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Development of polythene films for food packaging activated with an antilisterial bacteriocin from *Lactobacillus curvatus* 32Y

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ABSTRACT

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Aims: The aims of this work were to (i) use a bacteriocin produced by *Lactobacillus curvatus* 32Y active against *Listeria monocytogenes* to activate polythene films by different methods, (ii) implement a large-scale process for antilisterial polythene films production and (iii) verify the efficacy of the developed films in inhibiting the growth of *L. monocytogenes* during the storage of meat products.

Methods and Results: The film was made active by using the antilisterial bacteriocin 32Y by *Lact. curvatus* with three different procedures: soaking, spraying and coating. The antimicrobial activity of the activated films was tested in plate assays against the indicator strain *L. monocytogenes* V7. All the used procedures yielded active polythene films although the quality of the inhibition was different. The coating was therefore employed to develop active polythene films in an industrial plant. The antimicrobial activity of the industrially produced films was tested in experiments of food packaging involving pork steak and ground beef contaminated by *L. monocytogenes* V7 at roughly 10^3 CFU cm^{-2} and gram respectively. The results of the challenge tests showed the highest antimicrobial activity after 24 h at 4°C, with a decrease of about 1 log of the *L. monocytogenes* population.

Conclusions: Antimicrobial packaging can play an important role in reducing the risk of pathogen development, as well as extending the shelf life of foods.

Significance and Impact of the Study: Studies of new food-grade bacteriocins as preservatives and development of suitable systems of bacteriocin treatment of plastic films for food packaging are important issues in applied microbiology and biotechnology, both for implementing and improving effective hurdle technologies for a better preservation of food products.

Keywords: active packaging, bacteriocin-treated polythene films, food preservation, food-grade bacteriocin, *Listeria monocytogenes*.

INTRODUCTION

In recent years there is an increasing demand of minimally processed foods with fresh like quality; moreover, modern distribution systems require an adequate way to extend the shelf life of foods. Numerous types of food packaging can be used in combination with food preservation techniques in order to extend the effectiveness of the food preservation chain. One of the key technological measures needed during storage is the preservation of the food from microbial spoilage and contamination/proliferation of pathogenic micro-organisms.

Active packaging is defined as an intelligent or smart system that involves interactions between package or package components and food or internal gas atmosphere and complies with consumer demands for high quality,

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fresh-like and safe products (Labuza and Breene 1989). Antimicrobial active packaging is being increasingly experimented because it is believed to have a significant potential in improving food safety and prolonging the shelf life of food products (Vermeiren *et al.* 1999; Appendini and Hotchkiss 2002; Quintavalla and Vicini 2002). In fact, active packaging performs some desired roles other than providing an inert barrier between the product and external conditions, and combines advances in food technology, microbiology, biotechnology, packaging and material science, in an effort to meet the consumer demand for appreciated and safe products (Miltz *et al.* 1997).

The binding of antimicrobial agents directly to polymeric packaging is an exciting development, which allows industry to combine the preservative functions of antimicrobials with the protective functions of the pre-existing packaging concepts. Many preservatives, such as sorbic acid, plant extracts, silver-substituted zeolite, lysozyme and chlorine dioxide, have been successfully incorporated in packaging materials to confer antimicrobial activity in food packaging (Vermeiren *et al.* 1999; Appendini and Hotchkiss 2002; Quintavalla and Vicini 2002).

The use of bacteriocins and other biologically derived antimicrobials in packaging material is attracting increasing interest recently, and patents have been filed in the area (Wilhoit 1996, 1997; Ming *et al.* 1997; Siragusa *et al.* 1999).

The aims of this work were to (i) use a bacteriocin produced by *Lactobacillus curvatus* 32Y active against *Listeria monocytogenes* to activate polythene films by different methods, (ii) implement a large-scale process for antilisterial polythene films production and (iii) verify the efficacy of the developed films in inhibiting the growth of *L. monocytogenes* during the storage of meat products.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus curvatus 32Y (Mauriello *et al.* 1999) and *L. monocytogenes* V7 were maintained in storage at −20°C in 30% glycerol. *Lact. curvatus* 32Y was grown in MRS broth (Oxoid) at 30°C and *L. monocytogenes* V7 in TSB (Oxoid) at 37°C prior to their use in the experiments.

Preparation of the bacteriocin 32Y solution

Variable volumes of overnight MRS broth cultures of *Lact. curvatus* 32Y were used depending on the scope of the preparation. The broth cultures were centrifuged at 18 000 *g* for 30 min, the supernatant was mixed with 5% (w/v) of Amberlite XAD 16 (Oxoid) and the mixture was stirred at room temperature for 30 min (Villani *et al.* 2001). The mixture of supernatant broth and Amberlite resin was then used to pack a low-pressure chromatographic column (1·5 × 20 cm). After repeated column washings with deionized water the bacteriocin was eluted by using 1/10 the initial volume of a solution of 70% isopropanol and 30% 10 mmol l^{-1} acetic acid. The partially purified bacteriocin 32Y solution (PPBAC), showing an activity of 102 400 AU ml^{-1} against *L. monocytogenes*V7, was stored at 4°C prior to use. The concentration of the bacteriocin solution, expressed in arbitrary units (AU) per millilitre, was determined by an agar diffusion assay as described by Villani *et al.* (1994).

