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Soybean protein films. Characterization and Potential as Novel Delivery Devices of *Duddingtonia flagrans* Chlamydospores

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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 10wt.%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 5wt.%DAS and plasticized with 30wt.% glycerol exhibited the best compromise between solubility (only 29%), homogeneous morphology and adequate tensile strength (2.50 ± 0.43MPa) 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.

Keywords: Soybean protein concentrate; Film; Dissolution properties; Biological control; D. flagrans chlamydospores

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 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; Waller et al., 2001b). 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. (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 faeces 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 (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

92 successfully released *in vivo* in cannulated sheep and formulation did not affect the
93 predatory activity of *D. Flagrans*.

94 Within this context biopolymers from agricultural sources are becoming an
95 interesting alternative not only as biodegradable films, suitable for food packaging, but
96 also as specialized polymeric items which require mechanical and controlled-release
97 properties (Gómez-Martínez et al., 2009). Food proteins show great promise for
98 developing and engineering a range of new GRAS (generally recognized as safe)
99 matrices with the potential to incorporate drugs or other therapeutic compounds and
100 provide controlled release via the oral route (Chen et al., 2006). Among the potential
101 candidates, soy proteins represent one of the cheapest and most abundant biological
102 feed-stocks available in large quantities at low cost, and their use for developing CRD
103 offers numerous advantages, such as non-citotoxicity, good processability and
104 biodegradability which make them economically competitive (Peles and Zilberman,
105 2012).

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

116 Soy proteins can be transformed into films by thermoplastic processing and
117 dissolution – dehydration procedure called casting (Hernandez-Izquierdo and Krochta,
118 2008). During dissolution stage pH level of the film forming solution affects protein
119 charge and unfolding degree which determine the type of interactions (i.e. disulfide
120 bridges, hydrogen and hydrophobic interactions (Hernandez-Izquierdo and Krochta,
121 2008; Park et al., 2002) developed during the dehydration stage. In general, pure
122 globular protein films provide the potential to control transport of oxygen, aroma, oil,
123 and flavor compounds, however, by themselves, form brittle films, which limits their
124 application (Sothornvit and Krochta, 2001). Plasticizers are necessarily used in protein
125 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; Rhim et al., 1998) and compression molding protein films (Martucci and Ruseckaite, 2009) and its low citotoxicity as reported by Wilson (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 24ºC. 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 one 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. (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 39ºC in a thermostatic bath assisted by

194 shaking (Gyrotory Water Bath Shaker Model G76, New Brunswick, USA), to promote a
195 constant agitation simulating ruminal movements. The RF was sieved (mesh size 1 mm)
196 and kept at 39ºC (ruminal temperature).
197
198 *2.2. Film preparation*
199 Film formulation involved a mixture of food grade SPC, glycerol and DAS as
200 cross-linking agent. None of these materials have any known antihelmintic or fungicidal
201 activity. Film-forming solutions (FFS) were obtained by dispersing SPC powder in
202 constantly stirred buffer phosphate pH 10 (5 g SPC / 100 mL buffer solution) to provide
203 a 3.25% protein (wt/V) solution. Subsequently, glycerol (Gly) was added to SPC slurry
204 in appropriate amounts to reach 30, 40 and 50% (wt/wt SPC dry basis) and cross-linking
205 agent DAS was incorporated at 0, 5 and 10% (wt/wt SPC dry basis). The slurry was
206 stirred for 30 min at 70°C and subsequently sonicated in an ultrasonic bath (Test Lab,
207 160 W, 40 KHz) to remove bubbles. Finally the FFS were poured onto leveled Teflon-
208 coated Petri dishes. The target film thickness was 150 µm, and the quantity of each FFS
209 used was calculated so that the solid content (c.a., SPC, DAS, Gly) was the same (i.e.,
210 approximately 35 ml / 150 cm^2, for SPC - 30% Gly - 0% DAS). Samples were left to
211 dry in an air-circulating oven (DKN 400, USA) at 35ºC until reaching constant weight
212 (approximately 20 h). The dried films were peeled-off from the plates and kept in a
213 laboratory environmental chamber for 48 h at 25°C and 65 ± 2 % relative humidity
214 (RH) before testing. DF-loaded films were prepared similarly. Once obtained the FFSs
215 temperature was decreased to 30ºC (to preserve the spore's activity) and a suspension of
216 *D. flagrans* chlamydospores in distilled water (c.a., 70,875,000 chlamydospores in 20
217 mL of distilled water) was added and gently stirred for 15 min. After this time, casting
218 and drying procedures were performed similarly than for free-films. Free films were
219 labelled as **SPC-XGly-YDAS**, where X and Y are the percentages of glycerol and DAS
220 respectively, while DF-loaded films was labelled as **DF-SPC-XGly-YDAS.**
221
222 *2.3. Film characterization*
223 *2.3.1. Film thickness*. Film thickness was measured with a manual micrometer (0-25 ±
224 0.01 mm, Bta. China). Measurements were done at ten random points taken along the
225 rectangular films and an average value was taken. For tensile test, opacity and moisture
226 absorption experiments, three measurements were done on each specimen.
227

2.3.2. Conditioning. Prior testing, film samples were pre-conditioned in laboratory environmental chamber for 48 h at 25 ± 2 ºC 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) x 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) to

L*=100 (white); a* = - 80 (green) to a* = 100 (red); and b* = - 80 (blue) to b* = 70 (yellow). The total color difference (E), 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 ± 1ºC 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 24h immersion in distilled water (DW) (Rhim et al., 1998). Three samples were carefully weighed (±0.0001g, ALC-210.4, Acculab Sartorius, USA) and subsequently dried in an air-circulating oven at 105 ±1ºC for 24h 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 25ºC for 24h. Insoluble dry matter was determined recovering the immersed samples and drying them in an air-circulating oven (DKN 400, USA) at 105ºC for 24h. 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 ($\%_b$) 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 25ºC (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 39ºC 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:

$$\%\ WU\ or\ RFU = \frac{W_t - W_0}{W_0} \cdot 100 \quad (3)$$

where W_t is the sample weight at time t and W_0 is the initial weight. Reported results were the average of 3 values.

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

$$FU_t = FU_{eq} - FU_{eq} \cdot \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} \exp\left[\frac{-(2n+1)^2\pi^2 t}{4} \cdot \left(\frac{D_{WRV}}{L^2}\right)\right] \quad (4)$$

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:

$$\%\,WL = \frac{W_0 - W_{td}}{W_0} \cdot 100 \quad (5)$$

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 48h. 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:

$$\%\,CR = \frac{Chl_{total} - Chl_{released}}{Chl_{total}} \cdot 100 \quad (6)$$

where Chl_{total} is the total concentration of chlamydospores in the films (undigested chlamydospores) and $Chl_{released}$ 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.

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 wt.%, 1wt.% and 5wt.%) in the presence of free-life nematodes *Panagrellus* spp. As can be seen in Figure 1 mycelial growth and trapping structures were noticed after 8h 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. (Waller et al., 2001b) demonstrating that viability was preserved after exposure to temperature and pressure during processing into delivery tablets or under ruminal gases 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. 2 a-c) and light - barrier properties (Table 1). With an increase in glycerol concentration from 0 to 30wt.% 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. 2 d-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. 2 f). Similar behavior was already observed in DAS-cross-linked – plasticized -gelatin films obtained by compression molding (Martucci and Ruseckaite, 2009).

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; Rhim et al., 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; Rhim et al., 1998). DAS produced yellow-brownish films when reacted with gelatin (Martucci and Ruseckaite, 2009), SPI (Rhim et al., 2000; Rhim et al., 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.

