PAPER

Anti-listerial activity of coatings entrapping living bacteria†

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Polyvinyl alcohol (PVOH) based coatings entrapping either living bacteriocin-producer *Enterococcus casseliflavus* IM 416K1 bacteria or Enterocin 416K1 have been prepared and applied to poly(ethylene terephthalate) (PET) films. The antimicrobial activity of coated PET films was evaluated against *Listeria monocytogenes* NCTC 10888 by qualitative agar diffusion assays and by direct contact with artificially contaminated food samples (würstel and seasoned cheese) stored at 4 °C and 22 °C. Anti-listerial activity of both coatings was observed for both tests. However, the live-enterococcus doped coatings showed a much more remarkable anti-listerial activity than enterocin doped ones. Interestingly, live-enterococcus doped coatings lead to a strong decrease of *L. monocytogenes* viable counts even at 22 °C, indicating that they are able to contrast efficiently the fast *L. monocytogenes* growth occurring at this temperature in würstel samples. In this respect, they can be considered smart coatings, being able to be responsive towards an accidental rise of temperature during food storage. The capability of bacteria to survive for a long time can also assure a long lasting antibacterial activity.

Introduction

The food-borne diseases are a widespread and growing public health and economic problem. It has been estimated that about 30% of people in industrialized countries suffer from a food-borne disease each year and at least two million people die from diarrhoeal disease worldwide.[1]

Recent modifications in food production and processing practices and the ever-changing eating habits of the consumers are important factors for the food-borne infections.[2] For example, the trend towards consumption of minimally processed, ready-to-eat foods and refrigerated or frozen food products has affected the incidence of listeriosis,[3] an infection associated with a mortality up to 30%, even when an adequate antimicrobial treatment is administered. *Listeria monocytogenes* is a ubiquitous pathogen that has become an important cause of human food-borne infections worldwide and that primarily affect immuno-compromised individuals and pregnant women.[4] 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. Moreover, this microorganism can be found within biofilms in food processing plants as well as *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas* spp.,[5,6] this last genus including opportunistic pathogens as *P. aeruginosa* and spoilage bacteria commonly found in food industries. Indeed, microbial food spoilage is a field of global concern and it is estimated that as much as 25% of all food produced is lost post-harvest due to microbial activity.[7] There is therefore still a need for new methods of reducing or eliminating food-borne pathogens and spoilage bacteria.

A possible approach proposed to reduce the bacterial growth in foods is the use of antimicrobial active packaging obtained by the incorporation of additives into packaging systems.[8–10] In this respect, one possible attractive approach is the application of functional coatings to the substrates typically used in food packaging.[11,12]

Chemical additives are frequently used in food packaging to limit the microbial growth, however, their employment is less and less accepted by the consumers and limited by more and more restrictive laws. To overcome this hurdle, one possibility is the incorporation in food packaging of natural antimicrobial substances such as bacteriocins[13] (nisin is the most popular one), enzymes (such as lysozyme), chitosan and spice extracts, and many studies have shown these "natural" preservatives to be effective against microorganisms.[12–18] Most of these compounds have been entrapped in different food packaging materials and the retention of their antibacterial activity after inclusion has usually been demonstrated.

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Following this type of approach, in a previous study we evaluated the anti-listerial activity of an organic–inorganic hybrid coating entrapping Enterocin 416K1,[12] the bacteriocin produced by *Enterococcus casseliflavus* IM 416K1, a strain isolated from Italian sausages,[19,20] a food widely used in snacks. However, as it is typical of this approach, the enterocin is released over time and the antibacterial activity of the coating will progressively decrease. This can be a drawback that could in principle be overcome by the entrapment in the food packaging of bacteriocin-producer living microorganisms able to secrete bacteriocin continuously over time, if suitably fed. This novel approach should result in a more efficient and prolonged anti-listerial activity, and even in smart antibacterial coatings, able to contrast adverse environmental conditions, such as an unexpected increase of the temperature, if the increase of the growth rate of enterocin production with temperature is equal or faster than that of the pathogen bacteria. This work describes the preparation of such a kind of coating and its anti-listerial behaviour under various conditions. For this purpose, living *E. casseliflavus* IM 416K1 has been entrapped in PVOH-based coatings applied to PET films, and the anti-listerial activity of these coated films has been tested by qualitative agar diffusion assays and by direct contact with artificially contaminated food samples and compared to that of films coated with enterocin-doped coatings.

