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Gelatine based films added with bacteriocins and a flavonoid ester active against food-borne pathogens

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ABSTRACT

Active films, based on food grade gelatine added with bacteriocins and/or the flavonoid ester prunin laurate as antimicrobial compounds, were prepared. The films were characterized by water vapor permeability, mechanical property measurements and scanning electron microscopy. Film antibacterial activity was determined by the agar diffusion and direct contact microplate techniques against *Listeria monocytogenes* 01/155, *Staphylococcus aureus* ATCC29213 and *Bacillus cereus* 1. Physical properties of gelatine films were not significantly affected by active compound incorporation. Antibacterial effect on each strain was dependent on the active compound incorporated into the film, but in all cases a synergistic inhibitory action was observed when both antimicrobial compounds were added. Thus, the results of this study suggest that gelatine based active films here presented could provide an alternative strategy for food biopreservation.

Industrial relevance: This work proposes the elaboration of active films based on food grade gelatine and the addition of enterocins A, B and P synthesized by *Enterococcus faecium* SM21 and the flavonoid ester, prunin laurate, as antimicrobial active compounds. Films were prepared using a simple and low cost methodology and their application can be adapted to different kinds of food systems. The inclusion of the antimicrobial compounds on the film matrix did not alter their functional properties. The obtained films were active against *L. monocytogenes*, *S. aureus* and *B. cereus*, providing an alternative tool for food preservation, especially in the case of foods susceptible to contamination by these pathogens. Although the inhibitory effect of active films was different for each indicator strain, in all cases a synergistic inhibition effect was observed when both active compounds, ES and PL, were added to gelatine films. This synergistic effect between both antimicrobial compounds offers a novel potential *hurdle technology* for food preservation.

1. Introduction

The growing demand for high quality and extended shelf-life foods, along with environment protecting policies, has encouraged research and development of biodegradable edible films and coatings to be used in food packaging (Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011; Tharanathan, 2003). Films are based on substances of natural origin (polysaccharides, proteins, polyesters, lipids) that form a continuous matrix. They can also act as a carrier for active compounds such as antimicrobials, antioxidants, and flavors; becoming "active films" (Cagri, Ustunol, & Ryser, 2004). Thus, "active packaging" satisfies an additional function besides merely protecting and enveloping the food; it also acts by modifying and controlling food surface conditions. These "active packages" can act in combination with other preservation methods, enhancing and improving the conservation action (Kuorwel et al., 2011).

Bacteriocins are antimicrobial peptides synthesized by various lactic acid bacteria which present antimicrobial activity against other related species (Jack, Tagg, & Ray, 1995). They have GRAS (Generally Recognized As Safe) status; and those particularly synthesized by the genus *Enterococcus* are called enterocins (Eijsink, Skeie, Middelhoven, Bente Brurberg, & Nes, 1998). Due to their anti-*Listeria*

Abbreviations: CFS, cell free supernatant; UA, arbitrary units; ES, enterocin solution; PL, prunin laurate; Gel, gelatine film (control); Gel/ES, gelatine film added with ES; Gel/PL, gelatine film added with PL; Gel/ES/PL, gelatine film added with ES and PL.

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monocytogenes effect, they gained particular interest as potential natural and safe biopreservatives in the food industry (Gálvez, Abriouel, López, & Omar, 2007). Additionally, *Citrus* flavonoids are natural compounds with beneficial properties on human health Benavente García & Castillo, 2008; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). Although they do not present per se relevant antibacterial properties, the incorporation of aliphatic chains to their structures significantly enhances flavonoid antimicrobial activity, mainly against Gram positive bacteria (Céliz, Audisio, & Daz, 2010; Céliz, Daz, & Audisio, 2011).