426 *3.2.3. Residual moisture content and soluble matter*

427 SPC, DAS and glycerol easily absorbed moisture from the environment due to
428 their hydrophilic nature (Martucci and Ruseckaite, 2009; Rhim et al., 1998; Yu et al.,
429 2010). The presence of free polar – side chain groups from the protein fraction plus the
430 polysaccharide portion are the main factors determining the moisture absorption of SPC
431 (de la Caba et al., 2012). On the other hand, the presence of carbonyl as well as
432 hydroxyl groups from the polysaccharide backbone of DAS can bind water molecules
433 and favor moisture retention (Rhim et al., 1998; Yu et al., 2010). Cross-linking degree
434 was reported as a feasible way of controlling water uptake and solubility of soybean
435 protein and gelatin films (Martucci and Ruseckaite, 2009; Rhim et al., 2000; Rhim et
436 al., 1998). Therefore, cross-linking SPC with DAS is expected to reduce hydrophilic
437 sites reducing the water uptake capacity of the resultant films, as previously reported for
438 other protein films (Gennadios et al., 1998; Martucci and Ruseckaite, 2009; Rhim et al.,
439 2000; Rhim et al., 1998; Yu et al., 2010).

440 As anticipated, MC values after conditioning for 48h at 65%RH and ambient
441 temperature increased with glycerol content ($P < 0.05$) owing to the fact that there are
442 additional hydroxyl groups within the matrix, favoring the moisture retention (Martucci
443 and Ruseckaite, 2009; Rhim et al., 1998) (Table 2). MC is also related to the total void
444 or porous occupied by water molecules in the microstructure, therefore an increment in
445 such parameter could indicate increased void volume in the final films (Jiang et al.,
446 2010). The effect of DAS on MC seems to depend on the amount of glycerol, as
447 concluded from the analysis of results in Table 2. The incorporation of 5%DAS into
448 SPC – 30Gly formulation did not induce significant differences in MC values, while
449 rising glycerol content increased substantially this parameter ($P < 0.05$) even compared
450 with films without DAS. The inclusion of 10% of DAS in SPC-30Gly did not provoke
451 significant differences on MC ($P < 0.05$). When glycerol level ranges from 40-50 %,
452 SPC-5DAS films had many additional hydrophilic hydroxyl groups which
453 counterbalance the effect of cross-linking and the impact on MC was negligible
454 (Martucci and Ruseckaite, 2009; Rhim et al., 1998). In general, DAS-induced cross-
455 linking decreases water uptake by soy proteins since protein amino groups are not yet
456 available to bind water by hydrogen bonding (Rhim et al., 1998). However for a certain
457 DAS level (i.e., ~5% in the present case) cross-linking reaction with soy protein reached
458 a saturation point as already reported by SPI-DAS films (Rhim et al., 2000; Rhim et al.,

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; Rhim et al., 1998). The idea of measuring the soluble matter by the ‘‘dry’’ and ‘‘wet’’ methods was to evaluate the effect of drying the samples at 105ºC before testing. Table 2 presents the soluble fraction of films. SPC films without DAS maintained their structural integrity after 24h 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 105ºC 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; Rhim et al., 1998).

3.2.4. *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.

Figure 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

493 the elastic region defining the elastic modulus. Table 2 shows the effect of DAS and
494 glycerol levels on tensile strength (TS) and percentage of elongation at break ($\%_b$). For
495 control films (0 wt.% DAS) the addition of increasing amounts of glycerol did not
496 induced significant changes ($P > 0.05$) in TS and $\%_b$ values. The addition of 5%DAS
497 provoked an increase in $\%_b$ ($P < 0.05$) values without significant changes in TS. This
498 last observation demonstrates that cross-linking with DAS did not induce severe
499 restrictions within SPC-Gly matrix as usually occurs with short-chain dialdehydes
500 (Gennadios et al., 1998; Martucci et al., 2012; Martucci and Ruseckaite, 2009; Rhim et
501 al., 2000; Rhim et al., 1998). For DAS content as low as 5 wt %, the cross-linking effect
502 is counterbalanced by its plasticizing ability exerted by its hydroxylated polymeric
503 backbone plus that of glycerol and their mutual aptitude to attract water. This
504 observation agreed well with the low cross-linking degree and the high MC values
505 depicted by these films (Table 2). The increased MC values of such films when
506 comparing with those without DAS indicates that more water molecules can exert their
507 plasticizing effect on SPC films. The importance of MC on the mechanical and barrier
508 properties of protein films has been extensively discussed in the literature (Gennadios et
509 al., 1998; Martucci et al., 2012; Martucci and Ruseckaite, 2009; Rhim et al., 2000;
510 Rhim et al., 1998). Our results differ from previous data reported by Gennadios et al.,
511 (Gennadios et al., 1998) for SPI-50Gly and different DAS concentrations, showing an
512 improvement in TS together with a reduction in $\%_b$ up to 15% DAS. This discrepancy
513 might be associated to the higher MC values of SPC-based films owing to the
514 polysaccharide content in SPC.

515 Increasing DAS up to 10%, gave rise to increased TS values accompanied by
516 small changes in $\%_b$ for SPC films containing 30% and 40% glycerol. Rising glycerol
517 content up to 50% led to a detrimental effect on tensile properties, as concluded from
518 the decreased TS and $\%_b$ values observed (Table 2), presumably due to uneven cross-
519 linking within the sample owing to phase separation at such high DAS content.
520 According to TOM observations, phase separation seems far less likely to have occurred
521 at 5%DAS (Fig. 2 e) but the average size of dispersed particles was small enough to
522 obtain transparent and mechanically resistant films. With further increasing DAS
523 content large particles or aggregates were observed (Fig. 2, f). The larger diameter and
524 inhomogeneous distribution of the dispersed phase could be the main thing responsible
525 for the decreased tensile properties at high DAS content. This result agreed well with

526 that reported for gelatin films cross-linked with DAS (Martucci and Ruseckaite, 2009).
527 Authors evidenced a reduction in tensile properties for 30%DAS ascribed to the
528 presence of a dispersed phase mainly constituted by DAS.

529 The suitable use of SPC films as drug release devices strongly depends on their
530 favorable mechanical properties and integrity in wet environments. Clearly, based on
531 target properties, SPC-30Gly-5DAS films appeared as the best candidates. This
532 formulation gives rise to transparent and low – colored films, ensuring film
533 homogeneity which is critical for moisture uptake and release; TSM value as low as
534 29%, indicating that at least 70% of the components participate of the network and are
535 not soluble. Concerning tensile properties, SPC-30Gly-5DAS films were found to meet
536 an adequate compromise between TS and %e, to ensure stiffness and ductility with
537 moderate elongation. For all these reasons this formulation was chosen to evaluate the
538 release of *D. flagrans* chlamydospores *in vitro* so as to determine its suitability as
539 potential delivery device. It is speculated that SPC-30Gly-5DAS formulation could
540 result in films with controlled swelling, weight loss and release profiles during *in vitro*
541 studies.
542
543 *3.2.5. In vitro studies*

544 *D. flagrans*-SPC films were processed by suspended chlamydospores into SPC-
545 30Gly-5DAS film forming solution and transformed into films upon drying at 35°C.
546 Processing and drying temperatures were selected from previous studies reported in the
547 literature reported by Waller et. al., (Waller et al., 2001a) on spores viability exposed to
548 different temperatures. Authors found that chlamydospores were capable of surviving
549 pressures of several tones when incorporated into matrices and pressed into tablets for
550 the manufacture of prototype intra-ruminal controlled release devices. The presence of
551 spores within the SPC produced films was evidenced by SEM observations (Figure 4).
552 *D. flagrans* spores can be easily recognized as irregular beads homogeneously
553 distributed within the protein matrix, without any apparent loss in viability as concluded
554 from qualitative viability experiments (Fig. 1).

