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

1. Introduction

Diseases caused by the consumption of contaminated food represent a significant health problem and economic damage. It has been estimated that about 30% of people in industrialized countries suffer from a foodborne disease each year and at least two million people die from diarrhoeal disease worldwide (WHO, 2007). The economic damage caused by illness due to contaminated meat (poultry, pork, beef, deli and other meats) and produce is $6.65 billion, and $1.44 billion, respectively (Batz et al., 2012). Recent changes in processing technologies and food production seem to have increased the occurrence of food borne infections. In particular, the trend toward consumption of mildly processed refrigerated foods, most attractive for the consumers, arouses concern. Preferences in food consumption are increasingly geared towards fresh-like foods that are ready-to-eat (RTE) or easy to prepare, making minimal processed refrigerated foods the most rapidly growing segments of the food processing industry. On the other hand, this new kind of refrigerated fresh foods presents many safety and quality complications, especially those with extended shelf-life, more susceptible to microbial contamination for the absence of chemical preservatives (Jol et al., 2005; Coulomb, 2008). Food processing that increases the shelf-life of refrigerated foods without including effective barriers to pathogenic and spoilage bacteria, greatly enhances the risk of unsafe or poor quality products, with a consequent commercial damage for loss of food products and possible consequences for the health of consumers. Actually, the most critical microbial problem due to the trend towards the consumption of minimally processed RTE and refrigerated foods is the increase of infectious diseases, caused by psychrotrophic microorganisms, such as *Listeria monocytogenes* (Rocourt and Bille, 1997; Goulet et al., 2008; Jeddi et al., 2014). Outbreaks and sporadic cases of listeriosis have been associated with the contamination of various food items, including milk, soft cheese, meat and meat products, vegetables, seafood products, RTE foods (CAC, 2007), and cantaloupes (Lomonaco et al., 2013), as the ubiquitous nature of the pathogen allows easy access to food products during various phases of production, such as processing, manufacturing and distribution (White

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et al., 2002). Since refrigeration is one of the most common ways to increase the shelf-life of foods, the ubiquity and the psychrotrophy of *L. monocytogenes* make its control extremely difficult (Gandhi and Chikindas, 2007). As chemical additives are less accepted by the consumers and limited by more restrictive laws, a widely used alternative approach to conventional food preservation methods is the use of natural antimicrobials such as bacteriocins from lactic acid bacteria (LAB). Nisin, the most popular one (Muriana, 1996), is a small heat–stable bacteriocin classified as a lantibiotic (Holzapfel et al., 1995), produced by some strains of *Lactococcus lactis*, active against Gram-positive bacteria, including listeria, and recognized as safe (GRAS) for use as biopreservative in food systems. The anti-listerial activity of nisin has been well studied and applied in a variety of foods, including vegetable, meat and dairy products (Irkin and Esmer, 2015). Other bacteriocins could be of interest as natural anti-listerial compounds. Many enterocins (bacteriocins produced by enterococci), have already demonstrated considerable potentiality for food preservation applied to food products in numerous ways in form of purified or semi-purified extracts (Aymerich et al., 2000; Giraffa, 2003; Ananou et al., 2005; Marcos et al., 2008; Iseppi et al., 2008; Gálvez et al., 2009). In several studies enterococci were tested *in situ* as protective cultures (Sabia et al., 2003; Cocolin et al., 2007; Pingitore et al., 2012; Coelho et al., 2014; Devi et al., 2014; Hassanzadazar et al., 2014; Vandera et al., 2017). In this context food-packaging industries are developing packaging concepts for maintaining food safety and quality, in particular in minimally processed foods. Given that food contamination usually starts at the food surface, a variety of barriers to microbial growth, as the incorporation of additives into packaging systems, has been developed (Suppakul et al., 2003; Kerry e al., 2006; Joerger, 2007; Iseppi et al., 2008; Neetoo et al., 2008; Irkin and Esmer, 2015; Malhotra et al., 2015; Damania et al., 2016). One drawback of this approach is that the antibacterial activity decreases with time due to the progressive depletion of antibacterial additive in the packaging film. A possible way to overcome this problem is the inclusion of living microorganisms able to produce bacteriocins in the film. This approach has been proposed very rarely in the literature (Altieri et al., 2004; Iseppi et al., 2011).

The aim of this paper is to extend the previous study on the antibacterial effectiveness of film packaging entrapping living bacteria (Iseppi et al., 2011). In particular, this study compares, *in vitro* and directly on food, the anti-listerial activity of living *Enterococcus casseliflavus* IM 416K1 entrapped in PVOH-based coatings applied to PET films to that of commercial nisin and enterocin 416K1 included in similar PVOH-based coatings. In addition, the effect of the break of the cold chain conditions on antibacterial activity of these packaging films has been investigated. For this purpose, food samples (precooked chicken fillets) stored at refrigeration temperatures (4 °C) were put at 30 °C for 24 h in order to. simulate cold chain break conditions.

