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Soybean protein films. Characterization and potential as novel delivery devices of *Duddingtonia flagrans* chlamydospores

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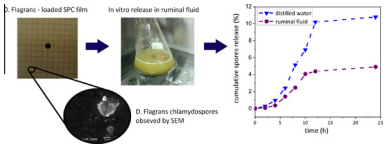
HIGHLIGHTS

- Soybean protein concentrate-based films were produced and characterized as potential vehicles of *Duddingtonia flagrans*.
- Viability of spores contained in films was verified.
- The best film composition was determined from mechanical, optical and swelling properties.
- Chlamydospores release was evaluated in ruminal fluid in vitro.
- This delivery system would be non-toxic and economically favorable.

GRAPHICAL ABSTRACT



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ABSTRACT

This study was aimed at assessing the potential use of soybean protein concentrate (SPC) – based film as vehicle for the delivery of *Duddingtonia flagrans* chlamydospores for the biological control of gastric nematodes in ruminals. Glycerol and dialdehyde starch (DAS) were used as plasticizer and cross-linking agent, respectively. Films were obtained by casting and characterized in terms of their physico-mechanical properties. The impact of cross-linking extent on moisture absorption, swelling and tensile properties of the resultant films was evaluated. The adequate tensile properties and stability in wet environment of SPC films cross-linked with 10 wt.% DAS was counterbalanced with the two-phase morphologies developed, irrespective of glycerol content, limiting their potential application as delivery devices. SPC films cross-linked with 5 wt.% DAS and plasticized with 30 wt.% glycerol exhibited the best compromise between solubility (only 29%), homogeneous morphology, adequate tensile strength (2.50 ± 0.43 MPa) and elongation at break (18.72 ± 2.34%) and swelling profile. The preliminary results of the release of *D. flagrans* chlamydospores in ruminal fluid revealed a slow release profile attaining 4.9% in a period of 24 h. Overall, these results substantiated the potential use of DAS-cross-linked SPC films as viable carrier matrix for *D. flagrans* release applications.

1. Introduction

Gastrointestinal infection produced by nematode parasites among pasture-grazed ruminant livestock is a great deal of concern since control failure leads to reduced production and profits. Pasture management and anthelmintic therapy are the main strategies traditionally used to control internal parasites (Ojeda-Robertos et al., 2009; Sagüés et al., 2011; Waller, 1997; Waller et al., 2001a), however, anthelmintic resistance is by far the main drawback of chemical treatment (Panchadcharam, 2004; Waller, 1997). This issue has encouraged the search for novel methods including the biological control by the use of nematophagous fungi

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against the free living stages of the parasite on pasture (Waller, 1997; Waller et al., 2001a). Biological control offers an effective, renewable and environmentally sound alternative in the reduction of infective larva populations of nematodes in pastures (Larsen, 1999). Among the promising nematode trapping fungi, *Duddingtonia flagrans* was identified as an exceptional antagonist of such parasite by producing prolific trapping networks and thick walled resting spores called chlamydospores (Waller, 1997; Waller et al., 2001a,b). Different strategies were proposed to deliver chlamydospores into the animal including oral administration (Ojeda-Robertos et al., 2009), incorporated with the animal feed (Waller et al., 2001b), within mineral (Waller et al., 2001b) and energetic blocks (Sagüés et al., 2011). The success of all the above mentioned alternatives was found to be dependent on the voluntary and variable intake of the animals which lead to unpredictable results (Waller, 1997; Waller et al., 2001b). In such situation the development of controllable release devices (CRD) is a more feasible approach.

At the present, rather less effort has been spent on the development of CRD for *D. flagrans* spores. In their seminal work, Waller et al. (2001b) reported the manufacture by compression of tablets from sucrose esters containing *D. flagrans* spores. Chlamydospores preserve their viability after processing and during storage. *In vivo* studies revealed the presence of viable chlamydospores on the surface of the CRD, and also in feces of fistulated sheep, for up to 3 weeks. It was also suggested the necessity of reducing production costs of such devices in order to make it competitive with chemical prophylaxis. Sagüés (2012) reported the first results on massif cylindrical CRD based on soybean protein isolated (SPI) containing *D. flagrans* chlamydospores processed by cold-extrusion. Authors stated that spores were successfully released *in vivo* in cannulated sheep and formulation did not affect the predatory activity of *D. flagrans*.

Within this context biopolymers from agricultural sources are becoming an interesting alternative not only as biodegradable films, suitable for food packaging, but also as specialized polymeric items which require mechanical and controlled-release properties (Gómez-Martínez et al., 2009). Food proteins show great promise for developing and engineering a range of new GRAS (generally recognized as safe) matrices with the potential to incorporate drugs or other therapeutic compounds and provide controlled release via the oral route (Chen et al., 2006). Among the potential candidates, soy proteins represent one of the cheapest and most abundant biological feed-stocks available in large quantities at low cost, and their use for developing CRD offers numerous advantages, such as non-citotoxicity, good processability and biodegradability which make them economically competitive (Peles and Zilberman, 2012).

Soy proteins are commercially available in different grades including soybean protein isolate (SPI, >90% protein), soybean flour (SF, about 50% protein) and soy protein concentrate (SPC, 65–70% protein) which is economically more favorable than SPI and has higher protein content than SF (Park et al., 2002). Protein fraction in SPC is composed by four fractions namely 2S, 7S, 11S, and 15S. The fractions 7S and 11S are the largest and most important globular storage protein, β-conglycinin and glycinin, respectively. The carbohydrate fraction in SPC (about 15%) (Ciannamea et al., 2010) is mostly composed by non starch polysaccharides, including 8–10% cellulose and pectic polysaccharides (linear hetero-polysaccharides which contain free or esterified galacturonic acid–based units) (de la Caba et al., 2012).

Soy proteins can be transformed into films by thermoplastic processing and dissolution – dehydration procedure called casting (Hernandez-Izquierdo and Krochta, 2008). During dissolution stage pH level of the film forming solution affects protein charge and unfolding degree which determine the type of interactions (i.e. disulfide bridges, hydrogen and hydrophobic interactions (Hernandez-Izquierdo and Krochta, 2008; Park et al., 2002) developed during the dehydration stage. In general, pure globular protein films provide the potential to control transport of oxygen, aroma, oil, and flavor compounds, however, by themselves, form brittle films, which limits their application (Sothornvit and Krochta, 2001). Plasticizers are necessarily used in protein films because they reduce brittleness by lowering the inter-molecular interactions between adjacent protein chains (Hernandez-Izquierdo and Krochta, 2008; Sothornvit and Krochta, 2001). Among various plasticizers, glycerol has shown greater miscibility with protein and plasticizing effect (Sothornvit and Krochta, 2001). However, the level of plasticizer has to be optimized since it greatly impact barrier and tensile properties (Park et al., 2002; Sothornvit and Krochta, 2001). One way to enhance moisture repellency of protein films is by introducing covalent bonds through physical, enzymatic and chemical routes (Park et al., 2002). Soybean proteins have many side-chain groups (i.e. NH2, COOH, OH) able to react with a variety of chemical reagents. Aldehydes such as glutaraldehyde (Caillard et al., 2008), glyoxal (Vaz et al., 2004), and formaldehyde (Chen et al., 2008) have been successfully used to stabilize soy protein films, however, their suspected toxicity have restricted their applications in food and pharmaceutical applications (Huang-Lee et al., 1990). Polymeric dialdehyde starch (DAS) is a cross-linking agent derived from natural sources (Yu et al., 2010). The potential applications of DAS are based on its well reported cross-linking ability in solution-casting (Gennadios et al., 1998; Rhim et al., 2000, 1998) and compression molding protein films (Martucci and Ruseckaite, 2009) and its low citotoxicity as reported by Wilson (1959).