Procedures for antimicrobial polythene films preparation

Three methods of film treatment with bacteriocin 32Y were tested.

Soaking. Samples of coupled polythene-oriented polyamide (PE-OPA) films (2 × 2 cm) were soaked in PPBAC diluted in phosphate buffer 50 mmol l^{-1} pH 7·00 at concentration of 0, 6400, 12 800 and 25 600 AU ml l^{-1} for 10 min and 1, 6 and 8 h. After the soaking, the films were air-dried and assayed for antimicrobial activity against *L. monocytogenes* V7 as described below.

Spray-coating. The PPBAC diluted in 70% isopropanol at a concentration of 6400 AU ml l^{-1} was sprayed onto PE-OPA film samples (20 × 20 cm). The films were then treated with warm air in order to let the solution dry and promoting a homogenous distribution of the bacteriocin onto the surface of the plastic film. After the treatment the films were assayed for antimicrobial activity against *L. monocytogenes* V7 as described below.

Industrial production of bacteriocin-coated PE-OPA films. The coating procedure was also used to produce bacteriocin-coated PE-OPA films in an industrial plant. A solution of PPBAC in 70% isopropanol was prepared with an activity of 6400 AU ml l^{-1} against *L. monocytogenes* V7 and used in a large-scale coating plant; a scheme is depicted in Fig. 1. A 46 cm wide PE-OPA film (300 m in length) was employed for the activation at a speed of 25 m min^{-1}. Briefly, PPBAC solution was spread on the PE-OPA film in thin layer by a spreading roll that dipped in a vessel containing the antimicrobial solution. The isopropanol fraction of the PPBAC solution was immediately evaporated in a warm air tunnel at 70°C. Eventually, the activated film was cooled at room temperature and collected in a reel. The antimicrobial activity of the activated PE-OPA films was tested, as described below, soon after the treatment and after 24 h, 1, 2, and 3 weeks and 1, 2 and 3 months during which the film reel was kept at room temperature.