2. Materials and methods

2.1. Bacterial strains

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

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

2.2. Enterocin 416K1 biosynthesis at different temperatures

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

Enterocin IM 416K1 shows a bactericidal activity against *L. monocytogenes*, as already demonstrated in our previous study (Sabia et al., 2002). CFS was collected by centrifugation (10.000 rpm, for 10 min at 4 °C), separated from the cellular pellet, dialyzed against 30 mmol/l sodium acetate buffer (pH 5.3) and filter sterilized (0.45 µm pore-size filter; Millipore Corp., Bedfort, Mass.). The antimicrobial titer of enterocin 416K1 was defined as the reciprocal of the highest dilution producing a distinct inhibition of the indicator lawn and expressed in terms of arbitrary units per millilitre (AU/ml) according to Mayr-Harting et al. (1972).

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

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

i) Enterocin 416K1 from an overnight culture at 30 °C in MRS broth of *E. casseliflavus* IM 416K1 was collected and treated as previously described.
ii) The pellet of *E. casseliflavus* IM 416K1, washed twice with sterile Ringer's solution, was maintained at refrigeration temperature and added to 5 ml of fresh MRS broth just before the coating preparation.
iii) 6,6 mg of nisin powder (kindly supplied by Handary; Nisin Ap, > 38,000 IU/mg, Handary, Bruxelles) was placed in a graduated cylinder and 0.02 M hydrochloric acid was added to the 100 ml mark obtaining a nisin concentration of 2500 IU/ml (Neetoo et al., 2008). The antibacterial activity of the nisin solution was evaluated by an agar well diffusion assay as previously reported for enterocin 416K1.

2.4. Preparation and application of coatings to PET substrate

Poly (ethylene terephthalate) thin films (PET, 80 µm thick; Enhance 80 Laminating Pouches, Fellowes Leonardi Spa, Italy) were used as polymer substrate for coatings. In order to avoid any surface contamination, PET films were washed with methanol and accurately dried just before coating application. Partially hydrolyzed polyvinyl alcohol (PVOH, Mowiol 4–88, Mw ≈ 31,000 g mol^{-1}, 86.7–88.7 mol% hydrolysis), 3-(triethoxysilyl)propyl isocyanate (ICPTES, 95%), glacial acetic acid, potassium acetate and diethyl ether were supplied by Sigma-Aldrich (Milano, Italy) and used as received without further purification.

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

The preparation of the aqueous coating solutions was carried out as

follows: PVOH-Si (2 g) was dissolved in bidistilled water (24 ml) and then a CH_3COOH/CH_3COOK buffer solution (6 ml, pH 4.5) was added as catalyst. The mixture was then added with 2 ml of *E. casseliflavus* IM 416K1 in MRS broth (5×10^9 CFU ml^{-1}) under stirring just before application of the solution to the PET substrate. The same procedure was used to prepare films coated with PVOH-Si alone (undoped, used as negative control) and PVOH-Si entrapping enterocin 416K1 or nisin (by adding 2 ml of deionised water or 2 ml of dialyzed CFS or 2 ml of nisin solution/suspension to the PVOH-Si solution). Coated films have been prepared by applying PVOH-Si aqueous solutions (alone or added with *E. casseliflavus* IM 416K1 bacteriocin-producer bacteria, enterocin 416K1 or nisin) onto PET films (120 cm^2) using a roll-coater (K Hand Coater, R K Print Coat Instruments Ltd.) with a calibrated wire-wound applicator (bar number 4). The water was allowed to evaporate at room temperature overnight. During water evaporation hydrolysis and condensation reactions of silica alkoxide occurred, as attested by extraction tests performed on the coating after crosslinking. A quite homogeneous distribution of *E. casseliflavus* IM 416K1 within the matrix was confirmed using microscopy techniques (Scanning Electron Microscope Quanta-Fei 200 in ESEM mode). SEM microscopy was also used to obtain information about the thickness of the coatings.

The coated films prepared for the test were 120 cm^2 surface area with a uniform single-layer coating about 10 μm thick. They were transparent and flexible, therefore particularly suitable for food packaging applications. The prepared PVOH coating applied to the PET substrate is not fragile and shows a fairly good adhesion.

2.5. Antibacterial activity evaluation of the doped coated films

The antibacterial activity was evaluated against *L. monocytogenes* ATCC 19117 using two different methods: (i) qualitative evaluation by a modified agar diffusion assay (ii) quantitative evaluation (*L. monocytogenes* viable counts), in artificially contaminated precooked chicken fillets packaged with doped and undoped coated films.

For the qualitative evaluation 2×2 cm^2 samples of live-*Enterococcus*, enterocin and nisin doped films were placed onto TSA plates seeded with 10^7 CFU of an overnight culture of *L. monocytogenes* ATCC 19117. Undoped coated films were also tested as negative control. The plates were incubated at 30 °C and 22 °C for 24 h and at 4 °C for 5 days. The antagonistic activity was evidenced by a clear zone of inhibition in the indicator lawn around the coated film.

