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Controlled delivery of propionic acid from chitosan films for pastry dough conservation

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ABSTRACT

A study was undertaken to evaluate the feasibility of using chitosan active films designed to control the release of an antimicrobial agent. In this context, the efficacy of propionic acid as both a medium for chitosan dissolution and an antimicrobial agent was examined. This work was focused on studying the potential application of chitosan active films developed to conserve a pastry dough product, modeling the release profile and evaluating the effectiveness of the active agent by extending the product shelf life.

The kinetic release was analyzed in a simulant and in a food matrix. Fitting parameters were lower in the pastry dough than in the buffer, since the food matrix limited the active compound diffusion as well as the film swelling.

The advantages of the active chitosan system were the capacity of control antimicrobial release to the pastry dough surface and their ability to be an effective carrier of propionic acid, with a consequent improvement from the technological point of view.

1. Introduction

Hydrophilic matrices have been used as controlled delivery systems in the last decades, due to their low cost and the simple technology associated. In spite of developing numerous investigations on drug release from hydrophilic matrices, the mechanism that drives this mass transfer process is still a matter of debate. The incorporation of active agents into polymeric systems results in a variety of release profiles with different stages. In some cases, the additive release has been described as a simple matrix diffusion process, with degradation occurring at a later stage, post active substance release (Gallagher and Corrigan, 2000).

The use of packaging films based on antimicrobial polymers would be more efficient by keeping high concentrations of the active substance on the food surface while preventing its migration, thereby maintaining a critical concentration for an extended period of time (Han and Floros1998; Ouattara et al., 2000a).

Chitosan is one of the few natural cationic polysaccharides that can be derived from crustacean or fungi, having antimicrobial properties against some bacteria, fungi and yeast (Kong et al., 2010; Rabea et al., 2003).

The selection of an antimicrobial agent depends on its activity against a target microorganism. The growths of potential microorganisms that can spoil food products are predictable due to the food product characteristics such as pH, water activity, composition, as well as storage conditions. The direct incorporation of additives in packaging films is a convenient methodology by which antimicrobial activity can be achieved.

Chemical preservatives, mainly organic acids and its salts (sorbates, benzoates and propionates), are used to inhibit the microbial growth (Ouattara et al., 2000a,b) and to increase the shelf life of fresh dough combined with refrigerated storage, because these products are packaged without a thermal treatment (Silveira et al., 2007).

Propionic acid, naturally found in various foods including butter, cheese and dough, categorized as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration, is used as an antimicrobial and flavoring agent.

Taking into consideration that the antimicrobial activity mainly depends on the diffusion of the antimicrobial agent, is crucial to evaluate its release kinetic from the polymer matrix. Korsmeyer–Peppas'model may be used to describe the Fickian and non-Fickian release behavior of swelling-controlled systems, which swell until reaching moderate equilibrium, and are prepared by incorporation of active agent in a hydrophilic, initially glassy polymer (Sangsuwan et al., 2009). Furthermore, the overall process was described by Gallagher and Corrigan (2000) through two phenomenological independent contributions: the initial fast release or "burst" phase which is the result from the rapid dissolution of the active compound domains at the solid–liquid surface. The second phase of active compound release is controlled by a polymer degradation

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mechanism. Several approaches are reported on active compounds release modeling (Aldana et al., 2012; Flores et al., 2007; Siepmann and Peppas, 2001).

This research emphasizes the use of the propionic acid as both a medium for the dissolution of chitosan and an antimicrobial agent to be used in the conservation of pastry dough. To the best of our knowledge there have been no reports about the use of a chitosan film as a carrier of propionic acid to prevent microbial growth on pastry dough.

This work was focused on:

(i) Analyzing the antimicrobial capacity of chitosan active films.
(ii) Studying the potential application of chitosan active films developed to conserve a dough product, evaluating the behavior of the active compound released and its effectiveness in extending the product shelf life.
(iii) Knowing the transport mechanism of the active antimicrobial compound and modeling the kinetics of this process mathematically.

2. Materials and methods

2.1. Reagents

Commercial chitosan from crab shells with a minimum deacetylation degree of 75% was purchased from Sigma (St. Louis, MO, USA). Analytical grade propionic acid (Pr) was purchased from Anedra (Buenos Aires, Argentina).

2.2. Film preparation

Chitosan solution of 1.5%(w/w) was prepared by solubilization in 2.5%(v/v) propionic acid solution at 20 °C under continuous agitation for 12 h approximately, followed by a vacuum filtration to eliminate insoluble materials. Chitosan films with propionic acid addition (CHPr) were obtained by casting 20 g of filmogenic solutions onto Petri dishes (9 cm diameter) and drying at 37 °C in an oven until reaching constant weight (approximately 36 h).

2.3. Film properties

2.3.1. Physicochemical properties

Moisture content of films was determined by measuring their weight loss, upon drying in an oven at 105 °C until constant weight (dry sample weight). Samples were analyzed at least in triplicate. Film color was evaluated by using a Minolta CR 400 (Osaka, Japan) as was described in a previous work (Rivero et al., 2010).