The direct introduction is the standard method for the addition of antimicrobials into food products. However, the interaction of the antimicrobial substances with various food components reduces its effectiveness against pathogens. Therefore, the use of alternative methods to reduce such interactions could improve the activity of the compound and its stability in complex food systems (Were, Bruce, Davidson, & Weiss, 2004). The incorporation of antimicrobial compounds into food coatings is an interesting strategy since only the necessary amount of the agent will be used and will not constitute a direct food additive (Gennadios, Hanna, & Kurth, 1997). Also, the additive is concentrated in the food area where microbial contamination occurs, i.e. on the surface of the food, and a controlled diffusion of the additive into the food is possible. In this regard, antimicrobial protein based films offer desirable mechanical, gas barrier, and optical properties, as well as nutritional extra value (Rawdkuen, Suthiluk, Kamhangwong, & Benjakul, 2012). Gelatine, a natural biopolymer derived from collagen of animal origin, particularly forms thin, flexible, and transparent biodegradable films; providing environmental compatibility as well as longer shelf life of foods when used as bio-packaging (Kavoosi, Dadfar, & Purfard, 2013; Ku & Song, 2007). Diverse natural antimicrobials, such as chitosan (Gómez Estaca, Gomez Guillen, Fernandez Martin, & Montero, 2011; Pereda, Ponce, Marcovich, Ruseckaite, & Martucci, 2011), lysozyme (Bower, Avena Bustillos, Olsen, Mchugh, & Bechtel, 2006), essential oils (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Gómez Estaca, de López Lacey, López Caballero, Gómez Guillén, & Montero, 2010) and nisin (Ku & Song, 2007), have been included in gelatine based "active" films.

In the present work, the effect of the incorporation of enterocins A, B and P synthesized by *Enterococcus faecium* SM21 and the flavonoid ester, prunin laurate, on antimicrobial and functional properties of gelatine based edible films was analyzed. Also, the impact of the immobilization of these compounds into the film on their antimicrobial activity against potentially pathogenic foodborne microorganisms, such as *L. monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*, was evaluated.

2. Materials and methods

2.1. Strains and culture conditions

E. faecium SM21, isolated from bee intestine (GenBank access code EU428012), was used as bacteriocin (enterocins A, B and P) producing strain (Audisio, Torres, Sabaté, Ibarguren, & Apella, 2011). *L. monocytogenes* 01/155 (MCA INIQUI-CONICET, Salta, Argentina), *S. aureus* ATCC29213 (American Type Culture Collection, USA) and *B. cereus* 1 (Institute of Microbiology "Dr. Carlos Malbrán", Buenos Aires, Argentina), were used as indicator strains. The cultures were activated by successive transfers in Brain–Heart Infusion broth (BHI, Britania, Argentina) or in MSC broth (medium without casein, designed in the laboratory: peptone 1% w v^{-1} meat extract, 0.5% w v^{-1} soybean, 0.3% w v^{-1} meat extract, 1% w v^{-1} glucose, 0.1% v v^{-1} Tween 80, pH 6.5) (Ibarguren, 2010) and incubated at 37 °C for 16–20 h. When agarized media were required, 1.5% w v^{-1} agar (Britania, Argentina) was added to the culture media. All strains were maintained at −20 °C in BHI broth with the addition of 10% v v^{-1} glycerol.

2.2. Enterocin solution

E. faecium SM21 was cultured in MSC broth (37 °C, 16 h) and the cells were removed from the fermentation broth by centrifugation (10,000 *g*, 20 min, 10 °C) and filtration (0.22 μm pore size). The resulting cell-free supernatant (CFS) was considered the enterocin solution (ES) and was kept at 4 °C until used to prepare the different edible films.

The antimicrobial titre of the ES was determined by the serial dilution method (Daba et al., 1991), using *L. monocytogenes* 01/155 as the indicator strain. Briefly, *Listeria* cells were grown overnight in BHI broth and 100 μL of this sensitive strain suspension was sieved in molten BHI agar cooled to 45 °C. The inoculated medium was introduced onto Petri dishes and dried for 30 min. The ES was two-fold serially diluted in sterile distilled water and aliquots (20 μL) of each dilution applied in 5 mm wells made in the BHI agar plate. The plate was incubated at 25 °C for 20 h and the presence of inhibition halos was observed. The suspension titre was expressed in arbitrary units per milliliter (AU mL^{-1}) and calculated as follows: (1000) / (Vs. D), where Vs: bacteriocin volume tested (μL), and D: the highest dilution that still inhibited cell growth. This determination was performed in triplicate. The final titre determined for the ES against *L. monocytogenes* 01/155 was 800 AU mL^{-1}.