The quantitative evaluation of the antimicrobial coating effectiveness was determined in samples of precooked and not dressed chicken fillets, typically used for chicken salad preparations, purchased from a supermarket on the first day of shelf-life (14 days). The microbial contamination of the precooked chicken fillets was determined on 10 g of this product on the same day of purchasing. The samples showed a microbial load of 30 CFU/g. No growth was observed on MacConkey agar plates (Oxoid). On the same day, a pH value of 6.5 was measured on the surface of the chicken fillets. The samples were surface contaminated by a 5 min immersion in a 10^8 CFU/ml suspension of an overnight culture of *L. monocytogenes* ATCC 19117 diluted in sterile saline solution (NaCl 0.85%) that resulted, after removing the excess fluid, in a final absorption of about 10^4 CFU/g in food samples. Portions of samples (25 g) were singly packaged in doped and undoped films. Food samples were wrapped with the PET coated films, with the PVOH-Si layer in contact with the food. In order to obtain a good contact between food and films, a particular care was used during the wrapping step. Finally, the ends of the wrapped films were tightly closed with appropriate clips. The samples were stored at room and refrigeration temperature (22 °C and 4 °C). At regular intervals (0, 1, 3, 4, 7, 10 and 14 days) the food samples were unwrapped and placed in sterile plastic bag. A particular care was used to avoid to damage the coating and to remove parts of it during the separation of the coated films from foods. At visual inspection, no coating fragments were removed from the PET coated film after it was peeled off from the food. Subsequently, 225 ml of buffered peptone water (Oxoid) were added to the unwrapped food and the samples were homogenized for 1 min in a laboratory blender (Stomacher Lab Blender, Seward Medical, London, UK). Serial tenfold dilutions of the obtained suspensions were spread in triplicate on Palcam agar (Oxoid) and plates were incubated aerobically at 37 °C for 48 h. In all negative samples the residual homogenates were filtered (0.45 μm pore-size filter; Millipore Corp., Bedford, MA) to recover the uncounted listeria. Colonies of *L. monocytogenes* ATCC 19117 were enumerated and results expressed as log CFU/g.

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

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

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

3. Results

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

E. casseliflavus IM 416K1 shows a good growth at 22 °C and 30 °C. At both temperatures the microorganism started to produce bacteriocin with inhibitory activity against *L. monocytogenes* ATCC 19117 already at the 4th hour (320 AU/ml) and after 12 h of incubation the inhibitory activity was 2560 AU/ml. The maximum activity of enterocin 416K1 has been recorded (3200 AU/ml) after 16 h of incubation and this value remained constant until the end of the experiment (48 h). At refrigeration temperature (4 °C), due to slower growth of *E. casseliflavus*, we detected a lower inhibitory activity (1280 AU/ml) at the end of experiment (48 h).

The antimicrobial titer of nisin solution (2500 IU/ml) exhibited by agar well diffusion assay against *L. monocytogenes* ATCC 19117 was found to be 2560 AU/ml.

3.2. Features of the coated films

Polyvinyl alcohol (PVOH) is a hydrophilic polymer which can be swelled/dissolved by water; this specific characteristic of PVOH can allow to create a friendly environment for the survival of bacteria and for this reason it has already been used to entrap living microorganisms (Lozinsky, and Plieva, 1998; Doria-Serrano et al., 2001; Szczesna and Galas, 2001; Liu et al., 2009).

In order to entrap living bacteria, the hydrophilic polymer has been first modified by replacing some –OH groups of PVOH (about 5%) with alkoxysilane groups (PVOH-Si) and then crosslinked in an aqueous solution containing bacterial nutrients (MRS broth) by exploiting the mild sol-gel chemistry. Details and reaction scheme are reported in a previous paper (Iseppi et al., 2011).

A thin layer of PVOH-Si sol-gel solution was applied to PET films by roll-coating deposition (before crosslinking of alkoxysilane groups) and allowed to react at room temperature overnight in order to achieve crosslinking. At the end of this process the PET film substrate is coated with a PVOH-Si crosslinked layer (about 10 μm thick) swollen by a water-MRS broth solution, with a gel fraction of about 84% and a

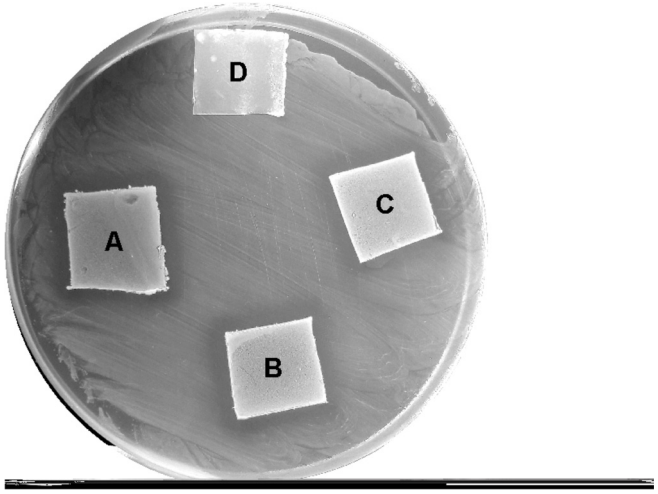

Fig. 1. Antibacterial activity evaluation by agar diffusion assay of the doped coated films against *L. monocytogenes* ATCC 19117 after 5 days incubation at 4 °C: (A): live-*Enterococcus*-doped film; (B): enterocin-doped film; (C): nisin-doped film; (D): undoped-coated film (control). The antagonistic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

swelling ratio in water of 56% (Iseppi et al., 2011). This crosslinked hydrophilic layer show good mechanical properties (not brittle) and a fairly good adhesion to the PET substrate (no evidence of coating detachment was observed by SEM, even after repeated manual bendings of the coated film). Interestingly, no evidence of big cracks or coating-fragment detachments was observed in the coated films recovered after unwrapping food samples. Accordingly, a possible release/migration of live cells from the coating to the food surface can be reasonably excluded. Finally, it has to be emphasized that the presence of water and nutrients within the crosslinked hydrophilic layer led to an environment suitable for the cell surviving/feeding and for an easy diffusion and release of antibacterial substances such as bacteriocins.