To the best of our knowledge this is the first time that such an approach is proposed for food packaging.

Experimental

E. casseliflavus IM 416K1 and Enterocin 416K1 preparation

E. casseliflavus 416K1 is a strain isolated from Italian sausages[20] (Italian sausages by Villani), a commercial popular food, widely used to prepare snacks. It belongs to the microbial flora which gives to the product organoleptic characteristics as well as an adequate maturation.

Crude filtrate supernatant fluid (CFSF) from a 24 h culture at 30 °C in MRS broth (De Man, Rogosa and Sharpe, Oxoid Milan, Italy) of *E. casseliflavus* IM 416K1[19] was collected by centrifugation (10 000 rpm, for 10 min at 4 °C) and separated from the cellular pellet. While the CFSF was dialyzed against 30 mmol l^{-1} sodium acetate buffer (pH 5.3) and filter sterilized (0.45 µm pore-size filter; Millipore Corp., Bedford, Mass.), the pellet, washed twice with sterile Ringer's solution, was maintained at refrigeration temperature and added to 5 ml of fresh MRS broth just before coating preparation. Dialyzed CFSF was two fold concentrated by ultrafiltration through 3 kDa molecular mass exclusion membranes (Diaflo Ultrafiltration Membranes, Amicon Corporation, Beverly, MA, USA).

The inhibitory activity of the concentrate Enterocin 416K1 was quantitatively determined as arbitrary units (AU ml^{-1}) by an agar well diffusion assay,[21] using *L. monocytogenes* NCTC 10888 as indicator, and was found to be 2560 AU ml^{-1}.

Coating preparation

Food packaging grade poly(ethylene terephthalate) thin films (PET, 30 µm thick; 3M Flip-Frame RS 7113) were used as received, 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, $M_w \approx 31\,000$ g mol^{-1}, 86.7–88.7 mol% hydrolysis), 3-(triethoxysilyl)propyl isocyanate (ICPTES), glacial acetic acid, potassium acetate and diethyl ether were supplied by Sigma-Aldrich and used as received without further purification. *N,N*-Dimethylformamide (DMF), supplied by Sigma-Aldrich, was distilled on calcium hydride before use.

In order to allow crosslinking of PVOH under mild conditions (after application of the coating to the PET substrate), commercial polyvinyl alcohol (PVOH) was chemically modified by introducing a limited fraction of trialkoxysilane groups along the chains (PVOH-Si). This chemical step was carried out as follows: 12.0 g of PVOH was dissolved in 120 ml of DMF at 110 °C, then the mixture was cooled at 45–50 °C and 3.0 g of ICPTES were added (corresponding to a molar ratio of about 1 : 20 with respect to the overall amount of vinyl alcohol and vinyl acetate monomeric units). The reaction was carried out for 1 hour at 50 °C under stirring. The triethoxysilane functionalized polymer (PVOH-Si) was recovered by precipitation in diethyl ether and then dried at 80 °C under dynamic vacuum for 2 hours. ^{1}H-NMR spectroscopy was used to estimate the composition of the alkoxysilane-modified copolymer (spectra and assignments are reported in the ESI†). The analysis was performed at 50 °C on a Bruker Avance 400 MHz instrument by using Me$_2$SO-d$_6$ as solvent.

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$_3$COOH/CH$_3$COOK 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 (by adding 2 ml of deionised water and 2 ml of two-fold concentrated dialyzed CFSF to the PVOH-Si solution).

Application of coatings to PET substrate

Coated films have been prepared by applying PVOH-Si aqueous solutions (alone or added with either *E. casseliflavus* IM 416K1 bacteriocin-producer bacteria or Enterocin 416K1) onto PET films (10.5×30 cm^2) using a roll-coater (K Hand Coater, RK 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 also 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 coating.

