2 ml of deionised water or 2 ml of dialyzed
173 CFS or 2 ml of nisin solution/suspension to the PVOH-Si solution). Coated films have been
174 prepared by applying PVOH-Si aqueous solutions (alone or added with *E. casseliflavus* IM
175 416K1 bacteriocin-producer bacteria, enterocin 416K1 or nisin) onto PET films (120 cm^2)
176 using a roll-coater (K Hand Coater, R K Print Coat Instruments Ltd.) with a calibrated wire-

177 wound applicator (bar number 4). The water was allowed to evaporate at room
178 temperature overnight. During water evaporation hydrolysis and condensation reactions of
179 silica alkoxide occurred, as attested by extraction tests performed on the coating after
180 crosslinking. A quite homogeneous distribution of *E. casseliflavus* IM 416K1 within the
181 matrix was confirmed using microscopy techniques (Scanning Electron Microscope
182 Quanta-Fei 200 in ESEM mode). SEM microscopy was also used to obtain information
183 about the thickness of the coatings.
184 The coated films prepared for the test were 120 cm^2 surface area with a uniform single-
185 layer coating about 10 µm thick. They were transparent and flexible, therefore particularly
186 suitable for food packaging applications. The prepared PVOH coating applied to the PET
187 substrate is not fragile and shows a fairly good adhesion.
188

189 ### 2.5. *Antibacterial activity evaluation of the doped coated films*

190 The antibacterial activity was evaluated against *L. monocytogenes* ATCC 19117 using two
191 different methods: (i) qualitative evaluation by a modified agar diffusion assay, (ii)
192 quantitative evaluation (*L. monocytogenes* viable counts) in artificially contaminated
193 precooked chicken fillets packaged with doped and undoped coated films.
194 For the qualitative evaluation 2 x 2 cm^2 samples of live-*Enterococcus*, enterocin and nisin
195 doped films were placed onto TSA plates seeded with 10^7 CFU of an overnight culture of
196 *L. monocytogenes* ATCC 19117. Undoped coated films were also tested as negative
197 control. The plates were incubated at 30°C and 22°C for 24h and at 4°C for 5 days. The
198 antagonistic activity was evidenced by a clear zone of inhibition in the indicator lawn
199 around the coated film.
200 The quantitative evaluation of the antimicrobial coating effectiveness was determined in
201 samples of precooked and not dressed chicken fillets, typically used for chicken salad

202 preparations, purchased from a supermarket on the first day of shelf-life (14 days). The
203 microbial contamination of the precooked chicken fillets was determined on 10 g of this
204 product on the same day of purchasing. The samples showed a microbial load of 30
205 CFU/g. No growth was observed on MacConkey agar plates (Oxoid). On the same day, a
206 pH value of 6.5 was measured on the surface of the chicken fillets. The samples were
207 surface contaminated by a 5 min immersion in a 10^8 CFU/ml suspension of an overnight
208 culture of *L. monocytogenes* ATCC 19117 diluted in sterile saline solution (NaCl 0.85%)
209 that resulted, after removing the excess fluid, in a final absorption of about 10^4 CFU/g in
210 food samples. Portions of samples (25 g) were singly packaged in doped and undoped
211 films. Food samples were wrapped with the PET coated films, with the PVOH-Si layer in
212 contact with the food. In order to obtain a good contact between food and films, a
213 particular care was used during the wrapping step. Finally, the ends of the wrapped films
214 were tightly closed with appropriate clips. The samples were stored at room and
215 refrigeration temperature (22°C and 4°C). At regular intervals (0, 1, 3, 4, 7, 10 and 14
216 days) the food samples were unwrapped and placed in sterile plastic bag. A particular care
217 was used to avoid to damage the coating and to remove parts of it during the separation of
218 the coated films from foods. At visual inspection, no coating fragments were removed from
219 the PET coated film after it was peeled off from the food. Subsequently, 225 ml of buffered
220 peptone water (Oxoid) were added to the unwrapped food and the samples were
221 homogenized for 1 min in a laboratory blender (Stomacher Lab Blender, Seward Medical,
222 London, UK). Serial tenfold dilutions of the obtained suspensions were spread in triplicate
223 on Palcam agar (Oxoid) and plates were incubated aerobically at 37°C for 48h. In all
224 negative samples the residual homogenates were filtered (0.45 μm pore-size filter;
225 Millipore Corp., Bedford, MA) to recover the uncounted listeria. Colonies of *L.*
226 *monocytogenes* ATCC 19117 were enumerated and results expressed as log CFU/g.