3.3. Qualitative antibacterial activity evaluation by agar diffusion assay

Fig. 1 shows, as an example, the qualitative antibacterial activity evaluation at 4 °C of the doped coated films against *L. monocytogenes* ATCC 19117 by direct contact in plate. The activity is revealed in the doped coated films (A,B,C) by a clear inhibition zone in the indicator lawn on the growth of the pathogen around the fragments tested. Indeed, in Fig. 1, inhibition clear zones (three/four mm wide) are evident around the 2 × 2 cm^2 A, B and C film samples, whereas no activity against *L. monocytogenes* ATCC 19117 is present around the negative control undoped coated film (D). Similar tests were performed at 30 °C with similar results. While there were no apparent differences between nisin and enterocin-doped films, a slightly higher activity was observed for the live-*Enterococcus*-doped films at all temperatures tested and in particular at 30 °C.

These results demonstrate that the treatment used for the entrapment of living bacteria and for the incorporation of enterocin 416K1 and nisin in PVOH-based coatings applied to plastic is compatible with the viability of the microorganisms and do not affect the anti-listerial activity of the two bacteriocins. Furthermore, they suggest that antibacterial products are released in the indicator lawn for all the doped films.

3.4. Quantitative antibacterial activity evaluation of the doped coated films

Fig. 2(a and b) show the mean values of *L. monocytogenes* ATCC

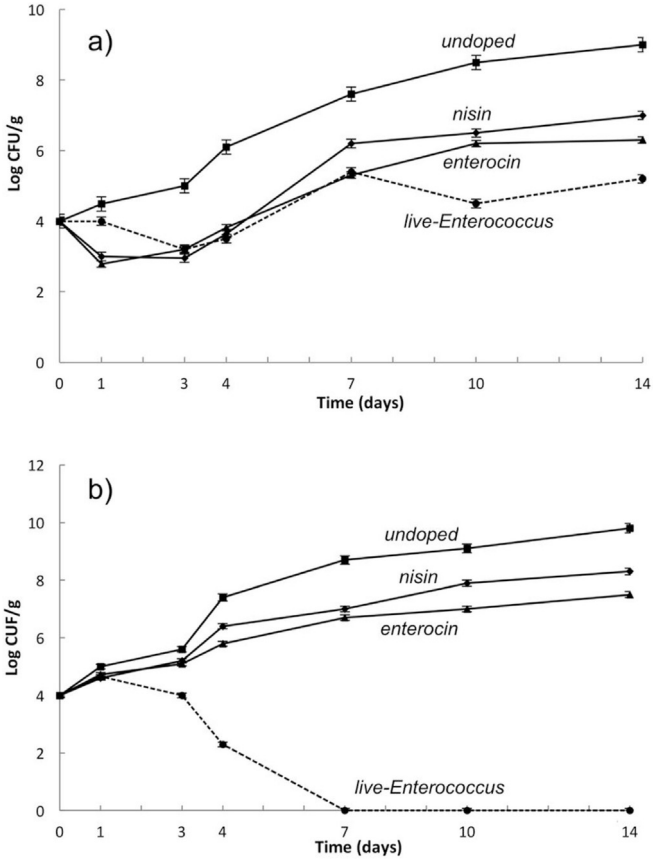

Fig. 2. (a, b). *L. monocytogenes* ATCC 19117 viable counts (log CFU/g) observed in the contaminated chicken fillets samples packaged in live-*Enterococcus*-doped film [●], enterocin-doped film [▲], nisin-doped film [♦] and undoped-coated film [■], stored at 4 °C (a) and at 22 °C (b). Error bars represent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

19117 counts (log CFU/g) detected in artificially contaminated chicken fillets samples wrapped with doped coated films during storage at 4 °C (Figs. 2a) and 22 °C (Fig. 2b). The results observed for undoped coated films are also reported in the same figures for comparison. At both temperatures the undoped film was not able to inhibit the *L. monocytogenes* growth. When the chicken fillets were stored at 4 °C (Fig. 2a), in samples packaged with undoped coated films *L. monocytogenes* counts increase up to more than 8 log CFU/g to the tenth day and 9 log CFU/g at the end of the trial (14th day). In comparison, samples packed with nisin- and enterocin-doped films show a gap of about 2 and 2.5 log CFU/g, respectively, almost constant until the end of the experiment ($p < 0,01$). In samples packaged with live-*Enterococcus*-doped films, the behavior is similar to that observed for nisin- and enterocin-doped films up to the 7th day; however a better antibacterial activity was observed in the last part of the test. The difference with respect to the undoped films is of about 4 log CFU/g from the 10th to the 14th day ($p < 0,01$). Of course, this means that there is a significant decrease of *L. monocytogenes* counts also with respect to samples packaged with nisin or enterocin-doped films.