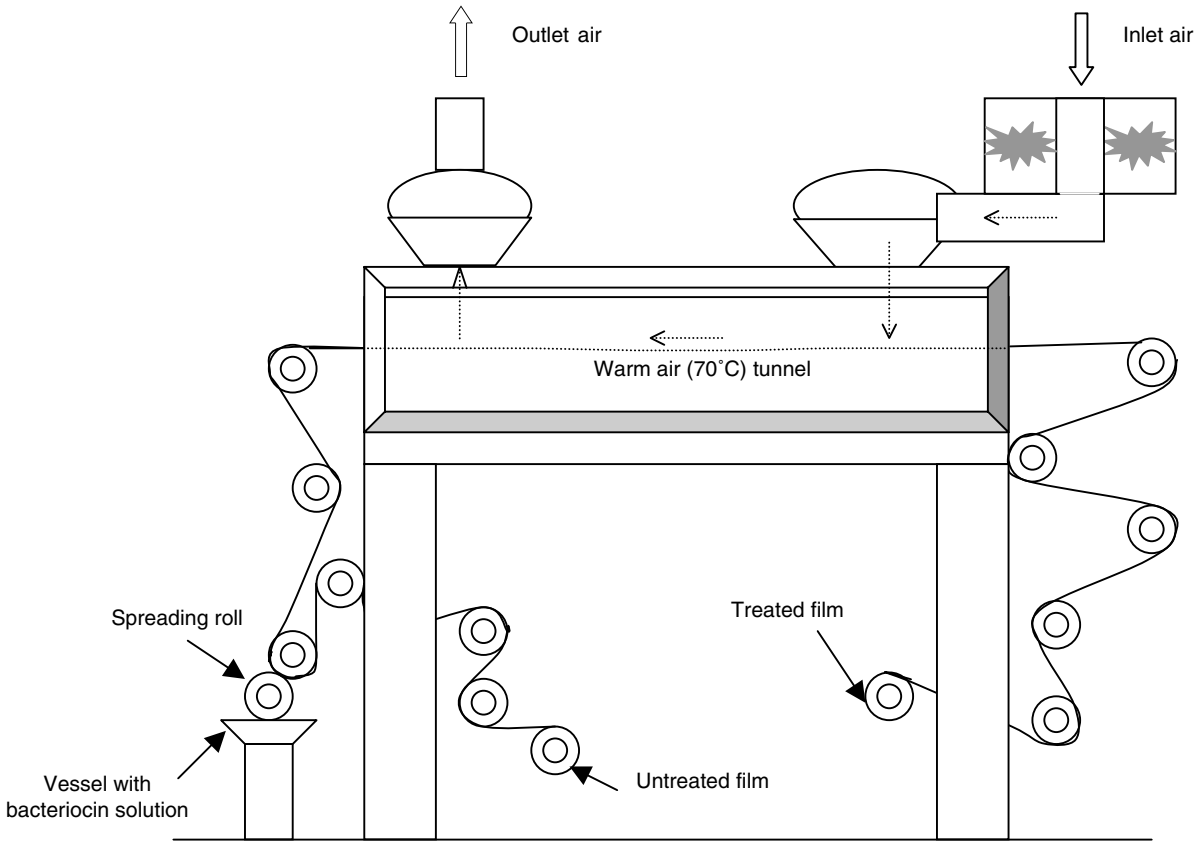

Fig. 1 Industrial plant for PE-OPA film treatment

Antimicrobial activity assay of the developed PE-OPA films

After the above treatments of activation with the bacteriocin 32Y the PE-OPA films were assayed for antimicrobial activity against the indicator *L. monocytogenes* V7. Samples (2 × 2 cm) of the treated PE-OPA films were located onto the surface of a BHI (Oxoid) soft (0·75%) agar plates seeded with 2·5% of an overnight culture of *L. monocytogenes* V7. The treated face of the film was in contact with the agar, untreated films were also assayed as negative controls. The plates were incubated at 37°C for 16 h and the antagonistic activity was evaluated by observing a clear zone of growth inhibition in correspondence of the active PE-OPA film.

In order to resemble a superficial development of *Listeria* on the surface of food products and the antimicrobial effect of the developed films on superficial growth of listeria, a further inhibition assay was performed. A quantity of 0·1 ml of a suspension containing about $1{\cdot}0 \times 10^5$ CFU of *L. monocytogenes* V7 were spread plated on BHI agar plates, the active face of the bacteriocin-treated PE-OPA film was located in contact with the agar surface and the Petri dish was incubated at 37°C for 16 h. After the incubation, the antimicrobial activity was revealed by the absence of growth of the indicator *Listeria* in the part of the plate in contact with the activated PE-OPA film.

Adsorption and release of the Bac 32Y from the PE-OPA films

For adsorption assays, 20 μl of PPBAC were spotted on the surface of samples of untreated PE-OPA films, removed after 1, 2, 3, 5 and 10 min and tested for antimicrobial activity on TSB agar plates inoculated with *L. monocytogenes* V7 as above described. For bacteriocin release assays, the simulating solution chosen was water, as recommended by analytic method defined in Repubblica Italiana, DM 21 Marzo, 1973, which indicates water as simulating solution to be used for release experiments regarding meat products. Therefore, 20 μl of sterile deionized water were spotted onto the surface of industrially developed PE-OPA films and removed for every 5 min for 1 h. Afterwards, both film and water spots were assayed for antimicrobial activity against *L. monocytogenes* V7.

Antilisterial activity of bacteriocin 32Y coated films during the storage of meat products

The industrially developed antilisterial PE-OPA films were used in challenge tests of control of *L. monocytogenes* growth during the storage of meat products. Pork steaks were superficially spiked with a 2 ml suspension of *L. monocytogenes* V7 at $1{\cdot}0 \times 10^6$ CFU ml^{-1}, the steaks were then packed with the active PE-OPA films and stored at 4°C. Steaks packed with untreated films, and unpacked steaks were included in the analysis as controls. After 0, 24, 48 and 72 h of storage, selective viable counts of listeria on Oxford agar (Oxoid) were performed on four pieces (3·8 cm^2 each) of pork steak 10-fold diluted in a quarter Ringer solution (Oxoid). The experiments were performed in triplicate and the results were expressed as CFU cm^{-2}. Similarly, minced beef was contaminated at 1% with a suspension of *L. monocytogenes* V7 at $1{\cdot}0 \times 10^6$ CFU ml^{-1} and 45 g hamburgers were prepared 1 cm thick. The hamburgers were packed on both faces with the antilisterial PE-OPA films and stored at 4°C. Hamburgers unpacked and packed with untreated films were included in the analysis as controls. After 0, 24, 48 and 72 h of storage, selective viable counts of *Listeria* on Oxford agar were performed and the results were expressed as CFU g^{-1}.