555 The bulk properties of films such as water absorption are strongly influenced by
556 the internal characteristics of the material such as the extent of cross-linking, thickness,
557 the hydrophilic/hydrophobic balance, conformation of chains, etc. Because these
558 parameters are influenced by many factors involved in the manufacture process, and

559 also the environmental conditions to which the films are inevitably exposed, it is
560 important to consider them when evaluating the absorption behavior of the films.
561 Ruminal fluid uptake behavior of DF–SPC-30Gly-5DAS films over time was
562 investigated in ruminal fluid (pH 6.0-6.5) under gentle shaking and the weight gain
563 against immersion time are presented in Figure 5. During the first 2h, films displayed a
564 sharp absorption and then samples gain weight slowly up to 10h when sorption rate
565 started to decrease owing to some extent of matrix hydrolysis and / or biodegradation in
566 ruminal fluid. Weight loss profiles determined during fluid uptake (Figure 6) revealed
567 that DF-loaded films followed essentially the same degradation pattern as free ones and
568 no significant differences in weight loss were observed regardless of the immersion
569 medium. The great dispersion of experimental data could be consequence of the
570 difficult in recovering all the fragments of the disintegrated materials, especially at late
571 stages of the process. Results suggest that microbial population in ruminal fluid has
572 slight effect on weight loss pattern of SPC-30Gly-5 DAS films with and without spores,
573 at least during the time of the experiment (i.e. 12 h).
574 Experimental absorption data were fitted to eq. (4) and the values of the
575 maximum fluid uptake at the equilibrium (FU_{eq}) and the apparent diffusion coefficient
576 (D_{app}/L^2) for each fluid are summarized in Table 3. Good agreement was achieved
577 indicating the validity of this model for this system (see R^2 in Table 3). It was found that
578 DF-loaded SPC films absorbed more ruminal fluid than free-films (i.e. 108% *vs* 90%,
579 respectively) but at slightly lower rate, according to the predicted D_{app} values, expressed
580 per the square of thickness (i.e., D_{app}/L^2. 0.415 h^{-1} *vs* 0.528 h^{-1}). This effect was less
581 pronounced in distilled water (Table 3) suggesting that medium composition influences
582 swelling profiles. The swelling ability of SPC films at pH values 6-6.5 (ruminal fluid)
583 and 5.8 (distilled water) could be the result of the electrostatic repulsions between
584 carboxyl groups from glutamic and aspartic acids which are in their ionized state at such
585 pH values. Osmotic pressure should increase inside the film due to the higher
586 concentration of free H^+ and promote the fluid uptake [10]. Furthermore, electrostatic
587 repulsion between carboxyl groups should cause macromolecular chain relaxation,
588 increasing the swelling ratio. As a general rule, the release of a biological material or a
589 drug from dry devices requires a rehydration process. Soy protein films are thought to
590 behave as pH sensitive devices (Chen et al., 2008), due to the presence of acidic (e.g.
591 carboxyl) and basic (e.g. amine) groups on the polypeptide chains, which either accept
592 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).

The preliminary results of the release of chlamydospores from SPC-30Gly-5DAS in ruminal fluid and distilled water performed at 39ºC are presented in Figure 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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References

Caillard, R., Remondetto, G.E., Mateescu, M.A., Subirade, M., 2008. Characterization of Amino Cross-Linked Soy Protein Hydrogels. Journal of Food Science 73**,** C283-C291.

Ciannamea, E.M., Stefani, P.M., Ruseckaite, R.A., 2010. Medium-density particleboards from modified rice husks and soybean protein concentrate-based adhesives. Bioresource Technology 101**,** 818-825.

Cordis, S.A., 2010. Internal Technical Report.

Chen, L., Remondetto, G., Rouabhia, M., Subirade, M., 2008. Kinetics of the breakdown of cross-linked soy protein films for drug delivery. Biomaterials 29**,** 3750-3756.

Chen, L., Remondetto, G.E., Subirade, M., 2006. Food protein-based materials as nutraceutical delivery systems. Trends Food Science & Technololgy 17**,** 272-283.

de la Caba, K., Peña, C., Ciannamea, E.M., Stefani, P.M., Mondragon, I., Ruseckaite, R.A., 2012. Characterization of soybean protein concentrate—stearic acid/palmitic acid blend edible films. Journal of Applied Polymer Science 124**,** 1796-1807.

Gennadios, A., Handa, A., Froning, G.W., Weller, C.L., Hanna, M.A., 1998. Physical Properties of Egg White−Dialdehyde Starch Films†. Journal of Agricultural and Food Chemistry 46**,** 1297-1302.

Gómez-Martínez, D., Partal, P., Martínez, I., Gallegos, C., 2009. Rheological behaviour and physical properties of controlled-release gluten-based bioplastics. Bioresource Technology 100**,** 1828-1832.

Hernandez-Izquierdo, V.M., Krochta, J.M., 2008. Thermoplastic Processing of Proteins for Film Formation—A Review. Journal of Food Science 73**,** R30-R39.

Huang-Lee, L.L.H., Cheung, D.T., Nimni, M.E., 1990. Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. Journal of Biomedical Materials Research 24**,** 1185-1201.

Huang, X., Brazel, C.S., 2001. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. Journal of Controlled Release 73**,** 121-136.

Irissin-Mangata, J., Bauduin, G., Boutevin, B., Gontard, N., 2001. New plasticizers for wheat gluten films. European Polymer Journal 37**,** 1533-1541.

Jiang, Y., Li, Y., Chai, Z., Leng, X., 2010. Study of the Physical Properties of Whey Protein Isolate and Gelatin Composite Films. Journal of Agricultural and Food Chemistry 58**,** 5100-5108.

Khomutov, L.I., Lashek, N.A., Ptitchkina, N.M., Morris, E.R., 1995. Temperature—composition phase diagram and gel properties of the gelatin—starch—water system. Carbohydrate Polymers 28**,** 341-345.

Larsen, M., 1999. Biological control of helminths. International Journal for Parasitology 29**,** 139-146.

Maltais, A., Remondetto, G.E., Subirade, M., 2010. Tabletted soy protein cold-set hydrogels as carriers of nutraceutical substances. Food Hydrocolloids 24**,** 518-524.

Martucci, J.F., Accareddu, A., Ruseckaite, R., 2012. Preparation and characterization of plasticized gelatin films cross-linked with low concentrations of Glutaraldehyde. Journal of Materials Science 47**,** 3282-3292.

681 Martucci, J.F., Ruseckaite, R.A., 2009. Tensile properties, barrier properties, and
682 biodegradation in soil of compression—Molded gelatin-dialdehyde starch films.
683 Journal of Applied Polymer Science 112**,** 2166-2178.
684 Nuthong, P., Benjakul, S., Prodpran, T., 2009. Effect of some factors and pretreatment
685 on the properties of porcine plasma protein-based films. LWT - Food Science
686 and Technology 42**,** 1545-1552.
687 Ojeda-Robertos, N., Torres-Acosta, J., Ayala-Burgos, A., Sandoval-Castro, C., Valero-
688 Coss, R., Mendoza-de-Gives, P., 2009. Digestibility of Duddingtonia flagrans
689 chlamydospores in ruminants: in vitro and in vivo studies. BMC Veterinary
690 Research 5**,** 1-7.
691 Panchadcharam, C., 2004. Problems in the control of nematode parasites of small
692 ruminants in Malaysia. Faculty of Veterinary Medicine and Animal Science >
693 Dept. of Biomedical Sciences and Veterinary Public Health, pp. 48.
694 Park, S.K., Hettiarachchy, N.S., Ju, Z.Y., Gennadios, A., 2002. Formation and
695 properties of soy protein films and coatings. In: Gennadios, A., (Ed.), Protein-
696 Based Films and Coatings. CRC Press, New York, pp. 123–138.
697 Peles, Z., Zilberman, M., 2012. Novel soy protein wound dressings with controlled
698 antibiotic release: Mechanical and physical properties. Acta Biomaterialia 8**,**
699 209-217.
700 Poulin, J.-F., Caillard, R., Subirade, M., 2011. β-Lactoglobulin tablets as a suitable
701 vehicle for protection and intestinal delivery of probiotic bacteria. International
702 Journal of Pharmaceutics 405**,** 47-54.
703 Rhim, J.W., Gennadios, A., Handa, A., Weller, C.L., Hanna, M.A., 2000. Solubility,
704 Tensile, and Color Properties of Modified Soy Protein Isolate Films†. Journal of
705 Agricultural and Food Chemistry 48**,** 4937-4941.
706 Rhim, J.W., Gennadios, A., Weller, C.L., Carole, C., Hanna, M.A., 1998. Soy protein
707 isolate-dialdehyde starch films. Industrial Crops and Products 8**,** 195-203.
708 Sagüés, M., Fusé, L., Fernández, A., Iglesias, L., Moreno, F., Saumell, C., 2011.
709 Efficacy of an energy block containing Duddingtonia flagrans; in the control of
710 gastrointestinal nematodes of sheep. Parasitology Research 109**,** 707-713.
711 Sagüés, M.F., 2012. Control biológico de nematodos parásitos de rumiantes mediante
712 hongos nematófagos: optimización de cultivos y su administración a través de
713 polímeros de soja y bloques energéticos. Facultad de Ciencias Veterinarias.
714 UNCPBA, Tandil, Argentina.
715 Sothornvit, R., Krochta, J.M., 2001. Plasticizer effect on mechanical properties of
716 [beta]-lactoglobulin films. Journal of Food Engineering 50**,** 149-155.
717 Vaz, C.M., van Doeveren, P.F.N.M., Dias, G.R., Reis, R.L., Cunha, A.M., 2004.
718 Controlled Delivery Achieved with Bi-Layer Matrix Devices Produced by Co-
719 Injection Moulding. Macromolecular Bioscience 4**,** 795-801.
720 Waller, P.J., 1997. Sustainable helminth control of ruminants in developing countries.
721 Veterinary Parasitology 71**,** 195-207.
722 Waller, P.J., Faedo, M., Ellis, K., 2001a. The potential of nematophagous fungi to
723 control the free-living stages of nematode parasites of sheep: towards the
724 development of a fungal controlled release device. Veterinary Parasitology 102**,**
725 299-308.
726 Waller, P.J., Knox, M.R., Faedo, M., 2001b. The potential of nematophagous fungi to
727 control the free-living stages of nematode parasites of sheep: feeding and block
728 studies with Duddingtonia flagrans. Veterinary Parasitology 102**,** 321-330.
729 Wilson, R.H., 1959. Utilization and Toxicity of Dialdehyde- and Dicarboxyl-Starches.
730 Proc Soc Exp Biol Med 102**,** 735-737.