Samples packaged with undoped films stored at 22 °C (Fig. 2b), show a rapid increase in listeria viable counts, ranging from about 7.5 log CFU/g, after four days, to nearly 10 log CFU/g at the end of the test (14th day). Compared to undoped control, samples packaged with nisin-doped film show 1 log CFU/g reduction after four days and 1,5 log CFU/g at the end of the experiment ($p < 0,05$). Compared to control, samples packaged with enterocin-doped film show a delay of the *L. monocytogenes* growth with a reduction of 1,4 log viable counts at the 4th day, value that reached 2.3 log CFU/g at the end of test ($p < 0,05$).

A significantly different behavior was observed for samples packaged with live-*Enterococcus*-doped films. After 4 days a reduction in listeria counts of about 5 log, 4 log and 3.5 log CFU/g was observed compared to control, nisin-doped and enterocin-doped films, respectively. This gap increases as the test time increases, until the total disappearance of *L. monocytogenes* from the 7th day. The total killing of listeria in samples packaged with live-*Enterococcus*-doped film is confirmed by lack of colonies on Palcam agar after filtration of the residual food homogenate.

A further interesting information concerns some features of samples. At a visual inspection, samples packaged with the antibacterial films result less deteriorated and show a minor spoilage compared to the undoped control and some organoleptic properties (texture, color and odor), were found to be better with a particularly relevant difference for samples packaged with the live-*Enterococcus* doped films stored at 22 °C. These observations suggest that the doped plastic films are able to interact with the growth of spoilage microorganisms, in addition to their capability to inhibit *L. monocytogenes* ATCC 19117 growth.

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

In order to investigate the effects of cold chain breaking, chicken fillets samples stored at refrigerated temperatures (4 °C) were incubated at 30 °C for 24 h on the third or on the seventh day. The average values of the *L. monocytogenes* ATCC 19117 counts (log CFU/g) are reported in Fig. 3(a and b).

Incubation for 24 h at 30 °C on the third day induces a sharp increase of *L. monocytogenes* ATCC 19117 counts (about 2 log) in all samples, wrapped both in doped and undoped films. During the following days of incubation at 4 °C there is a progressive increase of *L. monocytogenes* ATCC 19117 counts for samples wrapped in undoped and nisin- and 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 ($p < 0,01$).

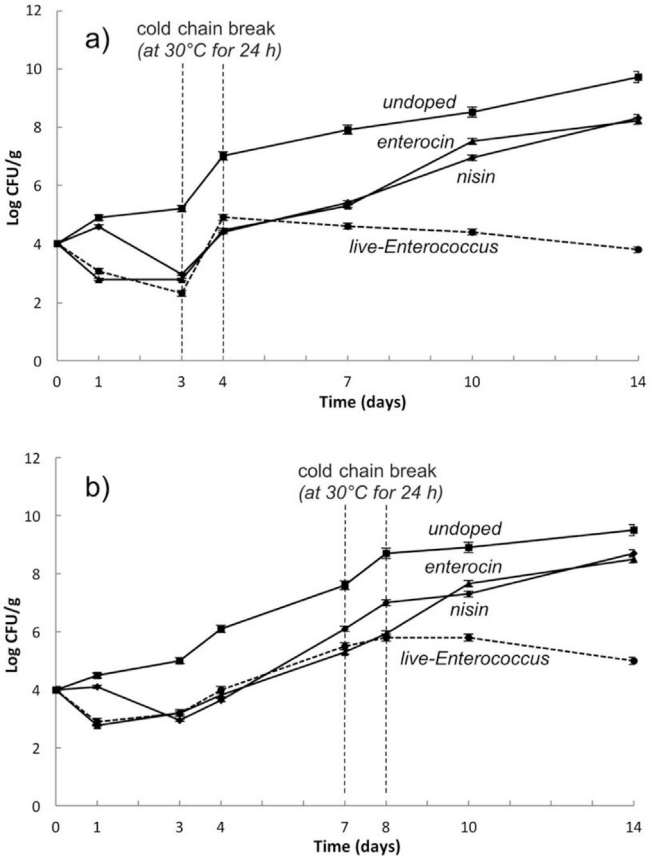

Fig. 3. (a, b). *L. monocytogenes* ATCC 19117 viable counts (log CFU/g) observed in the contaminated chicken fillets samples packaged in live-*Enterococcus*-doped film [●], enterocin-doped film [▲], nisin-doped film [♦] and undoped-coated film [■] stored at 4 °C and subjected to simulated cold chain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

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

In the last few years, fresh or minimally processed products have been included in the trend of the industrialized Western food model, mainly because of changes in the human lifestyle and their tendency towards convenience and spending less time on preparing food (Ragaert et al., 2007; Abadias et al., 2008; Gómez-Govea et al., 2012). The shelf-life of these foods is generally shorter than previously, to avoid product quality decay and increase in microbial contamination. Refrigeration is the most used way to prevent or delay the growth of spoilage and pathogenic microorganisms and to extend the shelf-life of RTE foods. Anyway if during the food production a temperature abuse occurs, unpredicted growth of microbial contamination may occur. Although the processes related to the food industry are widely regulated by a series of practices (European Standard EC, 2073/2005; Gómez-Govea et al., 2012), their application doesn't guarantee the absence of breakage in the cold chain of food production (Rodríguez et al., 2011). For example, it is reported that a slight abuse of temperature of refrigerated RTE foods increases greatly the risk that *L. monocytogenes*, will grow to a level that could cause human disease (Kotzekidou, 2013).