2.3. Prunin laurate solution

Prunin laurate ester (PL) was synthesized according to Céliz and Daz (2011). The process basically consists in a transesterification reaction catalyzed by a commercial lipase (Novozym 435, Sigma-Aldrich) in an organic solvent (acetone) using the flavonoid prunin as alcoholic group and vinyl laurate ester as acyl donor group. The reaction was carried out until prunin was converted totally to their corresponding lauryl ester, following the conversion by HPLC (*ca.* 24 h at 50 °C). Then, the solvent was removed on a rotary evaporator at room temperature. The solid residue was washed with hexane to eliminate vinyl laurate excess. The ester was recovered by dissolving the solid in dimethyl sulfoxide, filtering and evaporating the solvent under a hood. The purity of the ester was close to 100% determined by HPLC.

2.4. Film preparation

The commercial bovine food-grade gelatine type A (bloom 125) was obtained from Dulfix (Buenos Aires, Argentina). The films were prepared by dissolving gelatine 5% w v^{-1} in hot water with the addition of glycerol as plasticizer in a 10% w w^{-1} ratio relative to polymer. Prior to the film casting and drying, the following substances, alone or in combination, were added to the polymeric matrix, in order to obtain active films: i) ES to reach a final concentration of 63 UA cm^{-2} of dry film (ES from *E. faecium* SM21, 800 AU mL^{-1}), and ii) PL ethanol solution to obtain a final concentration of 0.039 μmol cm^{-2} of dry film (from 25 mM stock solution). Also, films based on gelatine, pure solvent (distilled water), and glycerol were prepared to be used as control. Films were dried at 37 °C and stored at room temperature in closed containers. In summary, the following films were prepared: Gelatine (Gel, control), Gelatine with ES (Gel/ES), Gelatine with PL (Gel/PL), and Gelatine with ES and PL (Gel/ES/PL).

2.5. Film characterization

Films were characterized in terms of their water vapor permeability, mechanical properties and morphology by scanning electron microscopy. Water vapor permeability was determined through ASTM E-96 "Standard test methods for water steam transmission of materials" by continuous measurements of weight loss using a precision balance connected to a computer. Mechanical properties were determined according to ASTM D882 "Standard test methods for tensile properties of thin plastic sheeting" using a computer QTS Texture Analyzer

(Brookfield, England). The film microstructure was studied by previous fracturing with liquid nitrogen and metallizing with gold plasma, using a Scanning Electron Microscope JSM 6480 JEOL Model LV (Jeol Ltd., Japan).

2.6. Antimicrobial film activity

The possible interactions between the polymer matrix and the antimicrobial compounds were analyzed by evaluating the antimicrobial activity of the active films against *L. monocytogenes* 01/155, *S. aureus* ATCC29213 and *B. cereus* 1.

2.6.1. Agar diffusion technique

The activity of the antimicrobial compounds immobilized on the gelatine films was determined against the selected pathogenic strains using an adaptation of the agar diffusion method (Ibarguren, Vivas, Bertuzzi, Apella, & Audisio, 2010). Fragments of 1 cm^2 of each film were cut, sterilized under UV light, and placed over solidified BHI agar. The agar was previously inoculated with each pathogen in order to obtain a lawn (10^7–10^8 cfu mL^{-1}) of the strain. After 24 h incubation at 37 °C the presence of halos of inhibition was evaluated.

2.6.2. Microplate direct contact

Sterile 12 well cell culture microplates (BD Falcon™) were used. Fragments of 1 cm^2 of each film, previously sterilized under UV light, were placed in each well in contact with 3 mL of peptone water suspensions (*ca.* 10^5 cfu mL^{-1}) of each indicator strain. The viability of each pathogen was computed during 24 h of contact (0, 2, 4, 6 and 24 h) by plate counts using BHI agar (Britania, Argentina). The plates were incubated at 37 °C for 24 h. Suspensions of each indicator strain in peptone water and in contact with films made without the addition of active substances were used as controls of each pathogen growth. Also, pathogen cell response to an amount of free ES and free PL, equivalent to that incorporated to the 1 cm^2 fragment of each film, was evaluated.