The research on soybean protein films as monolithic drug delivery devices is still limited to a few publications (Chen et al., 2008; Peles and Zilberman, 2012), and no information regarding their use as vehicle for delivering chlamydospores has been reported. Consequently, in the present work we explore the potential of SPC films with DAS as cross-linking agent and glycerol as plasticizer as monolithic CRD of *D. flagrans* chlamydospores under in vitro conditions. To evaluate SPC films as CRD, it is necessary to have an overall understanding of their physic-chemical properties. An ideal film for CRD should be homogeneous, mechanically strong such it do not fracture during processing or storing but sufficiently ductile to be shaped into the desired dosage form, c.a. a cylinder able to potentially be administrated to the animal via an esophagogastric probe to the rumen where spores would be released. Therefore, SPC films were evaluated in terms of opacity, morphology, cross-linking density, total soluble matter and tensile properties. The most suitable SPC–Gly–DAS formulation for delivering chlamydospores was obtained by correlating tensile properties and film stability with swelling behavior in distilled water and ruminal fluid. Viability of spores in the presence of DAS was also qualitatively evaluated. Preliminary study on the in vitro release of chlamydospores is also reported.

2. Materials and methods

2.1. Materials

Soybean protein concentrate (SPC, Solcom S 110), isoelectric point (pI) near 4.5, with an average particle size passing through 100 mesh and 7% moisture, 69% protein, 1.05% fat, 3.5% fibers, 6% ashes and about 15% non starch polysaccharides (NSP, mainly cellulose, non cellulose polymers and pectin polysaccharides) as mean composition (Cordis, 2010), was obtained from Cordis SA (Villa Luzuriaga, Buenos Aires, Argentina). Dialdehyde Starch (DAS) with 81.8% starch oxidation was supplied by Sigma–Aldrich (St. Louis, MO, USA) and used without further treatment. Glycerol

(Gly, 98%) analytical grade and buffer phosphate pH 10 were purchased from Anhedra (Buenos Aires, Argentina).

2.1.1. Fungal material

The trial was carried out on the Experimental Farm, Faculty of Veterinary Sciences, located in Tandil (Buenos Aires, Argentina (37°17′34″S, 59°5′W)). A local isolate of *D. flagrans*, previously isolated from the same site where the trial was carried out, was used for the experiments. Chlamydospores of this fungus were harvested from 2 weeks cultures grown on pure agar cultures at 24 °C. Following this, chlamydospores were gently rinsed off with sterile water and counted using a Neubauer haematocytometer to estimate the number of chlamydospores per ml of water. The number of spores was 70,875,000 in 20 ml of distilled water. Viability of spores in the presence of DAS was verified prior to their use in the different formulations. For this purpose a qualitative technique was used, which is based on the spore's ability to germinate and form trapping structures in the presence of *Panagrellus* spp, using water-agar medium with varying amounts of DAS (0, 0.24, 1, and 5 wt.%). Petri plates were incubated at 25 °C for 1 week. Samples were periodically observed by optical microscopy to evidence the presence of tridimensional structures. Characterized chlamydospores were preserved for further incorporating into SPC-based films.

2.1.2. Ruminal fluid

In vitro experiments were carried out in ruminal fluid (RF) prepared according to Sagüés et al. (2011)). RF consisted of 120 ml of synthetic saliva, 1.5 g of alfalfa (*Medicago sativa L.*) and 30 ml of ruminal fluid collected from a Holando Argentino cow through a ruminal fistula. The RF was kept into an Erlenmeyer flask and was sealed by airtight rubber stoppers supplied with an outlet valve to release the gas, and incubated at 39 °C in a thermostatic bath assisted by shaking (Gyrotory Water Bath Shaker Model G76, New Brunswick, USA), to promote a constant agitation simulating ruminal movements. The RF was sieved (mesh size 1 mm) and kept at 39 °C (ruminal temperature).

2.2. Film preparation

Film formulation involved a mixture of food grade SPC, glycerol and DAS as cross-linking agent. None of these materials have any known antihelmintic or fungicidal activity. Film-forming solutions (FFS) were obtained by dispersing SPC powder in constantly stirred buffer phosphate pH 10 (5 g SPC/100 mL buffer solution) to provide a 3.25% protein (wt/V) solution. Subsequently, glycerol (Gly) was added to SPC slurry in appropriate amounts to reach 30, 40, and 50% (wt/wt SPC dry basis) and cross-linking agent DAS was incorporated at 0, 5, and 10% (wt/wt SPC dry basis). The slurry was stirred for 30 min at 70 °C and subsequently sonicated in an ultrasonic bath (Test Lab, 160 W, 40 kHz) to remove bubbles. Finally the FFS were poured onto leveled Teflon-coated Petri dishes. The target film thickness was 150 μm, and the quantity of each FFS used was calculated so that the solid content (c.a., SPC, DAS, Gly) was the same (i.e., approximately 35 ml/150 cm^2, for SPC – 30% Gly – 0% DAS). Samples were left to dry in an air-circulating oven (DKN 400, USA) at 35 °C until reaching constant weight (approximately 20 h). The dried films were peeled-off from the plates and kept in a laboratory environmental chamber for 48 h at 25 °C and 65 ± 2% relative humidity (RH) before testing. DF-loaded films were prepared similarly. Once obtained the FFSs temperature was decreased to 30 °C (to preserve the spore's activity) and a suspension of *D. flagrans* chlamydospores in distilled water (c.a., 70,875,000 chlamydospores in 20 mL of distilled water) was added and gently stirred for 15 min. After this time, casting and drying procedures were performed similarly than for free-films. Free films were labeled as **SPC–XGly–YDAS**, where X and Y are the percentages of glycerol and DAS, respectively, while DF-loaded films was labeled as **DF–SPC–XGly–YDAS.**

2.3. Film characterization

2.3.1. Film thickness

Film thickness was measured with a manual micrometer (0–25 ± 0.01 mm, Bta. China). Measurements were done at ten random points taken along the rectangular films and an average value was taken. For tensile test, opacity and moisture absorption experiments, three measurements were done on each specimen.

2.3.2. Conditioning

Prior testing, film samples were pre-conditioned in laboratory environmental chamber for 48 h at 25 ± 2 °C and 65 ± 2% RH.