2.3.2. Water vapor permeability

Assays were performed using a modified ASTM method E96 (1995) as described in a previous work using a specially designed cell with silica-gel (Mali et al., 2002). After steady-state conditions were reached, changes in the weight of the cell were recorded to the nearest 0.0001 g as a function of time, leading to the gain weight (GW, g m^{-2}) when it was divided by the cell area. Water vapor transmission rate (WVTR, g s^{-1} m^{-2}) was calculated from the slope of the straight line by linear regression ($R^2 > 0.99$). Water vapor permeability (WVP) was calculated considering the WVTR, the partial vapor pressure gradient across the film (1753.55 Pa at 20 °C) and the film thickness (m), and expressed as g Pa^{-1} s^{-1} m^{-1}. Each informed value corresponds at least to four determinations.

2.3.3. FT-IR spectroscopy

The Fourier transform infrared (FT-IR) spectra of active CHPr films were recorded in an IR spectrometer (Vertex 50, Bruker, Germany) in the wavenumber range of 4000–400 cm^{-1} by accumulation of 64 scans at 4 cm^{-1} resolution.

2.3.4. Dynamic mechanical analysis (DMA)

DMA assays were conducted in a dynamic-mechanical thermal equipment Q800 (TA Instruments, New Castle, USA) using a clamp tension with a liquid N_2 cooling system as it was described in a previous work (Rivero et al., 2012). Multi-frequency sweeps (5, 10 and 15 Hz) at a fixed amplitude (7 μm) from −90 to 200 °C at 5 °C min^{-1} were carried out, with an isotherm of 15 min at −90 °C.

2.3.5. Tensile stress–strain

For quasi-static test in uniaxial condition, a preload force of 1 N and a constant force ramp rate of 0.3 N min^{-1} were applied to record the stress–strain curves until rupture from film sample strips or up to 18 N, using the same DMA and procedure to fix them to the clamps as in the case of dynamic tests. Tests were carried out at 25 °C. In order to calculate the elastic modulus at large deformations (E_C), stress–strain curves were fitted to the following equation:

$$\sigma_v = E_C \varepsilon_v e^{-\varepsilon_v K} \quad (1)$$

where, ε_v and σ_v are the true strain and the true stress, respectively, E_C is the elastic modulus; K is a constant and it is regarded as a fitting parameter. From Eq. (1), the relationship between the stress (σ_v) and the true deformation (ε_v) corresponds to the elastic module (León et al., 2009). Samples were analyzed at least in triplicate.

2.4. Antimicrobial capacity

2.4.1. Inoculum preparation

Isolates of *Staphylococcus aureus* (ATCC 25923), *Salmonella* spp. *Candida* spp. and *Penicillium* spp. were obtained from Catedra de Microbiologia, Universidad Nacional de La Plata, Argentina.

Candida spp. was growth in broth malta containing malt extract (1%, Biokar, France) and yeast extract (2%, Biokar, France). *S. aureus* (ATCC 25923) and *Salmonella* spp were grown in a nutrient broth (Merck, Germany). All culture were incubated at 37 °C for 12 h until reaching concentrations of 10^8 CFU ml^{-1}, determined by optical density (OD). Then, dilutions 1:10 were prepared from these inocula with sterile peptone water (Oxoid) to obtain concentrations of 10^7 CFU ml^{-1}.

The inoculum of *Penicillium* spp. was prepared by growing the fungi on agar malta slants composed by malt extract (1%, Biokar, France), yeast extract (2%, Biokar, France) and agar (2%, Merck, Germany) for 7 days at 30 °C. After incubation, 10 ml of 0.01% (w/v) sodium lauryl sulfate (SLS) in 1% (w/w) sodium chloride solution were added to the tubes and spores were loosened by gently scraping with a spatula, and serial dilutions were made (Molina and Giannuzzi, 1999). The cells were counted in a haemocytometer and diluted to a concentration of 10^5 spores ml^{-1}. Besides, a dilution 1:10 was also prepared with sterile peptone water (Oxoid).

2.4.2. Antimicrobial test

The antimicrobial capacity of the active films was determined by using the agar diffusion method described by Pranoto et al. (2005).

The inocula previously described were tested using 1 and 3 cm diameter film disks which were placed by pressing them to ensure contact with the agar surface. The average thickness of the studied films was 45 μm.

The disks were deposited on petri dishes with Nutrient agar (Merck) for trials with *S. aureus* and *Salmonella* spp. and with Agar Malta (malt extract 1%, yeast extract 2%, glucose 1% and agar 2%) for *Candida* spp. and *Penicillium* spp. previously planted from

100 μl of the corresponding inoculum. The tests were performed in duplicate to ensure reproducibility of the results. Visual observations were conducted in all cases, photographs were taken, and inhibitory zones of the films were measured at 24, 48, 72 and 96 h of incubation at 37 °C. The photographs were processed with software Image J, which was used to quantify the inhibition halo. The data were expressed in surface units (mm^2), subtracting the film area from the inhibition zone (Padgett et al., 1998).

2.5. Diffusion experiments

2.5.1. Diffusion in a buffer simulant

The antimicrobial agent was quantified by HPLC (Agilent Waters, USA). Sigma–Aldrich (USA) propionic acid (chromatographic quality) was used for release assays. The diffusion of the antimicrobial active compound was performed in a phosphate buffer (pH 6.5), at 4 °C, condition chosen to simulate the characteristics of selected food system to apply the antimicrobial film (pastry dough).