2.7. Statistical analyses

GraphPad Prism 5 Software (GraphPad Software Inc., USA) was used for all statistical analyses. Analyses of variance (ANOVA) linear and nonlinear regressions were applied. The significance levels used were $P < 0.05$. All assays were carried out at least in triplicate.

3. Results and discussion

The inclusion of two types of antimicrobial substances, enterocins and a flavonoid ester, in edible gelatine films for their potential application as food bioprotectors was studied. Gálvez et al. (2007) emphasize the advantages of this application method, since the carrier acts as reservoir and diffusor of the concentrated antimicrobial molecules, ensuring a gradual and continuous supply of the additives into the food. Besides, the immobilizing agent protects antimicrobials from inactivation or interaction with food components. Moreover, the precise location of the compounds on the food surface requires relatively smaller amounts than when they are applied throughout the food, which means a reduction in the process costs (Balciunas et al., 2013; Gálvez, Abriouel, Benomar, & Lucas, 2010).

On this basis, active gelatine films, added with enterocins A, B and P synthesized by *E. faecium* SM21 and the flavonoid ester PL, were designed. These additives were selected since in previous assays, enterocins showed a clear anti-*L. monocytogenes* effect (Audisio et al., 2011), while the flavonoid ester presented inhibition against *S. aureus* and *B. cereus* (Céliz et al., 2010, 2011). Different combinations of the antimicrobials were considered in the film preparation, in order to analyze possible synergistic or adverse effects on their antimicrobial activity.

3.1. Film characterization

Gelatine based films can be produced by drying thermally reversible gelatine gel formed from cross-linking between amino and carboxyl components of amino acid residue side groups. Gelatine forms clear, flexible and oxygen-impermeable films when cast from aqueous solutions in the presence of plasticizers (Gennadios, McHugh, Weller, & Krochta, 1994). Edible coatings with gelatine reduce oxygen, moisture and oil migration, or can carry antioxidant or antimicrobial agents (Krochta & De Mulder-Johnston, 1997). Mechanical and barrier properties of these hydrophilic films are affected by film water content and the polymer structure and molecular weight. The results of the functional properties (tensile strength, elongation and water vapor permeability) of films prepared with and without antimicrobial additives are presented in Table 1. No significant differences were found between the mechanical properties of the different films, showing an average tensile strength value of 4.33 MPa and elongation at break of 30.73%. Film water vapor permeability was neither affected by the addition of the antimicrobial compounds, showing an average value of 2.64×10^{10} g m^{-1} s^{-1} Pa^{-1}. Ma et al. (2012) reported TS of 6.1 MPa and WVP of 5.61 g m^{-1} Pa^{-1} s^{-1} for films based on bovine hide gelatine type B (Bloom 150) and plasticized with glycerol; and Carvalho et al. (2008) informed TS of 3.8 MPa and WVP of 3.33 g m^{-1} Pa^{-1} s^{-1} for films based on skin gelatine from the Atlantic halibut (*Hippoglossus hippoglossus*). These values are in the same range of those presented in Table 1. Similar behavior was previously observed when enterocin solution was added to films based on goma brea, gluten, and even gelatine (Ibarguren et al., 2010). Film morphology was observed by scanning electron microscopy. The micrographs revealed that the films were dense (non-porous) and no differences were detected in the matrix structure by the addition of the enterocins, the flavonoid product, or both components (Fig. 1). Compatibility between components was enough to avoid detectable phase separation. Also, good miscibility and distribution of the antimicrobial substances in the gelatine matrix were observed.

Therefore, it can be concluded that the addition of these substances at the concentrations studied does not affect functional properties of films, since no deterioration in the mechanical and barrier properties was detected; a situation that favors their potential application.

3.2. Antimicrobial activity of films

The potential antimicrobial activity of the different films was analyzed through two different techniques.