RESULTS

The activation of PE-OPA films with the bacteriocin 32Y was performed by different methods. The soaking procedure yielded positive results; in fact, after immersion for different times of the PE-OPA films into bacteriocin 32Y solutions at different concentrations, the films showed to be always active against *L. monocytogenes* V7 in agar inhibition assays. In all the cases untreated films did not show any antimicrobial activity. An example of the detection of antimicrobial activity of the bacteriocin-soaked PE-OPA films is shown in Fig. 2a,b. There was no difference in inhibition intensity when films were treated for 10 min (Fig. 2a) and 1 h (Fig. 2b). Moreover, the growth inhibition area was not confined to the film area but irregularly spread across the plate (Fig. 2). In order to assess whether the bacteriocin was actually absorbed by the surface of the PE-OPA film or migrated from the cut margins into the film, another experiment was carried out. Aliquots of the PPBAC 32Y solution at 6400 AU ml^{-1} were spotted on the surface of the film for different contact times and the antimicrobial activity of the films was then tested. As shown in Fig. 3, the antimicrobial activity was observed in correspondence to the spot area and the intensity of the activity was the same, regardless of the bacteriocin solution contact time.

The same PPBAC 32Y solution at 64 000 AU ml^{-1} was sprayed on the surface of the plastic films and the results of the antimicrobial activity are shown in Fig. 4. Also in this case the spray-activated films showed antilisterial activity and it could be noted that the bacteriocin diffused from the plastic film as the inhibition zone was not confined to the film area. The spray-coated PE-OPA films were also assayed for activity against superficial growth of *L. monocytogenes* V7 on agar plates. As shown in Fig. 5, the superficial growth of

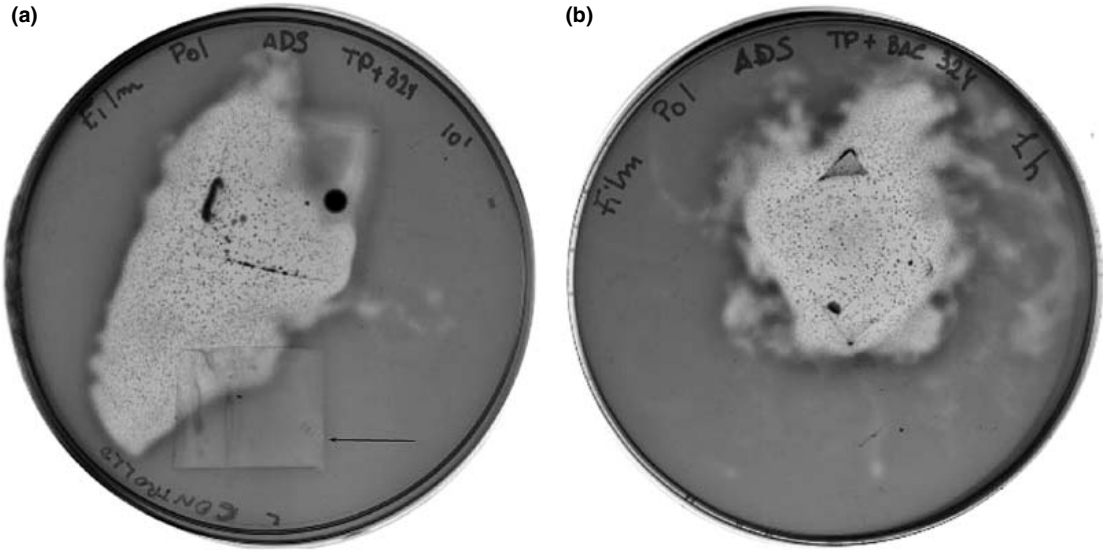

Fig. 2 Antimicrobial activity of bacteriocin-soaked PE-OPA films against the indicator strain *Listeria monocytogenes* V7. (a) Antimicrobial activity after 10 min soaking; (b) antimicrobial activity after 1 h soaking; the concentration of bacteriocin solution was 6400 AU ml^{-1}; the arrow indicates the untreated film used as control

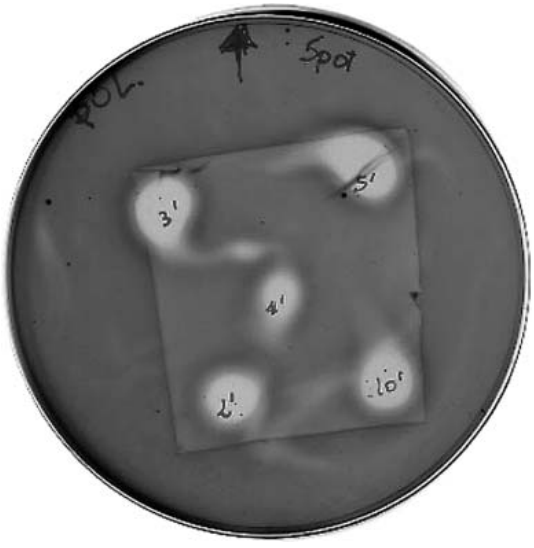

Fig. 3 Antimicrobial activity of PE-OPA films spotted with PPBAC 32Y solution at 6400 AU ml^{-1}. The inhibition zones correspond to 2, 3, 4, 5 and 10 min of PPBAC 32Y contact with the film

indicator strain was limited to the area surrounding the activated film that could clearly inhibit the development of the pathogen; in contrast, the pathogen could grew homogeneously on the surface of the plate and underneath the untreated PE-OPA film used as control (Fig. 5b).