2.3.3. Cross-linking extent

The extent of the amino groups involved in the cross-linking reaction was determined by UV–visible spectroscopy by using ninhydrin (2,2-dihydroxy-1,3-indanedione, NHN), in order to estimate the amount of free amino groups remaining after the chemical cross-linking reaction (Martucci et al., 2012). Ninhydrin forms a purple complex (Ruhemann's purple) with the α-amino functionality of proteins. The absorbance of the solution measured at 570 nm (the wavelength of the blue–purple color) is proportional to the amount of free amino groups left after the reaction with the cross-linking agent. Cross-linked SPC films were dried under vacuum at room temperature until constant weight. A precise amount of sample (100 ± 5 mg) was heated with ninhydrin solution (0.5 wt.%) for 20 min. The absorbance of this solution was recorded on a Shimadzu 1601 PC spectrophotometer at 570 nm (Tokyo, Japan). The cross-linking extent (%) was expressed as the percentage of free amino groups reacted by the following equation:

$$\text{Crosslinking extent } (\%) = \frac{(\text{NHN reactive amine})_g - (\text{NHN reactive amine})_{rg}}{(\text{NHN reactive amine})_g} \cdot 100 \quad (1)$$

where $(\text{NHN reactive amine})_g$ is the total amount of amino groups in the SPC film, and $(\text{NHN reactive amine})_{rg}$ is the amount of free amine groups present in the SPC film after the cross-linking reaction.

2.3.4. Light barrier properties and color parameters

Visible light – barrier properties of films were determined by measuring their light absorption at wavelength ranging from 400

to 800 nm, using a UV–Visible spectrophotometer (Shimadzu 1601 PC, Japan) according to the method described elsewhere (Irissin-Mangata et al., 2001). Rectangular strips of films were placed directly in the spectrophotometer test cell and air was used as reference. Film opacity was calculated as the area under the recorded curve and was expressed as absorbance units (AU) × nm.

Color parameters were assessed using a portable colorimeter (Novi Bond RT 500, Germany) with a measuring area of 8 mm of diameter. Film samples were placed on a white plate, and the Hunter Lab color scale was used to measure color: $L^* = 0$ (black)–$L^* = 100$ (white); $a^* = -80$ (green)–$a^* = 100$ (red); and $b^* = -80$ (blue)–$b^* = 70$ (yellow). The total color difference (ΔE), was calculated using the following equation:

$$\Delta E = \sqrt{(\Delta a^*)^2 + (\Delta b^*)^2 + (\Delta L^*)^2} \quad (2)$$

The results are the average of four readings.

2.3.5. Moisture content and total soluble matter (TSM)

Three films samples of each film were weight and dried in an air circulating oven at 105 ± 1 °C for 24 h. MC was calculated on dry basis and reported as the average of three replicates. *TSM* was expressed as the percentage of film dry matter solubilized after 24 h immersion in distilled water (DW) (Rhim et al., 1998). Three samples were carefully weighed (±0.0001 g, ALC-210.4, Acculab Sartorius, USA) and subsequently dried in an air-circulating oven at 105 ± 1 °C for 24 h to determine their initial dry matter. Afterward, samples were immersed in 30 mL of DW with traces of sodium azide (0.02%) to prevent microbial growth, and stored at 25 °C for 24 h. Insoluble dry matter was determined recovering the immersed samples and drying them in an air-circulating oven (DKN 400, USA) at 105 °C for 24 h. Dry soluble matter was calculated subtracting the insoluble dry matter weigh to the initial dry matter.

2.3.6. Tensile properties

Tensile tests were performed on an INSTRON 4467 Universal Test Machine (Buckinghamshire, England) equipped with a 0.5 KN load cell, at a crosshead speed of 3 mm/min at room temperature according to the procedure described in ASTM D1708-02a. Tensile strength (TS), elongation at break ($\%\varepsilon_b$) and elastic modulus (E) were calculated as the average of ten replicates.

2.3.7. Transmission optical microscopy (TOM)

Iodine stained SPC-DAS films were observed by using a Leica DMLB (Wetzlar, Germany) microscope, with crossed polarizer, provided with a video camera Leica DC 100.

2.3.8. Scanning electron microscopy (SEM)

The films failure and external surface (upper and lower) were observed with a Jeol JSM-6460LV (Tokyo, Japan) scanning electron microscope using 10 kV as accelerating voltage. Prior to the observation, the surfaces were sputter-coated with a gold layer of about 100 Armstrong to avoid charging under the electron beam.

2.3.9. Spores germination in the presence of DAS

Viability of spores contained in the respective DAS-containing formulations was verified prior to their use in the different experimental procedures. For this purpose a qualitative technique was used, which is based on the spore's ability to germinate and to form trapping structures in the presence of *Panagrellus* spp. Incubations were performed in water agar Petri dishes containing 0.24%, 1% and 5% DAS at 25 °C (Mechanical Convection Oven DKN 400), until evidencing the presence of tridimensional network.

2.3.10. In vitro water and ruminal fluid capacity of free and chlamydospores loaded-SPC films

The fluid absorbing capacity of films over a period of 24 h was determined gravimetrically. Square – shaped samples (area 2 cm^2) of films produced with the selected formulation with and without spores were carefully pre-weighed and then, immersed in Erlenmeyer flasks containing 150 ml of either distilled water with 0.02% of sodium azide (pH 5.8) and RF (pH 6–6.2). Both set of flasks were kept at 39 °C in a thermostatic bath (Gyrotory Water Bath Shaker Model G76, New Brunswick Scientific Edison, USA) under orbital shaking, to simulate ruminal conditions. Samples were periodically removed from the media, gently blotted with a tissue paper and weighed again. Fluid uptake, as expressed as water uptake (WU) and ruminal fluid uptake (RFU) at time t was calculated as:

$$\%\mathrm{WU} \quad \text{or} \quad \mathrm{RFU} = \frac{W_t - W_o}{W_o} \cdot 100 \quad (3)$$

where W_t is the sample weight at time t and W_0 is the initial weight. Reported results were the average of three values.

Experimental data were fitted to Fick's second law equation:

$$\mathrm{FU}_t = \mathrm{FU}_{eq} - \mathrm{FU}_{eq} \cdot \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left[\frac{-(2n+1)^2 \pi^2 t}{4} \cdot \left(\frac{D_{app}}{L^2}\right)\right] \quad (4)$$

where FU_t is the fluid uptake at time t, FU_{eq} is the maximum fluid uptake at the equilibrium, L is the thickness of the film and D_{app} is the apparent diffusion coefficient.

2.3.11. In vitro weight loss profiles of chlamydospores – loaded-SPC films Weight

Loss profile as a function of immersion time was studied on dry samples after swelling experiments. Samples were dried at ambient temperature until constant weight. The percentage of weight loss (%WL) was calculated as follows:

$$\%\mathrm{WL} = \frac{W_o - W_{td}}{W_o} \cdot 100 \quad (5)$$

where W_0 is the initial dry weight and W_{td} is the dry weight at time t.

2.3.12. In vitro chlamydospores release study

Square-shape samples (duplicate, 2 cm^2) of spore – loaded SPC films were immersed in distilled water and ruminal fluid at 39 °C for 48 h. Release studies were performed in closed Erlenmeyer flasks containing 20 ml of distilled water and ruminal fluid, respectively. At each sampling time aliquots of each media were removed and the number of chlamydospores in each aliquot was determined from three independent counts using a Neubauer chamber. During the counting procedure chlamydospores were visually assessed. The chlamydospores release (CR) was calculated as:

$$\%\mathrm{CR} = \frac{\mathrm{Chl}_{total} - \mathrm{Chl}_{released}}{\mathrm{Chl}_{total}} \cdot 100 \quad (6)$$

where Chl_{total} is the total concentration of chlamydospores in the films (undigested chlamydospores) and $\mathrm{Chl}_{released}$ is the amount of chlamydospores released at time t.