In this context we have developed and tested an active food packaging, which has on the surface a thin coating containing living enterocin-producer bacteria, that, according to our previous results (Iseppi et al., 2011) should be able to delay or inhibit the bacterial growth when a cold chain break occurs. The anti-listerial activity of these doped films was compared with that of films doped with bacteriocins, nisin and enterocin 416K1 and with an undoped film, as a negative control.

The qualitative preliminary tests (Fig. 1) show that all the doped films are able to release antibacterial products to the surrounding culture substrate. Therefore it is expected that these doped films are able to release antibacterial products also when in contact with food. To

demonstrate their effectiveness, contaminated chicken food was packaged in undoped and differently doped films and the quantitative effects of their antibacterial activity evaluated under different storage conditions.

As shown in Fig. 2a, at 4 °C the antibacterial behavior of all doped films is better than that of undoped film (about 2 log CFU/g) and effective to reduce the *L. monocytogenes* contamination up to 2–3 days. In addition, for all doped films it can be observed that after 3 days, the *L. monocytogenes* contamination start to grow and become higher than the initial contamination. Of course this behavior can be easily explained as a consequence of a progressive reduction of the amount of antimicrobial bacteriocins release from the films. In the first days, when the amount of antimicrobial products in the films is the highest the release is fast, however as a consequence of the progressive decrease of the amount of antibacterial products in the films their release becomes slower and slower. While for undoped and bacteriocin-doped films the growth of *L. monocytogenes* contamination was continuous up to the end of the experiment, it has to be noticed that after seven days the *L. monocytogenes* contamination does not increase anymore in films doped with living bacteria. This behavior suggests that at 4 °C living bacteria are able to slowly produce enterocin, whose concentration does not decrease within the film and whose release is able to contrast the growth of *L. monocytogenes* contamination.

However, the more astonishing difference between films doped with living bacteria and the other doped films is that recoded at 22 °C. The increase of *L. monocytogenes* contamination after one day means that at 22 °C *L. monocytogenes* grows faster than its killing rate due to the release of antibacterial products. While an increase of *L. monocytogenes* contamination continues till to the end of the experiment for undoped and bacteriocin-doped films, in the case of films containing living bacteria, the contamination start to decrease after few days and *L. monocytogenes* counts is reduced to zero after seven days. In our opinion, the most probable way to explain these results is to assume that living bacteria are able to produce enterocin, and that the rate of production of enterocin grows faster than the growth rate of *L. monocytogenes*. Probably also the pH decrease due to the growth of *E. casseliflavus*, added in MRS broth to the coating, could be a further element for the full inactivation of *L. monocytogenes* (Sabia et al., 2003). These results suggest that an accidental increase in storage temperature, and thereby, an increased growth of *L. monocytogenes*, would be counteracted by the use of packaging films containing living bacteria. To verify this hypothesis, in a second step of this study the anti-listerial activity of the three different doped-films was evaluated by cold chain break simulation (at 30 °C for 24 h) on the 3rd or 7th day of incubation.

The results of Fig. 3a and b shows that while the cold-chain breaking is deleterious for undoped and bacteriocin-doped films, it is less dangerous when food is wrapped with live-*Enterococcus*-doped films. In this latter case, after 24 h at 30 °C, the *L. monocytogenes* counts start to decrease progressively confirming that this kind of antibacterial film is very effective to contrast bacterial growth.

All these results extend and confirm our previous results obtained in a study of the anti-listerial effect of films containing living bacteria on contaminated wurstel and seasoned cheese samples (Iseppi et al., 2011).

In conclusion, this study shows that it is possible to entrap live *Enterococcus casseliflavus* IM 416K1 bacteria in PVOH-based coatings. The entrapped cells are able to survive and to produce anti-listerial products over long time, making this doped-film a very efficient preservative system. To our knowledge this innovative approach has never been proposed by other authors.

Finally, as suggested by the results of anti-listerial activity at 22 °C (Fig. 2b), this study seems to open a new approach to food preservation. In fact, the use of live bacteriocin producer strains doped films could not require refrigeration to prevent spoilage and bacterial growth.

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References

Abadias, M., Usall, J., Anguera, M., Solsona, C., Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. Int. J. Food Microbiol. 123, 121–129.

Altieri, C., Sinigaglia, M., Corbo, M.R., Falcone, P., Del Nobile, M.A., 2004. Use of entrapped microorganisms as biological oxygen scavengers in food packaging applications. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 37 (1), 9–15.