As the bacteriocin 32Y showed good potential for the development of antimicrobial PE-OPA films, an industrial procedure of coating was developed in order to produce 32Y-activated PE-OPA films in an industrial plant. The activation of the PE-OPA films did not alter their mechanical properties nor influenced its transparency and appearance. Moreover, the activated films did not show solvent release as detected by head space gas chromatography analysis (data not shown). Interestingly, the coated films displayed a clear and stable antilisterial activity during 3 months after the treatment. The heat treatment at 70°C did not affect the bacteriocin activity; this had been already demonstrated by treating at different temperature the PPBAC 32Y solution without loss of antimicrobial activity in terms of AU ml^{-1} (data not shown). As shown in Fig. 6, the antimicrobial activity of the bacteriocin-coated PE-OPA films was tightly confined to the area of the film and was not diffused across the plate as did the antimicrobial activity of soaked and sprayed films.

The coated films were also subjected to experiment release of the bacteriocin as a consequence of prolonged contact of the film with water spots. The water spots collected after different times (every 5 min for 1 h) did not show any antimicrobial activity in agar plate assays. However, the bacteriocin-coated PE-OPA films, after being assayed for bacteriocin release in water spots, displayed loss of antilisterial activity in correspondence of the zones where the water spots were left (data not shown).

The industrially developed antimicrobial films were also used in challenge tests of storage of meat products artificially contaminated by *L. monocytogenes* V7. The results of the viable counts of *Listeria* on pork steaks at different times of storage at 4°C are reported in Fig. 7. The film was washed prior to viable counts and the washing liquid was plated on Oxford agar plates yielding only occasionally viable listeria

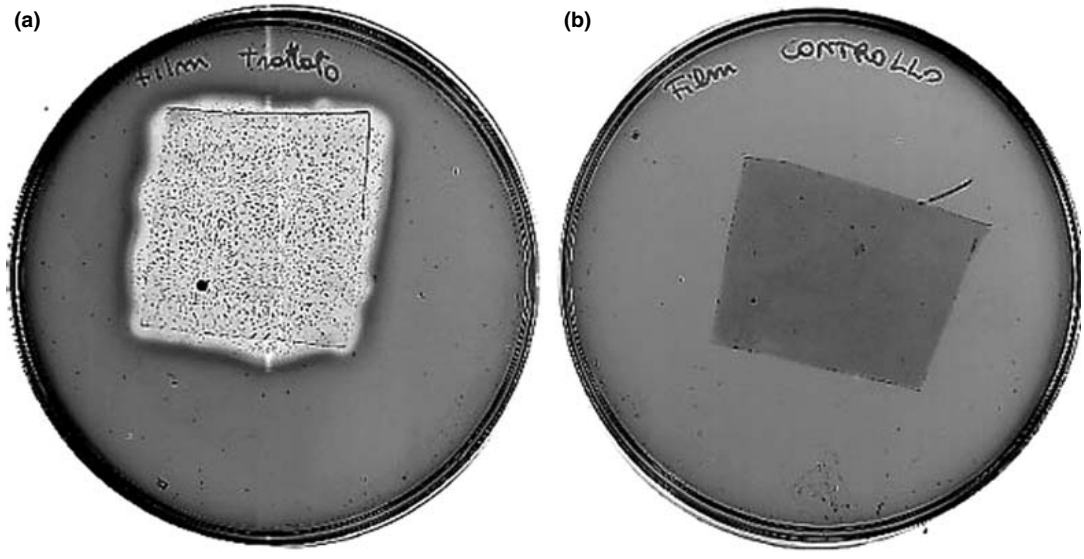

Fig. 4 Antimicrobial activity of the PE-OPA film spray activated with PPBAC 32Y solution at 6400 AU ml^{-1} against the indicator strain *Listeria monocytogenes* V7: (a) treated film; (b) untreated film