731 Yu, J., Chang, P.R., Ma, X., 2010. The preparation and properties of dialdehyde starch
732 and thermoplastic dialdehyde starch. Carbohydrate Polymers 79**,** 296-300.
733
734

735 **Caption of the figures**

736

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

739 **Figure 2.** (a-c) Macroscopic appearance of SPC-30Gly films with increasing DAS
740 content (d-f) Optical transmission microscopy of the films after staining with iodine
741 solution.

742 **Figure 3.** Representative stress *vs* strain curves of SPC-30Gly-DAS films.

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

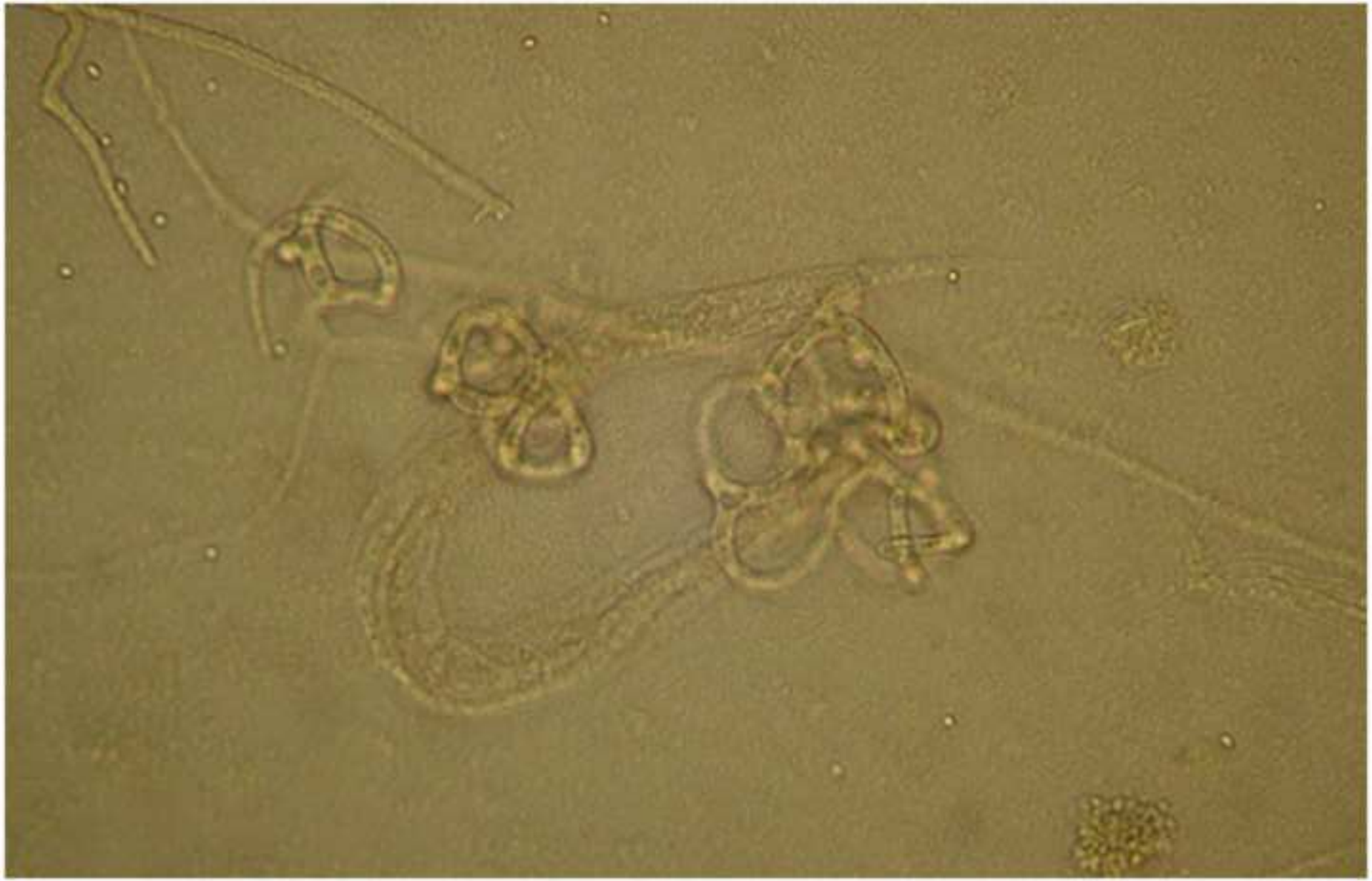
744 **Figure 5**. Fluid absorption profiles of free and *D. flagrans* - loaded SPC-30Gly-5DAS
745 films in (a) distilled water and (b) ruminal fluid.