Antibacterial activity evaluation of the coatings

The antibacterial activity of coated films was evaluated against *L. monocytogenes* NCTC 10888 using two different methods: (i)

qualitative evaluation by a modified agar diffusion assay and (ii) quantitative evaluation of *L. monocytogenes* viable counts in artificially contaminated food samples (würstel and seasoned cheese) after contact with coated films for various times.

(i) For the qualitative evaluation 2×2 cm^2 samples of the live-enterococcus-doped film and the enterocin-doped film were placed onto Tryptic Soy agar (TSA, Oxoid) plates seeded with 10^5 CFU overnight cultures of *L. monocytogenes* NCTC 10888. The plates were incubated at 22 °C for 24–48 h and at 4 °C for 5 days and the antagonistic activity was quantified by a clear zone of inhibition in the indicator lawn in contact with the coated plastic film and around the same. Undoped coated films were also tested as negative control. Experiments were carried out in triplicate.

(ii) The quantitative evaluation of the antimicrobial coating effectiveness towards real foods was determined in würstel and seasoned cheese food samples. In particular, seasoned Tuscan Pecorino cheese and casing-free chicken-meat Vienna sausage (würstel) were used as food samples; their surfaces were contaminated by a 5 min immersion in a 10^6 CFU ml^{-1} suspension of an overnight *L. monocytogenes* NCTC 10888 culture diluted in sterile saline solution (NaCl 0.85%) that resulted in a final absorption of about 10^4 CFU g^{-1} *L. monocytogenes* in food samples.

A portion of samples (25 g) were surface contaminated and singly packaged in doped and undoped coated films and stored at 22 °C and 4 °C. At regular intervals (0, 1, 4, 7 days), the packaged food was removed from the coating (during food removal a particular care was put to avoid to scrape and remove part of the coating) and the food samples were placed in sterile plastic bags, added with 225 ml of buffered peptone water (Oxoid) and homogenised for 1 min in Stomacher (Lab Blender, Seward, London, UK). Serial tenfold dilutions of the obtained suspensions were spread in triplicate on Palcam agar added with selective supplement (Oxoid) and plates were incubated aerobically at 37 °C for 48 h. When necessary, the suspensions were filtered (0.45 mm pore-size filter; Millipore Corp.) for the recovery of the residual listeria cells. Colonies of *L. monocytogenes* were enumerated and results expressed as log CFU g^{-1}. 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* NCTC 10888 were analyzed with a *t*-test for paired data. The statistical probability equal to or less than 0.05 was considered significant.

Results

Antimicrobial film preparation

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.[22–27] In order to exploit PVOH hydrophilicity by avoiding at the same time its dissolution, PVOH has to be crosslinked.[28–32] For this purpose, and in order to exploit the mild sol–gel chemistry for crosslinking, we modified the PVOH molecular structure by including alkoxysilane groups

along PVOH chains (PVOH-Si), according to the reaction reported in Scheme 1.

^{1}H-NMR allowed to estimate the fraction of trialkoxysilane-modified vinyl alcohol moieties. The resulting modified polymer (PVOH-Si) had about 4.5 mol% of trialkoxysilane-modified VOH units, in good agreement with a theoretical amount of 5 mol%, expected from a complete reaction of ICPTES. Spectra of partially hydrolyzed PVOH, ICPTES and PVOH-Si with the relative assignments[33–35] are reported as Fig. S1–S3 in the ESI†.

In the presence of water and acid catalyst, alkoxysilane groups can react through hydrolysis and condensation reactions, leading to insoluble crosslinked PVOH. After overnight reaction at room temperature in the presence of CH$_3$COOH/CH$_3$COOK solution as catalyst, the crosslinked PVOH had a gel fraction of about 83.5% and a swelling ratio in water of 56%. As described below, at this crosslinking density, the environment is suitable for the cell surviving/feeding.

Roll-coating of the original solution (before crosslinking) on PET films allowed preparation of PET coated films of 150 cm^2 with a uniform single-layer coating of 10 μm thick. The coated films were transparent and flexible, as required for food packaging applications. The as prepared PVOH coating applied to the untreated PET substrate shows good mechanical properties (it is not brittle) and a fairly good adhesion (no evidence of coating detachment was observed after repeated manual bendings). However, the coating is removed when submitted to the severe Scotch Tape Test and can be peeled off if suitably scratched. Coating removal is easier when PVOH is highly swollen with water.