Film pieces of 4 × 4 cm, were placed on a support and immersed in 200 ml of the buffer solutions, which were kept under agitation. Samples were taken from the buffer solution periodically, and the concentrations of propionic acid were quantified by HPLC, using a 0.009 N sulfuric-acid solution as the mobile phase, at a flow rate of 0.7 ml min^{-1} at a pressure of 700 psi. The samples were injected in a column Aminex HPX – 87 H (BIORAD, USA) operated at 60 °C, and the detection was done at 214 nm.

Taking into consideration that propionic acid is highly volatile and that part of it is lost during the drying process, the remaining concentration in the film was determined by HPLC immediately after completing the drying stage.

2.5.2. Application of chitosan films with propionic acid on pastry dough conservation

Commercial available pastry dough products contain preservatives (propionate salts, benzoate and sorbate). Therefore, pastry dough without additives was prepared to evaluate the influence of the propionic acid released from the separator films on the shelf life.

For dough preparation, commercial wheat flour (Type 0000, Código Alimentario Argentino, 2004) and shortening (Molinos Río de La Plata SACIFI, Buenos Aires, Argentina) were purchased from a local market, 62.5 and 10.6% (w/w), respectively, were used. An addition of 1.8% NaCl analytical grade and 25% distilled water was made.

To prepare the pastry dough, dry ingredients were mixed, and then water was added until forming dough, which was kneaded by hand, wrapped in polyethylene film and kept refrigerated at 4 °C for about 1 h. After that, dough was placed in a designed acrylic tray and was extended to obtain a 3 mm thick sheet. Dough disks of 40 mm in diameter were cut for assays.

Fig. 1 shows a scheme of the different types of systems analyzed in this work: pastry dough in contact with CHPr, CH and PD960 films.

Chitosan films formulated with propionic acid (CHPr) were used as separator films between pastry dough in order to extend the shelf life of the product. This active agent, as a result of mass transfer phenomenon, was released from the film matrix to pastry dough by a concentration gradient. Chitosan films were previously conditioned at 20 °C and 65% RH for 60 days with the purpose of analyzing only the inherent antimicrobial ability of this biopolymer, without the interference of the acid. This result was determined by HPLC as it was described previously.

PD 960 films (CRYOVAC®) based on polyethylene, used as a control, were used as both separator films and external packaging (Fig. 1).

Propionic acid released from CHPr films to pastry dough was determined at different times of being in contact at 4 °C. The used protocol was the following: dough disks were dissolved in a pH 6.5 phosphate buffer. The obtained suspension was filtered using 0.45 μm filters and the active agent was quantified by HPLC. All measurements were performed at least in triplicate.

The concentration of propionic acid quantified in the dough product respect to the amount of the active agent remaining in the film after the drying process was considered to determine the percentage of propionic acid released to pastry dough in function of time.

2.6. Shelf life product evaluation

2.6.1. Microbiological analysis

For microbial evaluation, formulated systems and controls were stored at 4 °C. After 7, 14, 20, 26 and 30 days, dough samples were removed from the packaging, and fungi and yeasts counts were carried out. To perform the test at each time, 10 g pastry dough was homogenized in a Stomacher Model Seward 400 (England) with 90 ml of 1% sterile peptone water during 60 s; 100 μl of the homogenate or necessary dilutions were inoculated in YGC media (Yeast extract Glucose Chloramphenicol, Merck) and the plates were incubated 8 days at 30 °C. Viable microorganisms were determined by counting the number of colonies formed, the results being expressed as CFU g^{-1}dough; determinations were performed at least in duplicate.

2.6.2. Microbial growth modeling

Mathematical models allow analyzing the effect of different chemical preservatives on microbial growth parameters. One of the most recommended models (Oteiza et al., 2003) is the Gompertz equation whose expression is:

$$\log N = \log N_0 + a \cdot \exp\left(-e^{-\left(\frac{x-x_0}{b}\right)}\right) \quad (2)$$

where log N is the decimal logarithm of microbial count (log (CFU g^{-1})) at time t; log N_0 is the asymptotic log count as time decreases indefinitely (approximately equivalent to the log of the initial level of microorganisms) (log (CFU g^{-1})); a is the count increment as time increases indefinitely, that is number of log cycles of growth (log (CFU g^{-1})); x_0 is the time required to reach the maximum growth rate (days); and $(1/b)$ is the specific growth rate at time x_0 (day^{-1}).

2.7. Statistical analysis

Systat-software (SYSTAT, Inc., Evanston, IL, USA) version 10.0 was used for all statistical analysis. Analysis of variance (ANOVA), linear and non-linear regressions and Fisher LSD mean comparison test were applied. The significance level used was 0.05.