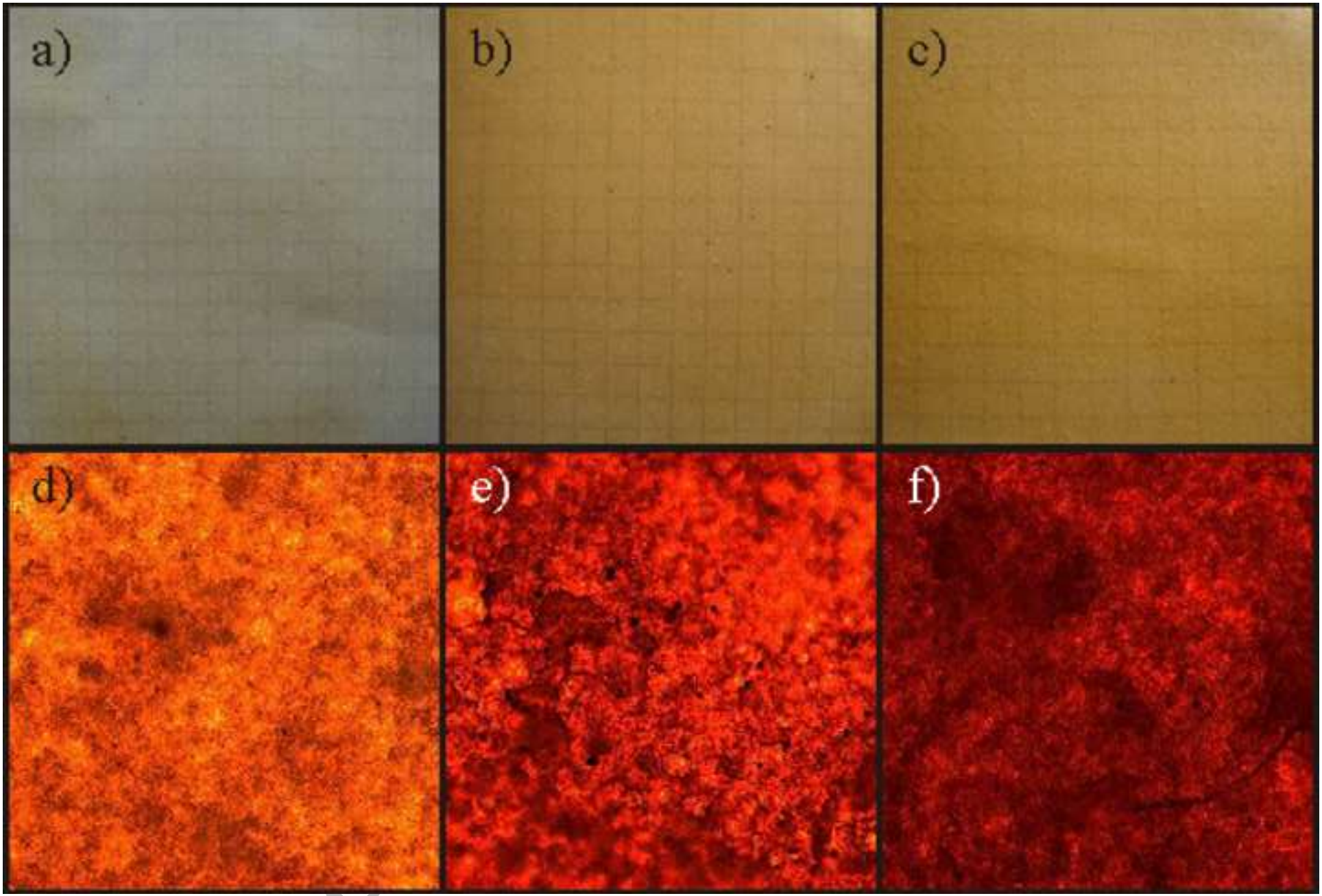
746 **Figure 6**.Weight loss of DF-loaded and free - SPC-30Gly-5DAS films in (a) distilled
747 water and (b) ruminal fluid.

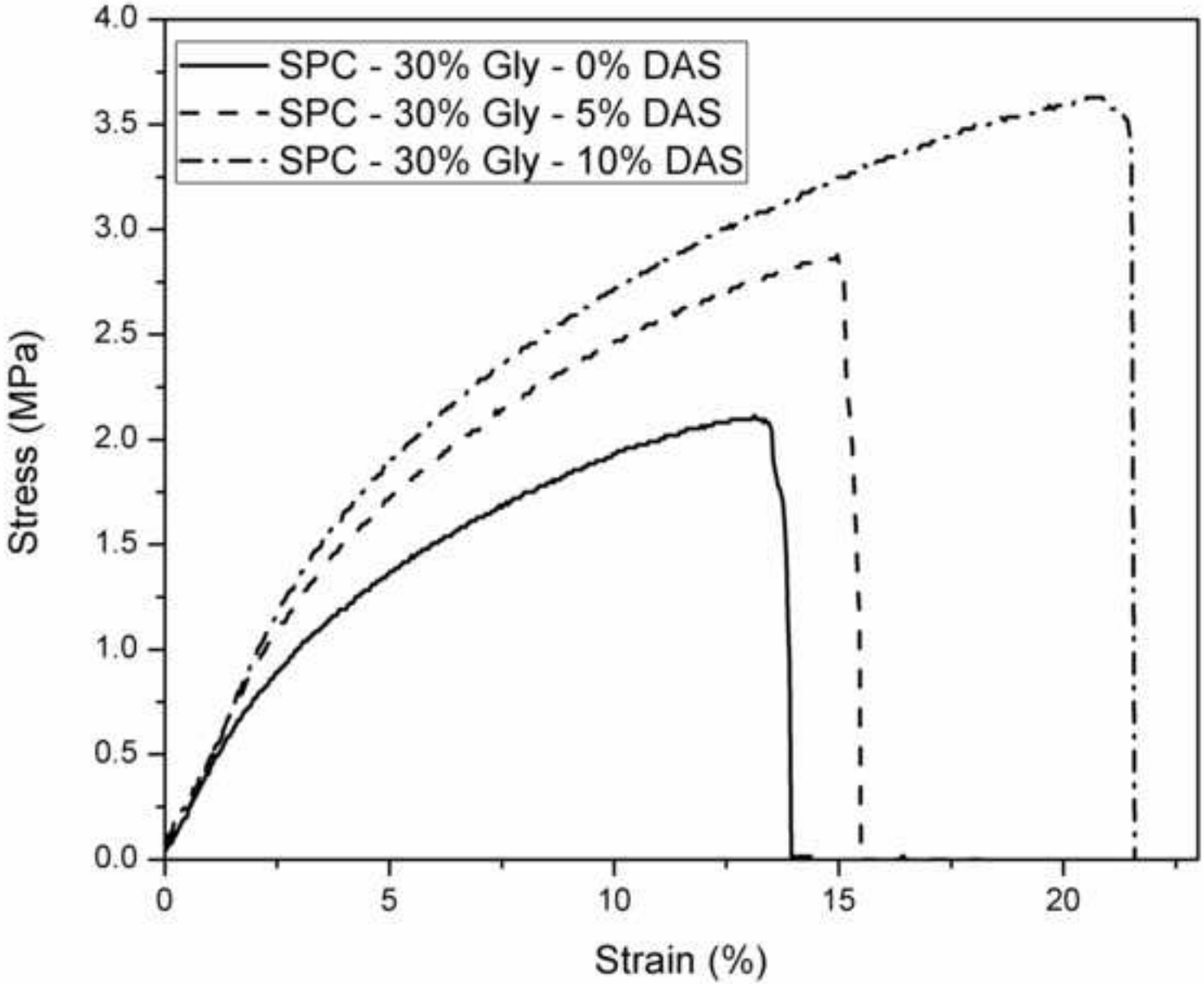
748 **Figure 7.** *In vitro* release of D. *flagrans* from SPC films in ruminal fluid at 39ºC