Fig. 1a and b show ESEM (surface and cross-sectional) pictures from which it is possible to see the presence of *E. casseliflavus* IM 416K1 cells well entrapped in the coating with a quite homogeneous distribution. Bacteria were found to be still present after repeated washings of the surface with running water.

An important concern is about the ability of *E. casseliflavus* IM 416K1 cells to survive within the coating during preparation and storage. Several evidences suggest that they are able to survive either in the absence or in the presence of feeding products. A first evidence is that living bacteria are able to grow when the film is incubated in contact with a nutrient substrate, as indicated by the growth of small colonies of *Enterococci*, probably on the coating surface in contact with the agar medium (sample 1 in Fig. 2a and sample 2 in 2b and S4 in the ESI†). A second evidence is given by the observed anti-listerial activity which, as discussed in the following, demonstrates that *E. casseliflavus* IM 416K1 is able to produce enterocin for a while when entrapped within the coating.

A further important observation is that *E. casseliflavus* IM 416K1 doped films showed very similar antimicrobial efficiency both just after preparation and after various times of storage at 4 °C as tested (up to one month) by the qualitative evaluation

Scheme 1 Reaction scheme of the preparation of triethoxysilane functionalized polyvinyl alcohol [PVOH-Si].
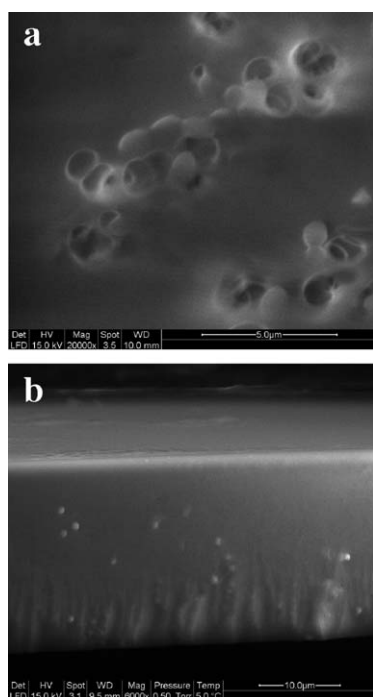
Fig. 1 ESEM electron microscope images showing the presence of *E. casseliflavus* IM 416K1 cells on the surface (a) and within the coating (b) cross-sectional view of the fractured coating.

method. This means that the capability of *E. casseliflavus* IM 416K1 to produce enterocin is not adversely affected by storage at 4 °C even in the absence of feeding. At present, we have still not investigated if bacteria are able to growth when entrapped within the coating. However all the above reported evidences confirm that the chemical environment within the coating and the treatments employed for its preparation/manipulation do not negatively affect the livability of *E. casseliflavus* IM 416K1 cells.

Qualitative antibacterial activity evaluation of enterocin- and live-enterococcus-doped films