3. Results and discussion

3.1. Film properties

The film visual properties finally determine the consumer acceptability of the packaged products, thus the film color evaluation is required. In general, polysaccharide films are colorless, though chitosan films prepared with propionic acid exhibited a slightly yellow appearance; the value of color differences (ΔE) was 3.52 ± (0.15). The films were transparent, smooth and homogeneous; they showed good structural integrity and were easily removed from the acrylic plate. The WVP and the moisture content were 9.83×10^{-11} ± (0.77) g s^{-1} m^{-1} Pa^{-1} and 22.83 ± (0.41) g water/100 g of dry films, respectively. These results were similar to

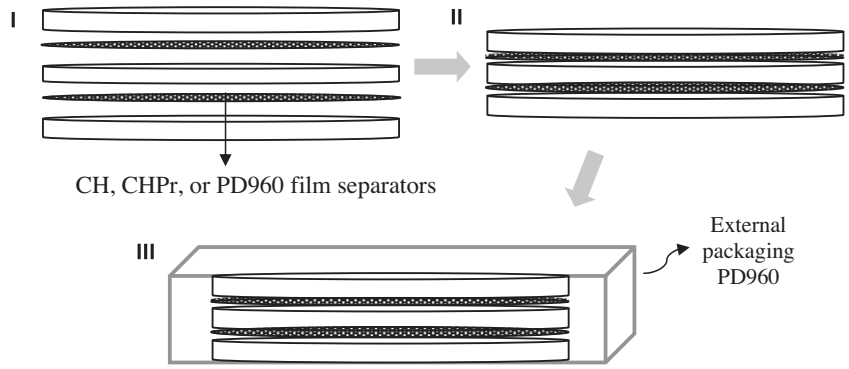

Fig. 1. Scheme of the different types of systems analyzed: pastry dough in contact with CHPr, CH and control PD960 films.

those reported by Rivero et al. (2012) for chitosan films prepared with acetic acid, which is the common solubilization medium of chitosan. In the same way, Caner et al. (1998) informed similar WVP values using 1% propionic acid concentration in the film preparation.

Furthermore, the mechanical properties were evaluated by uniaxial tension tests through DMA. The stress–strain curves showed the mechanical responses of the films (Fig. 2a). The model used to estimate the elastic modulus fitted the experimental data satisfactorily, the obtained value being on average 1722.7 MPa.

Concerning tan δ curves obtained by DMA, α relaxation associated with glass transition temperature T_g was around 120 °C (Fig. 2b). Quijada-Garrido et al. (2007) indicated that the discrepancies in T_g values could be due to the different preparation of the chitosan films, the acid used for film formulation, the degree of neutralization, and the thermal treatment employed.

On the other hand, Wu et al. (2001) reported that polymer small molecules systems may manifest a new transition at temperatures above T_g, assigned to the dissociation of intermolecular hydrogen bonds between the polymer and the additive incorporated. This explanation could justify the appearance of a transition at about 170 °C, as can be seen in Fig. 2b.

Fig. 3 shows FTIR spectra obtained for CHPr films, which were tested immediately after the drying process. FTIR spectra exhibited bands characteristic of the presence of the acid such as those located at 1420 and 1380 cm^{-1} (Kim et al., 2006). Spectra also revealed the appearance of a weak signal at 1715 cm^{-1} (corresponding to the extension mode of C=O) confirmed through the second derivative technique which is applied as an enhancement resolution method to verify the peak positions (Rivero et al., 2010). Higher amount of propionic acid was added (10% v/v), with the purpose of visualizing this band with higher definition and verifying its location. The higher the acid concentration, the higher the peak intensity, as can be observed in the insert of Fig. 3.

FTIR spectra showed the split of the peak at 1320 cm^{-1} attributed to the amide III (Kumirska et al., 2010), and the appearance of the band at 1460 cm^{-1} characteristic of the CH_2 group. In the same way, Kim et al. (2006) found similar results for chitosan films prepared with propionic acid.

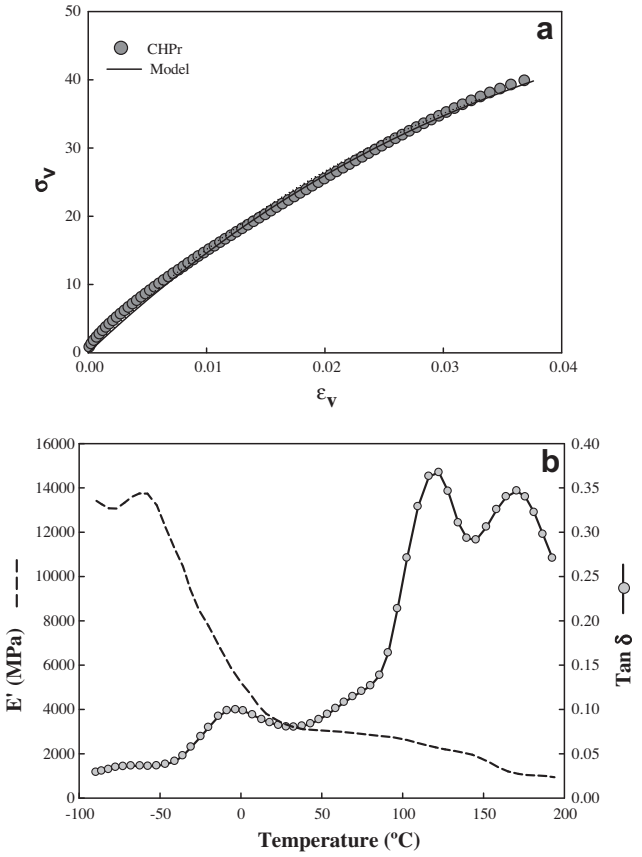

Fig. 2. (a) Tensile stress strain behavior of chitosan active films (CHPr). Continuous lines indicate data fitting to Eq. (1), (b) dynamic mechanical analysis spectrum showing the storage modulus, E′ (——) and the tangent of the phase angle, tan δ(-∘-) of CHPr.

