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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