2.3.13. Statistical analysis

Statistical analysis of results was performed using Origin Pro 8 (Origin Lab Co.). Tukey's test ($P < 0.05$) was used to detect differences among mean values of film properties.

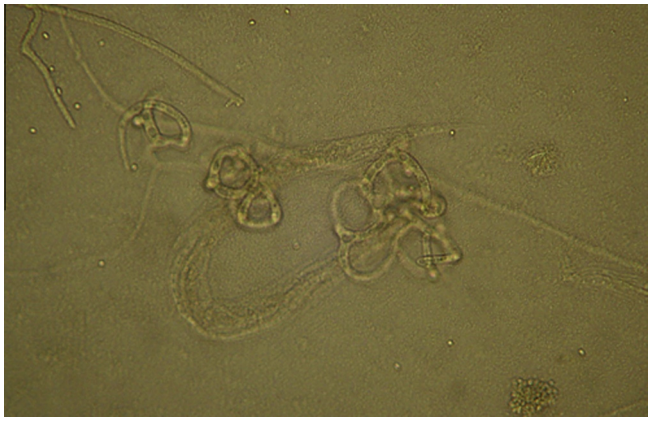

Fig. 1. Optical microscopy photograph of nematode trapped in the tridimensional network of *D. flagrans* hyphae.

3. Results and discussion

3.1. Viability of chlamydospores in the presence of DAS

Prior to incorporate *D. flagrans* chlamydospores to formulations containing DAS as cross-linking agent, spores germination and growth was assessed qualitatively by TOM observations of samples recovered periodically during one week of incubation in water–agar medium containing varying DAS concentrations (0.24, 1, and 5 wt.%) in the presence of free-life nematodes *Panagrellus* spp. As can be seen in Fig. 1 mycelial growth and trapping structures were noticed after 8 h of incubation signifying that spores viability was not significantly affected by this reagent. This result is in accordance with those reported by Waller et al. (2001b) demonstrating that viability was preserved after exposure to temperature and pressure during processing into delivery tablets or under ruminal gasses such as methane and carbon dioxide.

3.2. Structural and functional characterization of blank SPC–Gly–DAS films

Free-chlamydospores SPC films with different Gly and DAS contents were studied to evaluate their water resistance, swelling capability in distilled water and mechanical properties in order to select the most suitable formulation for delivering *D. flagrans* in ruminal fluid.

3.2.1. Optical properties, morphology and cross-linking extent

All the produced films are transparent as find out by naked eye observation (Fig. 2a–c) and light – barrier properties (Table 1). With an increase in glycerol concentration from 0 to 30 wt.% there was a progressive increase in film transparency ($P < 0.05$) owing to the fact that plasticizer interferes in protein chain-to-chain interaction lowering the film absorbance in the visible light region of the spectrum (i.e., 400–800 nm) (Nuthong et al., 2009). The addition of DAS increased significantly opacity values ($P < 0.05$) (Table 1), nevertheless for DAS contents lower than 10 wt.%, films can be considered transparent. Since no significant differences ($P > 0.05$) in film thickness were detected (Table 1), it was assumed that this reduction in film transparency could be consequence of some degree of phase separation due to the limited thermodynamic compatibility between protein and polysaccharide (Khomutov et al., 1995) or by an ineffective mixing during the dissolution stage of film formation. Optical microscopy performed on iodine stained films confirmed the existence of two-phase morphologies (Fig. 2d–f) where protein – rich domains constitute the continuous phase and the dispersed particles are mainly composed by DAS, since amylose – type chains constituting the vast majority of DAS, gave rise to the characteristic starch iodine complex. A qualitative analysis of TOM micrographs revealed that components compatibility reduced with DAS content according to greater concentration of dispersed phase (Fig. 2f). Similar behavior was already observed in DAS-cross-linked – plasticized – gelatin films obtained by compression molding (Martucci and Ruseckaite, 2009).

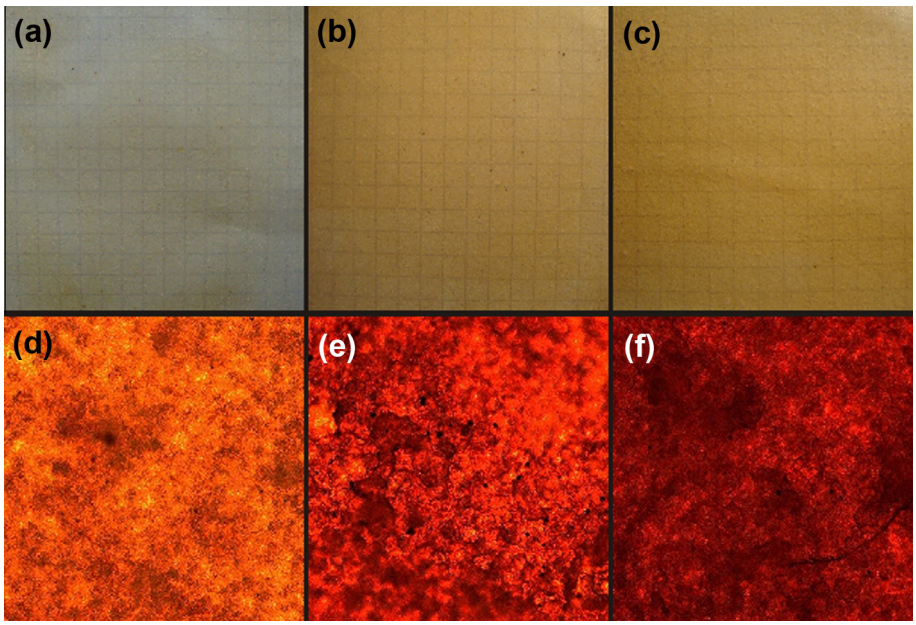

Fig. 2. (a–c) Macroscopic appearance of SPC-30Gly films with increasing DAS content, and (d–f) optical transmission microscopy of the films after staining with iodine solution.

Table 1
Thickness, opacity and color parameters of SPC-Gly-DAS films.