Ananou, S., Garriga, M., Hugas, M., Maqueda, M., Martínez-Bueno, M., Galvez, A., Valdivia, E., 2005. Control of *Listeria monocytogenes* in model sausages by enterocin AS-48. Int. J. Food Microbiol. 103, 179–190.

Aymerich, M.T., Garriga, M., Ylla, J., Vallier, J., Monfort, J.M., Hugas, M., 2000. Application of enterocins as biopreservatives against *Listeria innocua* in meat products. J. Food Protect. 63, 721–726.

Batz, M.B., Hoffmann, S., Morris Jr., J.G., 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. J. Food Protect. 75 (7), 1278–1291.

CAC Guidelines on the Application of General Principles of Food Hygiene to the Control of Listeria Monocytogenes in Ready-to-eat Foods – CAC/GL 61–2007.

Cocolin, L., Foschino, R., Comi, G., Fortina, M.G., 2007. Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goat milk. Food Microbiol. 24, 752–758.

Coelho, M.C., Silva, C.C., Ribeiro, S.C., Dapkevicius, M.L., Rosa, H.J., 2014. Control of *Listeria monocytogenes* in fresh cheese using protective lactic acid bacteria. Int. J. Food Microbiol. 17 (191), 53–59.

Coulomb, D., 2008. Refrigeration and cold chain serving the global food industry and creating a better future: two key IIR challenges for improved health and environment. Trends Food Sci. Technol. 19, 413–417.

Damania, P., Patel, R., Shaw, R., Kataria, R.P., Wadia, A., 2016. Development of antimicrobial packaging materials for food preservation using bacteriocin from *Lactobacillus casei*. Microbiol. Res. 7, 23–28.

Devi, S.M., Ramaswamy, A.M., Halami, P.M., 2014. In situ production of pediocin PA-1 like bacteriocin by different genera of lactic acid bacteria in soymilk fermentation and evaluation of sensory properties of the fermented soy curd. J. Food Sci. Technol. 51, 3325–3332.

Doria-Serrano, M.C., Ruiz-Trevino, F.A., Rios-Arciga, C., Hernández-Esparza, M., Santiago, P., 2001. Physical characteristics of poly(vinyl alcohol) and calcium alginate hydrogels for the immobilization of activated sludge. Biomacromolecules 2, 568–574.

European Commission, 2005. Commission Regulation of 15 November 2005 on microbiological criteria for foodstuffs, 2073/2005/EC. In: Official Journal, L 388/1, 22/12/2005.

Gálvez, A., Dubois-Dauphin, R.R., Ghalfi, H.H., Campos, D., Thonart, P., 2009. Description of two *Enterococcus strains* isolated from traditional Peruvian artisanal-produced cheeses with a bacteriocin-like inhibitory activity. Biotechnol. Agron. Soc. Environ. 13, 349–356.

Gandhi, M., Chikindas, M.L., 2007. Listeria: a foodborne pathogen that knows how to survive. Int. J. Food Microbiol. 113 (1), 1–15.

Giraffa, G., 2003. Functionality of enterococci in dairy products. Int. J. Food Microbiol. 88, 215–222.

Gómez-Govea, M., Solís-Soto, L., Heredia, N., García, S., Moreno, G., Tovar, O., Isunza, G., 2012. Analysis of microbial contamination levels of fruits and vegetables at retail in Monterrey, Mexico. J. Food Agric. Environ. 10, 152–156.

Goulet, V., Hedberg, C., Le Monnier, A., de Valk, H., 2008. Increasing incidence of listeriosis in France and other European countries. Emerg. Infect. Dis. 14, 734–740.

Hassanzadazar, H., Ehsani, A., Mardani, K., 2014. Antibacterial activity of *Enterococcus faecium* derived from Koopeh cheese against *Listeria monocytogenes* in probiotic ultrafiltrated cheese. Vet. Res. Forum 5 (3), 169–175.

Holzapfel, W.H., Geisen, R., Schillinger, U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24, 343–362.

Irkin, R., Esmer, O.K., 2015. Novel food packaging systems with natural antimicrobial agents. J. Food Sci. Technol. 52 (10), 6095–6111.

Iseppi, R., Pilati, F., Marini, M., Toselli, M., de Niederhäusern, S., Guerrieri, E., Messi, P., Sabia, C., Manicardi, G., Anacarso, I., Bondi, M., 2008. Anti-listerial activity of a polymeric film coated with hybrid coatings doped with Enterocin 416K1 for use as bioactive food packaging. Int. J. Food Microbiol. 123, 281–287.

Iseppi, R., de Niederhäusern, S., Anacarso, I., Messi, P., Sabia, C., Pilati, F., Toselli, M., Degli Esposti, M., Bondi, M., 2011. Anti-listerial activity of coatings entrapping living bacteria. Soft Matter 7, 8542–8548.

Jeddi, M.Z., Yunesian, M., Gorji, M.E., Noori, N., Pourmand, M.R., Khaniki, G.R., 2014. Microbial evaluation of fresh, minimally-processed vegetables and bagged sprouts from chain supermarkets. J. Health Popul. Nutr. 32, 391–399.

Joerger, R.D., 2007. Antimicrobial films for food applications: a quantitative analysis of their effectiveness. Packag. Technol. Sci. 20 (4), 231–273.