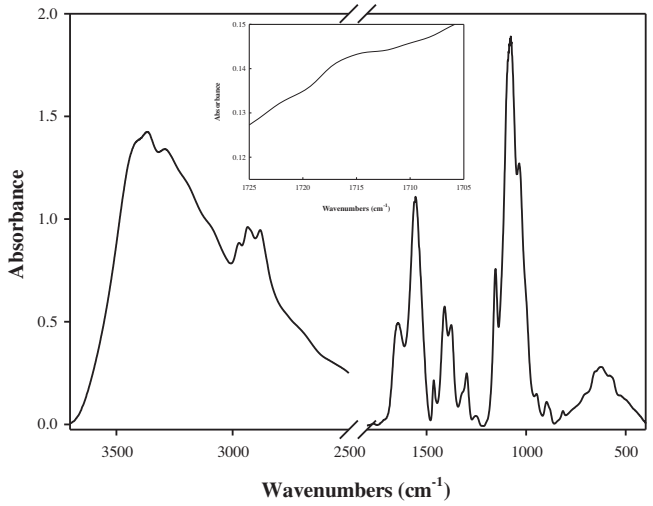

Fig. 3. FTIR spectra of: CHPr films in the 3700–400 cm^{-1} wavenumber region. Insert shows the 1725–1705 cm^{-1} enlarged region of chitosan films prepared with 10% (v/v) of propionic acid.

The bands located at 2930 (ν_{AS}) and 2970 (ν_S) cm^{-1} corresponded to symmetric and antisymmetric CH_2 vibrations of carbohydrate ring. These peaks underwent a shift compared to those obtained for chitosan films prepared with acetic acid (Rivero et al., 2010). This fact was attributed to the increase in chain length of the acylated derivatives (Fig 3). Similar results were found by Hu et al. (2007) working on chitosan matrices.

3.2. Antimicrobial capacity

The film inhibitory capacity was assessed on *S. aureus* which is recognized as a result of the inadequate operator handling while *Salmonella* spp. has been associated with serious food contamination and poisoning.

On the other hand, it was expected that the predominant microorganisms were fungi and yeasts. Therefore *Penicilium* spp. and *Candida* spp. were used to complete the evaluation of the antimicrobial properties of the active films.

The antimicrobial activity of chitosan against a variety of bacteria (Gram-negative and Gram-positive) and fungi, owing to its polycationic nature is well known (Kong et al., 2010; Rabea et al., 2003).

Halo inhibition test is a means to study the antibacterial ability of samples by measuring the halo size caused by growth inhibition. In this sense, when the active CHPr films are placed on the culture media, it is expected that additives will diffuse from the polymer matrix into the agar in a radial form, producing a clear zone of growth inhibition around the active films.

CHPr films inhibited the microbial growth by contact with the culture medium, regardless of the microorganism tested. In all cases, the most diluted concentration of inocula allowed a better visualization of the film inhibitory effect.

Fig. 4 shows the agar plate containing CHPr films for all microorganisms assayed. This active matrix showed a strong bacteriostatic action and demonstrated to be effective in the inhibition of microbial growth.

Table 1 and Fig. 4a and b show that chitosan active films exhibit a stronger antibacterial activity against Gram-positive bacteria than Gram-negative one as can be observed through the inhibition zones of *S. aureus* and *Salmonella* spp., respectively. A similar trend was described by No et al. (2002), Kong et al. (2010) stressed that the mechanism of antibacterial action is a complicated process that differs between Gram-type bacteria due to different cell surface characteristics.

In the case of *S. aureus* (Fig. 4b) an inhibition halo was developed and remained until 48 h and for *Candida* spp. (Fig. 4c) the inhibition was complete for the assayed period (96 h). Similarly, CHPr films showed antifungal activity against *Penicilium* spp. based on the inhibition halo test (Fig. 4d and Table 1).

3.3. Release models

Before the application on pastry dough, the propionic acid diffusion from chitosan film to a phosphate buffer was studied to determine the maximum release of the compound.

The release mechanism was determined by fitting the first 60% of the release profile data with the semiempirical equation proposed by Korsmeyer et al. (1983).

$$\frac{M_t}{M_\infty} = F_T = kt^n \tag{3}$$

where M_t/M_∞ is the release fraction of antimicrobial compound at time t, k is a constant and n (the release exponent) is a parameter indicative of the mechanism of transference of the active agent. If the n value is 0.5, the release mechanism follows Fickian diffusion (diffusion-control), $n = 1$ is obtained for case-II transport (relaxation/erosion-control), while values ranged between $0.5 < n < 1$ are related to anomalous transport mechanism corresponding to the superposition of both phenomena (Peppas and Brannon-Peppas 1994).