227

228 2.6. *Antibacterial activity evaluation of the doped coated films after cold chain break*

229 *simulation*

230 During the shelf-life period (14 days), the samples of chicken fillets stored at refrigerated
231 temperature (4°C) were submitted to a temperature abuse simulating a cold chain break.
232 The simulation was carried out incubating the samples at 30°C for 24h on the third or the
233 seventh day of storage and then restoring the pre-existing conditions. At selected times
234 the samples were assessed for *L. monocytogenes* ATCC 19117 counts as described
235 above.

236 All experiments were carried out in triplicate. The means, expressed as log bacterial count,
237 were plotted against the incubation time (days) and the standard deviation was reported as
238 error bars. The rates of decline of *L. monocytogenes* ATCC 19117 were analyzed with a t-
239 test for paired data. The statistical probability equal to or less than 0.05 was considered
240 significant.

241

242 **3. Results**

243

244 3.1. *Enterocin 416K1 biosynthesis at different temperatures and antimicrobial titer of nisin*

245 *solution*

246 *E. casseliflavus* IM 416K1 shows a good growth at 22°C and 30°C. At both temperatures
247 the microorganism started to produce bacteriocin with inhibitory activity against *L.*
248 *monocytogenes* ATCC 19117 already at the 4th hour (320 AU/ml) and after 12h of
249 incubation the inhibitory activity was 2560 AU/ml. The maximum activity of enterocin
250 416K1 has been recorded (3200 AU/ml) after 16h of incubation and this value remained
251 constant until the end of the experiment (48h). At refrigeration temperature (4°C), due to

252 slower growth of *E. casseliflavus*, we detected a lower inhibitory activity (1280 AU/ml) at
253 the end of experiment (48h).
254 The antimicrobial titer of nisin solution (2500 IU/ml) exhibited by agar well diffusion assay
255 against *L. monocytogenes* ATCC 19117 was found to be 2560 AU/ml.
256

257 3.2. *Features of the coated films*

258 Polyvinyl alcohol (PVOH) is a hydrophilic polymer which can be swelled/dissolved by
259 water; this specific characteristic of PVOH can allow to create a friendly environment for
260 the survival of bacteria and for this reason it has already been used to entrap living
261 microorganisms (Lozinsky, and Plieva, 1998; Doria-Serrano et al., 2001; Szczesna and
262 Galas, 2001; Liu et al., 2009).
263 In order to entrap living bacteria, the hydrophilic polymer has been first modified by
264 replacing some –OH groups of PVOH (about 5 %) with alkoxysilane groups (PVOH-Si)
265 and then crosslinked in an aqueous solution containing bacterial nutrients (MRS broth) by
266 exploiting the mild sol-gel chemistry. Details and reaction scheme are reported in a
267 previous paper (Iseppi et al. 2011).
268 A thin layer of PVOH-Si sol-gel solution was applied to PET films by roll-coating deposition
269 (before crosslinking of alkoxysilane groups) and allowed to react at room temperature
270 overnight in order to achieve crosslinking. At the end of this process the PET film substrate
271 is coated with a PVOH-Si crosslinked layer (about 10 μm thick) swollen by a water-MRS
272 broth solution, with a gel fraction of about 84% and a swelling ratio in water of 56% (Iseppi
273 et al. 2011). This crosslinked hydrophilic layer show good mechanical properties (not
274 brittle) and a fairly good adhesion to the PET substrate (no evidence of coating
275 detachment was observed by SEM, even after repeated manual bendings of the coated
276 film). Interestingly, no evidence of big cracks or coating-fragment detachments was

277 observed in the coated films recovered after unwrapping food samples. Accordingly, a
278 possible release/migration of live cells from the coating to the food surface can be
279 reasonably excluded. Finally, it has to be emphasized that the presence of water and
280 nutrients within the crosslinked hydrophilic layer led to an environment suitable for the cell
281 surviving/feeding and for an easy diffusion and release of antibacterial substances such as
282 bacteriocins.
283

284 ### 3.3. *Qualitative antibacterial activity evaluation by agar diffusion assay*

