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Original article

# Edible films with anti-*Listeria monocytogenes* activity

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**Summary** Edible films based on wheat gluten, gelatin and brea gum were prepared with incorporation of ca. 500 UA $cm^{-2}$ of enterocin with anti-*Listeria monocytogenes* action, synthesised by *Enterococcus faecium* CRL1385. The analyses of the different edible films revealed that they did not show any significant changes in their functional and physicochemical features after the enterocin incorporation ($P$ value $< 0.05$). Gelatin and brea gum films had a higher solubility in water than wheat gluten films. *Listeria* cell growth were not inhibited by edible matrices *per se*; while a similar inhibition to that of free enterocin was observed in gelatin films after 2 h and remained unchanged for the duration of the assay. Wheat gluten films showed a lower enterocin–matrix interaction and a limited bactericidal action was observed for 2 h, after which the activity disappeared. In contrast, brea gum was found to interact with the enterocin molecule inhibiting its anti-*Listeria* activity. Edible gelatin and wheat gluten films with enterocin can be potentially used to control *Listeria monocytogenes* contamination in food products.

**Keywords** Antagonistic activity, brea gum, edible film, enterocin, gelatin, *Listeria monocytogenes*, wheat gluten.

## Introduction

In the late few years, growing demand for fresh, natural and minimally processed food has led attention towards the use of natural alternatives, such as lactic bacteria bacteriocins, rather than chemical compounds as biopreservatives (Deegan *et al*., 2006). Biopreservation deals with the use of antagonistic microorganisms or their metabolic products to inhibit or control undesirable bacteria in food so as to improve its safety and/or prolong its shelf-life (Chen & Hoover, 2003). Bacteriocins are peptidic antimicrobial compounds synthesised by different bacteria with bactericidal activity against other generally related species. Bacteriocins synthesised by lactic bacteria are particularly interesting in food preservation because of their GRAS condition. There are many ways to incorporate bacteriocins into food: (i) inoculation with the bacteriocin producer strain, provided it is considered safe and harmless to human health (GRAS), (ii) addition of purified or partially purified bacteriocins as a food supplement or (iii) use of a previously fermented ingredient with a bacteriocin producer strain (Holzapfel *et al*., 1995; Schillinger *et al*., 1996; Devlieghere *et al*., 2004). Direct introduction is the standard application of antimicrobials to food products. However, the interaction between these compounds and different food components reduces the antagonistic effect on the target cells. Hence, the analysis of alternative methods that decrease these interactions may improve antimicrobial activity and stability in complex systems (Were *et al*., 2004).

Biodegradable active films are an innovative alternative: they incorporate compounds that inhibit or delay pathogen growth (Vermeiren *et al*., 1999; Bertuzzi *et al*., 2006, 2007). These films wrap and protect food by creating an insulating barrier against humidity and oxygen. Additionally, they may prolong its shelf-life by retaining crispness, taste and appearance (McHugh & Krochta, 1994). The inclusion of bacteriocins in food coatings is an interesting antimicrobial delivery mechanism since only the minimal amount of bacteriocins will be used and the agent is not a direct food additive (Gennadios *et al*., 1997). It is important to formulate films or coatings in such a way that favour homogeneous distribution of antimicrobial additives on food surfaces. Interactions between the antimicrobial compound and the film forming material will affect its activity, availability and rate of release. Furthermore, the matrices that can be selected for film formation have varied characteristics that depend on their composition and physicochemical properties (Guiga *et al*., 2009).

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The aim of this work was to study the anti-*Listeria monocytogenes* effect of enterocins synthesised by *Enterococcus faecium* CRL1385 (Audisio *et al*., 1999, 2001) supported on gelatin, wheat gluten and brea gum edible films, through their physicochemical characteristics and the determination of their resulting biological activity. In order to establish if the interaction between enterocin and film forming polimer modifies the antilisteria effect of the enterocin in a permanent way.

