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Properties of biopolymer dispersions and films used as carriers of the biocontrol agent *Candida sake* CPA-1

A. Marín*, L. Atarés, M. Cháfer, A. Chiralt

Instituto de Ingeniería de Alimentos para el Desarrollo, Departamento de Tecnología de Alimentos, Universitat Politècnica de València, 46022, Valencia, Spain

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ABSTRACT

The use of biocontrol agents (BCA) for controlling plant diseases is an alternative to reduce the use of pesticides. Their performance can be improved when applied in combination with coatings. Films and coatings formulated from different biopolymers were characterized as to their barrier and optical properties to analyse their impact on fruit when applied as carriers of the BCA *Candida sake* CPA-1. The properties of the film-forming dispersions were more affected by the type of polymer than by the incorporation of surfactants. Sodium caseinate formed the thickest coatings, but these were very thin in every case, which led to there being no predicted relevant effect on the gas exchanges of the fruit. The cell viability in the films was good during film drying, especially in the case of protein films; however, it decreased after storage.

1. Introduction

The use of living agents to control pests or plant pathogens or biological control, is considered as a reliable alternative to pesticide use (Droby, Wisniewski, Macarisin, & Wilson, 2009). The formulation of coatings containing living agents, for biological control purposes (biocontrol agents: BCA), represents an interesting means of applying this kind of preservation method. The coatings constituents can help to keep the microorganisms alive, by acting as nutrients, and to protect them from environmental damage, favouring their adhesion to the plant (Marín et al., 2016).

Antimicrobial edible films can be formulated via the incorporation of different compounds in the formulation of film-forming dispersions (FFDs) (Suppakul, Miltz, Sonneveld, & Bigger, 2003). Some microorganisms, such as lactic acid bacteria (LAB), have been also used for the obtaining of antimicrobial films, due to their ability to produce metabolites effective against some foodborne bacteria (Sánchez-González, Quintero Saavedra, & Chiralt, 2013). Other microorganisms which can act as microbial antagonists are yeasts, which have received considerable attention as controlling agents of diseases caused by molds in fruits (Sui, Wisniewski, Droby, & Liu, 2015). There are few studies dealing with coatings as carriers of antagonistic yeasts (Aloui, Licciardello, Khwaldia, Hamdi, and Restuccia (2015); González-Estrada et al., 2015; Fan et al., 2009).

Candida sake CPA-1 is one of the most studied antagonistic yeasts, due to its ability to control grey mold caused by *Botrytis cinerea* (Calvo-Garrido, Viñas, Elmer, Usall, & Teixidó, 2013). Competition for nutrients and space is the proposed mechanism whereby CPA-1 is able to inhibit fungal diseases. This mode of action requires the presence of a high number of cells on the fruit to ensure their efficiency. Their application in edible coatings based on different hydrocolloid improved the cell viability and their effectiveness, as has been reported recently (Marín et al., 2016).

The selection of the coating forming agents (CFAs) is necessary both to ensure their ability to be carriers of BCAs, as well as to confer suitable properties to the coatings. Therefore, both cell viability and coating functional properties must be taken into account in BCA formulations with CFAs. Barrier or optical properties, which could affect the exchanges of water and gases of the plant or its appearance, should be analysed to identify suitable formulations. Biopolymers such as hydroxypropylmethylcellulose (HPMC), corn starch (S), sodium caseinate (NaCas) and pea protein (PP), have been studied as CFAs (Jiménez, Fabra, Talens, & Chiralt, 2012; Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2009). They have shown good compatibility with *C. sake* when

* Corresponding author.
E-mail address: anmargo6@upvnet.upv.es (A. Marín).

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applied on grapes (Marín et al., 2016). The use of surfactants in the coating formulations could improve the adherence on the fruit and modulate the film properties (Ortega-Toro, Jiménez, Talens, & Chiralt, 2014).

This work was undertaken to determine the properties of different coatings, compatible with the BCA *C. sake* CPA-1, in order to predict their effects when applied on the fruit. The hydrocolloid FFD and films were based on HPMC, S, NaCas or PP with or without surfactants with different hydrophilic-lipophilic balance (oleic acid: OA, Span 80: S80 and Tween 85: T85). The properties of the FFD relevant to their stability and application on the plant and the barrier and optical properties of the films were analysed. Likewise, the viability of the BCA in the films was studied.

2. Materials and methods

2.1. Materials

HPMC (molecular weight: ~86 kDa, viscosity: 2.6–5.6 mPa·s, 2%) NaCas (molecular weight: ~23 kDa), surfactants and streptomycin sulphate were supplied by Sigma–Aldrich (Madrid, Spain). Native corn S and PP with a purity of 85–90% were purchased from Roquette Laisa España, S.A., (Valencia, Spain) and glycerol, magnesium nitrate-6-hydrate ($Mg(NO_3)_2$), phosphorus pentoxide (P_2O_5) and potassium iodide (KI) from Panreac Química, S.L.U (Barcelona, Spain).

2.2. Preparation of the film forming dispersions (FFDs)

FFDs were prepared by dispersing the biopolymers in deionized water. HPMC (2% wt.) was heated until 80 °C and maintained under magnetic stirring at 25 °C overnight. No plasticizer was required to obtain adequate films, as previously reported by other authors (Villalobos, Hernández-Muñoz, & Chiralt, 2006). S (2% wt.) was stirred at 95 °C for 30 min to induce starch gelatinization. NaCas and PP (4% wt.) were dispersed at 25 °C for 2 h. Glycerol was incorporated as plasticizer in S, NaCas and PP FFDs at a hydrocolloid:glycerol mass ratio of 1:0.25, according to previous studies (Fabra, Jiménez, Atarés, Talens, & Chiralt, 2009; Jiménez et al., 2012) and surfactants were added at a mass ratio of 1:0.1, also on the basis of previously reported studies (Jiménez et al., 2012; Ortega-Toro et al., 2014). FFDs were homogenized with a Ultraturrax T25 (Janke and Kunkel, Germany) at 13,600 rpm for 4 min and sterilized at 121 °C. Each film forming dispersion was prepared at least in triplicate for its characterization.

2.3. Characterization of the FFDs

2.3.1. Density, pH, particle size and ζ-potential

Density (ρ) was measured with a pycnometer, using water as reference. A pH-meter (GLP +21 Crison Instruments SA, Barcelona, Spain) was used to determine the pH. Both tests were performed at 25 °C in triplicate.

The droplet size distribution, volume-length mean diameter ($D_{4,3}$) and volume-surface mean diameter ($D_{3,2}$) of the polymer aggregates or surfactant droplets were measured by using a laser diffractometer (Mastersizer 2000; Malvern Instruments, Worcestershire, UK). Three samples of each FFD were measured in quintuplicate.

ζ-potential was measured in triplicate using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK).