Fig. 2a (sample 3) shows the inhibitory activity of the concentrate Enterocin 416K1 against *L. monocytogenes* NCTC 10888. Fig. 2a (samples 1 and 2) and 2b (samples 2 and 3) show the results of the qualitative evaluation of antibacterial activity of enterocin-doped films and live-enterococcus-doped films against listeria by direct contact at 22 °C and 4 °C. PVOH undoped coatings (not reported in Fig. 2) did not show any appreciable antibacterial activity. Instead, the antibacterial activity of both coated films is revealed by a clear inhibition zone in the indicator lawn in contact with and around the films. Of course, the inhibiting effects of live-enterococcus-doped coatings against *L. monocytogenes* in a given place around the films depend on the relative rate of several phenomena that occur during the tests, namely: *L. monocytogenes* growth; *E. casseliflavus* IM 416K1 growth; enterocin production by *E. casseliflavus* IM 416K1; enterocin diffusion out of the coating and within the agar medium; enterocin killing capability. The relative rate of these phenomena can affect the inhibition zone size. The comparison of the inhibition zone around the enterocin-doped and live-enterococcus-doped films shows that the inhibitory activity is similar at 4 °C, while the
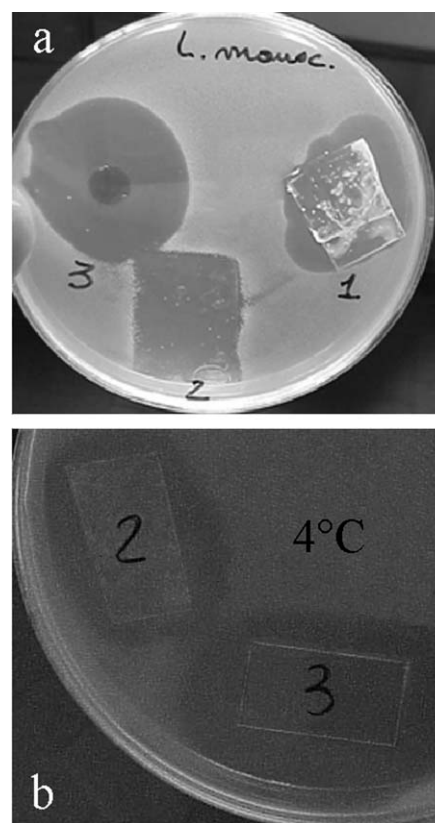
Fig. 2 Qualitative agar well diffusion tests to evaluate antibacterial activity of enterocin-doped and live-enterococcus-doped films against *L. monocytogenes* NCTC 10888 by direct contact at 22 °C (a) and 4 °C (b) of incubation. (a) 1: live-enterococcus-doped film; 2: enterocin-doped film; and 3: concentrate Enterocin 416K1 [CFSF]. (b) 2: live-enterococcus-doped film; and 3: enterocin-doped film.

activity of the live-enterococcus doped film appears significantly higher at 22 °C. The apparently lower activity of enterocin-doped coatings at 22 °C can be explained assuming that the metabolism of *L. monocytogenes* NCTC 10888 is higher at 22 °C than at 4 °C, and that the effect of the temperature on the diffusion rate of enterocin out of the films in the surrounding medium is negligible compared to that on the bacteria growth rate. Based on these reasonable assumptions, the results observed for live-enterococcus-doped films suggest that also the metabolism of *E. casseliflavus* IM 416K1 is significantly increased by rising the temperature from 4 °C to 22 °C.

Finally, the absence of colonies of bacteria in the agar medium, far from the film surface, suggests that bacteria are not able to move away from the film surface.

Quantitative evaluation of antibacterial activity in artificially contaminated foods

In a previous paper it has been shown that when inoculated in Italian sausages at 10^5 CFU g^{-1}, *E. casseliflavus* IM 416K1 is able to reduce the content of *L. monocytogenes* NCTC 10888 from 10^4 CFU g^{-1} to 0 CFU g^{-1} in 10 days.[19]

Fig. 3(a,b) and 4(a,b) report the mean values of the *L. monocytogenes* viable counts (log CFU g^{-1}) detected in the contaminated seasoned cheese and würstel samples, respectively,

packaged either with doped (live-enterococcus-film and enter-ocin-film) or undoped (control) coated films, and after storage at 22 °C (Fig. 3a and 4a) and 4 °C (Fig. 3b and 4b). In all cases, the undoped film was not able to inhibit the listeria growth.

In contrast, in seasoned cheese samples packaged with enterocin-doped films and stored at 22 °C (Fig. 3a), *L. monocytogenes* viable counts decreased 0.7 log during the first 24 h and showed a reduction of 3.6 log compared to the control ($p < 0.05$), after 7 days. Under the same conditions, seasoned cheese packaged with the live-enterococcus-doped film showed a decline less evident in the first 3 days, but the bacteria reduction was of 4.3 log after 7 days, compared to the control ($p < 0.01$).