The agar diffusion method revealed that control films (i.e. without addition of bioactive compounds) had no inhibitory activity against bacterial indicator strains (Fig. 2A_1, B_1 and C_1). Thus, gelatine per se has no inhibitory effect but could act as suitable matrix to support the antimicrobial substances here considered. The films added with ES or PL, in turn, presented an indicator strain dependent inhibition, but in all cases bioactive films were obtained (Fig. 2). *L. monocytogenes* 01/155 was clearly inhibited by the films prepared with ES. The PL ester supported on gelatine did not inhibit this pathogen and the combination with ES apparently did not modify the inhibition exerted by the ES

Table 1
Mechanical and barrier properties of gelatine edible films with and without addition of antimicrobial compounds.

Film composition	Tensile strength (MPa)	Elongation (%)	Water vapor permeability 10^{10} g m^{-1} s^{-1} Pa^{-1}
Gel	3.25 ± 0.82	48.52 ± 6.45	2.356 ± 0.619
Gel/ES	4.16 ± 0.79	30.04 ± 7.74	2.862 ± 0.430
Gel/PL	3.36 ± 1.15	47.58 ± 5.31	1.979 ± 0.521
Gel/ES/PL	5.02 ± 1.85	35.90 ± 4.84	2.568 ± 0.252

Gel: gelatine; ES: enterocin solution; PL: prunin laurate.

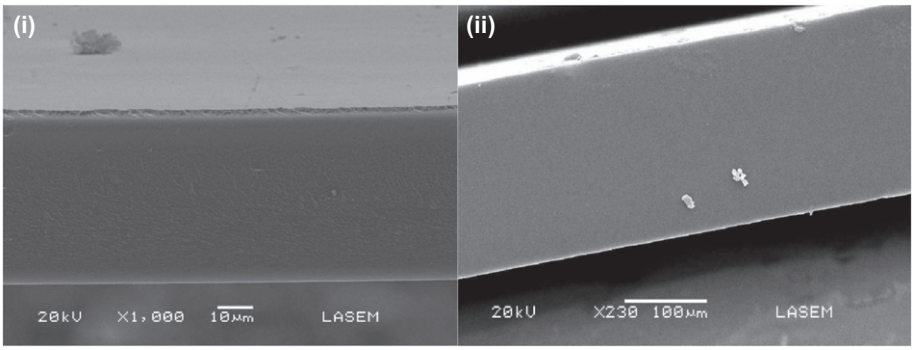

Fig. 1. SEM microphotographs of the fracture section of gelatine based films: (i) control film (without additives), (ii) active film with a final concentration of *ca.* 64 UA enterocin cm^{-2} and 0.039 μmol prunin laurate cm^{-2}.

acting individually (Fig. 2A). *S. aureus* and *B. cereus* ATCC29213 exhibited similar inhibition profiles (Fig. 2B and C). Both pathogens were clearly inhibited by the films added with PL and ES/PL; while ES individually supported on gelatine films did not inhibit these microorganisms.

The analysis of direct contact between each pathogen and the different films confirmed that the cell growing patterns were dependent on both film additive and indicator strain (Fig. 3). As observed in the agar diffusion technique, the direct contact method showed no inhibitory effect of gelatine films on the tested pathogens. In general, the viability of cells in contact with the control gelatine film, without additives, was similar to the cell growth observed in suspensions of each indicator strain in peptone water (Fig. 3A_1, B_1 and C_1).

When *L. monocytogenes* 01/155 grew in contact with films added with ES, an immediate inhibitory effect was observed. This bactericidal effect was noticeable during the first 6 h of contact, with a subsequent decrease on the inhibitory effect. This behavior can be noticed by comparing the curves representing the pathogen growth in contact with ES active films and that corresponding to the pathogen growth in peptone water (Fig. 3A). The addition of PL to gelatine films, instead, exerted a lower inhibitory effect on this strain. The pathogen viability presented a slight decrease after 4 h of contact with the PL active film, but this inhibition was not maintained after 24 h of contact (Fig. 3A). The inhibition behavior was similar to that observed when the pathogen grew in contact with a free PL solution prepared with a concentration of PL equivalent to the 0.039 μmol cm^{-2} included in 1 cm^2 of active film.