Gly (%)	DAS (%)	Thickness (μm)	Opacity (AU nm)	L^*	a^*	b^*	ΔE
30	0	154 ± 16 a	955 ± 10 a	82.87 ± 1.43 a	0.83 ± 0.19 a	12.57 ± 2.59 a	21.30 ± 2.93 a
	5	156 ± 13 a	1110 ± 3 bc	75.47 ± 2.22 b	6.51 ± 1.96 b	38.56 ± 4.63 b	50.59 ± 5.28 b
	10	154 ± 17 a	1110 ± 24 bc	69.04 ± 0.89 c	12.01 ± 0.66 c	48.91 ± 0.80 c	63.18 ± 0.56 c
40	0	156 ± 32 a	968 ± 24 a	82.47 ± 1.91 a	0.87 ± 0.25 a	12.66 ± 3.72 a	21.54 ± 4.15 a
	5	153 ± 10 a	1156 ± 1 d	77.71 ± 1.67 b	4.62 ± 1.12 b	34.21 ± 4.26 b	45.56 ± 4.66 b
	10	155 ± 13 a	1101 ± 18 b	70.21 ± 1.88 c	10.44 ± 1.82 c	47.64 ± 3.39 c	61.35 ± 4.05 c
50	0	159 ± 19 a	1001 ± 16 a	82.21 ± 1.57 a	0.90 ± 0.31 a	13.39 ± 2.81 a	22.32 ± 3.19 a
	5	156 ± 15 a	1154 ± 6 cd	75.91 ± 1.04 b	5.44 ± 1.02 b	37.34 ± 1.78 b	49.19 ± 2.07 b
	10	157 ± 16 a	1152 ± 17 cd	68.74 ± 0.86 c	11.75 ± 0.76 c	48.96 ± 1.28 c	63.29 ± 1.52 c

Mean values ± standard deviations. Any two means in the same column followed by the same letter are not significantly different ($P > 0.05$) by Tukey's Test.

As a general rule, homogeneous morphologies are desired in monolithic devices intended for drug delivery. In swellable monolithic systems, heterogeneous morphologies may induce "burst release". Fast release in a burst is used in certain drug administration strategies, however the negative effects of such burst can be pharmacologically dangerous and economically inefficient (Huang and Brazel, 2001).

On the other hand, the addition of increasing amounts of glycerol did not influence significantly ($P > 0.05$) color parameters (Table 1) but values differed considerably ($P < 0.05$) with DAS level. The incorporation of DAS up to 10% leads to a decrease in L^* values ($P < 0.05$), indicating darker films. The increase in film yellowness ($P < 0.05$) was evidenced by a greater value of b^*, accompanied by an increase in a^* (increasing redness). This increased yellow/brown coloration with DAS level gave indirect evidence of the cross-linking efficiency of DAS with soy protein. Cross-linking mechanism of DAS with soy proteins was postulated to be analogous to that of short chain aldehydes (Gennadios et al., 1998; Martucci and Ruseckaite, 2009; Rhim et al., 2000, 1998). It has been proposed that carbonyl groups react with exposed amine – side chain groups from proteins throughout the formation of conjugated Schiff's bases, which are colored intermediate products of the Maillard reactions (Rhim et al., 2000, 1998). DAS produced yellow–brownish films when reacted with gelatin (Martucci and Ruseckaite, 2009), SPI (Rhim et al., 2000, 1998) and egg-white protein (Gennadios et al., 1998).

In order to confirm the above assumption, the cross-linking extent of SPC films was estimated by using Eq. (1) and results are reported in Table 2. The addition of 10% DAS reduced the number of free-amino groups in about 50% irrespective of the glycerol content. Contrary, for 5% DAS, the percentage of cross-linking was reduced with glycerol content. At glycerol level of 50% and low DAS content such as 5%, there appears to be a dilution effect which prevent the aldehyde groups in the polymeric DAS from reaching every available reactive side-chain amino group on the protein fraction of SPC.

3.2.2. Residual moisture content and soluble matter

SPC, DAS and glycerol easily absorbed moisture from the environment due to their hydrophilic nature (Martucci and Ruseckaite, 2009; Rhim et al., 1998; Yu et al., 2010). The presence of free polar – side chain groups from the protein fraction plus the polysaccharide portion are the main factors determining the moisture absorption of SPC (de la Caba et al., 2012). On the other hand, the presence of carbonyl as well as hydroxyl groups from the polysaccharide backbone of DAS can bind water molecules and favor moisture retention (Rhim et al., 1998; Yu et al., 2010). Cross-linking degree was reported as a feasible way of controlling water uptake and solubility of soybean protein and gelatin films (Martucci and Ruseckaite, 2009; Rhim et al., 2000, 1998). Therefore, cross-linking SPC with DAS is expected to reduce hydrophilic sites reducing the water uptake capacity of the resultant films, as previously reported for other protein films (Gennadios et al., 1998; Martucci and Ruseckaite, 2009; Rhim et al., 2000, 1998; Yu et al., 2010).

As anticipated, MC values after conditioning for 48 h at 65% RH and ambient temperature increased with glycerol content ($P < 0.05$) owing to the fact that there are additional hydroxyl groups within the matrix, favoring the moisture retention (Martucci and Ruseckaite, 2009; Rhim et al., 1998) (Table 2). MC is also related to the total void or porous occupied by water molecules in the microstructure, therefore an increment in such parameter could indicate increased void volume in the final films (Jiang et al., 2010). The effect of DAS on MC seems to depend on the amount of glycerol, as concluded from the analysis of results in Table 2. The incorporation of 5% DAS into SPC – 30Gly formulation did not induce significant differences in MC values, while rising glycerol content increased substantially this parameter ($P < 0.05$) even compared with films without DAS. The inclusion of 10% of

Table 2
Cross-linking extent (%), residual moisture content (MC), total soluble matter (TSM), tensile strength (TS) and elongation at break (ε_b) of SPC–Gly–DAS films.

Gly (%)	DAS (%)	Cross-linking extent (%)	MC (%)	TSM[1] (%)	TSM[2] (%)	TS (MPa)	ε_b (%)
30	0	0	17.7 ± 0.7	34.7 ± 3.3^{a,b}	52.8 ± 3.8^{a}	2.27 ± 0.20^{a,b}	14.52 ± 2.18^{a}
	5	31	17.7 ± 1.0	29.2 ± 2.5^{a}	37.0 ± 5.4^{b,c}	2.50 ± 0.43^{b,c}	18.72 ± 2.34^{a,b,c}
	10	54	18.9 ± 0.8	32.0 ± 0.7^{a,b}	30.9 ± 5.6^{c}	3.22 ± 0.81^{c}	19.95 ± 5.61^{a,b,c}
40	0	0	20.4 ± 0.8	36.7 ± 3.4^{a,b}	46.7 ± 4.0^{a,b}	2.12 ± 0.47^{a,b}	15.82 ± 2.89^{a,b}
	5	16	29.5 ± 2.3	36.3 ± 3.9^{a,b}	39.8 ± 5.2^{a,b,c}	1.78 ± 0.55^{a,b}	22.37 ± 3.14^{c,d}
	10	53	26.2 ± 0.3	38.5 ± 0.1^{b}	35.5 ± 3.5^{b,c}	2.48 ± 0.67^{b,c}	23.45 ± 3.63^{c,d}
50	0	0	23.6 ± 1.2	39.6 ± 5.2^{b}	45.3 ± 1.1^{a,b}	1.73 ± 0.37^{a,b}	18.02 ± 1.65^{a,b,c}
	5	4	34.1 ± 1.8	38.3 ± 2.8^{b}	46.0 ± 5.1^{a,b}	1.55 ± 0.52^{a}	24.92 ± 1.81^{d}
	10	53	26.3 ± 1.0	33.3 ± 2.1^{a,b}	36.9 ± 2.1^{b,c}	1.61 ± 0.25^{a,b}	21.27 ± 5.56^{b,c,d}

Mean values ± standard deviations. Any two means in the same column followed by the same letter are not significantly different ($P > 0.05$) by Tukey's Test.
[1] TSM determined by dry method.
[2] TSM determined by wet method.