When seasoned cheese samples were stored at 4 °C (Fig. 3b) a significant decrease of *L. monocytogenes* viable counts was observed, similar for the two differently doped films up to the 3rd day, while over the same time *L. monocytogenes* viable counts increased to 5 log CFU g^{-1} in the control sample. After 7 days, the decrease induced by live-enterococcus-doped films is again significantly higher (to 0.6 log, $p < 0.05$) than for enterocin-doped films (decreased to 2.6 log, $p < 0.01$).

In würstel samples stored at 22 °C (Fig. 4a) a strong and rapid growth of listeria viable counts was observed within 3 days for the undoped film. For the same würstel samples packaged with enterocin-doped films the growth was delayed until the 3rd day, however, after that time the listeria viable counts started to increase reaching 6.8 log after 7 days, with a limited difference compared to the control ($p = 0.075$). In contrast, in würstel samples packaged within live-enterococcus-doped films the listeria viable counts showed a strong decrease in the first three days (differences of 4.1 log compared to the sample packaged with the
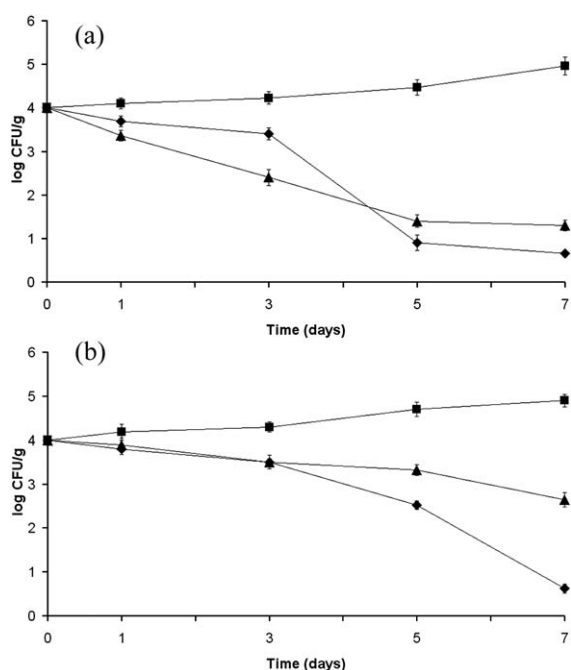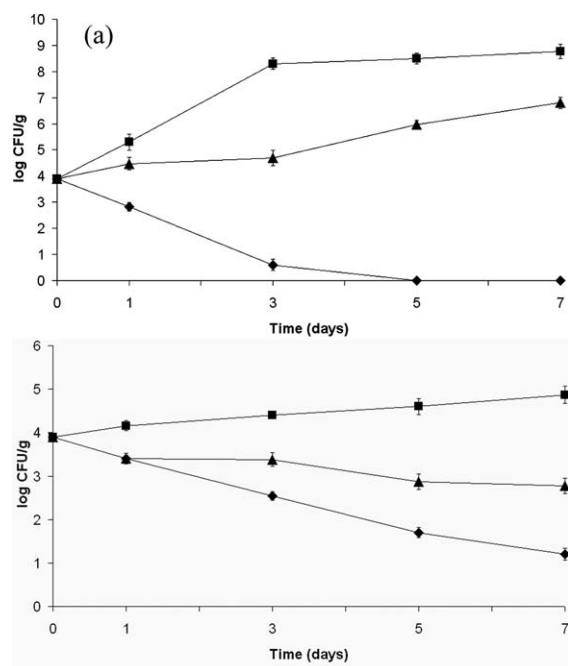
Fig. 4 *L. monocytogenes* NCTC 10888 viable counts observed in the contaminated würstel samples packaged in live-enterococcus-doped film [◆], enterocin-doped film [▲] and undoped PVOH-coated film [■] stored (a) at 22 °C and (b) at 4 °C. Error bars represent standard deviations. The rates of decline of *L. monocytogenes* NCTC 10888 analyzed with a *t*-test for paired data are significant [with the exception of listeria viable counts in the samples packaged with enterocin-doped film at 22°C].

enterocin-doped film and of 7.7 log compared to the undoped one) and still more in the following days, reaching differences of 6.8 and 8.7 log, relative to the other two samples ($p < 0.05$ and $p < 0.01$), after one week.