The obtained results revealed that complete transference from CHPr was achieved when the films were immersed into the buffer solution. The mathematical model Eq. (3) fitted the experimental data and adequately predicted the mechanism of propionic acid released from the film. The diffusion exponent obtained from the Eq. (3) was $n = 0.5$, confirming that the release of the antimicrobial active compound would be mainly governed by diffusion. Although, taking the matrix swelling into account these results suggested that the release of antimicrobial agent was controlled by two parallel mechanisms, one type Fickian and other associated to the high degree of the matrix swelling.

The complete hydration of the material takes a relatively long time to be achieved. Thus, the evolution from Fickian toward non-Fickian behavior in relation to the initial extent of hydration considers intermediate situations (Piron et al., 1997). This was consistent with a film that quickly swells much more than the original dry size. In spite of the equilibrium swelling of the matrix was reached after approximately 12 min, the Pr continued releasing for much longer time. This fact would be further proof that the release followed a combined mechanism of diffusion and polymer relaxation. Similar results were obtained by Ouattara et al. (2000a).

Experimental data were fitted with the model of Gallagher and Corrigan (2000) that estimates the total fraction of additive released at time t (F_{TOT}), which is given by the sum of active compound transferred by surface diffusion and that released by degradation of the polymer matrix as follows.

$$F_{TOT} = F_B(1 - e^{-K_B t}) + (1 - F_B)\left(\frac{e^{k\ t-k\ t_{MAX}}}{1 + e^{k\ t-k\ t_{MAX}}}\right) \tag{4}$$

where k_B is the burst rate constant, F_B is the total burst fraction at infinite time, t_{MAX} is the time for maximum rate and k the rate constant of the polymer degradation release phase.

The kinetic profile of this model is described as initial "burst effect" of a drug non-bound to the drug matrix, followed by slow release determined by the matrix swelling (Aldana et al., 2012; Gallagher and Corrigan, 2000). Fig. 5a shows the kinetic of release of propionic acid in phosphate buffer at 4 °C, and as it can be observed the model approximated the experimental points satisfactorily ($R^2 = 0.99$).

The parameters of the first ($K_B = 8.22\ h^{-1}$) and the second release stage ($k = 2.88\ h^{-1}$) were estimated from the model Eq. (4). The obtained release profile suggested that the process was driving by diffusion at the initial phase release (burst stage) and a slower second one, in which polymer swelling became the main mechanism of agent delivery ($K_B > k$). Similar results were reported by Proikakis et al. (2006) working on drug release from polylactic acid tablets.

The propionic acid release from the separator film to a real matrix food (pastry dough) was also studied. In this case, the Gallagher–Corrigan's model also allowed fitting the experimental data ($R^2 = 0.99$), obtaining the first and second stage parameters, $K_B = 0.029\ h^{-1}$and $k = 0.027\ h^{-1}$, respectively (Fig. 5b). As it would be expected the kinetic release fitting parameters were lower in the pastry dough than in the buffer, since the food matrix limited the active compound diffusion as well as the film swelling. Moreover, in the food matrix the fitting parameters exhibited comparable values (K_B–k), proving that both mechanisms had similar contributions to the overall process.

In the pastry dough both the time required to reach the second stage ($t_{MAX} = 240$ h) as well as that necessary to achieve the complete release (Fig. 5) were longer than those in the phosphate