2.3.2. Rheological behaviour

The rheological behaviour of FFDs was analysed in duplicate at 25 °C by means of a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a Z34DIN Ti type sensor system. Up and down curves of shear stress (σ) *vs*. shear rate ($\dot{\gamma}$) from 0 to 800 s^{-1} were obtained. Either the Ostwald de Waele or the Herschel-Bulkey models (Eqs. (1) and (2)) were fitted to the experimental data depending on whether the curves show yield shear stress (σ_y) or not. The consistency index (K), the flow behaviour index (n) and the apparent viscosities (η) at 100 s^{-1} were determined.

$$\sigma = K \cdot \dot{\gamma}^n \quad (1)$$

$$\sigma = \sigma_y + K \cdot \dot{\gamma}^n \quad (2)$$

2.3.3. Coating capacity of FFD on grape surface

The coating capacity of the formulations on the fruit surface was studied following a gravimetric method. Four replicates of bunches of grapes were coated with the FFDs by spraying them. Samples were weighed before and after pulverization and the FFD adhered mass on the grape surface was determined. To calculate the total adhered solids, the mass fraction of each FFD was considered. These values were used to estimate the thickness of the applied coatings. To this end, films of the different formulations, with different thicknesses were obtained by casting different amounts of the FFDs, thus obtaining different surface solid densities (g/cm^2), which were correlated with the measured film thickness. Then, the surface solid density (SSD) on the grapes was estimated from the total adhered solids (TAS, mg/cm^2), considering a spherical geometry for the grapes (2.5 cm mean diameter) and a density of 1100 mg/cm^3. The surface solid density on the grapes was calculated by multiplying the TAS per the fruit density and dividing by the specific surface for a sphere ($S/V = 3/r$).

2.4. Film preparation

The mass of each FFD containing 1 g of solids was spread over 15 cm diameter polytetrafluorethylene plates (solid surface density: 5.6 mg/cm^2). Films were formed by drying for 48 h at 45% RH and 25 °C. Prior to characterization, the films were stored for 7 days in dessicators at 25 °C and 53%RH using an oversaturated solution of $Mg(NO_3)_2$. Films without surfactants were also prepared by adding cell culture suspensions as described in section 2.6. At least three films per formulation were obtained for characterizations of their different properties.

2.5. Characterization of the films

2.5.1. Optical properties

The gloss of the films was measured at an incidence angle of 60°, according to the ASTM standard D523 (ASTM, 1999), using a flat surface gloss meter (Multi-Gloss 268, Minolta, Germany) in three films per formulation. The transparency of the films was determined through the surface reflectance spectra from 400 to 700 nm with a spectrocolorimeter CM-3600d (Minolta Co., Tokyo, Japan). The Kubelka-Munk theory was applied in order to determine the transparency. Internal transmittance (T_i) was quantified using Eq. (3) in which R_0 is the reflectance of the film on an ideal black background. a and b parameters were calculated by Eqs. (4) and (5), where R is the reflectance of the sample layer backed by a known reflectance R_g. R_∞ (Eq. (6)) values were used to determine L^*, a^* and b^* values from the CIELab colour space, using D65 illuminant and 10° observer. From these values, whiteness index (WI) was obtained (Eq. (7)). All the measurements were taken in triplicate.

$$T_i = \sqrt{(a - R_0)^2 - b^2} \quad (3)$$

$$a = \frac{1}{2}\left(R + \frac{R_0 - R + R_g}{R_0 \cdot R_g}\right) \quad (4)$$

$$b = \sqrt{a^2 - 1} \quad (5)$$

$$R_\infty = a - b \quad (6)$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (7)$$

2.5.2. Thickness, moisture content and barrier properties

A digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain) was used to measure the thickness of four films per formulation. Measurements were taken at six points of each film.

Moisture content (MC) was determined gravimetrically. Four samples per formulation were dried for 24 h at 60 °C in a vacuum oven and then placed in a desiccator containing P_2O_5 at room temperature, until constant weight was reached.

The water vapour permeability (WVP) of the films was measured according to a modification of the ASTM E-96-95, based on that previously reported by McHugh, Avena-Bustillos, and Krochta (1993), at 25 °C and for a 53–100% RH gradient, generated by using an oversaturated solution of $Mg(NO_3)_2$ and distilled water. Measurements were taken in triplicate in each formulation by placing them on permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium), which were periodically weighed. The determination of the WVP was carried out with Eq. (8) and Eq. (9).

$$WVTR = \frac{P \cdot D \cdot \mathrm{Ln}\left[\frac{(P - p_2)}{(P - p_1)}\right]}{R \cdot T \cdot \Delta z} \quad (8)$$

where P, total pressure (atm); D, diffusivity of water through air at 10 and 25 °C (m^2/s); R, gas law constant ($82.0 \cdot 10^{-3}$ $m^3 \cdot atm/kmol \cdot K$); T, absolute temperature (K); Δz, mean stagnant air gap height (m); p_1, water vapour pressure on the solution surface (atm); p_2, corrected water vapour pressure on the film's inner surface (atm).

$$Permeance = \frac{WVTR}{(p_1 - p_2)} \quad (9)$$

The oxygen permeability (OP) was determined by triplicate at 53% RH and 25 °C using an OX-TRAN model 2/21 ML Mocon (Germany). The samples were conditioned in the cells of the equipment for 6 h and the transmission values were determined until the equilibrium was reached.

2.6. Candida sake incorporation to the films and viability over film storage

Strain CPA-1 of *C. sake* was originally isolated from the surface of apples by UdL-IRTA Centre (Lleida, Spain) and deposited at the "Colección Española de Cultivos Tipo" (CECT-10817). Cell production and formulation was carried out according to Cañamás et al. (2011).

C. sake was incorporated into each FFD in a concentration of $5 \cdot 10^7$ CFU per film and the films were obtained as described in section 2.4. After drying, they were stored in desiccators at 25 °C and 53% or 68% RH, using oversaturated solutions of $Mg(NO_3)_2$ or KI, to simulate two possible ambient conditions at field applications. Films stored at 53% RH were also characterized. Viability of *C. sake* was tested after drying and over storage time (7, 14 and 21 days) at both RH. The films were placed in sterile plastic bags containing 100 mL of deionized water with 0.01% (w/v) Tween 85 and homogenized for 6 min. Serial dilutions were made by duplicate and plated onto trypticase soy agar medium with streptomycin sulphate (0.5 g/L). Plates were incubated for 48 h at 25 °C.

2.7. Statistical analysis

Statistical Analyses were performed using Statgraphics Centurion XVI 16.1.17 (Manugistics Corp., Rockville, Md.) Principal Component Analysis (PCA) was carried out using Unscrambler 10.X software.