749

750






SPC - 30% Gly - 0% DAS
SPC - 30% Gly - 5% DAS
SPC - 30% Gly - 10% DAS
Stress (MPa)
Strain (%)
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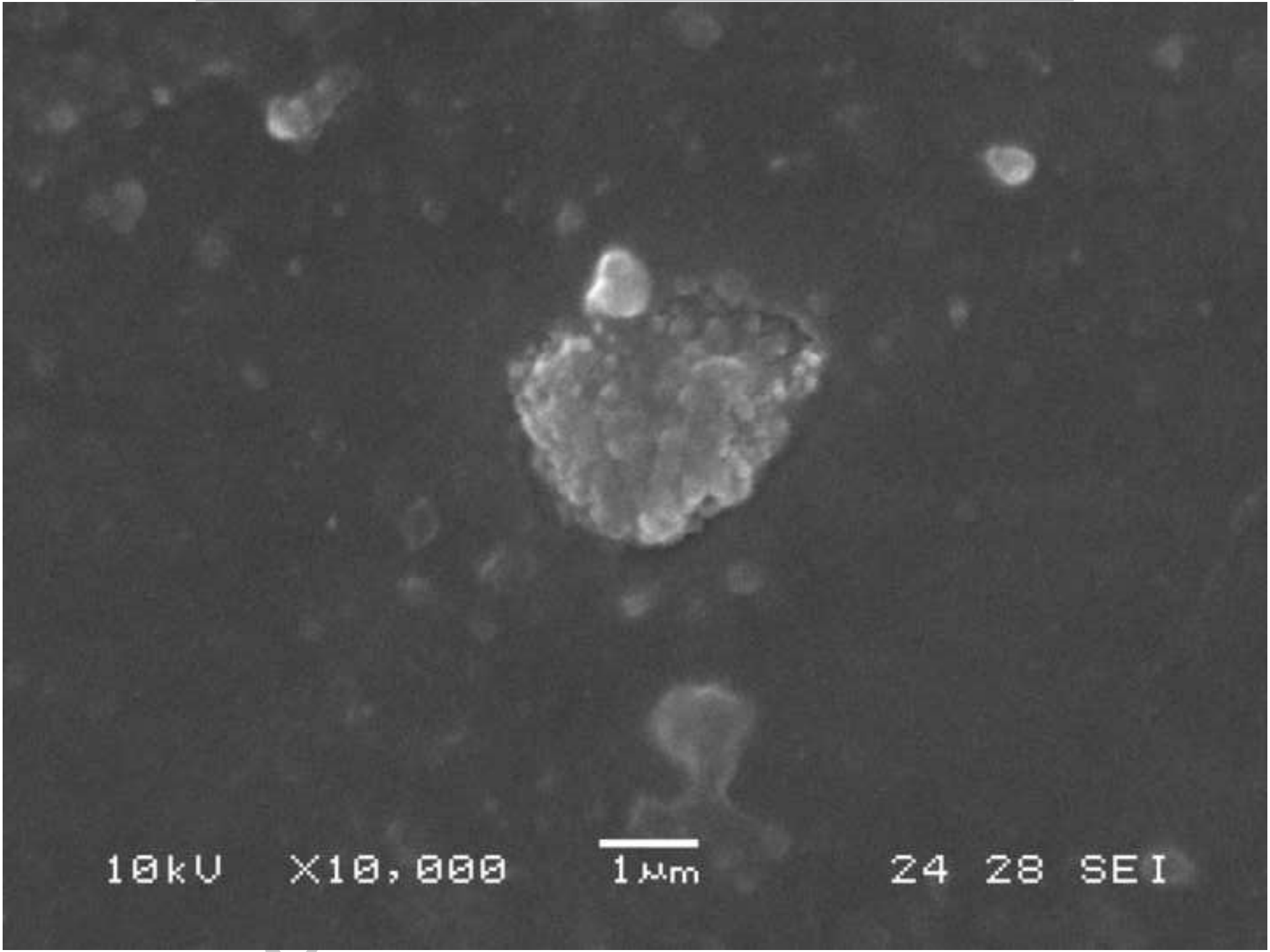