DAS in SPC-30Gly did not provoke significant differences on MC ($P < 0.05$). When glycerol level ranges from 40–50%, SPC-5DAS films had many additional hydrophilic hydroxyl groups which counterbalance the effect of cross-linking and the impact on MC was negligible (Martucci and Ruseckaite, 2009; Rhim et al., 1998). In general, DAS-induced cross-linking decreases water uptake by soy proteins since protein amino groups are not yet available to bind water by hydrogen bonding (Rhim et al., 1998). However for a certain DAS level (i.e., ~5% in the present case) cross-linking reaction with soy protein reached a saturation point as already reported by SPI-DAS films (Rhim et al., 2000, 1998). MC results gave experimental evidence of the efficiency of DAS as cross-linking agent for concentrations equal to 5%.

Water (in) solubility is one of the most important properties to control in polymeric delivery devices. The soluble fraction represents the percentage of polymer chains in the initial aqueous solution that does not participate in network formation. Reportedly the soluble fraction in plasticized cross-linked protein films could be mainly attributed to the loss of low molar mass compounds, such glycerol and short-chain polypeptides that could not be linked to the network (Rhim et al., 2000, 1998). The idea of measuring the soluble matter by the "dry" and "wet" methods was to evaluate the effect of drying the samples at 105 °C before testing. Table 2 presents the soluble fraction of films. SPC films without DAS maintained their structural integrity after 24 h soaking, suggesting that SPC proteins established some degree of physical cross-linking during the drying step, as stated by other authors (Gennadios et al., 1998; Martucci and Ruseckaite, 2009; Rhim et al., 1998). Films treated at 105 °C prior to TSM experiments produced films less soluble ($P < 0.05$), irrespective of glycerol content. It seems that heating treatment induced additional cross-linking such as disulfide bridges making the films more insoluble (Rhim et al., 2000). The reduced effect of adding 5% DAS on solubility agreed well with the low cross-linking extent attained by these films (Table 2). Increasing DAS reduced TSM values whatever the glycerol content and the method employed. The reduction in TSM observed in SPC films containing DAS was considered indirect evidence of DAS–SPC cross-linking in the films, as previously reported for protein-DAS treated films (Gennadios et al., 1998; Martucci and Ruseckaite, 2009; Rhim et al., 2000, 1998).

3.2.3. Tensile properties

The tensile testing of SPC–Gly–DAS films provides an idea on the strength and elasticity of the films given by the parameters like tensile strength (TS), elastic modulus (EM), and elongation at break (ε_b). A good film for delivering chlamydospores in rumen should be strong and ductile enough to be shaped in the desired dosage form.

Fig. 3 shows representative stress–strain curves obtained from tensile tests, for all the films under study. It can be observed that the deformation at room temperature, under an applied load, was typical of ductile plastics in terms of the stress and strain. These curves exhibited the typical deformation behavior; at low strains (lower than 10%) the stress increased rapidly with an increase in the strain and the slopes were in the elastic region defining the elastic modulus. Table 2 shows the effect of DAS and glycerol levels on tensile strength (TS) and percentage of elongation at break (%ε_b). For control films (0 wt.% DAS) the addition of increasing amounts of glycerol did not induced significant changes ($P > 0.05$) in TS and %ε_b values. The addition of 5% DAS provoked an increase in %ε_b ($P < 0.05$) values without significant changes in TS. This last observation demonstrates that cross-linking with DAS did not induce severe restrictions within SPC–Gly matrix as usually occurs with short-chain dialdehydes (Gennadios et al., 1998; Martucci et al., 2012; Martucci and Ruseckaite, 2009; Rhim et al., 2000, 1998). For DAS content as low as 5 wt.%, the cross-linking effect is counterbalanced by its plasticizing ability exerted by its hydroxylated polymeric backbone plus that of glycerol and their mutual aptitude to attract water. This observation agreed well with the low cross-linking degree and the high MC values depicted by these films (Table 2). The increased MC values of such films when comparing with those without DAS indicates that more water molecules can exert their plasticizing effect on SPC films. The importance of MC on the mechanical and barrier properties of protein films has been extensively discussed in the literature (Gennadios et al., 1998; Martucci et al., 2012; Martucci and Ruseckaite, 2009; Rhim et al., 2000, 1998). Our results differ from previous data reported by Gennadios et al. (1998) for SPI-50Gly and different DAS concentrations, showing an improvement in TS together with a reduction in %ε_b up to 15% DAS. This discrepancy might be associated to the higher MC values of SPC-based films owing to the polysaccharide content in SPC.

Increasing DAS up to 10%, gave rise to increased TS values accompanied by small changes in %ε_b for SPC films containing 30% and 40% glycerol. Rising glycerol content up to 50% led to a detrimental effect on tensile properties, as concluded from the decreased TS and %ε_b values observed (Table 2), presumably due to

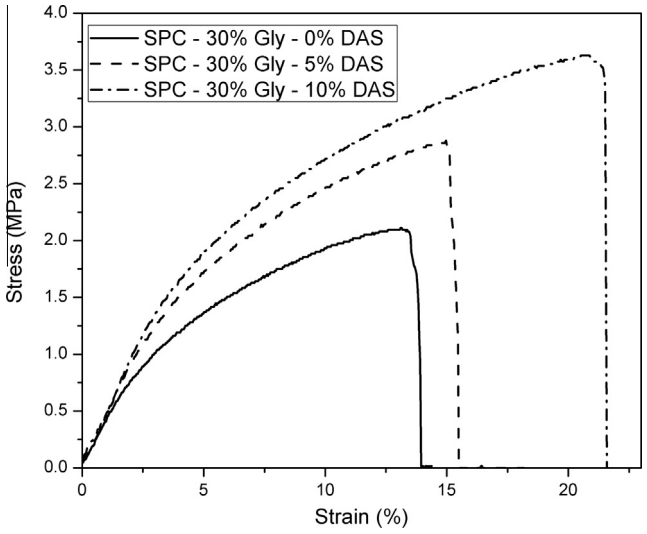

Fig. 3. Representative stress *vs.* strain curves of SPC–30Gly–DAS films.