3. Results and discussion

3.1. Properties of FFDs

3.1.1. Density, pH, particle size and ζ-potential

Table 1 shows the values of density, pH, average diameters of the particles and their ζ-potential of the FFDs. The density was always similar to that of water, given the low solid proportion. The highest values were found for the proteins, which were incorporated into the highest mass ratio (4% wt.). The pH values were in the neutral range, although NaCas FFDs were slightly more acid than the rest.

Neither HPMC FFD nor that with T85 could be characterized in their size distribution, since they did not reach the required obscuration level for measurement. In the other cases, polymer aggregates were formed, giving rise to measurable size particles.

Surfactants did not have a notable effect on the proteins or S. However, according to Table 1 surfactants affected $D_{4,3}$ and $D_{3,2}$ parameters of the HPMC FFD, and S80 yielded the greatest aggregates. Likewise, T85 reduced the aggregation of NaCas particles, showing a greater population of smaller particles. This indicates particular interactions between the different surfactants and polymers, which affected the compounds dispersion in water.

All particles were negatively charged in agreement with both the adsorption of the negative ions on neutral polysaccharides and the negatively-charged protein chains. Protein FFDs showed higher values of ζ–potential due to their ionisable groups. Generally, surfactant addition resulted in changes of the surface charge of the particles, thus indicating the interactions/adsorptions of these amphiphilic compounds with/on the polymer chains. This was remarkable for HPMC S80 and for all FFDs with proteins.

3.1.2. Rheological behaviour

All FFDs, except S with surfactants, exhibited a non-time dependent behaviour, below a limit shear rate ranging from 250 to 540 s^{-1}, where a change in the shear stress-shear rate relationship was observed. Fig. 1 shows the flow curves of S dispersions, where those containing surfactants exhibited time-dependent behaviour depending on the surfactant.

The Ostwald de Waele model was fitted to the experimental data up to the limit shear rate values. Table 2 shows the rheological parameters (K and *n*) of the FFDs, including the η at 100 s^{-1} and the highest shear rate value up until which the model was fitted (limit $\dot{\gamma}$). Repeatability of rheological behaviour was very high in all formulations, as deduced from the low values of the variation coefficients (VC) of rheological parameters obtained: lower than 3% in all cases for *n* values and lower than 10% for K values.

Both for HPMC and NaCas FFDs, the flow index was similar to those reported by other authors (Sánchez-González, Chiralt,

Table 1
Density (kg/m^3), pH, ζ-potential (mV) and mean particle size of the different film forming dispersions with and without surfactants (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
Density (kg/m^3)	WS	1003.3 ± 0.7^{b}	1005.0 ± 0.6^{ab}	1011.0 ± 2.0^{a}	1010.1 ± 1.1^{a}
	OA	1002.3 ± 0.4^{ab}	1004.9 ± 1.1^{a}	1010.0 ± 0.9^{a}	1008.9 ± 1.4^{a}
	S80	1001.0 ± 2.0^{a}	1006.4 ± 0.8^{b}	1010.7 ± 1.1^{a}	1008.7 ± 1.7^{a}
	T85	1003.1 ± 0.5^{ab}	1005.3 ± 0.6^{ab}	1010.6 ± 0.8^{a}	1010.2 ± 0.1^{a}
pH	WS	6.61 ± 0.15^{a}	7.10 ± 0.20^{b}	6.96 ± 0.08^{b}	7.84 ± 0.05^{d}
	OA	6.90 ± 0.05^{b}	6.28 ± 0.09^{a}	6.48 ± 0.09^{a}	7.01 ± 0.02^{a}
	S80	7.32 ± 0.04^{d}	7.24 ± 0.10^{b}	6.93 ± 0.03^{b}	7.55 ± 0.01^{b}
	T85	7.14 ± 0.09^{c}	7.15 ± 0.03^{b}	6.92 ± 0.04^{b}	7.71 ± 0.03^{c}
ζ-potential (mV)	WS	−7.9 ± 1.4^{a}	−10.0 ± 0.5^{a}	−18.1 ± 1.7^{a}	−19.9 ± 1.1^{a}
	OA	−7.5 ± 0.8^{a}	−12.8 ± 1.3^{c}	−34.9 ± 3.0^{c}	−26.2 ± 0.5^{d}
	S80	−19.0 ± 3.0^{b}	−11.5 ± 0.7^{b}	−24.5 ± 1.8^{b}	−23.2 ± 0.6^{c}
	T85	−24.0 ± 2.0^{c}	−9.9 ± 0.7^{a4}	−38.0 ± 4.0^{d}	−22.1 ± 0.3^{b}
$D_{4,3}$	WS	–	26.0 ± 12.0^{c}	47.4 ± 6.3^{d}	14.6 ± 1.4^{a}
	OA	2.6 ± 0.2^{a}	10.4 ± 0.6^{a}	34.0 ± 3.0^{b}	17.2 ± 0.8^{c}
	S80	24.9 ± 0.7^{b}	9.6 ± 0.5^{a}	42.6 ± 8.0^{c}	15.6 ± 0.9^{b}
	T85	–	16.0 ± 6.0^{b}	20.8 ± 2.0^{a}	14.6 ± 1.6^{a}
$D_{3,2}$	WS	–	8.3 ± 0.9^{d}	7.7 ± 0.9^{d}	7.9 ± 0.5^{a}
	OA	1.5 ± 0.2^{a}	4.1 ± 0.1^{a}	4.9 ± 0.2^{b}	9.8 ± 0.4^{b}
	S80	4.9 ± 0.2^{b}	5.7 ± 0.1^{c}	6.5 ± 1.1^{c}	7.7 ± 0.4^{a}
	T85	–	5.2 ± 0.1^{b}	4.4 ± 0.1^{a}	7.7 ± 0.5^{a}

Different superscripts (a-d) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.

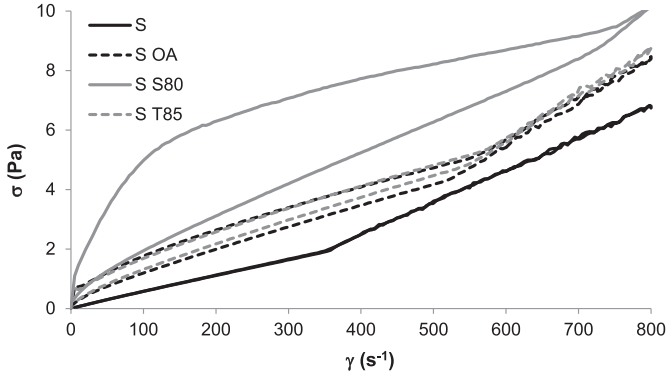

Fig. 1. Flow curves at 25 °C of the film forming dispersions based on starch (S) with and without surfactants. OA: oleic acid, S80: Span 80, T85: Tween 85.