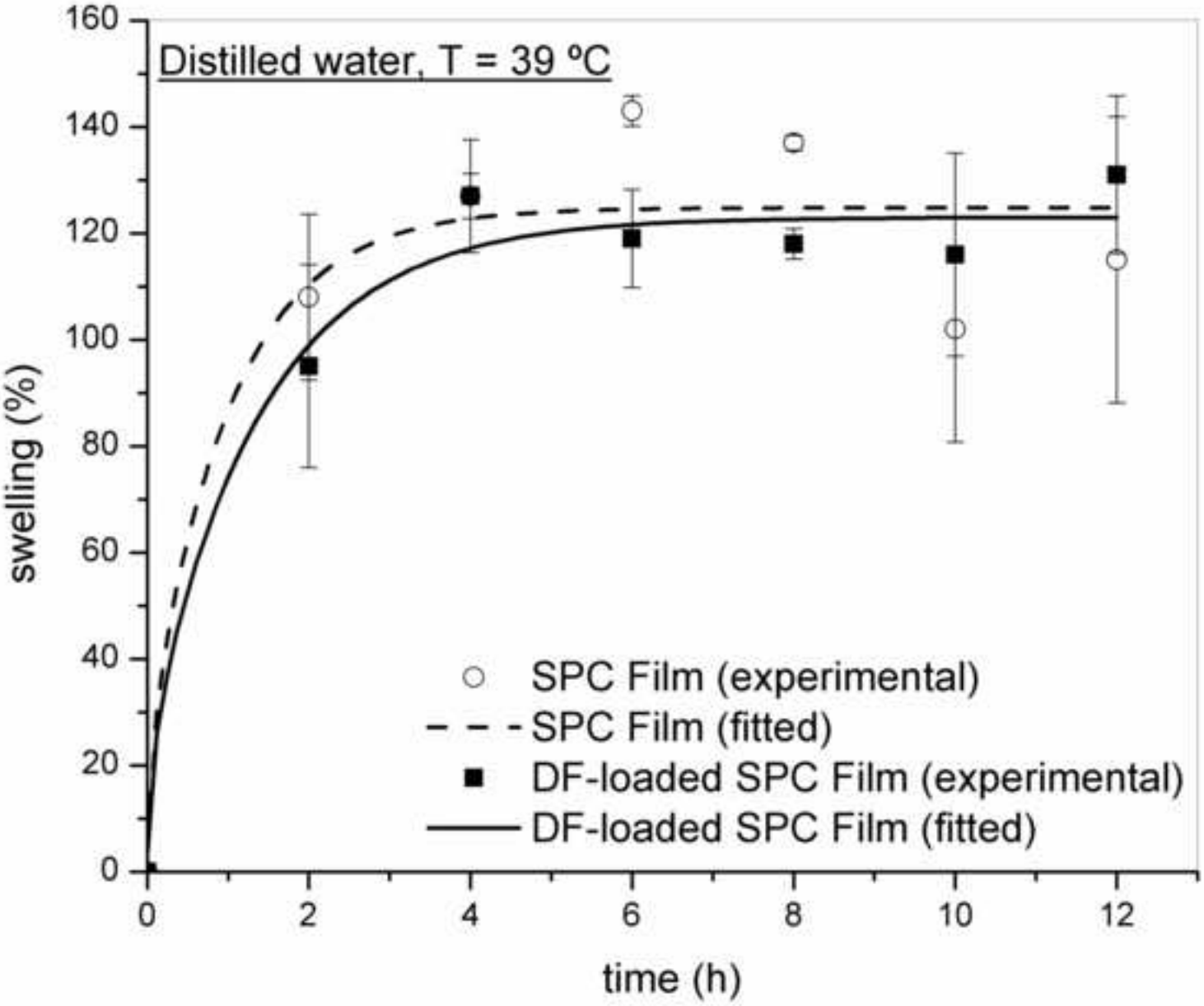
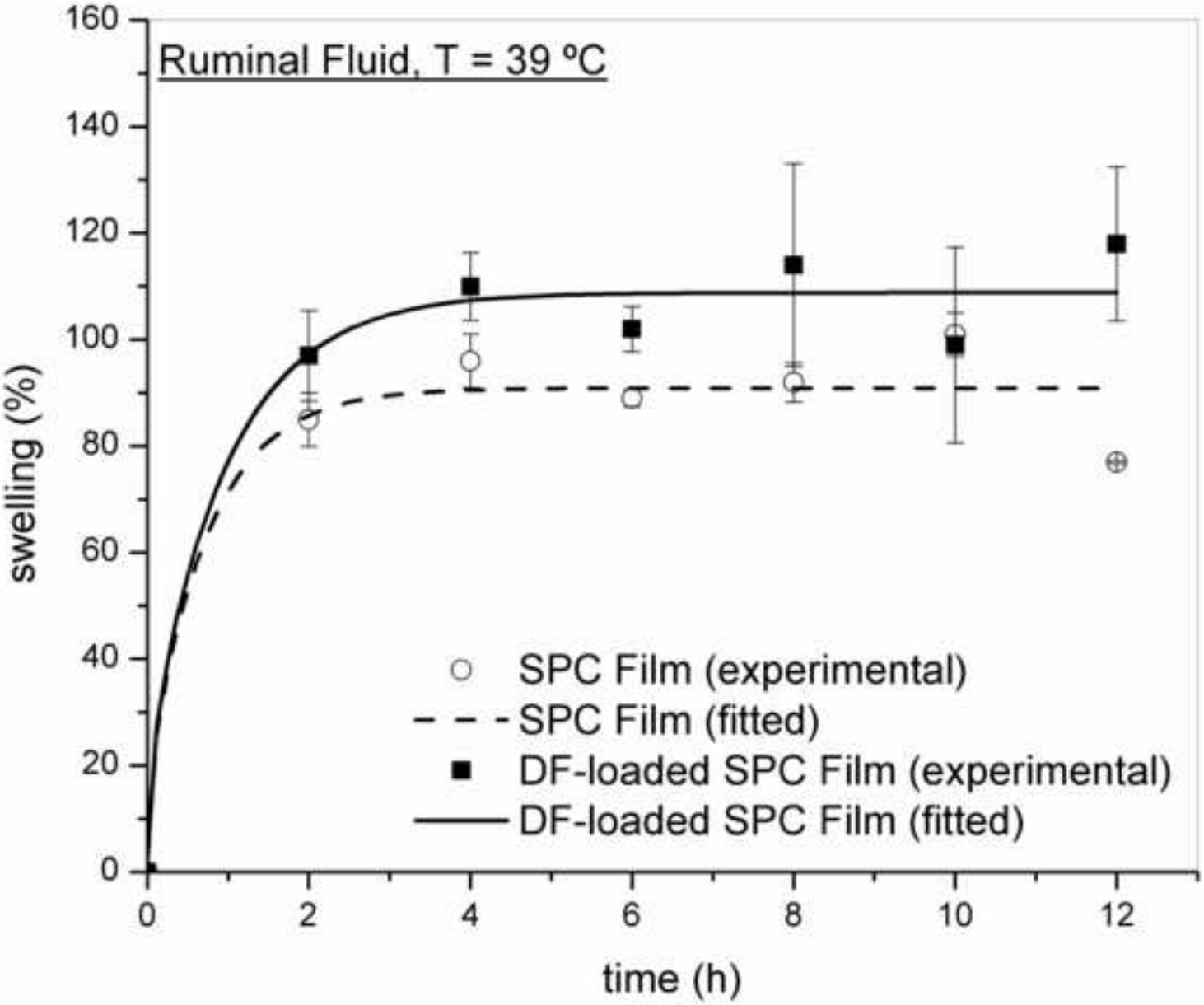
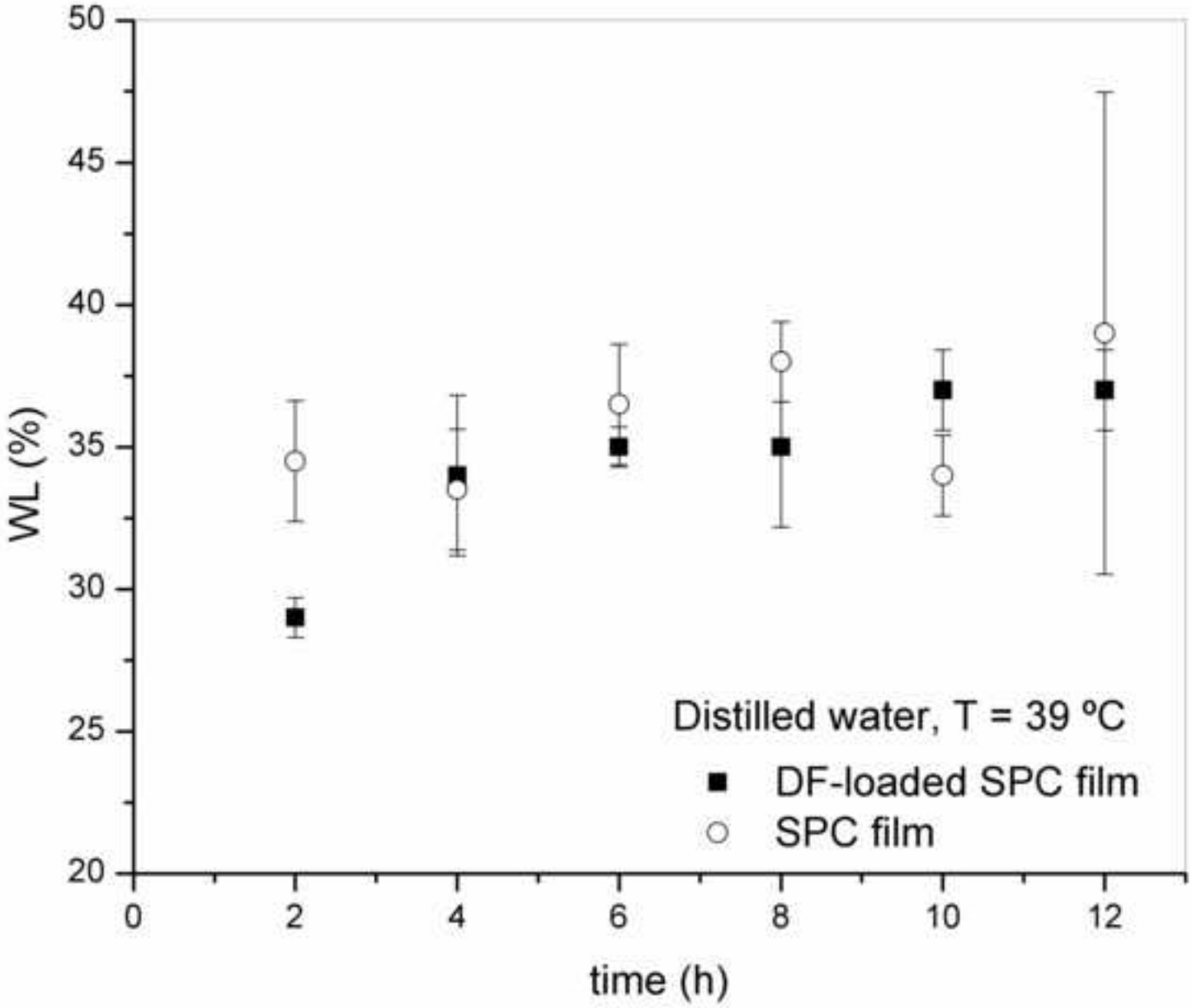
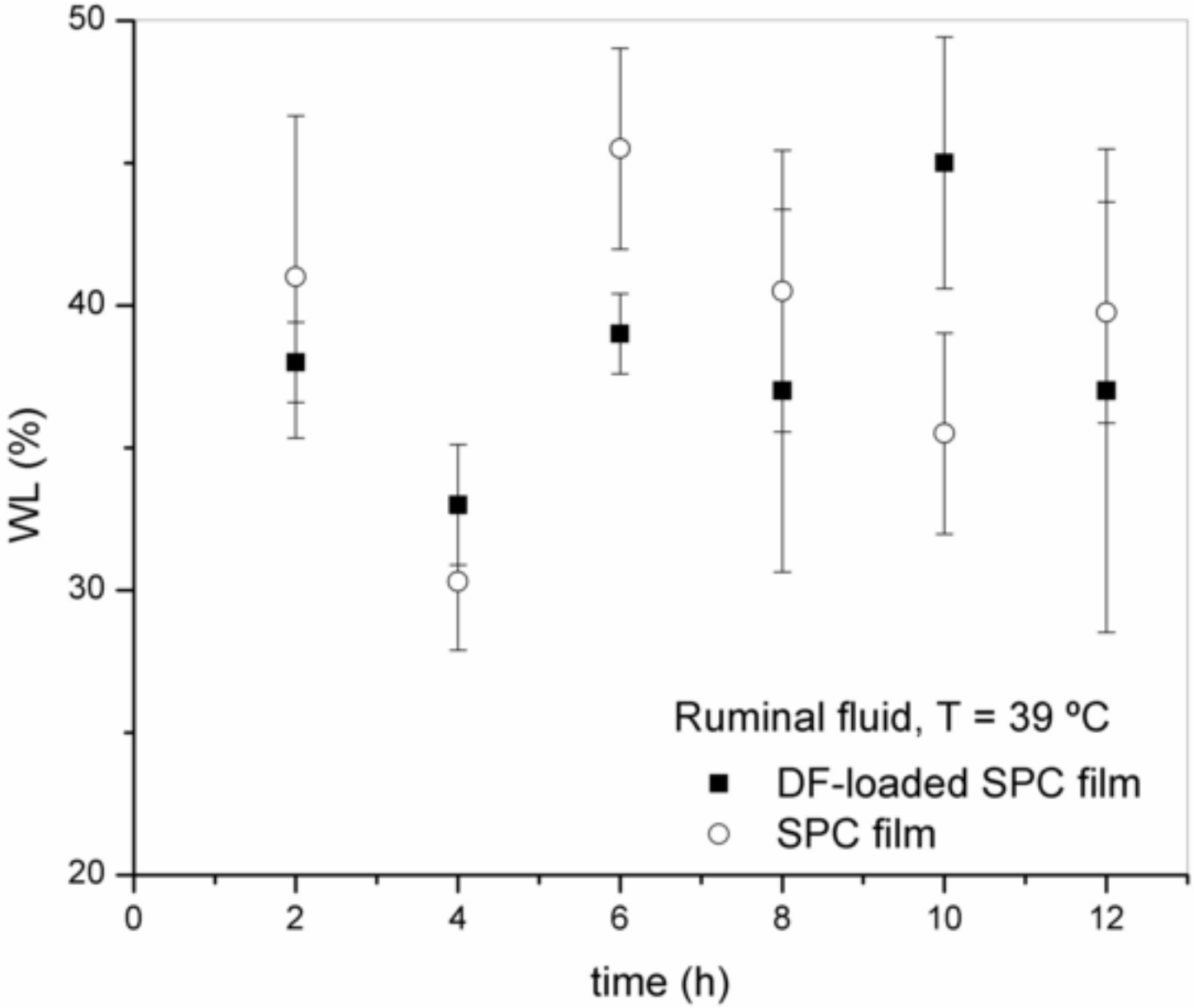
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
5
10
15
20