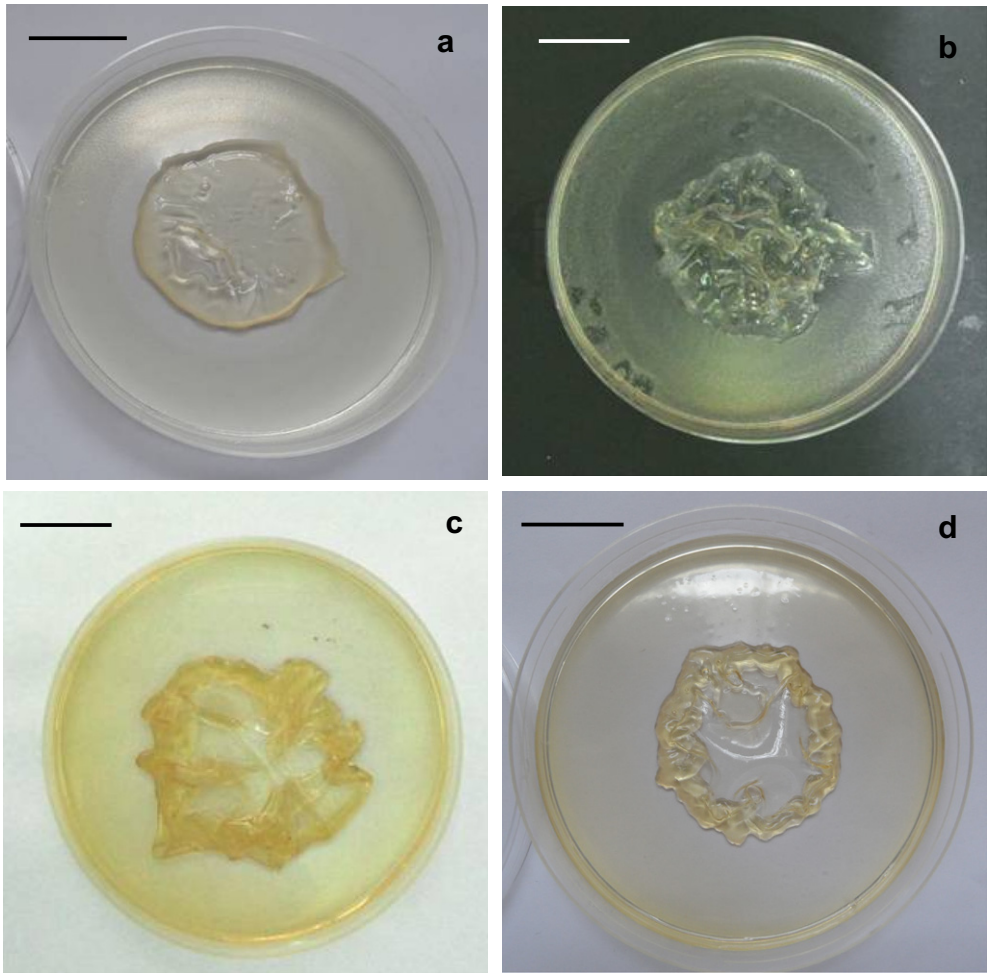

Fig. 4. Antimicrobial properties of CHPr films incubated at 37 °C against (a) *Salmonella* spp. (b) *S. aureus* (c) *Candida* spp. and (d) *Penicillium* spp. (Scale bar: 2 cm).

Table 1
Measures of inhibition halos developed of chitosan films prepared with propionic acid against inoculation of *Salmonella* spp., *S. aureus*, *Candida* spp. and *Penicillium* spp*.

Microorganism	Inhibition halos (cm)	
	Diluted inoculum (10^7 CFU ml^{-1})	Concentrated inoculum (10^8 CFU ml^{-1})
Salmonella spp.	1.02 (0.06)	0.45 (0.027)
S. aureus	1.99 (0.11)	0.57 (0.024)
Candida spp.	Complete inhibition	Complete inhibition
	Diluted inoculum (10^4 spores ml^{-1})	Concentrated inoculum (10^5 spores ml^{-1})
Penicillium spp.	Complete inhibition	1.47 (0.08)

* Measures were taken after 24 h incubation. The values in parentheses correspond to the standard deviation.

buffer (t_{MAX} = 1.3 h). These findings suggest that a more controlled and continuous release was attained, with a consequent improvement from the technological point of view.

3.4. Application of antimicrobial active films to pastry dough disk

When antimicrobial agent was measured in the pastry dough the propionic acid concentration did not exceed the maximum dose allowed (0.25 g Pr/100 g product) by the Código Alimentario Argentino (2004). Preliminary tests were conducted by assaying different propionic acid concentrations to select the effective dose that complies with the legal requirement.

The remaining propionic acid in the film after the drying process was on average 17% respect to the added acid. From this value, the amount of propionic acid present in the film could be calculated prior to the release assays.

The pastry dough pH decreased from 6.5 to 5.5 on average after 7 days in contact with the active film. This pH decrease was attributed to the propionic acid transferred to the pastry dough from the chitosan active matrix.

3.5. Shelf life of pastry dough

The shelf life of the pastry dough was defined as the time required for the microbial counts (in this case fungi and yeasts) to reach levels of 10^6 CFU g^{-1} and absence of pathogenic microorganisms. This limit of acceptability was based on the onset of food spoilage (Nopwinyuwonga et al., 2010). In previous work, a similar definition for shelf life in dough products was used (Oteiza et al., 2003).

Fig. 6 shows the counts of fungi and yeasts developed in the pastry dough in contact with the different systems during the storage at 4 °C. In the case of the CH films, counts reached levels of 10^6 CFU g^{-1} at 20 days of storage at 4 °C, while samples of pastry dough interspersed with PD960 films (control) required only 15 days to develop these counts. The counts rapidly increased reaching 10^7 CFU g^{-1} at 20 days of storage with a strong