Jol, S., Kassianenko, A., Wszol, K., Oggel, J., 2005. The cold chain, one link in Canada's food safety initiatives. Food Contr. 18, 713–715.

Kerry, J.P., O'Grady, M.N., Hogan, S.A., 2006. Past, current and potential utilization of active and intelligent packaging systems for meat and muscle-based products: a review. Meat Sci. 74, 113–130.
Kotzekidou, P., 2013. Survey of *Listeria monocytogenes, Salmonella* spp. and *Escherichia coli* O157:H7 in raw ingredients and ready-to-eat products by commercial real-time PCR kits. Food Microbiol. 35 (2), 86–91.
Liu, L., Shang, L., Guo, S., Li, D., Liu, C., Qi, L., Dong, S., 2009. Organic-inorganic hybrid material for the cells immobilization: long-term viability mechanism and application in BOD sensors. Biosens. Bioelectron. 25 (2), 523–526.
Lomonaco, S., Verghese, B., Gerner-Smidt, P., Tarr, C., Gladney, L., Joseph, L., Kats, L., Turnsek, M., Frace, M., Chen, Y., Brown, E., Meinersmann, R., berrang, M., Knabel, S., 2013. Novel epidemic clones of *Listeria monocytogenes*, United States, 2011. Emerg. Infect. Dis. 19, 147–150.
Lozinsky, V.I., Plieva, F.M., 1998. Poly(vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. Overview of recent research and developments. Enzym. Microb. Technol. 23 (3–4), 227–242.
Malhotra, B., Keshwani, A., Kharkwal, H., 2015. Antimicrobial food packaging: potential and pitfalls. Front. Microbiol. 6 (611), 1–9.
Marcos, B., Jofré, A., Aymerich, T., Monfort, J.M., Garriga, M., 2008. Combined effect of natural antimicrobials and high pressure processing to prevent *Listeria monocytogenes* growth after a cold chain break during storage of cooked ham. Food Contr. 19, 76–81.
Mayr-Harting, A., Hedges, A.J., Berkeley, R.C.W., 1972. Methods for studying bacteriocins. In: In: Norris, J.R., Ribbons, D.W. (Eds.), Methods in Microbiology, vol. 7a. Academic Press, New York, pp. 313–342.
Muriana, P.M., 1996. Bacteriocins for control of *Listeria* spp. in food. J. Food Protect. 59, 54–63.
Neetoo, H., Ye, M., Chen, H., Joerger, R.D., Hicks, D.T., Hoover, D.G., 2008. Use of nisin-coated plastic films to control *Listeria monocytogenes* on vacuum-packaged cold-smoked salmon. Int. J. Food Microbiol. 122, 8–15.
Pingitore, E.V., Todorov, S.D., Sesma, F., Franco, B.D., 2012. Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese. Food Microbiol. 32, 38–47.
Ragaert, P., Devlieghere, F., Debevere, J., 2007. Role of microbiological and physiological spoilage mechanisms during storage of minimally processed vegetables. Postharvest Biol. Technol. 44, 185–194.
Rocourt, J., Bille, J., 1997. Foodborne listeriosis. World Health Stat. Q. 50, 67–73.
Rodríguez, V., Amorrortu, I., Álvarez, M.J., 2011. Setting parameters in the cold chain. Tecnura 15, 71–81.
Rogers, A.M., Montville, T.J., 1991. Improved agar diffusion assay for nisin quantification. Food Biotechnol. 5, 161–168.
Sabia, C., Manicardi, G., Messi, P., de Niederhäusern, S., Bondi, M., 2002. Enterocin 416K1, an antilisterial bacteriocin produced by *Enterococcus casseliflavus* IM 416K1 isolated from Italian sausages. Int. J. Food Microbiol. 75, 163–170.
Sabia, C., de Niederhäusern, S., Messi, P., Manicardi, G., Bondi, M., 2003. Bacteriocin-producing *Enterococcus casseliflavus* IM 416K1, a natural antagonist for control of *Listeria monocytogenes* in Italian sausages ("cacciatore"). Int. J. Food Microbiol. 87, 173–179.
Suppakul, P., Miltz, J., Sonneveld, K., Bigger, S.W., 2003. Active packaging technologies with an emphasis on antimicrobial packaging and its applications. J. Food Sci. 68, 408–420.
Szczesna, M., Galas, E., 2001. Bacillus subtilis cells immobilised in PVA-cryogels. Biomol. Eng. 17, 55–63.
Vandera, E., Lianou, A., Kakouri, A., Feng, J., Koukkou, A.I., Samelis, J., 2017. Enhanced control of *Listeria monocytogenes* by *Enterococcus faecium* KE82, a multiple enterocin-producing strain, in different milk environments. J. Food Protect. 80 (1), 74–85.
White, D.G., Zhao, S., Simjee, S., Wagner, D.D., McDermott, P.F., 2002. Antimicrobial resistance of food-borne pathogens. Microb. Infect. 4, 405–412.
World Health Organization (WHO), 2007. Food Safety and Foodborne Illness. WHO, Geneva Fact Sheet no. 237 (reviewed March 2007).