González-Martínez, & Cháfer, 2011). Likewise, S dispersions without surfactants behaved similarly to that previously reported by Ortega-Toro et al. (2014).

Surfactant incorporation did not entail significant changes in the rheological behaviour of HPMC, NaCas and PP FFDs, despite the interactions deduced from the ζ−potential values. The flow curves of all these polymer dispersions exhibited two different trends below and above the limit shear rate, and the η sharply increased at 350 s^{-1} (S), 300 s^{-1} (HPMC) or 250 s^{-1} (NaCas and PP). This increase in η could be related to an increase in the hydrodynamic volume of the polymer chains due to the changes in their conformation and aggregation as a consequence of the shear flow.

Surfactant addition to S dispersions led to an increase in the η, promoting thixotropic behaviour. The greatest hysteresis area in flow curves was found for S S80 (1622 Pa s^{-1}) (Fig. 1). This effect could be attributed to the aggregation of amylose−lipid complexes formed through the helical conformation of amylose, entrapping hydrophobic chains of surfactants (Wokadala, Ray, & Emmambux, 2012). These aggregates cause an increase in the stress-strain relationship and can be disrupted during shear, thus causing thixotropic effects.

S S80 showed the lowest *n* (0.43) and the highest K, as well as the greatest thixotropic effects, which suggests a higher degree of amylose complex formation. In these cases, the Herschel-Bulkley model was fitted up to 540 and 520 s^{-1} in order to obtain yield stress values (Table 2).

Multifactorial ANOVA revealed that, the type of polymer and surfactant significantly ($p < 0.05$) affected the values of η. Nevertheless, in practical terms, apparent viscosity of FFDs was similar (3−4 mPa·s), except in the case of FFD based on S.

3.1.3. *Coating capacity of film forming dispersions on grape surface*

All samples were effectively and homogenously coated by spraying as revealed their complete surface wetting and the homogenous final sample gloss of the grapes, imparted by coatings. Table 2 shows the mass of FFD adhered to the fruit surface. S and NaCas dispersions exhibited a similar coating capacity, regardless of the presence of surfactants, this being about 5−8 mg FFD/g grapes, whereas PP showed less coating capacity. HPMC FFDs were better spread and retained on the grape surface. No significant effect of surfactants was observed, except in HPMC, where OA and S80 reduced the coating capacity, and in PP, where T85 produced the same effect. Therefore, despite the expected action of surfactants on the contact angle and adhesion forces, no notable effect was observed in practical terms. Despite the higher viscosity of S dispersions at low shear rates, no greater retention of the surface coating against gravitational drainage was observed.

From the adhered mass of the different FFDs, the total solid mass of the fruit coating was estimated by considering their respective concentrations (Table 2). The NaCas FFDs provided the highest values of adhered solid mass, and hence, the formation of the thickest coatings is expected in this case.

The solid surface density (g/cm^2) and film thickness were correlated with films prepared with different amounts of solids per surface unit. The slopes of the fitted straight lines ($r^2 > 0{,}98$) were 8.08, 6.52, 6.92 and 6.53, for coatings of HPMC, S, NaCas and PP. From these values and the mass of solids adhered to the grape surface, the expected thicknesses of the coatings were estimated, which were 0.8, 0,5, 1.2 and 0.6 μm for HPMC, S, NaCas and PP. To this estimation, grapes of 2.5 cm diameter and 1100 mg/cm^3 density were considered. The obtained thickness values indicate that

Table 2
Rheological parameters (n and K), apparent viscosity (mPa·s), highest shear rate value up until which the model was fitted (limit γ) and adherence on grapes surface of the different film forming dispersions with and without surfactants (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
n	WS	1.06^{a}	0.95^{b}	1.05^{a}	1.02^{a}
	OA	1.06^{a}	1.08^{bc}	1.05^{a}	1.02^{a}
	S80	1.06^{a}	0.43^{a}	1.04^{a}	1.03^{a}
	T85	1.05^{a}	1.15^{c}	1.04^{a}	1.02^{a}
K (Pa·s^{n})	WS	3.20^{a}	7.11^{a}	3.03^{a}	3.42^{b}
	OA	3.33^{b}	5.53^{a}	3.02^{a}	2.94^{a}
	S80	3.29^{ab}	626.90^{b}	3.35^{a}	3.54^{b}
	T85	3.30^{ab}	4.06^{a}	3.22^{a}	3.26^{ab}
η_{ap} at 100 s^{-1} (mPa·s)	WS	4.16 ± 0.01^{a}	5.70 ± 0.04^{a}	3.78 ± 0.03^{a}	3.70 ± 0.30^{b}
	OA	4.29 ± 0.06^{b}	15.50 ± 0.30^{b}	3.84 ± 0.09^{a}	3.20 ± 0.05^{a}
	S80	4.30 ± 0.03^{b}	46.10 ± 0.90^{c}	4.00 ± 0.30^{a}	4.00 ± 0.17^{b}
	T85	4.24 ± 0.01^{ab}	14.90 ± 0.30^{b}	3.80 ± 0.20^{a}	3.63 ± 0.07^{ab}
Limit $\dot{\gamma}$ (s^{-1})	WS	300	350	250	250
	OA	300	540	250	250
	S80	300	350	250	250
	T85	300	520	250	250
Adherence of FFD (mg/g grape)	WS	12.6 ± 1.1^{b}	7.7 ± 1.7^{a}	6.5 ± 0.2^{a}	4.0 ± 0.6^{b}
	OA	9.0 ± 2.0^{a}	5.0 ± 2.0^{a}	8.5 ± 1.6^{a}	4.1 ± 0.7^{b}
	S80	7.7 ± 1.4^{a}	7.0 ± 3.0^{a}	7.0 ± 3.0^{a}	4.0 ± 0.4^{b}
	T85	12.0 ± 3.0^{b}	7.3 ± 1.8^{a}	8.0 ± 3.0^{a}	2.7 ± 0.2^{a}
Adhrence of solids (mg/g grape)	WS	0.25 ± 0.04^{bc}	0.19 ± 0.04^{a}	0.33 ± 0.01^{a}	0.20 ± 0.03^{b}
	OA	0.20 ± 0.05^{ab}	0.14 ± 0.06^{a}	0.45 ± 0.08^{a}	0.20 ± 0.05^{b}
	S80	0.17 ± 0.03^{a}	0.18 ± 0.08^{a}	0.36 ± 0.16^{a}	0.20 ± 0.03^{b}
	T85	0.27 ± 0.06^{c}	0.20 ± 0.05^{a}	0.41 ± 0.18^{a}	0.15 ± 0.01^{a}

Different superscripts (a-c) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.

coatings represent a very thin layer on the fruit.