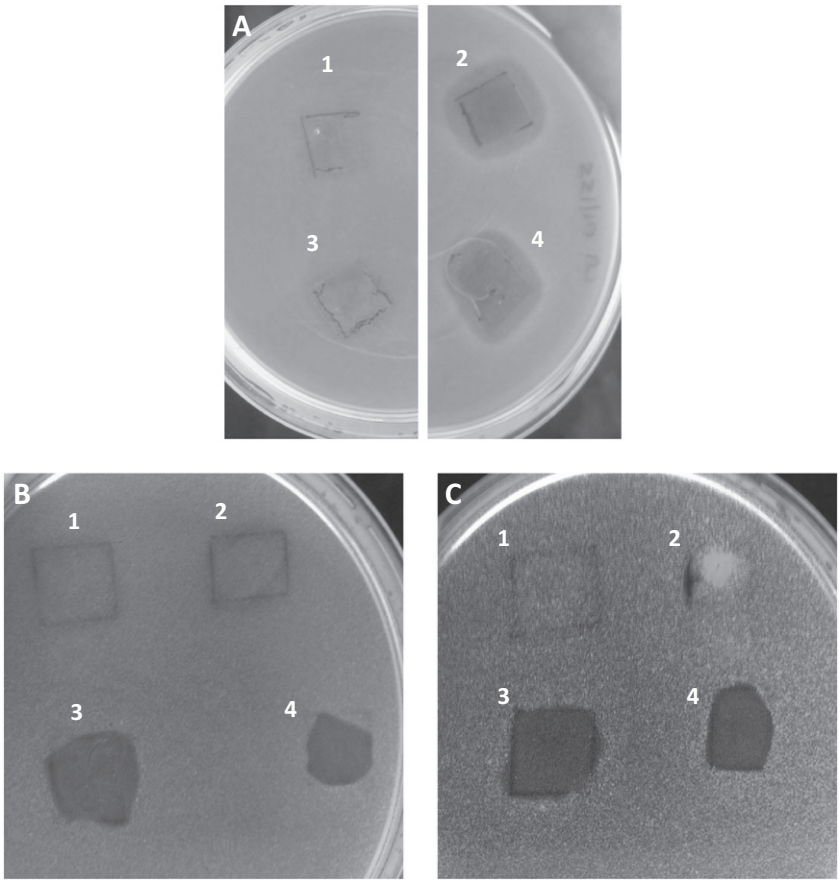

Fig. 2. Effect on cell growth of *E. faecium* SM21 enterocin solution (ES) (*ca.* 64 UA cm^{-2}) and/or prunin laurate flavonoid ester (PL) (0.039 μmol cm^{-2}) added to gelatine edible films, according to the agar diffusion technique. A) *L. monocytogenes* 01/155, B) *S. aureus* ATCC29213 and C) *B. cereus* 1. 1: control without additives (Gel); 2: Gel/ES; 3: Gel/PL; 4: Gel/ES/PL.