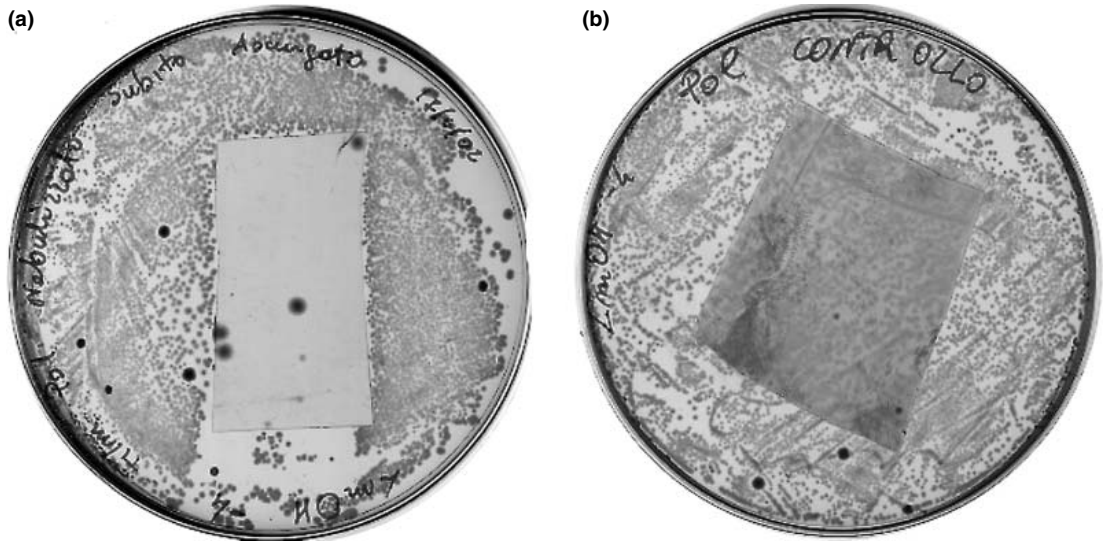

Fig. 5 Antimicrobial activity of the PE-OPA film spray activated with PPBAC 32Y solution at 6400 AU ml^{-1} against the indicator strain *Listeria monocytogenes* V7 spread inoculated on TSA plates: (a) treated film; (b) untreated film

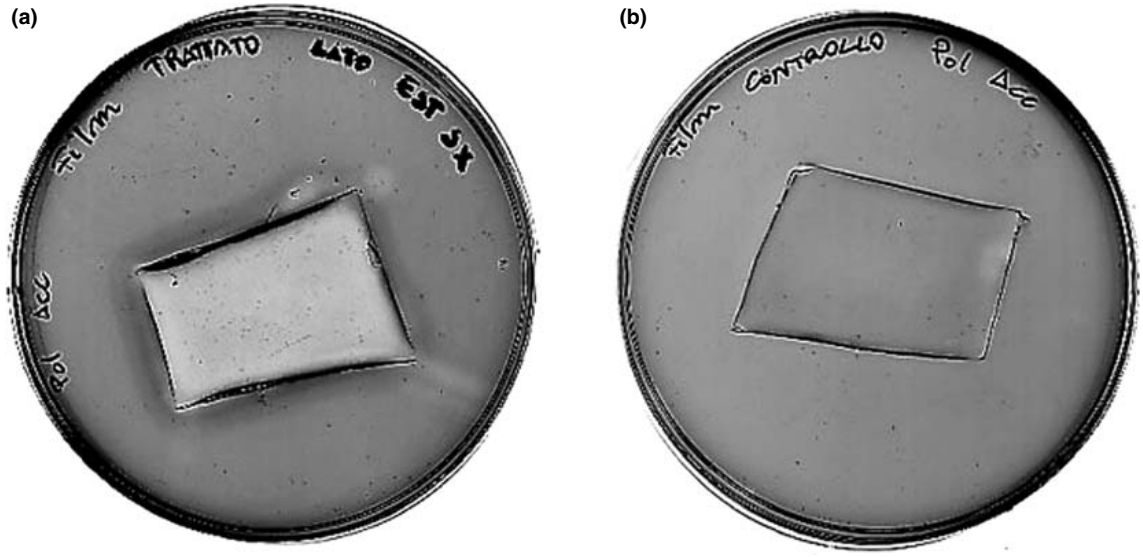

Fig. 6 Antimicrobial activity of the PE-OPA film coated in the industrial plant with PPBAC 32Y solution at 6400 AU ml^{-1} against the indicator strain *Listeria monocytogenes* V7: (a) treated film; (b) untreated film

counts after 48 h at 37°C. The trend of the *Listeria* population appeared to be the same during the storage of the pork steaks in PE-OPA films both with and without bacteriocin treatment. However, the viable counts of *Listeria* were lower during the whole period of storage when the pork steaks were packed with bacteriocin-activated films (Fig. 7). Good results were also obtained by storing hamburgers in bacteriocin-activated films. The initial load of *Listeria* was reduced of almost 1 log during the first 24 h of storage (Fig. 8). Moreover, the *Listeria* viable counts after 24 h of storage in activated films were kept lower than the counts of hamburgers packed with untreated films or unpacked hamburgers (Fig. 8).