285 Figure 1 shows, as an example, the qualitative antibacterial activity evaluation at 4°C of
286 the doped coated films against *L. monocytogenes* ATCC 19117 by direct contact in plate.
287 The activity is revealed in the doped coated films (A,B,C) by a clear inhibition zone in the
288 indicator lawn on the growth of the pathogen around the fragments tested. Indeed, in Fig.
289 1, inhibition clear zones (three/four mm wide) are evident around the 2 x 2 cm^2 A, B and C
290 film samples, whereas no activity against *L. monocytogenes* ATCC 19117 is present
291 around the negative control undoped coated film (D). Similar tests were performed at 30°C
292 with similar results. While there were no apparent differences between nisin and enterocin-
293 doped films, a slightly higher activity was observed for the live-*Enterococcus*-doped films
294 at all temperatures tested and in particular at 30°C.
295 These results demonstrate that the treatment used for the entrapment of living bacteria
296 and for the incorporation of enterocin 416K1 and nisin in PVOH-based coatings applied to
297 plastic is compatible with the viability of the microorganisms and do not affect the anti-
298 listerial activity of the two bacteriocins. Furthermore, they suggest that antibacterial
299 products are released in the indicator lawn for all the doped films.
300

301 ### 3.4. *Quantitative antibacterial activity evaluation of the doped coated films*

302 Figs. 2(a, b) show the mean values of *L. monocytogenes* ATCC 19117 counts (log CFU/g)
303 detected in artificially contaminated chicken fillets samples wrapped with doped coated
304 films during storage at 4°C (Fig 2a) and 22°C (Fig 2b). The results observed for undoped
305 coated films are also reported in the same figures for comparison. At both temperatures
306 the undoped film was not able to inhibit the *L. monocytogenes* growth. When the chicken
307 fillets were stored at 4°C (Fig. 2a), in samples packaged with undoped coated films *L.*
308 *monocytogenes* counts increase up to more than 8 log CFU/g to the tenth day and 9 log
309 CFU/g at the end of the trial (14th day). In comparison, samples packed with nisin- and
310 enterocin-doped films show a gap of about 2 and 2.5 log CFU/g, respectively, almost
311 constant until the end of the experiment ($p<0,01$). In samples packaged with live-
312 *Enterococcus*-doped films, the behavior is similar to that observed for nisin- and enterocin-
313 doped films up to the 7th day; however a better antibacterial activity was observed in the
314 last part of the test. The difference with respect to the undoped films is of about 4 log
315 CFU/g from the 10th to the 14th day ($p<0,01$). Of course, this means that there is a
316 significant decrease of *L. monocytogenes* counts also with respect to samples packaged
317 with nisin or enterocin-doped films.
318 Samples packaged with undoped films stored at 22°C (Fig. 2b), show a rapid increase in
319 listeria viable counts, ranging from about 7.5 log CFU/g, after four days, to nearly 10 log
320 CFU/g at the end of the test (14th day). Compared to undoped control, samples packaged
321 with nisin-doped film show 1 log CFU/g reduction after four days and 1,5 log CFU/g at the
322 end of the experiment ($p< 0,05$). Compared to control, samples packaged with enterocin-
323 doped film show a delay of the *L. monocytogenes* growth with a reduction of 1,4 log viable
324 counts at the 4th day, value that reached 2.3 log CFU/g at the end of test ($p<0,05$). A
325 significantly different behaviour was observed for samples packaged with live-
326 *Enterococcus*-doped films. After 4 days a reduction in listeria counts of about 5 log, 4 log

327 and 3.5 log CFU/g was observed compared to control, nisin-doped and enterocin-doped
328 films, respectively. This gap increases as the test time increases, until the total
329 disappearance of *L. monocytogenes* from the 7th day. The total killing of listeria in samples
330 packaged with live-*Enterococcus*-doped film is confirmed by lack of colonies on Palcam
331 agar after filtration of the residual food homogenate.
332 A further interesting information concerns some features of samples. At a visual
333 inspection, samples packaged with the antibacterial films result less deteriorated and show
334 a minor spoilage compared to the undoped control and some organoleptic properties
335 (texture, color and odor), were found to be better with a particularly relevant difference for
336 samples packaged with the live-*Enterococcus* doped films stored at 22°C. These
337 observations suggest that the doped plastic films are able to interact with the growth of
338 spoilage microorganisms, in addition to their capability to inhibit *L. monocytogenes* ATCC
339 19117 growth.
340

341 3.5. *Antibacterial activity evaluation of the doped coated films under simulated cold chain*
342 *break conditions*