The change of listeria viable counts with time in würstel samples stored at 4 °C is reported in Fig. 4b. Both doped coatings showed a significant antibacterial activity ($p < 0.01$) when compared to the undoped one. The effect of the live-enterococcus-doped film is again significantly higher than that of enterocin-doped one, even though the difference is less impressive than that observed at 22 °C.

It is finally interesting to note that after storage under different conditions and for various times some organoleptic characteristics of the food samples, like texture, colour and odour, were found to be better in the food samples packaged with doped films than for undoped control ones and these effects are particularly relevant for the live-enterococcus doped film at 22 °C.

This means that the interaction of *E. casseliflavus* IM 416K1 growth with the growth of spoilage microorganisms contributes to preserve the quality and to extend the shelf-life of the foods, even though it is possible that a direct antagonism, due to *E. casseliflavus* IM 416K1 growth, can operate in addition to the effect of bacteriocin.

Discussion

In a previous study it has been demonstrated that a polymer film coated with a hybrid coating entrapping Enterocin 416K1[12] has anti-listerial activity, and also other authors have observed

Fig. 3 *L. monocytogenes* NCTC 10888 viable counts observed in the contaminated seasoned cheese samples packaged in live-enterococcus-doped film [◆], enterocin-doped film [▲] and undoped PVOH-coated film [■] stored (a) at 22 °C and (b) at 4 °C. Error bars represent standard deviations. The rates of decline of *L. monocytogenes* NCTC 10888 analyzed with a *t*-test for paired data are significant.

similar results in reducing bacteria populations within food samples by wrapping up them in packaging containing bacteriocins.[13,17,36–41] However, the progressive release of entrapped enterocin leads to a progressive decrease of antibacterial activity of the doped coating, making such a type of approach less effective over long times and in particular at high temperature, when *L. monocytogenes* growth is fast (see Fig. 4a). The entrapment of living bacteria in suitable coatings, like live-enterococcus-doped films described in this work, can overcome this drawback and this novel approach seems an important step for a potential innovation in food storage systems. Even though entrapment of living microorganisms in plastic gels or coatings has already been reported,[42–45] to the best of our knowledge this is the first time that such an approach is proposed for antibacterial food packaging. Indeed, the entrapment of living bacteria that are able to maintain their liveability when in contact with foods and to produce continuously bacteriocin can result in several advantages. In this respect, the entrapment of *E. casseliflavus* IM 416K1 in PVOH-based coatings applied to plastic films for food packaging is interesting as it could generate continuously Enterocin 416K1 which has antimicrobial activity against various bacteria and in particular against *L. monocytogenes*.[12] As it has been reported in an our previous work,[46] the anti-listerial activity of *E. casseliflavus* 416K1 is due to bacteriocin production and not to other metabolic products released, as hydrogen peroxide or organic acids. Therefore, in this work, the antibacterial activity of *E. casseliflavus* IM 416K1-doped films has been compared to that of Enterocin 416K1-doped films. As shown by the above reported results, the *E. casseliflavus* IM 416K1-doped films showed in general a better antibacterial efficiency compared to that of Enterocin 416K1-doped films, and in particular, the antagonistic action of the live-enterococcus doped films against *L. monocytogenes* is much better than that of enterocin-doped films at 22 °C, as attested by both qualitative modified agar diffusion assays and quantitative evaluation of *L. monocytogenes* counts in previously contaminated foods. The effectiveness of live-enterococcus doped coatings is higher for würstel than for seasoned cheese food samples. There are several reasons that may explain this behaviour. In the case of seasoned cheese samples, the antagonistic competition due to indigenous bacteria contained in seasoned cheese is probably one of these reasons as it can contribute to reduce the *L. monocytogenes* growth rate, however a less effective *E. casseliflavus* IM 416K1 feeding (and in turn enterocin production) and a slower enterocin diffusion in the food cannot be excluded. On the other hand, for würstel the results can be explained by considering that it is a substrate where *L. monocytogenes* can grow easily and quickly at high temperature (see Fig. 4a), so that the release of enterocin from enterocin-doped coatings is effective only for the first few days, while it becomes progressively less effective in the subsequent days, resulting, after one week, in a *L. monocytogenes* viable count similar to that observed for undoped coatings (see Fig. 4a). In contrast, the strong progressive decrease of *L. monocytogenes* counts when the live-enterococcus-doped film was used to wrap contaminated würstel means that *E. casseliflavus* IM 416K1 bacteria are able to produce enterocin continuously and at a fast rate. It means also that live-enterococcus-doped films are responsive to temperature and able to contrast an accidental increase of temperature during food storage. This is a very interesting outcome because even in the case of cold-chain interruption the live-enterococcus-doped films would be able to inhibit the listeria growth. In this respect, live-enterococcus-doped films can be considered smart food packaging able to be responsive to the risk of a fast pathogen bacteria growth in contaminated foods. The continuous production of bacteriocin from living *E. casseliflavus* IM 416K1 bacteria can also allow to extend the antibacterial activity of live-enterococcus-doped coatings over times as long as the living time of bacteria. At present, we have observed a good antibacterial activity up to one month of storage, however we are confident that the living time can be much longer if required.