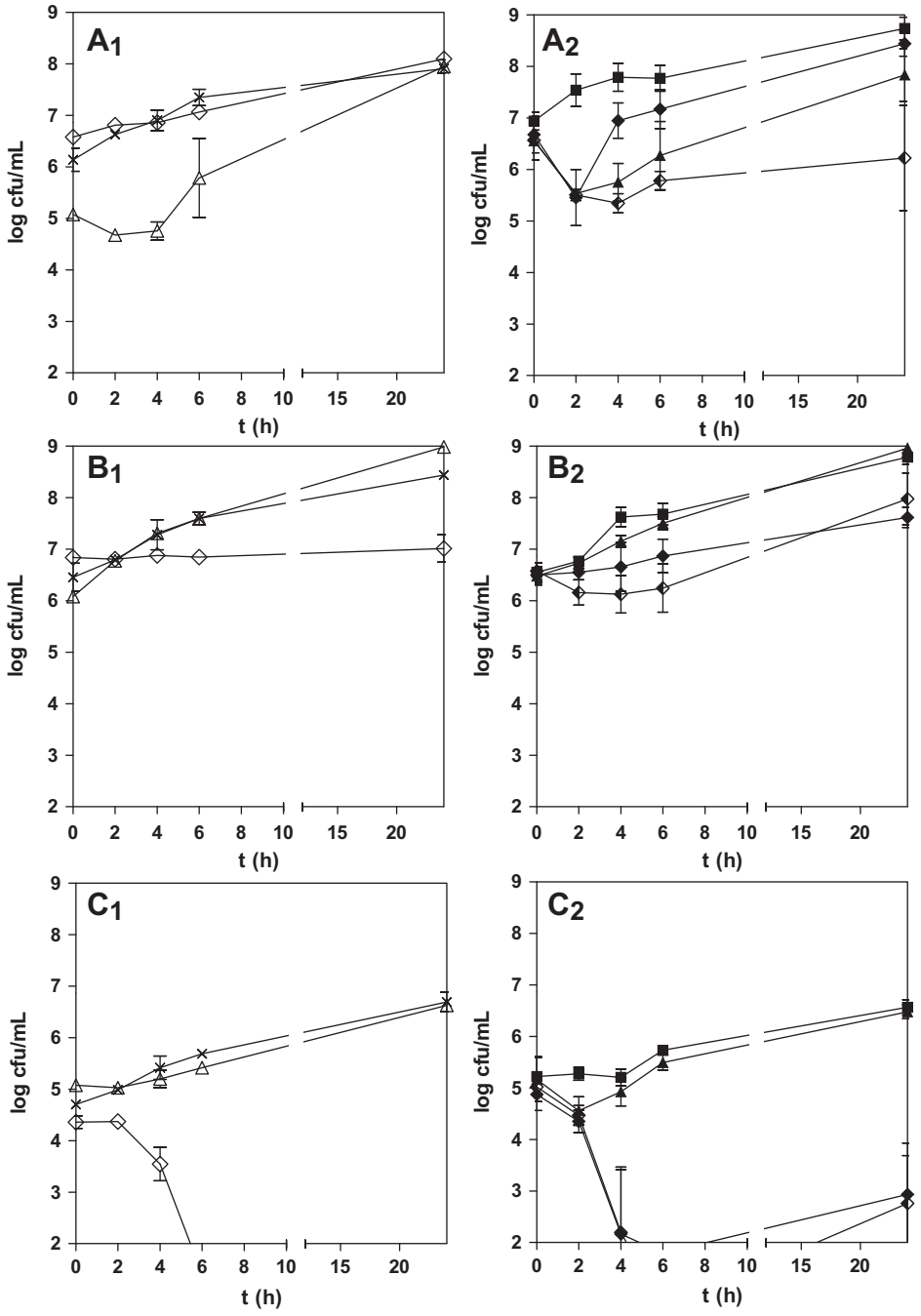

Fig. 3. Viability of (A) *L. monocytogenes* 01/155, (B) *Staphylococcus aureus* ATCC29213 and (C) *Bacillus cereus* 1 cells in contact with gelatine edible films prepared with enterocins synthesized by *Enterococcus faecium* SM21 (*ca.* 64 UA cm^2) (▲) Gel/ES, prunin laurate flavonoid ester (0.039 μmol cm^{-2}) (◆) Gel/PL, or both antimicrobials (◊) Gel/ES/PL; according to the direct microplate contact bioassay. Growth control in (x) peptone water, (■) gelatine films without additives (Gel), (◊) free ES (equivalent to 64 UA cm^{-2}) and (Δ) free PL (equivalent to 0.039 μmol cm^{-2}). All tests were conducted in triplicate.

However, when the pathogen was in direct contact with films containing ES and PL, a marked decrease in the viability of *L. monocytogenes* 01/155 from the beginning of the assay, was observed (Fig. 3B). After 24 h, a decrease of 3 log orders in viability in relation to control growth was observed. These results indicate that the ES/PL active film showed the highest inhibitory effect on this pathogen, with a bactericidal action on its growth. This inhibitory effect was even better than the antagonist activity exerted by a free ES equivalent to 63 UA cm^{-2}.