## Materials and methods

### Bacterial strains and culture media

The bacteria used were *Enterococcus faecium* CRL1385, a lactic acid bacterium that synthesises enterocins with anti-*Listeria* activity (Audisio *et al.,* 1999), and *Listeria monocytogenes* 01/155 (Instituto de Microbiología 'Dr. Carlos Malbrán', Buenos Aires, Argentina). *E. faecium* CRL1385 was routinely propagated in LAP-Tgm modified (Raibaud *et al.,* 1961): meat peptone 1%; yeast extract 1%; tryptein 1%; glucose 1%; Tween 80 0.1%, pH 6.5 at 37 °C for 12 h. *L. monocytogenes* 01/155 was cultured in a brain–heart infusion broth (BHI, Britania, Buenos Aires, Argentina) at 37 °C for 12 h. Stock cultures were stored at −20 °C in BHI broth containing 10% (w/v) glycerol.

### Enterocin solution preparation

To prepare the enterocin solution (ES), *E. faecium* CRL1385 was cultured in MSC (medium without casein: meat peptone 1%, soya extract 0.5%; meat extract 0.3%, glucose 1%, Tween 80 0.1%, pH 6.5) designed in our laboratory. The culture was incubated at 37 °C for 6 h. Cells were removed from the fermentation broth by centrifugation (10 000 ***g*** for 20 min at 10 °C). Then, the cell-free supernatant (CFS) (pH 5) was filter-sterilised (0.45 μm pore size) and the resultant ES was kept between 24 and 48 h at 4 °C until used to prepare the different edible films. Bacteriocin activity was determined in duplicate by the critical-dilution method (Daba *et al*., 1991), using *L. monocytogenes* 01/155 as the indicator culture. To do this, *Listeria* cells were grown overnight in BHI broth to reach mid-exponential phase. Cells were then harvested by centrifugation (5000 ***g*** for 10 min at 25 °C), resuspended in 0.1% (w/v) peptone water and the concentration adjusted to $10^6$ CFU $mL^{-1}$. Molten BHI agar was cooled to 45 °C and subsequently seeded with 1% (v/v) of the suspension. The inoculated medium was introduced onto Petri dishes and dried for 30 min. The ES was twofold serially diluted in sterile distilled water and aliquots (20 μL) of each dilution applied in 5 mm wells using a steril punch-tool on the BHI agar plate. The plate was incubated at 25 °C for 20 h, and the production of inhibition zones was observed. The suspension titre was expressed in arbitrary units per milliliter (AU $mL^{-1}$) and calculated as follows: $(1000)/(V_s \times D)$, where $V_s$: bacteriocin volume tested (μL), and $D$: the highest dilution that still inhibited cell growth. The final titre of ES measured with *L. monocytogenes* 01/155 as the sensitive strain, was 6400 AU $mL^{-1}$.

### Preparation of antibacterial edible films

Wheat gluten and gelatin films were prepared with food grade ingredients. Brea gum was obtained as an exudate of *Cercidium praecox* and purified in our laboratory before film elaboration.

Gluten films were prepared by diluting gluten in alcohol and water, heating to 50 °C and later acidifying until pH 4 with acetic acid following the Pochat-Bohatier *et al*. (2006) procedure. Both brea gum and gelatin films were prepared by simple water dissolution in a concentration of 5% and 4%, respectively. Glycerol was added as a plasticiser at a concentration of 20% (w/w) of dry matter. The resulting film forming solution was poured on plastic Petri dishes (9 cm diameter), after the incorporation of 5 mL of the *E. faecium* CRL1385 enterocin solution (ES), as explained above. The final titre of the enterocin on each film was ca. 500 UA $cm^{-2}$. Films made with culture medium, instead of ES, and pure solvent used as controls. The edible films were then placed in an air circulating oven at 35 °C and 50% of RH for 15 h. The dishes were then removed from the oven, films were peeled off and mantained in a desiccator cabinet with silica gel at 25–27 °C until used. Film transparency was consistently excellent.