A PCA was carried out, taking all the determined properties of FFDs into account, for the purposes of comparing them. Fig. 2 shows the typical plot of the two functions, PC1 and PC2, which explain 69% of the variance. The different FFDs were grouped by the type of polymer. PC1 allowed protein and polysaccharide FFDs to be differentiated and PC2 separated the FFDs of each polymer. The presence of surfactants particularly affected the HPMC samples, which group was more dispersed in the plot. Therefore, the behaviour of the FFDs was more affected by these compounds. The properties with the higher weight in the PC1 were ζ-potential (0.583), density (−0.534), solid adherence (−0.499) and viscosity (0.304). From the analysed properties of the FFDs, a good stability and ability to spraying could be deduced.

3.2. Properties of the films

3.2.1. Optical properties

As all the films had gloss values lower than 70 (Table 3), they could be considered as matt (Trezza & Krochta, 2000). The kind of

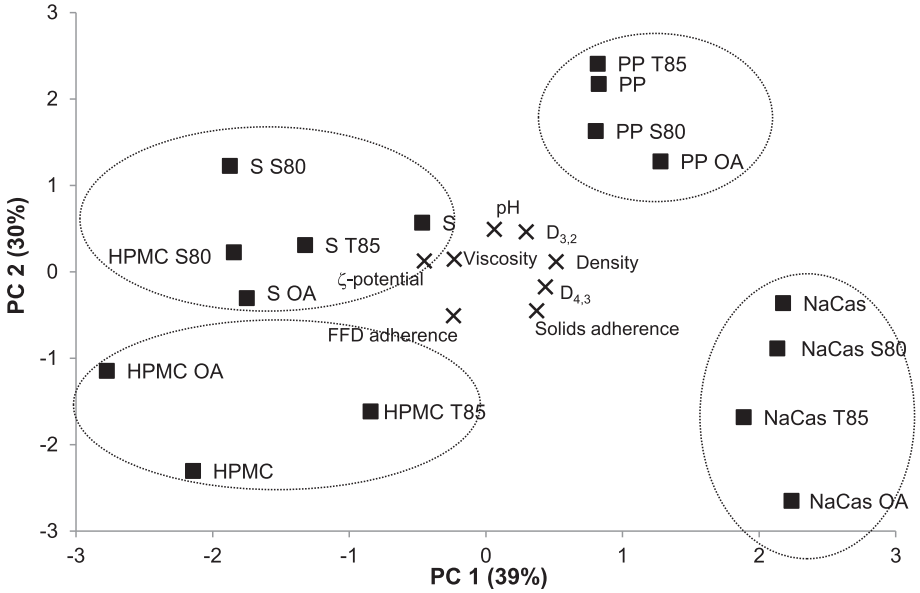

Fig. 2. Principal component analysis for properties of film forming dispersions. HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Table 3
Optical properties of the different films: gloss at 60°, colour coordinates (lightness (L^*), chrome (C^*_{ab}), hue (h^*_{ab}), whiteness index (WI) and internal transmittance (T_i) at 400 nm (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
Gloss (60°)	WS	39 ± 20^{b}	46 ± 14^{c2}	19 ± 8^{a}	52 ± 15^{bc}
	OA	7 ± 2^{a}	21 ± 8^{a}	48 ± 20^{b}	55 ± 13^{c}
	S80	7 ± 4^{a}	22 ± 6^{a}	22 ± 4^{a}	45 ± 13^{b}
	T85	12 ± 3^{a}	31 ± 10^{b}	14 ± 3^{a}	31 ± 10^{a}
L^*	WS	85.4 ± 0.2^{c}	85.5 ± 0.8^{b}	77.0 ± 0.8^{a}	68.0 ± 1.2^{a}
	OA	82.3 ± 0.6^{b}	83.2 ± 0.4^{a}	79.6 ± 1.0^{c}	70.5 ± 0.2^{a}
	S80	79.0 ± 3.0^{a}	85.0 ± 1.1^{b}	77.9 ± 0.7^{b}	67.3 ± 0.9^{a}
	T85	80.7 ± 0.4^{ab}	85.5 ± 0.4^{b}	77.0 ± 0.3^{a}	70.5 ± 5.0^{a}
C^*_{ab}	WS	4.0 ± 0.1^{a}	3.6 ± 0.1^{b}	15.2 ± 0.9^{a}	16.4 ± 0.2^{b}
	OA	4.8 ± 0.5^{a}	3.0 ± 0.3^{a}	15.3 ± 0.8^{a}	18.5 ± 0.7^{c}
	S80	4.4 ± 1.5^{a}	3.9 ± 0.6^{b}	15.1 ± 0.1^{a}	16.2 ± 0.1^{ab}
	T85	5.1 ± 0.8^{a}	4.0 ± 0.3^{b}	16.9 ± 0.3^{b}	15.7 ± 0.7^{a}
h^*_{ab}	WS	91.3 ± 1.3^{a}	100.1 ± 1.8^{b}	79.7 ± 1.3^{b}	80.7 ± 0.3^{a}
	OA	96.0 ± 1.3^{b}	97.7 ± 1.5^{a}	81.2 ± 0.6^{c}	79.7 ± 0.1^{a}
	S80	92.0 ± 3.0^{a}	98.8 ± 1.2^{ab}	78.0 ± 0.9^{a}	80.4 ± 0.5^{a}
	T85	90.2 ± 1.1^{a}	102.9 ± 0.8^{c}	78.8 ± 0.8^{ab}	83.0 ± 3.0^{b}
WI	WS	84.8 ± 0.2^{c}	85.3 ± 0.3^{c}	72.4 ± 1.1^{ab}	64.1 ± 1.2^{a}
	OA	81.7 ± 0.6^{b}	82.9 ± 0.3^{a}	74.5 ± 1.2^{c}	65.0 ± 1.1^{a}
	S80	79.0 ± 3.0^{a}	84.5 ± 0.9^{b}	73.3 ± 0.8^{b}	63.0 ± 0.1^{a}
	T85	79.8 ± 0.7^{ab}	84.9 ± 0.4^{bc}	71.8 ± 0.4^{a}	67.0 ± 5.0^{a}
T_i (400 nm)	WS	0.85 ± 0.01^{c}	0.84 ± 0.01^{c}	0.76 ± 0.01^{a}	0.66 ± 0.02^{ab}
	OA	0.84 ± 0.01^{b}	0.83 ± 0.01^{b}	0.77 ± 0.01^{b}	0.68 ± 0.01^{b}
	S80	0.83 ± 0.10^{a}	0.83 ± 0.01^{b}	0.76 ± 0.01^{a}	0.62 ± 0.02^{a}
	T85	0.84 ± 0.01^{ab}	0.82 ± 0.01^{a}	0.75 ± 0.01^{a}	0.67 ± 0.08^{ab}

Different superscripts (a-c) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.

polymer significantly affected the film gloss ($p < 0.05$). PP films showed the highest gloss values, which were comparable to those obtained by Sánchez-González et al. (2013). HPMC films with surfactants showed the lowest gloss values. The incorporation of all surfactants into HPMC and S matrices resulted in a significant gloss reduction ($p < 0.05$). Surfactant addition increased the heterogeneity and roughness of the film surface, thus reducing gloss (Jiménez et al., 2012). In protein matrices, OA incorporation resulted in a significant gloss increase, which could be attributed to this liquid lipid filling the gaps on the film surface, making it more even and glossier.