DISCUSSION

For active antimicrobial packaging to be effective, an adequate procedure of activation is necessary in order to

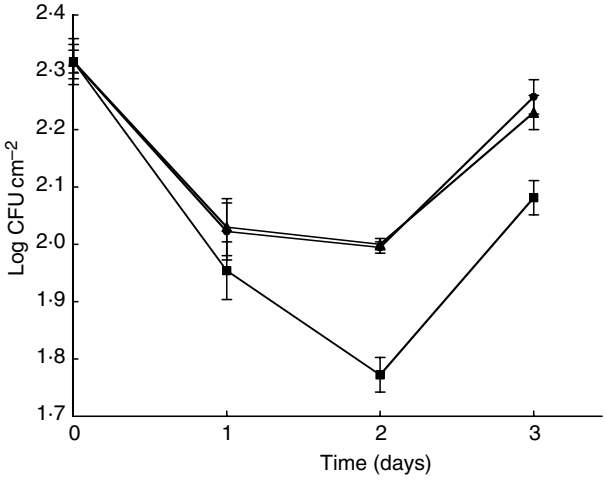

Fig. 7 Viable counts of listeria population during the storage at 4°C of pork steaks spiked with *Listeria monocytogenes* V7. (●) Unpacked steaks; (■) steaks packed with industrially developed bacteriocin-activated PE-OPA film; (▲)steaks packed with untreated film

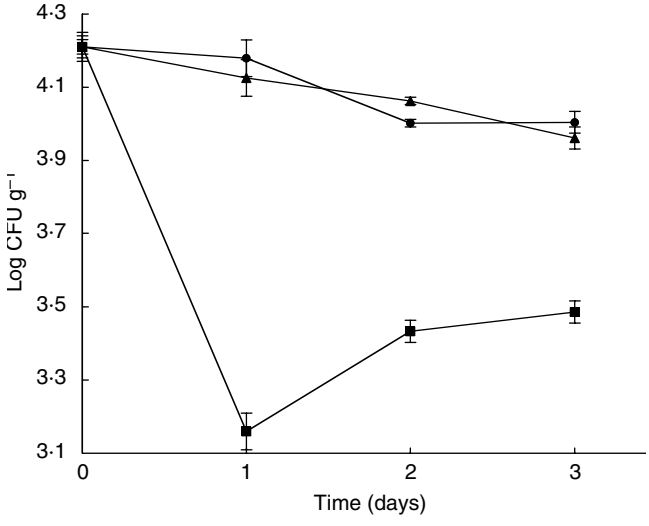

Fig. 8 Viable counts of listeria population during the storage at 4°C of hamburgers spiked with *Listeria monocytogenes* V7. (●) Unpacked hamburgers; (■) hamburgers packed with industrially developed bacteriocin-activated PE-OPA film; (▲) hamburgers packed with untreated film

assure that the antimicrobial is linked to the film and to keep the antimicrobial activity during the film shelf life. Moreover, the activated film has to exert its preservative antimicrobial potential during packed food storage.

In this study different methods were used to bind the bacteriocin 32Y from *Lact. curvatus* 32Y to PE-OPA films. All the activation procedures adopted were successful, although the results of the antimicrobial activity were substantially different. The soaking procedure proved to be effective. However, the distribution of the bacteriocin on the surface of the soaked PE-OPA films was shown to be not homogeneous and the results of the antilisterial activity suggested that the bacteriocin irregularly diffused from the film into the agar. The further test consisted of spotting the bacteriocin on the surface of the PE-OPA film, which demonstrated that even a quick contact of the bacteriocin with the surface of the film conferred activation. As it appears in Fig. 3, the bacteriocin seemed to migrate from the spots and it was not possible to determine whether the bacteriocin migrated into the film from the margins. This is an important characteristic of the substance to be employed because it is essential that preservatives have low diffusivity in their host film and remain at the surface of the food. In fact, diffusion into the food matrix may result in reduction of the preservative concentration at the surface increasing the possibility for the micro-organisms to escape their antimicrobial effect (Han and Floros 1997; Scannell *et al.* 2000). The spray coating also yielded positive results. Also in this case the bacteriocin did not prove to bind homogeneously and firmly to the surface of the film. The spray-coated films proved to be effective in containing the surface-development of *L. monocytogenes* V7 on agar plates (Fig. 5). As a matter of fact, this should be the function of an antimicrobial film used for fresh food packaging where the possible contamination of the food is on the surface and thus the prevention of the surface microflora is important for a long and safe food storage. Many studies have dealt with the activation of plastic films with bacteriocins. Several authors reported on the efficacy of antimicrobial films activated by nisin alone or in combination with other preservatives or chelators and by using different methods of activation (Siragusa *et al.* 1999; Scannell *et al.* 2000; Coma *et al.* 2001; Cutter *et al.* 2001). Some other authors (An *et al.* 2000; Kim *et al.* 2000; Lee *et al.* 2004) described a coating of low-density polythene films with bacteriocins different from nisin. However, the procedures used for the activation of the films were not shown to be adjustable to an industrial production, although the antimicrobial PE film proved to be effective in inhibiting the growth of *Listeria* and *Micrococcus* (An *et al.* 2000; Kim *et al.* 2000; Lee *et al.* 2004). Similarly, Ming *et al.* (1997) developed pediocin-coated casings that showed useful in controlling the growth of *L. monocytogenes* in meat and poultry products. Also in this case, the spray-mediated activation procedure did not exactly fit an industrial model of production. The spray-coating was also used by Natrajan and Sheldon (2000) to develop antimicrobial films activated with nisin.