### Edible film characterisation

Films with and without ES incorporation were characterised according to the following determinations:

*Film solubility in water*. Film solubility in water was measured as a percentage of dry matter of the film dissolved in water for a period of 1 h. The initial dry matter of each film was obtained after drying film specimens in desiccators containing $P_2O_5$ for a week. Samples were weighed and immersed in 50 mL of distilled water at 25 °C, sealed and agitated. Films not dissolved in water were separated by centrifuge (Sigma 4K10, Osterode, Germany) and dried to determine the weight of dry matter. Tests were performed in triplicate and the solubility was calculated as follows:

$$\text{Solubility}(\%) = \left(\frac{\text{Initial dry weight} - \text{Final dry weight}}{\text{Initial dry weight}}\right) \text{x}100$$

*Film thickness measurement*. The thickness of film was measured with an electronic micrometer (0.001 mm

accuracy, Fowler, Cole-Parmer Instruments Co., Illinois, USA). Reported thickness was the mean value of five measurements and it was used as the specimen thickness for water vapour permeability calculations and other calculations required.

*Water vapour permeability (P) determination*. The apparatus and methodology described in the ASTM E96 (ASTM, 1995) were used to measure the WVP of the film. Film specimens were conditioned for 48 h in a chamber at 25 °C and 52% relative humidity ($Mg(NO_3)_2$ saturated solution) before being analysed. Films were sealed on cups containing distilled water. Films were then placed in a desiccator cabinet with silica gel at 30 °C. A fan was used to maintain uniform conditions at all test locations over the specimens. Periodical weighings monitored the weight changes. Weight loss was plotted over time and when a steady state (straight line) was reached six more hours were recorded. The WVP was calculated from the slope ($G$) of a linear regression of weight loss versus time.

$$\mathrm{WVP} = \frac{G \cdot x}{A \cdot \Delta p}$$

where $x$ is the film thickness; $A$ is the area of exposed film and $\Delta p$ is the differential water vapour partial pressure across the film. This method uses Fick's first law and Henry's law to calculate film WVP and assumes that film solubility and diffusivity are constant.

*Colour measurement:* Samples were monitored for their surface colours by using a Hunter LAB colourimeter. Instrumental colour readings are $L$, $a$ and $b$. Before each measurement the equipment was calibrated using black and white. Measurements were taken as an average of at least three points of each sample.

*Microstructure characteristics of films*. The top and low surface and the fracture surface topography of the films were examined by scanning electron microscopy (SEM). Film samples were coated with gold under vacuum conditions (coating thickness 20 μm) in a metalizer Desk-IV. The samples were then studied and photographed in a JSM 6480 LV JEOL SEM (Tokyo, Japan).

### Bioactivity assay

a) *Agar diffusion technique*: Enterocin activity on the different films was evaluated against *Listeria monocytogenes* 01/155 using an adaptation of the agar diffusion technique (Millette *et al*., 2007). Strips of 1 $cm^2$ of bioactive films with ES incorporated (ca. 500 UA $cm^{-2}$) or controls with culture medium of each kind of film (brea gum, gelatin or gluten), previously sterilised under UV light, were placed on a *Listeria monocytogenes* lawn ($10^7$–$10^8$ CFU $mL^{-1}$) and the presence of inhibition halos was determined after incubation of 24 h at 37 °C.

b) *Direct microplate contact*: Cell culture microplates of 6 wells (BD Falcon™, BD Biosciences, New Jersey, USA) were used. A UV light sterilised 1 $cm^2$ strip of each film, was placed in each well with 5 mL of peptone water suspension of ca. $10^4$ CFU $mL^{-1}$of the indicator strain at 25 °C and the viability was determined after 0, 0.5, 1, 2, 4 and 24 h by plate counting with BHI agar. Strips of bioactive films with ES incorporated (ca. 500 UA $cm^{-2}$) or controls with culture medium were used. Also evaluated was *Listeria* cell response to an amount of free enterocin, equivalent to that incorporated to the 1 $cm^2$ strips of each film (ca. 500 UA $cm^{-2}$). Enterocin released in the reaction medium was also quantified at the same time periods by titre determination, according to the serial dilution method (Daba *et al*., 1991).