From CIE L*a*b* colour coordinates, C^*_{ab}, h^*_{ab} and WI were obtained and are shown in Table 3. The kind of biopolymer greatly affected the colour of the films. As compared to proteins, polysaccharides gave rise to lighter films with less saturated colour, more yellow and less red in hue. Consequently, the WI of protein films was lower. Surfactant incorporation led to a slight decrease in the L^* of HPMC films, which can be attributed to changes in the film structure.

Table 3 shows the values of T_i at 400 nm where the greatest differences among films were observed. The highest T_i values corresponded to HPMC and S without surfactants, which was probably caused by the high packing of polysaccharide chains giving rise to more homogeneous structures. The greatest opacity was found for PP films, as reported in previous studies (Sánchez-González et al., 2013).

The incorporation of surfactant caused a slight T_i decrease in HPMC and S films, which can be attributed to the presence of dispersed surfactant aggregates, this causing light dispersion and transparency decrease. Ortega-Toro et al. (2014) also observed the lipid separation in the starch matrix for S films with S80. Adding surfactants to NaCas films did not result in T_i modifications, other than a slight increase when OA was added. Particular interactions between NaCas and OA have been previously described (Fabra et al., 2009).

3.2.2. Thickness, moisture and barrier properties

Table 4 shows the values of thickness, moisture content, WVP and OP of the films. The film thickness ranged between 40 and 65 μm, despite the constant amount of solids per unit of surface area. The protein films were thicker than those of polysaccharide, which indicated the tighter packing of S and HPMC chains, giving rise to thinner films. Likewise, the incorporation of surfactants led to thicker films, in line with the effects of their interruption on the matrices. No significant effect was observed for PP and NaCas OA, in agreement with the better compatibility of amphiphilic molecules which led to a more compact packing.

HPMC films had significantly lower equilibrium MC than the rest, while S films exhibited the greatest water holding capacity. Regardless of the polymer, surfactant addition resulted in a significant ($p < 0.05$) decrease in the film's MC, coherently with their greater hydrophobic nature, which limited the water sorption capacity of the films. In HPMC, the effect of the surfactant was not significant due to the more hydrophobic nature of this hydrocolloid.

As shown in Table 4, HPMC films were the most efficient as water vapour barriers, coherently with their greater hydrophobicity, which limited the solubility of water molecules. The WVP values obtained were similar to those found by Sánchez-González et al. (2011). On the other hand, NaCas films showed the highest WVP. The effect of incorporating surfactants on the WVP depended on both the surfactant and the polymer. Generally, OA addition led to a significant WVP decrease, which was probably due to its greater hydrophobicity (Fabra et al., 2009). The rest of the surfactants did not significantly affect WVP, except in S matrices where a slight increase was observed, as Ortega-Toro et al. (2014) previously reported, probably due to the formation of a more open polymer network where water molecules could diffuse more easily.

Table 4 shows the values of OP of the films. The OP of HPMC could not be quantified since they were above the threshold sensitivity of the equipment. The S films exhibited better oxygen barrier properties than the protein films. In all cases, surfactant addition (especially OA) worsened the OP, which may be linked to the incorporation of a hydrophobic phase in the matrix where the oxygen solubility is enhanced.

A PCA was used to compare all the analysed properties of the films. Fig. 3 shows the PCA plot, where PC1 explained 54% of total variance and PC2 24%. Polysaccharide films were differentiated from protein films in terms of PC1 while HPMC and S films were differentiated by PC2. Optical parameters had the higher weight in the PC1 function, whereas barrier properties showed greater weight in the PC2 function.

Taking into account the obtained data, and considering their estimated thicknesses, the oxygen (OTR) and water (WTR) transmission rates of the coatings applied on grapes were obtained and plotted in Fig. 4. Due to the low thickness of the coatings, very high values of WTR and OTR were obtained, which will not imply serious restrictions for the water vapour and oxygen exchanges of the coated fruit. The location of the samples in the WTR-OTR map indicated that NaCas coatings will better limit water vapour and oxygen exchanges, mainly due to their higher coating capacity, whereas S without surfactants will be the most effective at limiting the exchange of oxygen.

It can be summarized that S was the best one for the purposes of reducing oxygen exchanges, whereas the HPMC coatings implied a better control of the water exchange. The incorporation of surfactants reduced the OP with no notable reduction of the WVP.

Table 4
Thickness (μm), equilibrium moisture content (g water/100 g dry film), water vapour permeability (WVP) and oxygen permeability (OP) of the different films (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
Thickness (μm)	WS	44 ± 1^{a}	42 ± 5^{a}	62 ± 6^{ab}	48 ± 3^{a}
	OA	48 ± 4^{ab}	47 ± 1^{b}	54 ± 6^{a}	51 ± 9^{a}
	S80	52 ± 5^{b}	59 ± 4^{c}	65 ± 2^{b}	54 ± 5^{a}
	T85	49 ± 4^{ab}	46 ± 2^{ab}	64 ± 7^{b}	56 ± 8^{a}
% moisture content (d.b.)	WS	5.0 ± 1.3^{a}	9.4 ± 0.8^{b}	8.3 ± 0.5^{b}	9.4 ± 0.5^{c}
	OA	3.6 ± 1.6^{a}	8.3 ± 0.5^{a}	8.0 ± 0.6^{b}	6.7 ± 0.4^{a}
	S80	3.6 ± 0.4^{a}	8.9 ± 0.7^{ab}	7.0 ± 0.6^{a}	6.9 ± 0.5^{a}
	T85	4.3 ± 1.5^{a}	8.4 ± 0.8^{ab}	6.2 ± 0.5^{a}	8.5 ± 0.4^{b}
WVP (g/Pa·s·m) × 10^{11}	WS	62 ± 17^{ab}	121 ± 7^{a}	196 ± 14^{b}	171 ± 5^{ab}
	OA	49 ± 3^{a}	152 ± 8^{b}	145 ± 17^{a}	130 ± 30^{a}
	S80	81 ± 13^{b}	178 ± 9^{c}	201 ± 14^{b}	156 ± 14^{ab}
	T85	68 ± 13^{ab}	160 ± 30^{bc}	211 ± 8^{b}	180 ± 50^{b}
OP (cm^3/Pa·s·m) × 10^{11}	WS	> L.D.[a]	16 ± 1^{a}	98 ± 2^{a}	150 ± 20^{a}
	OA	> L.D.[a]	132 ± 9^{c}	167 ± 2^{c}	244 ± 23^{b}
	S80	> L.D.[a]	106 ± 6^{b}	132 ± 12^{b}	156 ± 5^{a}
	T85	> L.D.[a]	114 ± 3^{b}	200 ± 14^{d}	173 ± 25^{a}

Different superscripts (a-c) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.
[a] > L.D. Above the detection limit (200 cm^3/m^2·day).