When the growth of *S. aureus* ATCC29213 was analyzed, an inhibitory effect was observed for systems in contact with PL active films. An average reduction of 1 log viability order was observed during the first 6 h of contact, which increased to *ca.* 2 log orders after 24 h of contact, similar to the inhibition effect exerted by free PL (Fig. 3B). When compared to the growth control in peptone water, the viability of the pathogen in contact with the film added only with ES was virtually the same during 24 h; in agreement with the inhibitory effect of the free ES. This showed that ES active films had no significant inhibitory effect on this strain. However, films containing ES and PL showed an interesting inhibitory effect, with a decrease of *ca.* 2 log order on the viability of *S. aureus* ATCC29213 during 24 h of contact (Fig. 3B).

In the case of *B. cereus* 1 a different behavior was observed (Fig. 3C). A similar inhibition pattern was noticed during the first 2 h of contact for the three active films tested (ES, PL and ES/PL), but it varied during the following 22 h. The inhibitory effect of the films added with ES

decreased and the suspended cells in contact with the active matrix reached viability values similar to the controls. This was expectable, since free ES did not inhibit this pathogen. However, films containing PL and ES/PL showed an interesting inhibitory effect, decreasing the viability of *B. cereus* from about 2 orders in the first hours of contact and up to 4 log orders after 24 h; in concordance with the antimicrobial profile of free PL (Fig. 3C).

The effect on enterocins and prunin laurate antimicrobial activity supported on the gelatine matrix was variable. In general, an initial inhibition of the three pathogen strains by ES active films, which decreased with contact time, was observed. This could be due to the concentration of ES included in the films which was not enough for pathogen inhibition or because the interaction with the gel matrix did not allow the liberation of these antimicrobial peptides. Previous tests of films prepared with gelatine and the addition of enterocins synthesized by *E. faecium* CRL1385 showed a positive inhibitory effect during 24 h of contact between the film and a suspension of *L. monocytogenes* (Ibarguren et al., 2010). Nonetheless, the gelatine matrix did not mainly interfere on the antimicrobial activity of the flavonoid PL. The films added with this substance inhibited sensitive strains, with exception of *L. monocytogenes* 01/155.

"Hurdle technology" refers to the manipulation of multiple factors (intrinsic and extrinsic) designed to prevent microbial contamination or control microorganism growth and survival in foods. A combination of conservation methods may work synergistically or at least provide better protection than a single method alone, which improves food safety and quality (Chen & Hoover, 2003; Deegan, Cotter, Hill, & Ross, 2006; Drider, Fimland, Héchard, McMullen, & Prévost, 2006; Gálvez et al, 2007; Schillinger, Geisen, & Holzapfel, 1996). Also, synergism between different antimicrobial effects enables the use of lower doses of each individual application Gálvez et al. (2007) In this work, a synergistic effect for the film prepared with the combination of ES and PL was observed. This combined film showed a higher inhibitory effect against the three pathogenic strains than the films prepared with each substance separately, in some cases even greater than the inhibition observed for free non-supported antimicrobials. The inclusion of both antimicrobials in the gel matrix probably promotes a cooperative retention of the antimicrobials also enabling their gradual release. This is an essential characteristic, critical for the design of bioactive films to be applied on food.

4. Conclusions

Active films based on food grade gelatine incorporated with enterocins synthesized by *E. faecium* SM21 and a flavonoid ester (prunin laurate) as antimicrobial compounds, were obtained. Films were prepared using a simple and low cost methodology and their application can be adapted to different kinds of food systems. The gelatine matrix results an adequate support for ES and PL since it did not affect antimicrobial activity of the active compounds. The films here obtained were active against *L. monocytogenes*, *S. aureus*, and *B. cereus*. Therefore, they provide an alternative potential tool for preserving foods which are susceptible to contamination by these pathogens. Although the inhibitory effect of each active film was indicator strain dependent, in general, a synergistic effect on each pathogen inhibition was observed when both active compounds, ES and PL, were added to gelatine films. The synergistic effect between enterocins and a flavonoid ester offers a novel potential strategy for *hurdle technology*.

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