343 In order to investigate the effects of cold chain breaking, chicken fillets samples stored at
344 refrigerated temperatures (4°C) were incubated at 30°C for 24h on the third or on the
345 seventh day. The average values of the *L. monocytogenes* ATCC 19117 counts (log
346 CFU/g) are reported in Figs. 3(a, b).
347 Incubation for 24 h at 30°C on the third day induces a sharp increase of *L. monocytogenes*
348 ATCC 19117 counts (about 2 log) in all samples, wrapped both in doped and undoped
349 films. During the following days of incubation at 4°C there is a progressive increase of *L.*
350 *monocytogenes* ATCC 19117 counts for samples wrapped in undoped and nisin- and
351 enterocin-doped films. In contrast, a significant reduction of *L. monocytogenes* ATCC

19117 counts is observed for samples wrapped with the live-*Enterococcus*-doped film, with a significant difference at the end of the trial of 6 log CFU/g compared to control and more than 4 log CFU/g compared to the samples packaged with the bacteriocins doped films ($p<0,01$).

The results of cold chain breaking occurring at the 7th day are shown in Fig. 3b. It can be observed that, after the simulated cold chain break, at the 7th day, the control sample and those packaged with nisin- and enterocin-doped films showed an increase in listeria viable counts (at the end of the test the difference among these samples reduce to less than 1 log CFU/g).

According to our data, when the cold chain break simulation occurs closer to the end of shelf-life, the quality and food safety control results more critical. In particular the action of the nisin and enterocin-doped films is insufficient to inhibit Listeria growth (probably the films partially lose their activity).

On the contrary, in samples packaged with live-*Enterococcus*-doped films we observed a remarkable decrease in listeria counts that reach a gap of 4.5 log CFU/g compared to control and about 3.5 log CFU/g compared to samples packaged with nisin- and enterocin-doped films ($p<0,01$) at the end of the trial.

The progressive decrease of *L. monocytogenes* ATCC 19117 counts when the live-*Enterococcus*-doped film was used to wrap the contaminated chicken fillets, means that *E. casseliflavus* IM 416K1 is able to produce enterocin continuously and at a relatively fast rate.

This outcome suggests also that live-*Enterococcus*-doped films are responsive to temperature and able to contrast a cold-chain break during food storage.

4. Discussion

377

378 In the last few years, fresh or minimally processed products have been included in the
379 trend of the industrialized Western food model, mainly because of changes in the human
380 lifestyle and their tendency towards convenience and spending less time on preparing food
381 (Ragaert et al., 2007; Abadias et al., 2008; Gómez-Govea et al., 2012). The shelf-life of
382 these foods is generally shorter than previously, to avoid product quality decay and
383 increase in microbial contamination. Refrigeration is the most used way to prevent or delay
384 the growth of spoilage and pathogenic microorganisms and to extend the shelf-life of RTE
385 foods. Anyway if during the food production a temperature abuse occurs, unpredicted
386 growth of microbial contamination may occur. Although the processes related to the food
387 industry are widely regulated by a series of practices (European Standard EC 2073/2005;
388 Gómez-Govea et al., 2012), their application doesn't guarantee the absence of breakage
389 in the cold chain of food production (Rodríguez et al., 2011). For example, it is reported
390 that a slight abuse of temperature of refrigerated RTE foods increases greatly the risk that
391 *L. monocytogenes*, will grow to a level that could cause human disease (Kotzekidou 2013).
392 In this context we have developed and tested an active food packaging, which has on the
393 surface a thin coating containing living enterocin-producer bacteria, that, according to our
394 previous results (Iseppi et al., 2011) should be able to delay or inhibit the bacterial growth
395 when a cold chain break occurs. The anti-listerial activity of these doped films was
396 compared with that of films doped with bacteriocins, nisin and enterocin 416K1 and with an
397 undoped film, as a negative control.
398 The qualitative preliminary tests (Fig 1) show that all the doped films are able to release
399 antibacterial products to the surrounding culture substrate. Therefore it is expected that
400 these doped films are able to release antibacterial products also when in contact with food.
401 To demonstrate their effectiveness, contaminated chicken food was packaged in undoped