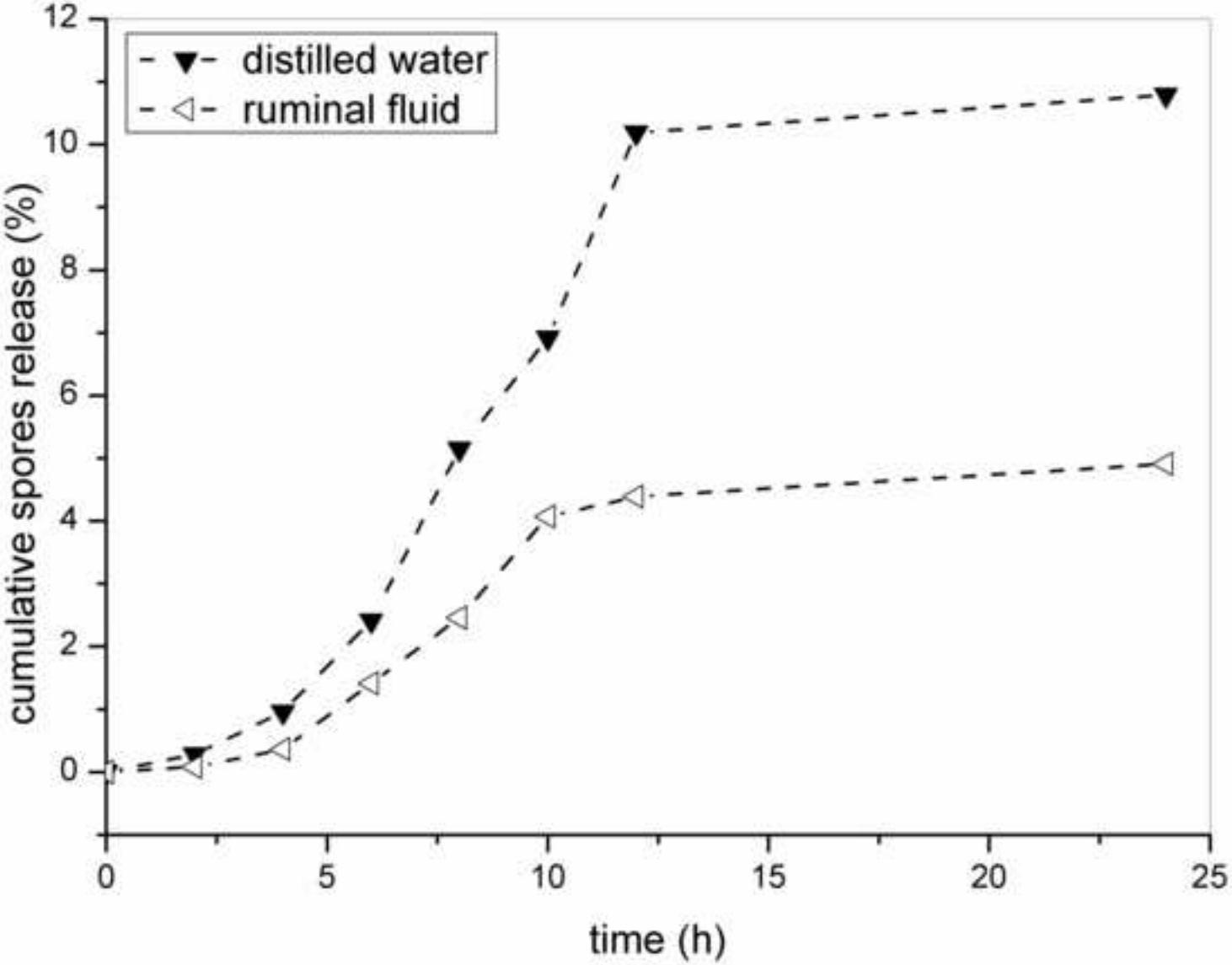



Distilled water, T = 39 ºC
swelling (%)
time (h)
0
20
40
60
80
100
120
140
160
0
2
4
6
8
10
12
SPC Film (experimental)
SPC Film (fitted)
DF-loaded SPC Film (experimental)
DF-loaded SPC Film (fitted)


Ruminal Fluid, T = 39 ºC
swelling (%)
time (h)
0
20
40
60
80
100
120
140
160
0
2
4
6
8
10
12
SPC Film (experimental)
SPC Film (fitted)
DF-loaded SPC Film (experimental)
DF-loaded SPC Film (fitted)







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

752

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

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

755

756

757 **Table 2.** Cross-linking extent (%), **r**esidual moisture content (MC), total soluble matter
758 (TSM), tensile strength (TS) and elongation at break (ε_b) of SPC - Gly – DAS films
759

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

760 Mean values ± standard deviations. Any two means in the same column followed by the
761 same letter are not significantly different ($P > 0.05$) by Tukey's Test.
762 a) TSM determined by dry method
763 b) TSM determined by wet method
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767 **Table 3.** Fick´s second law equation parameters (eq.4): diffusion coefficient/area
768 (D_{app}/L^2), fluid uptake at the equilibrium (FU_{eq}) and regression coefficients (R^2).
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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

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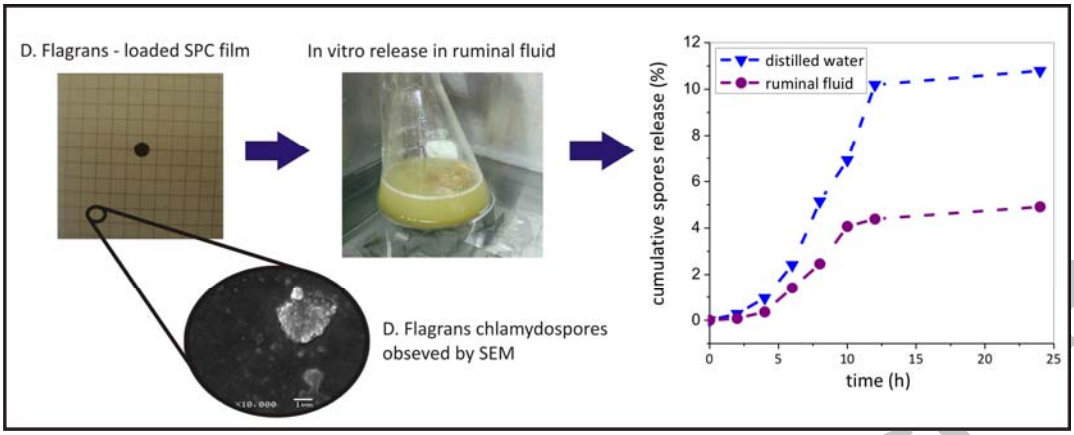

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- Soybean protein concentrate-based films were produced and characterized as potential vehicles of *D. Flagrans*.
- Viability of spores contained in films was verified.
- The best film composition was determined from mechanical, optical and swelling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