### Statistical analyses

GraphPad Prism 4 Software (GraphPad Software Inc., California, USA) was used for all statistical analyses. Analyses of variance (ANOVA) linear and non linear regressions were applied. The significance levels used were $P < 0.05$. All asays were carried out at least in triplicate.

## Results and discussion

Three different film forming materials were selected to incorporate *E. faecium* CRL1385 antilisterial enterocin: brea gum, gelatin and wheat gluten. Brea gum is an heteropolysaccharide formed by arabinose, xylose and uronic acids present in nature as a *Cercidium Praecox* exudate. In an aqueous solution it has a pH 4.0 and produces yellowish, very soluble films in water with good mechanical resistance and low oxygen permeability at a low relative humidity. Gelatin results from collagen hydrolysis, and glycine, proline, hidroxiproline and glutamic acid are the predominant among the 18 aminoacids that constitute its chemical composition. In an aqueous solution it has a pH 3.8–5.5 and edible coatings made with gelatin reduce moisture, oxygen and oil migration and is known to form clear, flexible, strong, and oxygen-impermeable films when cast from aqueous solutions in the presence of plasticisers (Gennadios *et al*., 1994, Lacroix & Cooksey, 2005). Wheat gluten, due to its markedly apolar character, complexity, and diversity of protein fractions, results in films with functional features, such as selective gas-barrier properties and rubber-like mechanical characteristics. Wheat gluten based coatings and films are homogeneous, transparent, mechanically strong, and relatively water resistant. They are biodegradable, biocompatible, and edible when food-grade additives are used (Guilbert *et al*., 2002).

It was found that edible films of gelatin, wheat gluten and brea gum did not show any significant changes ($P$ value $< 0.05$) in their physicochemical characteristics

analysed after the addition of enterocin. Gelatin and brea gum films showed high water solubility (78.3 and 73.5%, respectively), compatible with the affinity for water that these materials present. Wheat gluten films, on the contrary, presented low solubility values (23.5%). Brea gum and gelatin water vapour permeability values could not be measured because of their high water solubility. Wheat gluten films gave readings of $5.025 \times 10^{-11}$ g mm $s^{-1}$ $cm^{-2}$ $Pa^{-1}$ at 24 °C. Brea gum films were amber yellow, gluten films were whitish with a light reddish hue, while gelatin ones were transparent as shown by parameters *L*, *a* and *b* (Table 1). All these properties were coincident with data previously reported for these film forming materials (Gennadios *et al*., 1994; Micard *et al*., 2000).

**Table 1** Physicochemical properties of bioactive edible films

| Property/film | Wheat Gluten | Brea Gum | Gelatin |
|---|---|---|---|
| Water solubility (%) | 23.5 | 73.5 | 78.3 |
| Water vapour permeability (g mm $s^{-1}$ $cm^{-2}$ $Pa^{-1}$) | n/d | $5.025 \times 10^{-11}$ | n/d |
| Hunter color parameters | | | |
| *L* | 60.03 | 69.56 | 77.50 |
| *a* | 1.00 | −6.41 | −1.84 |
| *B* | 10.19 | 12.34 | 4.88 |

The microstructure of the films made with and without enterocin was homogeneous and compact (without pores) and showed no significative differences as well as other film properties analysed (Fig. 1). These results suggest that good miscibility exists between the enterocin and the three matrices analysed.