402 and differently doped films and the quantitative effects of their antibacterial activity
403 evaluated under different storage conditions.
404 As shown in Figure 2a, at 4°C the antibacterial behavior of all doped films is better than
405 that of undoped film (about 2 log CFU/g) and effective to reduce the *L. monocytogenes*
406 contamination up to 2-3 days. In addition, for all doped films it can be observed that after 3
407 days, the *L. monocytogenes* contamination start to grow and become higher than the initial
408 contamination. Of course this behavior can be easily explained as a consequence of a
409 progressive reduction of the amount of antimicrobial bacteriocins release from the films. In
410 the first days, when the amount of antimicrobial products in the films is the highest the
411 release is fast, however as a consequence of the progressive decrease of the amount of
412 antibacterial products in the films their release becomes slower and slower. While for
413 undoped and bacteriocin-doped films the growth of *L. monocytogenes* contamination was
414 continuous up to the end of the experiment, it has to be noticed that after seven days the
415 *L. monocytogenes* contamination does not increase anymore in films doped with living
416 bacteria. This behavior suggests that at 4°C living bacteria are able to slowly produce
417 enterocin, whose concentration does not decrease within the film and whose release is
418 able to contrast the growth of *L. monocytogenes* contamination.
419 However, the more astonishing difference between films doped with living bacteria and the
420 other doped films is that recoded at 22°C. The increase of *L. monocytogenes*
421 contamination after one day means that at 22°C *L. monocytogenes* grows faster than its
422 killing rate due to the release of antibacterial products. While an increase of *L.*
423 *monocytogenes* contamination continues till to the end of the experiment for undoped and
424 bacteriocin-doped films, in the case of films containing living bacteria, the contamination
425 start to decrease after few days and *L. monocytogenes* counts is reduced to zero after
426 seven days. In our opinion, the most probable way to explain these results is to assume

427 that living bacteria are able to produce enterocin, and that the rate of production of
428 enterocin grows faster than the growth rate of *L. monocytogenes*. Probably also the pH
429 decase due to the growth of *E. casseliflavus*, added in MRS broth to the coating, could be
430 a further element for the full inactivation of *L. monocytogenes* (Sabia et al., 2003). .These
431 results suggest that an accidental increase in storage temperature, and thereby, an
432 increased growth of *L. monocytogenes*, would be counteracted by the use of packaging
433 films containing living bacteria. To verify this hypothesis, in a second step of this study the
434 anti-listerial activity of the three different doped-films was evaluated by cold chain break
435 simulation (at 30°C for 24 h) on the 3th or 7th day of incubation.
436 The results of Figures 3a and 3b show that while the cold-chain breaking is deleterious for
437 undoped and bacteriocin-doped films, it is less dangerous when food is wrapped with live-
438 *Enterococcus*-doped films. In this latter case, after 24 h at 30°C, the *L. monocytogenes*
439 counts start to decrease progressively confirming that this kind of antibacterial film is very
440 effective to contrast bacterial growth.
441 All these results extend and confirm our previous results obtained in a study of the anti-
442 listerial effect of films containing living bacteria on contaminated wurstel and seasoned
443 cheese samples (Iseppi et al., 2011).
444 In conclusion, this study shows that it is possible to entrap live *Enterococcus casseliflavus*
445 IM 416K1 bacteria in PVOH-based coatings. The entrapped cells are able to survive and
446 to produce anti-listerial products over long time, making this doped-film a very efficient
447 preservative system. To our knowledge this innovative approach has never been proposed
448 by other authors.
449 Finally, as suggested by the results of anti-listerial activity at 22°C (Figure 2b), this study
450 seems to open a new approach to food preservation. In fact, the use of live bacteriocin

451 producer strains doped films could not require refrigeration to prevent spoilage and
452 bacterial growth.
453

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455

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598 **Figure captions**

599

600 Fig. 1. Antibacterial activity evaluation by agar diffusion assay of the doped coated films
601 against *L. monocytogenes* ATCC 19117 after 5 days incubation at 4°C: (A): live-
602 *Enterococcus*-doped film; (B): enterocin-doped film; (C): nisin-doped film; (D): undoped-
603 coated film (control). The antagonistic activity was revealed by a clear zone of inhibition
604 (three/four mm wide) in the indicator lawn around the 2 x 2 cm^2 A, B and C film samples,
605 whereas no activity against *L. monocytogenes* ATCC 19117 is present around the
606 negative control undoped coated film (D).

607

608 Fig. 2(a, b). *L. monocytogenes* ATCC 19117 viable counts (log CFU/g) observed in the
609 contaminated chicken fillets samples packaged in live-*Enterococcus*-doped film [●],
610 enterocin-doped film [▲], nisin-doped film [♦] and undoped-coated film [■], stored at 4°C
611 (a) and at 22°C (b). Error bars represent standard deviations.