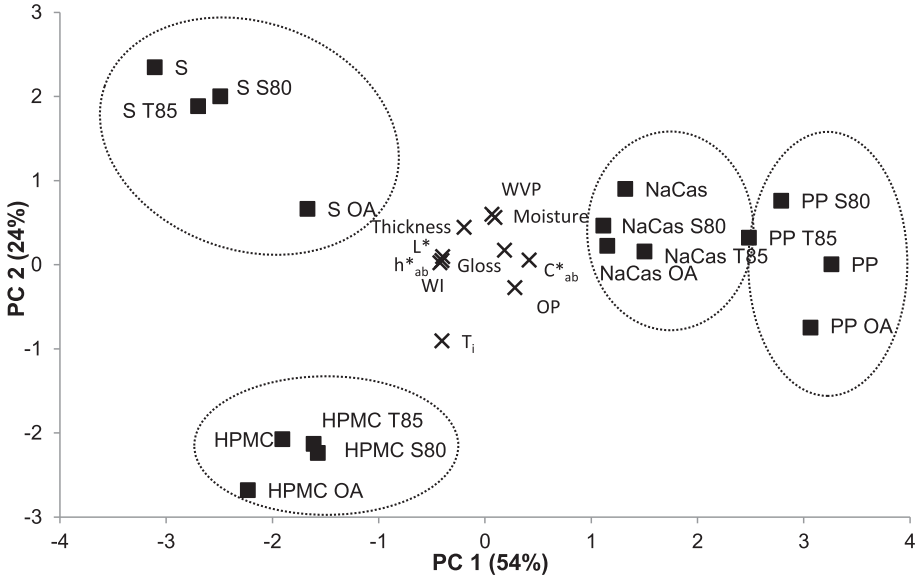

Fig. 3. Principal component analysis for properties of films. HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

3.3. Effect of the BCA incorporation on film properties

Fig. 5 shows the values of gloss, MC and barrier properties for films with and without cells. No great differences in barrier properties were observed as a result of cell incorporation, despite the fact that an increase in the MC occurred in some S and Nacas films. Whereas cells enhanced the barrier capacity in protein films, they slightly reduced it in polysaccharide films. Similar effects were previously observed when different microorganisms were added to biopolymer films (Aloui et al., 2015; Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010; Sánchez-González et al., 2013).

As shown in Fig. 5, cell incorporation implied a decrease in film gloss in the glossiest films (HPMC, S and PP), which could be attributed to the presence of cells on the film surface, introducing surface roughness and reducing the gloss. In NaCas films, this effect could not be relevant due to the low gloss value of these films.

3.4. The viability of Candida sake in the films

The viability of cells in the different matrices was studied in order to identify their ability as carriers of BCA, regardless of the fruit support. Table 5 shows the viability of *C. sake* (log CFU/cm^2) in the films both after the drying period (48 h) and storage (7 and 14 days) under 53 and 68% RH at 25 °C. In no case were any viable cells found after 21 days of storage. After the drying period, the cell viability was slightly higher in the protein films, which could be explained by the nutritional effect of free aminoacids. In fact, the population of *C. sake* in protein films after the film drying was higher than that inoculated (5.4 log CFU/cm^2), pointing to cell growth during the 48 h drying step. This trend agreed with that found in previous studies (Sánchez-González et al., 2013).

The statistical analysis did not reveal a clear pattern as regards the effect of surfactants on the cell viability after drying. In HPMC,

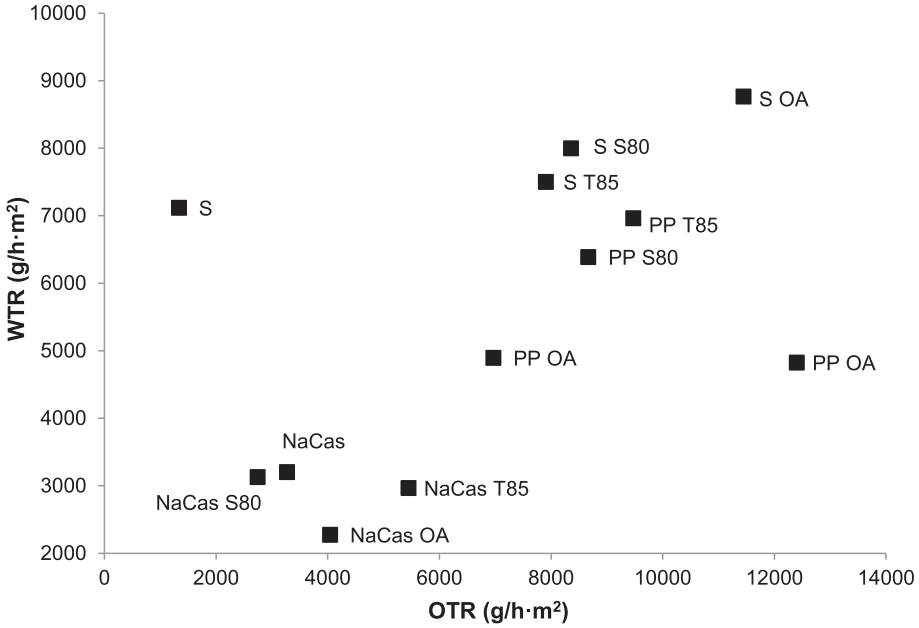

Fig. 4. Water and oxygen transmission rates of the coatings applied on the grape surface. S: starch, NaCas: sodium caseinate, PP: pea protein, OA: oleic acid, S80: Span 80, T85: Tween 85. HPMC films were not included because of their oxygen permeability was the highest and overcomed the threshold sensitivity of the used equipment.