According to the agar diffusion technique, the analysis of the antilisteria effect of the enterocins incorporated into the different films revealed that even though *L. monocytogenes* were inhibited in all cases, the effect was higher when they were held on gelatin (dh = 10 mm) rather than gluten (dh = 2 mm) or brea gum (dh = 0.5 mm) films. Control films with the culture medium did not inhibit listeria cells. This shows that the matrices used did not present *per se* any

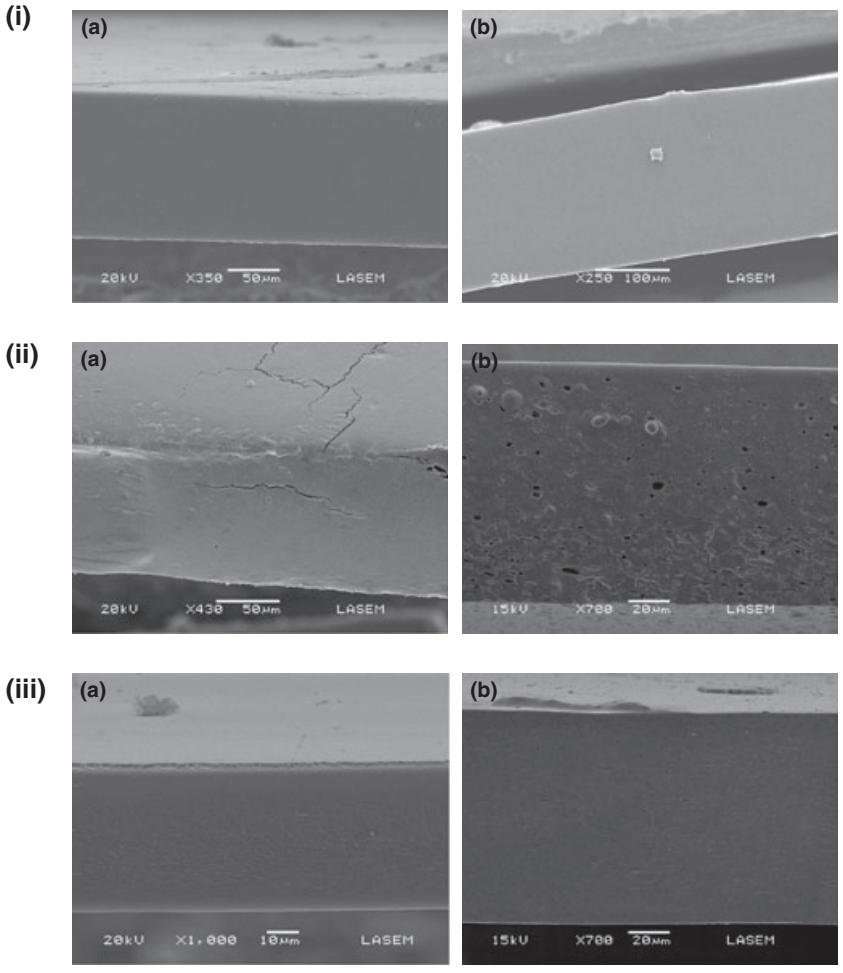


**Figure 1** SEM microscopy of the fracture section of edible films: (i) brea gum; (ii) wheat gluten, (iii) gelatin (a: control; b: with a final enterocin concentration of ca. 500 UA $cm^{-2}$).

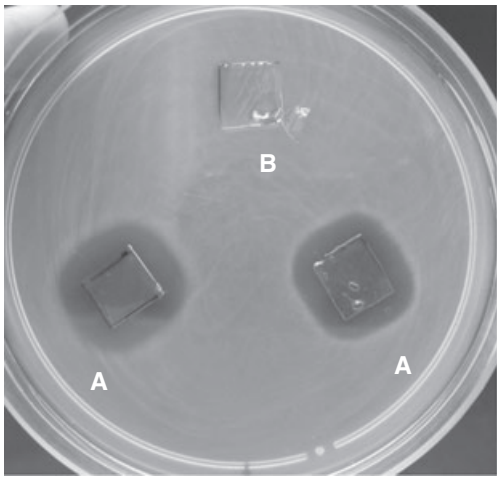


**Figure 2** Effect on *L. monocytogenes* 01/155 cells viability of *E. faecium* CRL1385 enterocin (ca. 500 UA $cm^{-2}$) incorporated on gelatin edible films according to the agar diffusion technique (A). B: control films without enterocin.