612

613 Fig. 3(a, b). *L. monocytogenes* ATCC 19117 viable counts (log CFU/g) observed in the
614 contaminated chicken fillets samples packaged in live-*Enterococcus*-doped film [●],
615 enterocin-doped film [▲], nisin-doped film [♦] and undoped-coated film [■] stored at 4°C
616 and subjected to simulated cold chain break conditions on the 3th (a) and 7th (b) day. Error
617 bars represent standard deviations.

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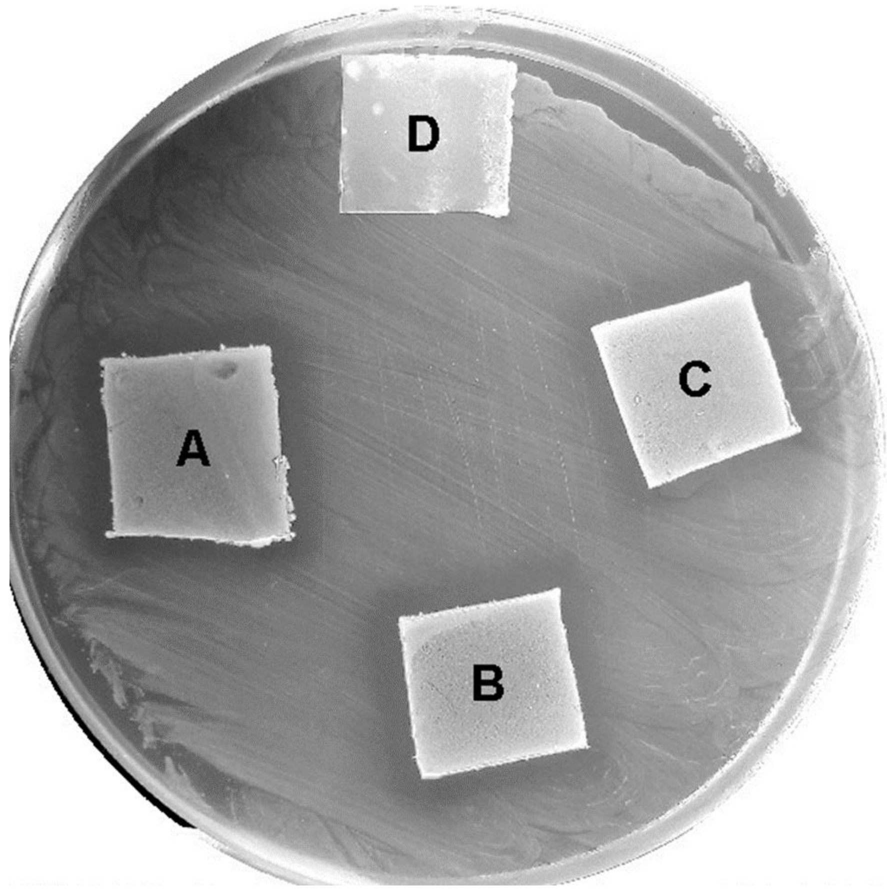