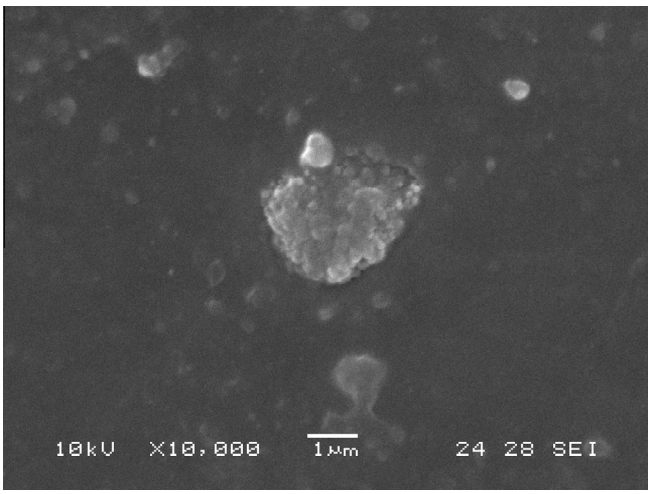

Fig. 4. SEM observation of *D. flagrans* spores in SPC films.

uneven cross-linking within the sample owing to phase separation at such high DAS content. According to TOM observations, phase separation seems far less likely to have occurred at 5% DAS (Fig. 2e) but the average size of dispersed particles was small enough to obtain transparent and mechanically resistant films. With further increasing DAS content large particles or aggregates were observed (Fig. 2f). The larger diameter and inhomogeneous distribution of the dispersed phase could be the main thing responsible for the decreased tensile properties at high DAS content. This result agreed well with that reported for gelatin films cross-linked with DAS (Martucci and Ruseckaite, 2009). Authors evidenced a reduction in tensile properties for 30%DAS ascribed to the presence of a dispersed phase mainly constituted by DAS.

The suitable use of SPC films as drug release devices strongly depends on their favorable mechanical properties and integrity in wet environments. Clearly, based on target properties, SPC–30Gly–5DAS films appeared as the best candidates. This formulation gives rise to transparent and low – colored films, ensuring film homogeneity which is critical for moisture uptake and release; TSM value as low as 29%, indicating that at least 70% of the components participate of the network and are not soluble. Concerning tensile properties, SPC–30Gly–5DAS films were found to meet an adequate compromise between TS and $\%e_b$, to ensure stiffness and ductility with moderate elongation. For all these reasons this formulation was chosen to evaluate the release of *D. flagrans* chlamydospores in vitro so as to determine its suitability as potential delivery device. It is speculated that SPC-30Gly-5DAS formulation could result in films with controlled swelling, weight loss and release profiles during in vitro studies.

3.2.4. In vitro studies

D. flagrans-SPC films were processed by suspended chlamydospores into SPC-30Gly-5DAS film forming solution and transformed into films upon drying at 35 °C. Processing and drying temperatures were selected from previous studies reported in the literature reported by Waller et al. (2001a) on spores viability exposed to different temperatures. Authors found that chlamydospores were capable of surviving pressures of several tones when incorporated into matrices and pressed into tablets for the manufacture of prototype intra-ruminal controlled release devices. The presence of spores within the SPC produced films was evidenced by SEM observations (Fig. 4). *D. flagrans* spores can be easily recognized as irregular beads homogeneously distributed within the protein matrix, without any apparent loss in viability as concluded from qualitative viability experiments (Fig. 1).

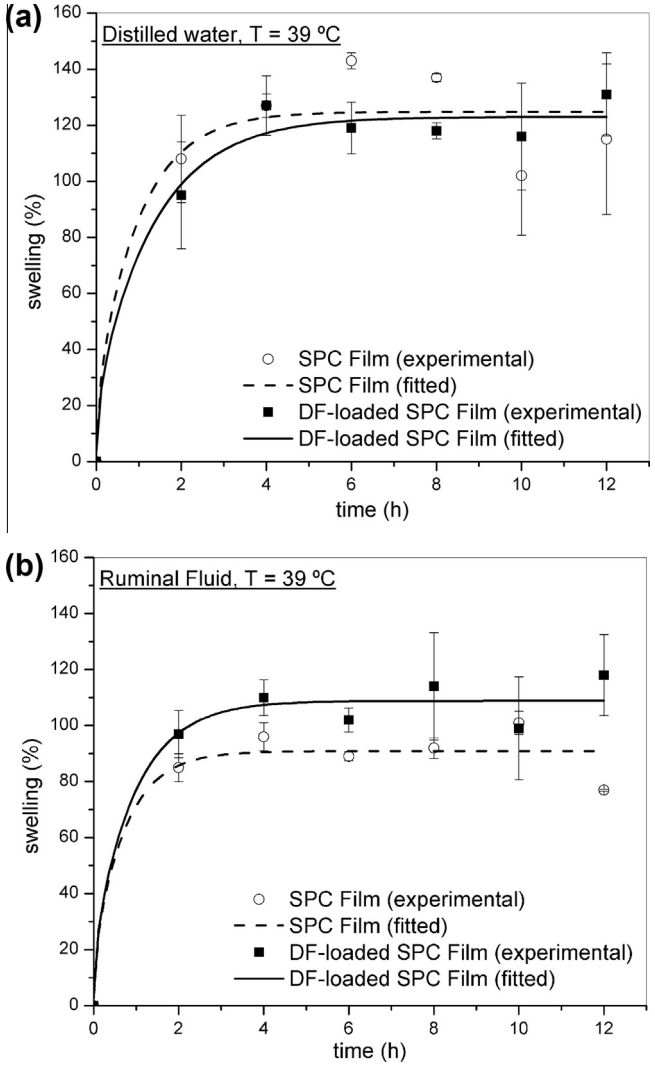

Fig. 5. Fluid absorption profiles of free and *D. flagrans* – loaded SPC–30Gly–5DAS films in (a) distilled water and (b) ruminal fluid.

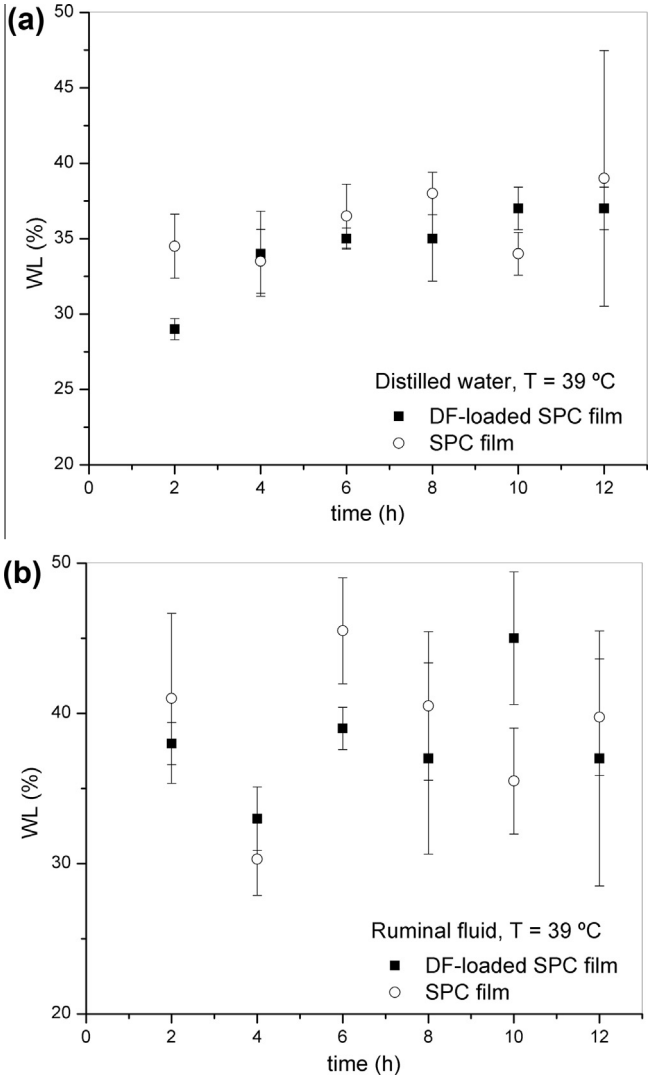

Fig. 6. Weight loss of DF-loaded and free – SPC–30Gly–5DAS films in (a) distilled water and (b) ruminal fluid.