antagonistic activity with the techniques used (Fig. 2 for gelatin films). In all cases there was inhibition both in the contact zone between agar, film and the zones that surround the film, contrary to what was reported by other authors for similar assays with starch and corn zein films (Sanjurjo *et al.*, 2006; Padgett *et al.*, 2000). A slight growth in the film/medium interface and a clear inhibition zone around the film were reported in those papers.

When the different edible films were placed in closer contact with *Listeria* cells, i.e. without the agar interface between *Listeria monocytogenes* and the film with enterocin, different results were detected. Inhibition was larger with the enterocin incorporated into the gelatin film and it registered a decrease of ca. 3 orders in the *Listeria* viable cell number after 24 h of contact (Fig. 3i). It is interesting to remark that the gelatin film lost its initial dimensions by swelling when in contact with the aqueous solution with the indicator strain cells. Although gluten films decreased *L. monocytogenes* 01/155 in ca. 2 viability orders in relation to the bacteria number of the control assay during the first 2 h. That effect did not continue through the remaining assay and it was reduced to only 1 order less in the listeria viable cell number after 24 h of contact (Fig. 3ii). No inhibition or differences were detected with brea gum films when they were compared with the control (Fig. 3iii).

The brea gum films were totally soluble in the reaction medium while those of wheat gluten kept their integrity during the experiment. Gelatin films, on the other hand, experienced swelling in the aqueous medium as stated before. These results are caused by polymer hygroscopicity, hydratability and solubility.

The pathogen behaviour in an amount of free enterocin, i.e. a dilution of ES in peptone water, equivalent to that found in the films mentioned before

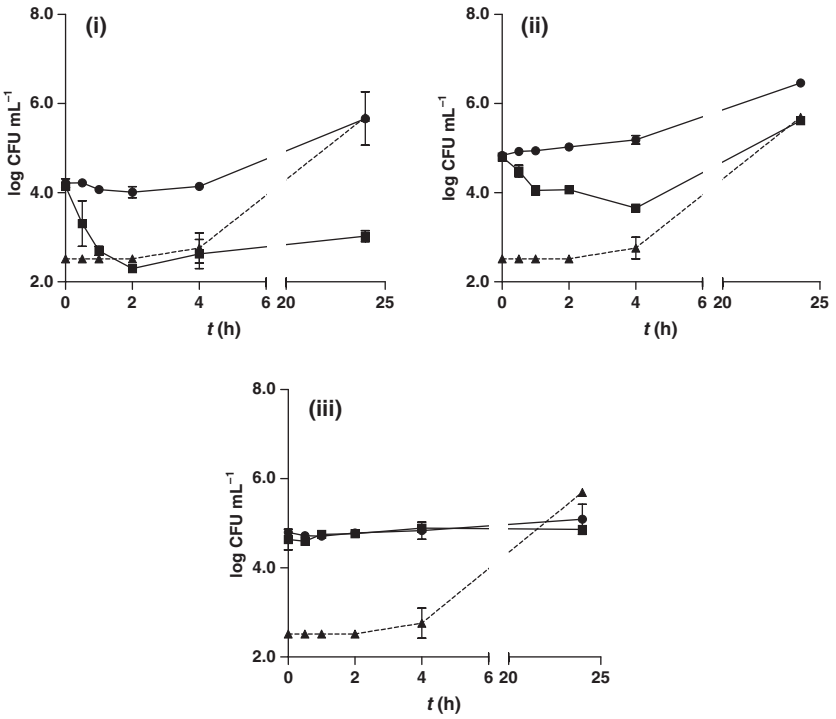


**Figure 3** Viability of *L. monocytogenes* 01/155 cells in contact with edible films (i) gelatin, (ii) wheat gluten, (iii) brea gum, prepared with (•) culture media (control) and (■) ES (ca. 500 UA $cm^{-2}$); and (▲, dotted lines) with free ES (equivalent to 500 UA $cm^{-2}$) according to the direct microplate contact bioassay. All tests were conducted in triplicate.