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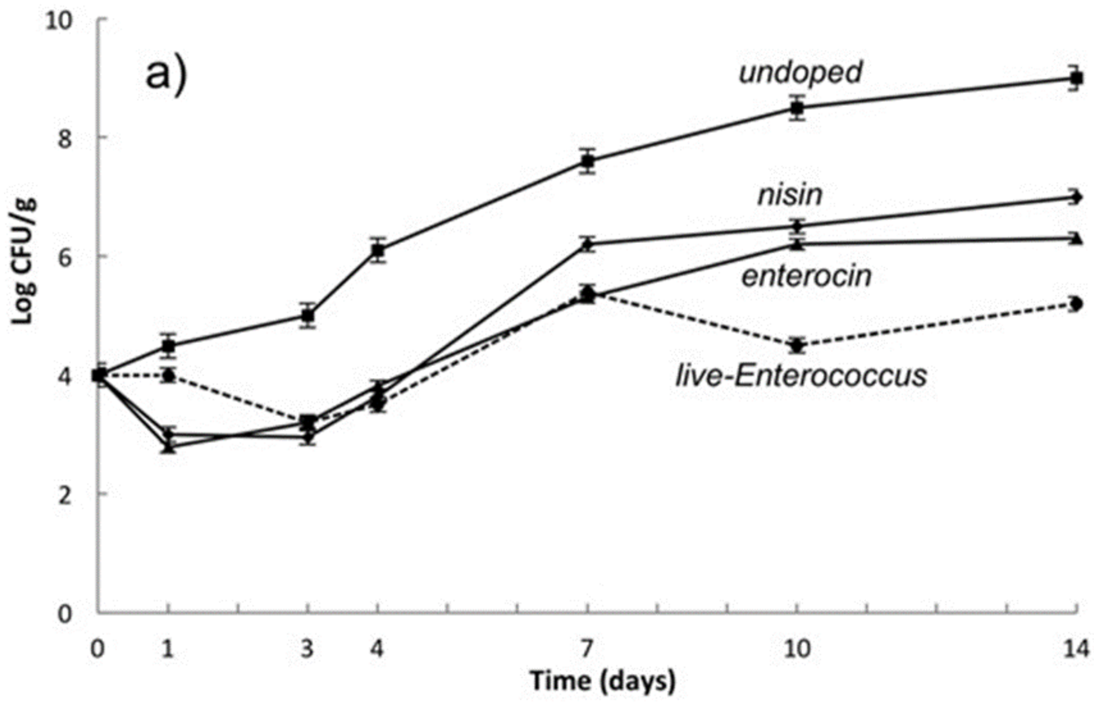