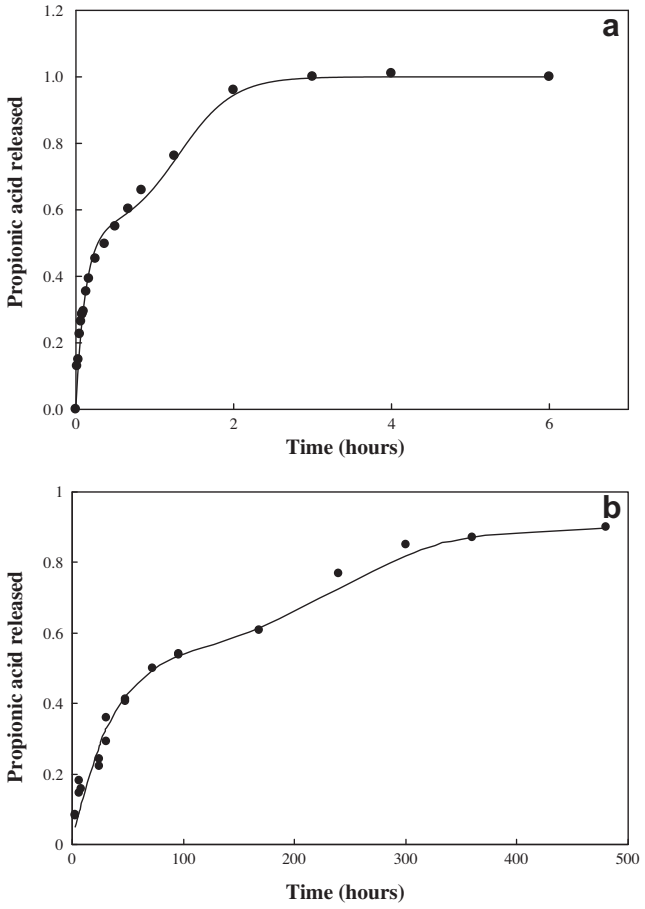

Fig. 5. Release profile of propionic acid from chitosan films prepared at 2.5% in: (a) phosphate buffer pH = 6.5 and (b) food matrix (●). Experimental data (––) were fitted to Eq. (4).

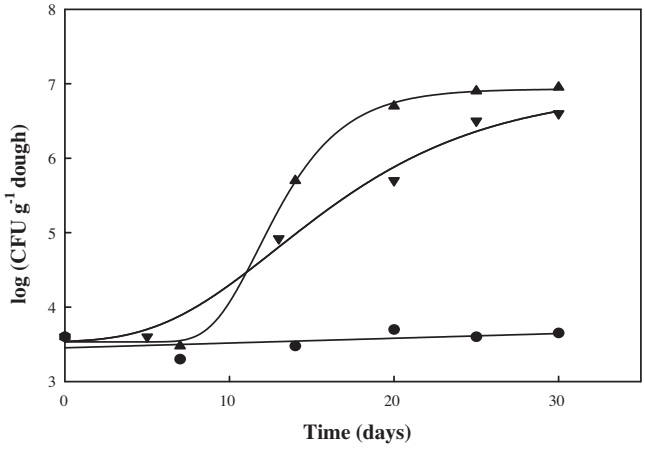

Fig. 6. Development of fungi and yeast in pastry doughs in contact with CHPr films (●), CH films (▼) and control synthetic films (▲) stored at 4 °C. Experimental data (––) were fitted to Eq. (2).

appearance of fungal growth that makes it rejectable to the consumer. Taking into account stored dough disk in contact with synthetic or CH films, microbial growth followed a type-sigmoide kinetic; hence its development was fitted using the Gompertz model Eq. (2), which allowed the prediction of the entire growth curve. Regression coefficients (R^2) were higher than 0.99 in both cases and the determined fitting parameters are shown in Table 2.

Table 2
Parameters estimated from the Gompertz equation with experimental data for the growth of fungi and yeasts in pastry dough with different films used as separators[a].

Parameters estimated from Gompertz	PD 960	CH films
a	3.39 (0.11)	3.30 (0.42)
b	2.85 (0.75)	5.99 (1.87)
x_0	11.76 (0.62)	13.73 (1.14)
y_0	3.53 (0.06)	3.54 (0.17)

[a] The values in parentheses correspond to the standard deviation.

From the Gompertz equation Eq. (2) and the calculated parameters, it was possible to estimate the time at which microbial counts reached this value in the case of dough in contact with control films (Table 2). Linear regression was used when CHPr films were put in contact with the pastry dough disks.

CHPr films in contact with the dough disk did not allow the development of microorganisms, reaching maximum count values of 10^4 CFU g^{-1}, even after 30 days of storage; so these films could be effective at doubling shelf life (Table 2). Microbial counts remained in the latency period, and there were no fungi or yeasts visible in the dough disks, showing a close relationship with the studied antimicrobial activity of the active films (inhibition halo test).

The results obtained in this work were in concordance with those obtained by Ouattara et al. (2000b), in which the use of antimicrobial films in processed meats turned out to be highly efficient. The antimicrobial agent slowly and gradually delivered from the packaging to the food surface, where it was kept in a concentration necessary to inhibit the growth of microorganisms.

4. Conclusions

Antimicrobial CHPr films applied to the pastry dough constituted an innovation within the biodegradable active packaging concept.

HPLC analysis allowed quantifying the retention fraction of the propionic acid in the films after the drying process. FTIR spectrum confirmed that the chitosan matrix effectively retained the propionic acid in the film structure. The developed films exhibited adequate mechanical and structural properties that allowed their use as supports for the control delivery of active agents.

By using the mathematical modeling of the antimicrobial agent release process, the kinetic profile could be predicted, revealing that the process was driven by a combination of both a diffusive process and another associated with the matrix swelling.

The incorporation of propionic acid in chitosan films was positive from the point of view of microbial development due to the growth inhibition of a wide spectrum of microorganism.

Films with propionic acid in contact with the pastry dough permitted the release of the acid from the chitosan matrix towards the product, extending its shelf life and maintaining the microbial growth in the latency period.

In summary, the advantages of the chitosan active system were its capacity of control release to the pastry dough surface and its possibility to substitute both separator synthetic films and chemical preservatives in the product.

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