Therefore, to date little work has been done to prove the suitability of the bacteriocin and of the activation system for a real production. In this study, a film coating procedure with bacteriocin 32Y was developed using an industrial

plant. The PE-OPA films used for the active packaging manufacture were checked for their necessary technological standard characteristics such as solderability, resistance to tensile stress, transparency, etc. before and after the activation treatment and they were always shown to keep their quality after being coated with bacteriocin 32Y. Only about 1 l of PPBAC 32Y solution at 6400 AU ml^{-1} were used to activate about 300 m of PE-OPA film and this proved the process to be not expensive as only 500 ml of *Lact. curvatus* 32Y in broth are needed to produce 1 l of PPBAC 32Y ready for the coating. The bacteriocin-coated films were active against *L. monocytogenes* V7 in agar plates assays and yielded clean, homogeneous and confined inhibition areas, suggesting that the bacteriocin was uniformly bound to the surface of the film and did not diffuse irregularly into the agar. Therefore, the procedure used for the large-scale PE-OPA film activation, including spreading of the bacteriocin on the surface of the film and immediate air drying of the bacteriocin solution by a hot air flux, did not negatively affect the antimicrobial potential of the bacteriocin 32Y. Experiments of migration of the bacteriocin in water, used as simulator of meat products according to the regulation 2002/72/CE, demonstrated that the coated films lost the antilisterial activity in correspondence of the water-treated zones, although no residual antimicrobial activity was registered in the water drops after their contact with the activated film up to 1 h. The concentration of the bacteriocin released in water is probably below the detection limit of the agar diffusion assay used to detect the antagonistic activity. However, migration and activity of the bacteriocins incorporated in food packaging and their effect on microbial development should be assessed *in vivo* in challenge tests directly performed in foods. Before applications to food products can be considered, it is important to first ascertain, insofar as is possible, the shelf life of the bioactive films. Previous studies have shown that bacteriocins retain their activity when applied to various surfaces (Daeschel and Mc Guire 1992; Bower *et al.* 1995a,b; Ming *et al.* 1997). Experiments used to qualitatively monitor the activity and stability of the bacteriocin coated PE-OPA films developed in this study demonstrated that the antilisterial activity was still stable after 4 months of film storage at room temperature. Moreover, the mechanical and standard required properties of the PE films were also stable during the storage proving to be unaffected by the bacteriocin treatment. Therefore, the developed active PE-OPA films, appearing suitable for a real production, were assayed for their antimicrobial activity against *L. monocytogenes* V7 in challenge tests involving storage of fresh meat products at refrigeration temperatures. The growth of the indicator during the storage of pork steaks was inhibited by both treated and untreated films and the effect of the activated films in providing an increasing reduction of *Listeria* population compared with the control was fully different only after the first 24 h. In contrast, a significant decrease in *Listeria* viable counts was registered during the first 24 h of storage of hamburgers packed with antimicrobial PE-OPA film. The overall effect of control of the growth of *Listeria* resulted better in hamburgers than pork steaks storage. This may be due either to the higher superficially concentrated contamination of *Listeria* on the pork steaks, which was more difficult to control, or to the nature of the meat products itself and their possible effect on bacteriocin release and action. In both cases, moreover, an increase in *Listeria* viable counts was registered after 48 h of storage, which may be due to the particular mechanism of action of bacteriocins that can inhibit as many cells as molecules available in the medium (Moll *et al.* 1999). Increasing the concentration of the bacteriocin in the coating solution may be also experimented with the aim of improving the preservative performance of the bacteriocin-coated PE-OPA films in storage of meat as well as other food products. Addition for further hurdle molecules such as EDTA, lisozima, citric acid, lactic acid, lauric acid into the coating solution may improve the antimicrobial performance of bacteriocin-activated films as reported in other studies (Natrajan and Sheldon 2000).

Antimicrobial packaging can play an important role in reducing the risk of pathogen development, as well as extending the shelf life of foods although it should not substitute for good quality raw materials and good manufactures practices. Studies of new food-grade bacteriocins as preservatives and development of suitable systems of bacteriocin treatment of plastic films for food packaging are important issues in applied microbiology and biotechnology, both for implementing and improving effective hurdle technologies for a better preservation of food products.

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