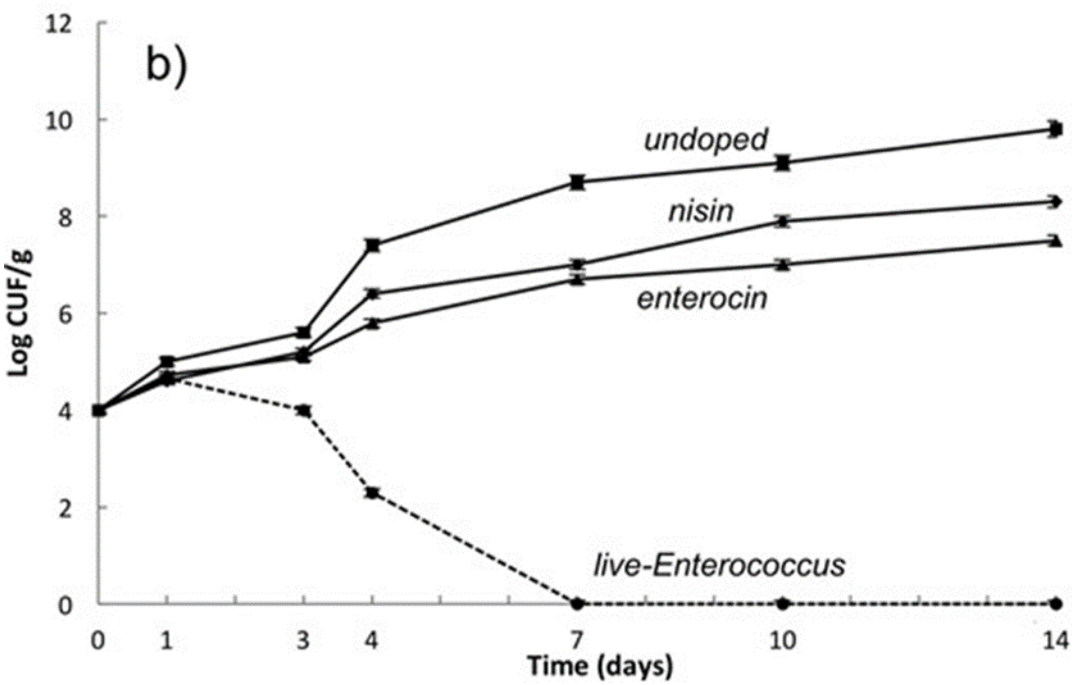

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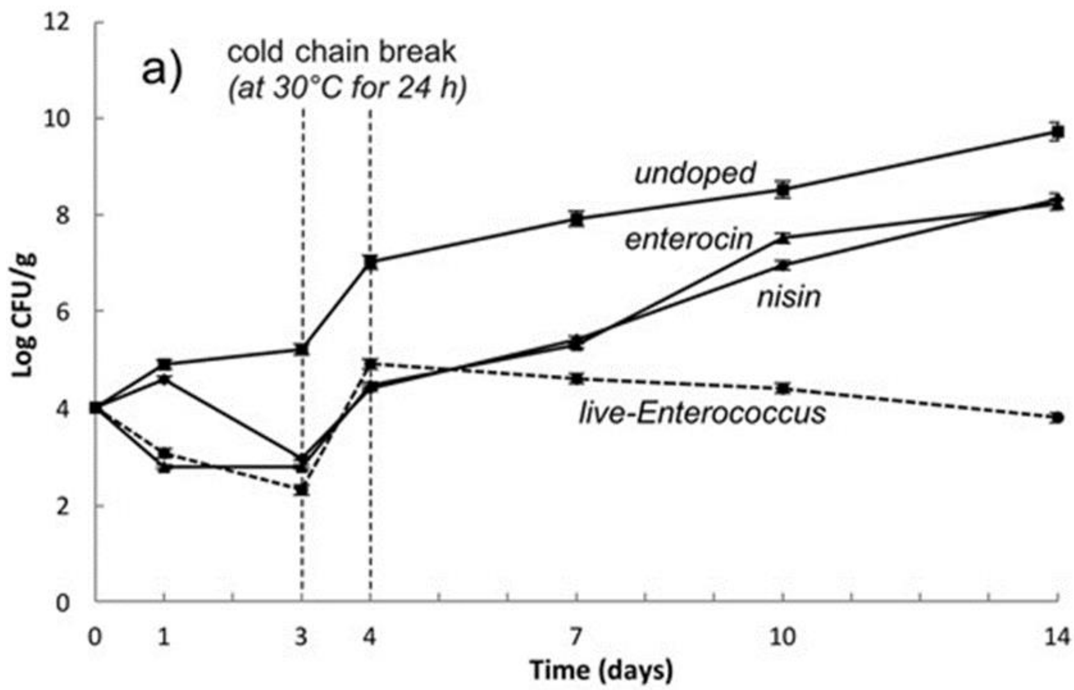

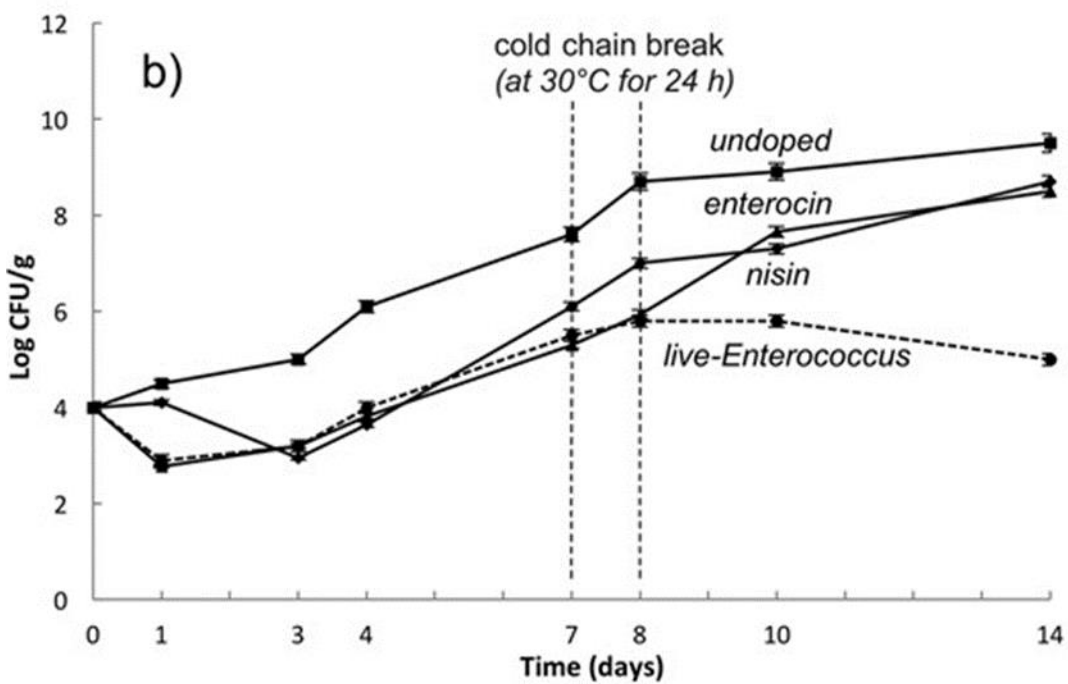

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