Of course, in view of a possible application to commercial foods, it is of great importance to know and understand what consequences could arise if for example a human was exposed to fairly large amounts of these bacteria. Indeed, it has been reported that *E. casseliflavus* bacteria can cause bacteremia in immunosuppressed patients,[47] are listed as being able to cause nosocomial disease[48] and can show an intrinsic resistance to some antibiotics.[49] However, we have to emphasize that *E. casseliflavus* IM 416K1 did not show any virulence character (hemolysins, gelatinase, *etc.*) or antibiotic resistance (unpublished author's data) and that there is a wide literature that reports that even the most harmless bacteria (like probiotics) can cause infections in immunosuppressed people.

In addition, we have to consider that *L. monocytogenes* is a psychrotrophic foodborne pathogen that represents a serious health hazard[50] and that the incidence of listeriosis is increasing in various countries.[51–53] So, it is clear from the literature that the biological hazard of *L. monocytogenes* is by far higher than that of *E. casseliflavus*.

Even though the investigation of the potential pathogenicity of *E. casseliflavus* IM 416K1 is out of the scope of the present work, we have to emphasize again that it is a strain isolated from Italian sausages,[20] a very popular commercial food, and we believe that the widespread use of this food can be considered as a preliminary indication of safety.

Finally, it has to be stressed that this is a general approach that can in principle be extended to other bacteriocin-producer bacteria and in this perspective this approach should be easily extended to promote antibacterial activity even for other foodborne pathogens.

Conclusions

This work demonstrates that it is possible to entrap living *Enterococus casseliflavus* IM 416K1 bacteria in PVOH-based coatings. It also demonstrates that, due to the mild conditions used to crosslink the coatings, entrapped *E. casseliflavus* IM 416K1 bacteria are able to survive the stage of coating production and application, as well as the storage in the absence of nutrients, and that they are able to produce bacteriocin over long times in different environmental conditions. The Enterocin 416K1 produced by the living bacteria either at 4 °C or at 22 °C is able to attack and kill efficiently *Listeria monocytogenes* NCTC 10888 making live-enterococcus-doped coatings very efficient anti-listerial systems. The antibacterial activity of coatings entrapping *E. casseliflavus* IM 416K1 is better than that of enterocin-doped coatings both in qualitative modified agar

assays and in contact with real foods, such as seasoned cheese and würstel. In particular live-enterococcus-doped coatings are very effective at high storage temperature (22 °C) in contact with listeria-contaminated würstel, where the *L. monocytogenes* growth is fast. In this case, the live-enterococcus-doped coating behaves like a smart coating being responsive to accidental critical storage conditions.

The continuous production of Enterocin 416K1 by *E. casse-liflavus* IM 416K1 entrapped in the coatings is also able to extend a very effective antibacterial activity over very long times.

Finally, it has to be emphasized that the entrapment of living bacteria in suitable coatings can, in principle, be extended to other bacteriocin-producer bacteria and to bacteria endowed with probiotic activity, and is therefore full of perspectives for future applications in the food and health industry.

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