Table 3
Fick's second law equation parameters (Eq. (4)): diffusion coefficient/area (D_{app}/L^2), fluid uptake at the equilibrium (FU_{eq}) and regression coefficients (R^2).

Medium	Sample name	D_{app}/L^2 (h^{-1})	FU_{eq} (%)	R^2
Distilled water	Free-SPC	0.39524	124.80	0.90243
	DF-Loaded SPC	0.28812	122.98	0.97470
Ruminal fluid	Free-SPC	0.53804	90.92	0.94440
	DF-loaded SPC	0.41350	108.85	0.96797

The bulk properties of films such as water absorption are strongly influenced by the internal characteristics of the material such as the extent of cross-linking, thickness, the hydrophilic/hydrophobic balance, conformation of chains, etc. Because these parameters are influenced by many factors involved in the manufacture process, and also the environmental conditions to which the films are inevitably exposed, it is important to consider them when evaluating the absorption behavior of the films.

Ruminal fluid uptake behavior of DF–SPC–30Gly–5DAS films over time was investigated in ruminal fluid (pH 6.0–6.5) under gentle shaking and the weight gain against immersion time are presented in Fig. 5. During the first 2 h, films displayed a sharp absorption and then samples gain weight slowly up to 10 h when sorption rate started to decrease owing to some extent of matrix hydrolysis and / or biodegradation in ruminal fluid. Weight loss profiles determined during fluid uptake (Fig. 6) revealed that DF-loaded films followed essentially the same degradation pattern as free ones and no significant differences in weight loss were observed regardless of the immersion medium. The great dispersion of experimental data could be consequence of the difficult in recovering all the fragments of the disintegrated materials, especially at late stages of the process. Results suggest that microbial population in ruminal fluid has slight effect on weight loss pattern of SPC–30Gly–5DAS films with and without spores, at least during the time of the experiment (i.e. 12 h).

Experimental absorption data were fitted to Eq. (4) and the values of the maximum fluid uptake at the equilibrium (FU_{eq}) and the apparent diffusion coefficient (D_{app}/L^2) for each fluid are summarized in Table 3. Good agreement was achieved indicating the validity of this model for this system (see R^2 in Table 3). It was found that DF-loaded SPC films absorbed more ruminal fluid than free-films (i.e. 108% *vs.* 90%, respectively) but at slightly lower rate, according to the predicted D_{app} values, expressed per the square of thickness (i.e., D_{app}/L^2. 0.415 h^{-1} *vs.* 0.528 h^{-1}). This effect was less pronounced in distilled water (Table 3) suggesting that medium composition influences swelling profiles. The swelling ability of SPC films at pH values 6–6.5 (ruminal fluid) and 5.8 (distilled water) could be the result of the electrostatic repulsions between carboxyl groups from glutamic and aspartic acids which are in their ionized state at such pH values. Osmotic pressure should increase inside the film due to the higher concentration of free H^+ and promote the fluid uptake [10]. Furthermore, electrostatic repulsion between carboxyl groups should cause macromolecular chain relaxation, increasing the swelling ratio. As a general rule, the release of a biological material or a drug from dry devices requires a rehydration process. Soy protein films are thought to behave as pH sensitive devices (Chen et al., 2008), due to the presence of acidic (e.g. carboxyl) and basic (e.g. amine) groups on the polypeptide chains, which either accept or release protons in response to changes in the pH of the medium (Caillard et al., 2008; Park et al., 2002; Peles and Zilberman, 2012). This behavior could strongly influence the release in different pH-dependent media by facilitating the entrance of water inside the network and chain relaxation allowing outward diffusion of the loaded molecules or spore (Caillard et al., 2008; Maltais et al., 2010; Poulin et al., 2011). Furthermore, the protein might degrade in the presence of microbial population or digestive enzymes (Sagüés, 2012). Both of these factors have been reported to affect the release behavior of encapsulated materials from whey-protein-based matrices (Poulin et al., 2011).

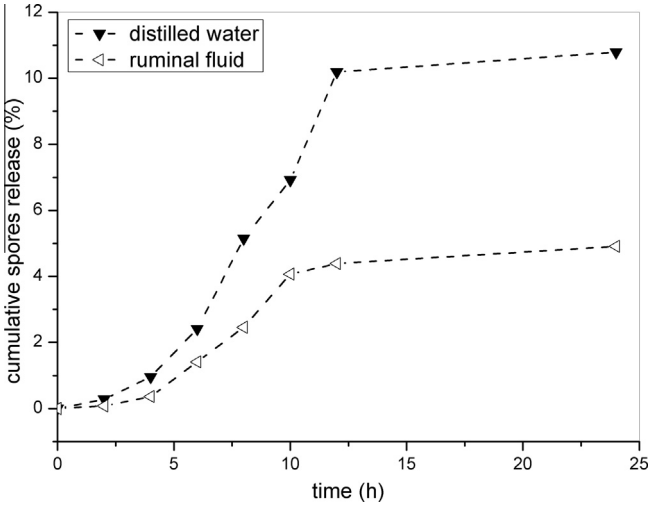

Fig. 7. *In vitro* release of *D. flagrans* from SPC films in ruminal fluid at 39 °C.

The preliminary results of the release of chlamydospores from SPC-30Gly-5DAS in ruminal fluid and distilled water performed at 39 °C are presented in Fig. 7. It can be seen than in both media the cumulative release profile in quite low, attaining about 10% in distilled water while this value reduced to 4.9% in ruminal fluid after 24 h. This result was consistent with the higher swelling ratio of the film in distilled water which accelerates the spores release. The slower release evidenced in ruminal fluid.

would be beneficial since spores should remain in rumen about 4 weeks according to the biological cycle of gastrointestinal nematodes (Sagüés, 2012). Therefore, this finding reflects the potential application of SPC-based films in delivering spores and encourages further studies on these systems.

4. Conclusions

Soybean – based films offer attractive properties to be applied as monolithic sustained released devices such as tunable physic–chemical properties by simple modifications, cost-effectiveness and broad regulatory acceptance. A series of SPC films plasticized with glycerol and cross-linked with DAS were produced and further studied to evaluate their suitability as monolithic sustained release devices of *D. flagrans* chlamydospores in ruminants. Films formulated as SPC–30Gly–5DAS exhibited an adequate compromise between homogeneous morphology, good stability in wet environment (c.a. only 29% of soluble matter) and target tensile properties with swelling profile. SPC–30Gly–5DAS films afforded low in vitro spores release, i.e. 4.9% for at least 24 h when exposed to fresh ruminal fluid correlating well with swelling profile. Studies are ongoing to extent the stability of SPC films in biotic medium such as ruminal fluid. Through careful selection of SPC film formulation, including more effective cross-linking agents and less hydrophilic plasticizers, the spores release rate and duration can be better optimized to maximize the delivery of spores in rumen. This work is currently in progress.

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