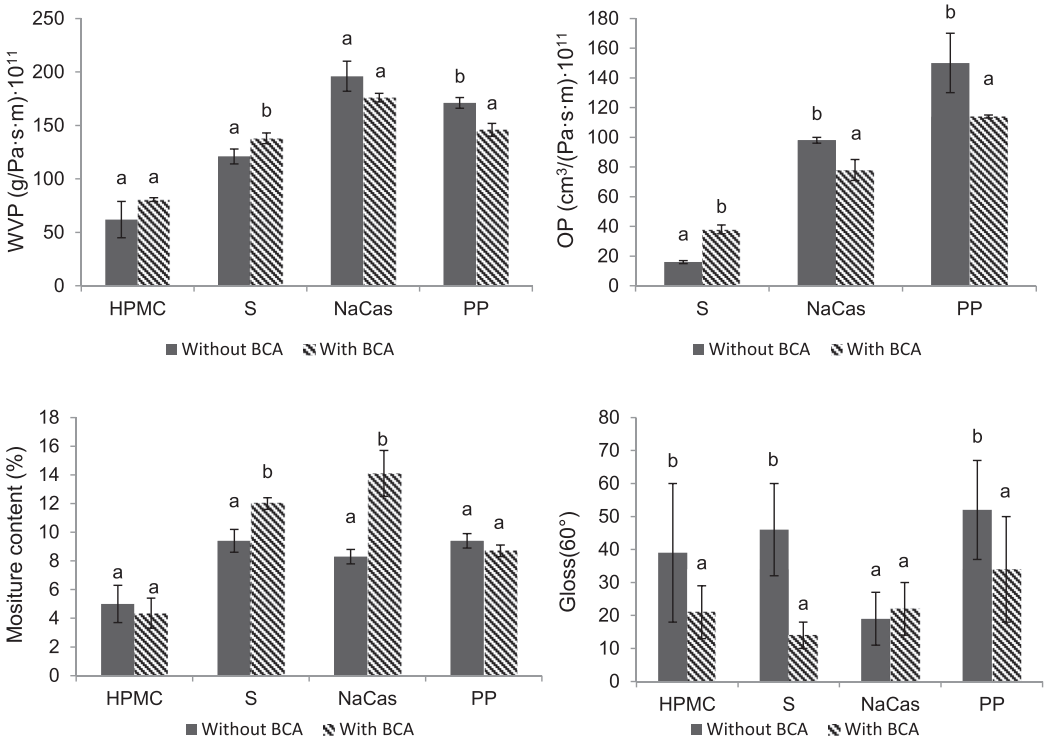

Fig. 5. Water vapour permeability (WVP), oxygen permeability (OP), moisture content (g water/100 g dry film) and gloss of the surfactant free films without and with the BCA (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, BCA: biocontrol agent. Different superscripts (a–b) for the same polymer indicate significant differences ($p < 0.05$) due to the incorporation of *Candida sake*.

S80 and T85 seemed to favour cell survival, while in S they provoked a decrease in cell population. Likewise, T85 and OA reduced cell viability in NaCas and PP, respectively.

After 7 and 14 days of storage, although the viability was very much reduced in HPMC and S formulations, protein films better maintained the *C. sake* viability. This could also be explained by the nutritional effect of proteins.

The ambient RH (water activity in the film), affected the yeast viability throughout storage. In S films, no cells were viable after 7 storage days either at 0.53 or 0.68 a_w. In HPMC and NaCas films, the yeast viability was maintained after 7 storage days at 53% RH, but drastically dropped at 68%. However, for PP films, the greatest

Table 5
Viability of *Candida sake* in the films (log CFU/cm^2) after film drying and 7 and 14 days of storage at 25 °C and 53% or 68% RH (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, OA: oleic acid, S80: Span 80, T85: Tween 85.

Formulation	log CFU/cm^2				
	After drying	7 days		14 days	
		53% RH	68% RH	53% RH	68% RH
HPMC	4.5 ± 0.5^{ab}	–	–	–	–
HPMC OA	4.2 ± 0.3^{a}	–	–	–	–
HPMC S80	5.6 ± 0.4^{c}	3.2 ± 0.1^{a}	–	–	–
HPMC T85	6.1 ± 0.1^{d}	4.6 ± 0.3^{b}	–	–	–
S	5.9 ± 0.1^{cd}	–	–	–	–
S OA	4.9 ± 0.5^{b}	–	–	–	–
S S80	4.9 ± 0.7^{b}	–	–	–	–
S T85	4.4 ± 0.3^{ab}	–	–	–	–
NaCas	7.1 ± 0.3^{ef}	7.1 ± 0.4^{c}	–	–	–
NaCas OA	6.7 ± 0.7^{e}	4.8 ± 0.5^{b}	–	–	–
NaCas S80	7.2 ± 0.5^{f}	6.3 ± 0.9^{c}	3.9 ± 0.1^{a}	–	–
NaCas T85	5.5 ± 0.2^{c}	–	–	–	–
PP	6.9 ± 0.1^{ef}	–	5.6 ± 0.5^{c}	–	3.8 ± 0.9^{a}
PP OA	5.9 ± 0.3^{cd}	–	4.3 ± 0.1^{ab}	–	–
PP S80	6.7 ± 0.3^{e}	–	5.1 ± 0.9^{bc}	–	3.4 ± 0.2^{a}
PP T85	8.8 ± 0.1^{ef}	4.7 ± 0.1^{b}	4.2 ± 0.5^{ab}	–	–

Different superscripts (a-f) within the same column indicate significant differences ($p < 0.05$) among formulations.

counts after 7 storage days were obtained at a_W 0,68 and they maintained cell survival after 14 storage days in some formulations.

These results suggest that, at a lower a_W the yeast could be in a latent state, due to the low water availability, prolonging its survival, whereas under more vital conditions (0.68 a_W), cells extenuate themselves fighting for survival in a water stressed medium without adequate nutrients. In S films, the greater availability of nutritive glucose could accelerate cell death due to the lack of water availability under both a_w conditions. This trend was similar to that found by Romano et al. (2014).

At a low a_w, the microbial cells remained viable in a latent state. On the contrary, with restricted, but greater, availability of water, vital cell activity occurs but the stress conditions result in cell death. The opposite effect observed in PP films points to specific survival mechanism for the cells in the chemical context of this protein. No clear tendencies in the role of surfactants on cell survival during storage were observed.

When the cell survival in the films was compared to that previously reported in coatings with similar composition applied on grapes (Marín et al., 2016), different trends were observed, which indicates that the fruit support affected cell viability. This could be explained by the fact that this yeast is naturally present in fruit surface. Therefore, when *C. sake* was present in its natural environment and supported in a thin coating it was able to better survive and multiply. However, when entrapped in a standalone film with thickness about 50 μm, its viability resulted compromised.

In conclusion, FFDs of polysaccharides and proteins, with and without surfactants, can be used as carriers of the BCA *C. sake* to be applied on grapes, at the same time as the coatings can modulate the exchange of gases, without introducing any negative effects on the product's appearance due to the great film transparency. NaCas permits a greater coating capacity, and so thicker coatings. The thickness and barrier properties of the matrices will determine the water vapour and gas exchanges, depending on the RH of the ambient/environment where the coatings are applied. Although the yeast's viability was better maintained in the PP films at higher a_W, in NaCas films this took place at lower a_W. The formulation of PP-NaCas blend films could be a good strategy with which to prolong yeast viability.

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