(ca. 500 UA $cm^{-2}$), showed an inhibition of ca. 2 orders in pathogen viability related to the control growth, during the first 4 h of contact. However, the inhibition disappeared after 24 h, since viability reached similar values to those for the *Listeria* growth controls. This behaviour is very similar to that showed by the enterocin supported on wheat gluten film. In spite of the inhibition measured on *Listeria* cells in contact with gelatin, wheat gluten and brea gum films, free enterocin could not be detected in the liquid medium where films were inmersed. Concentration was quantified by enterocin titrering at the same plating times. These results showed that the released enterocin was not available in the medium to affect other cells, once it had acted on a target cell.

Furthermore, brea gum was found to interact with the enterocin molecule inhibiting its activity (Fig. 3iii). Wheat gluten films, whose solubility is rather poor, seem to have a low enterocin/film material interaction and the bacteriocin release takes place in a short time. Hence, there is a limited bactericidal action, similar to that of the enterocin-free test that disappeared in 24 h (Fig. 3ii).

A similar inhibition to that of free enterocin was observed in gelatin films after 2 h and it remained unchanged for 24 h. The enterocin film material interaction that allowed a time controlled release of the antimicrobial, ensured an adequate concentration in the medium to affect pathogen viability (Fig. 3i). The slow antimicrobial release extended its effect for a longer period, achieving a better control through time.

It should be remarked that the three kind of films used in this work were all stored at 25–27 °C in silica gel for 6 months. Their anti-*Listeria* effect was analysed after this time period and the assays reproduced similar data obtained with freshly made films (see Fig. 4 for gelatin films). These results showed that active films are an excellent means of stabilising, supporting and delivering this enterocin. The films selected have the additional advantage of being made with highly available raw materials of low cost. Moreover, the manufacturing methods described in this work are simple and their application can be easily adapted to different kinds of food.

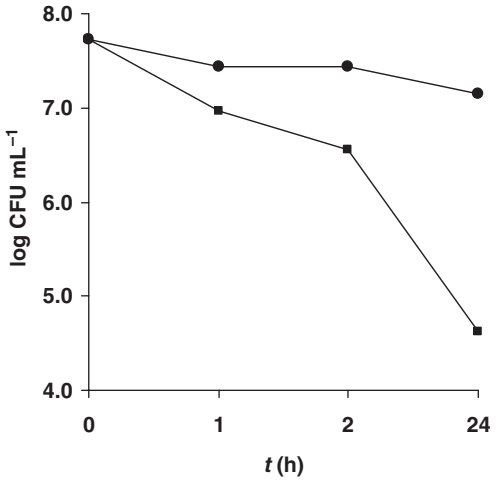


**Figure 4** Viability of *L. monocytogenes* 01/155 cells in contact with gelatin edible films stored 25–27 °C in a desiccator cabinet with silica gel for 6 months, elaborated with (•) media culture (control) and (■) ES (ca. 500 UA $cm^{-2}$) according to the direct microplate contact bioassay. All tests were conducted in triplicate.

Edible gelatin and wheat gluten films with enterocin made by *E. faecium* CRL1385 in a concentration of ca. 500 UA $cm^{-2}$, can control *Listeria* contamination with both superficial and aqueous medium activity. In addition, brea gum and the enterocin were found to be incompatible since antimicrobial activity was lost in the films. The use of antilisteria active films could be a very convenient alternative to the addition of antimicrobials to foods, such as ham, hard and semi-harde cheeses, with the advantage of concentrating the additive in the zone where the majority of the contamination takes place.

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