RESEARCH ARTICLE

Effect of the potassium sorbate and carvacrol addition on the properties and antimicrobial activity of tapioca starch – Hydroxypropyl methylcellulose edible films

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Tapioca starch (TS), hydroxypropyl methylcellulose (HPMC), and glycerol (GLY) based edible films with the addition of potassium sorbate (KS) and carvacrol (CARV) were prepared by casting technique. The effect of antimicrobial concentrations on the color, mechanical properties, solubility in water (SW), water vapor permeability (WVP), and antimicrobial effectiveness of films was analyzed and the composition was optimized by using a response surface methodology (RSM). The films were transparent and slightly yellow but with high L^* values (89–91). Potassium sorbate incorporation increased the color parameter values and presented an antagonistic interaction with CARV for b^* and yellow index (YI). A higher amount of CARV drastically decreased the stress at break, and increased the deformation and the SW of the films. The water vapor permeability ($1.1 \pm 0.1 \times 10^{-9}\,g^{-1}\,m^{-1}\,s^{-1}\,Pa^{-1}$) was not influenced by the different concentrations of antimicrobials. The film formulated with KS 0.30 g $100\,g^{-1}$ and CARV 0.50 g $100\,g^{-1}$ correctly satisfied the requests of mechanical strength, colorless, and appropriate bioavailability of antimicrobials for *Zygosaccharomyces. bailii*. This optimized film showed a higher surface hydrophobicity and exerted a highly improved antimicrobial barrier against *Z. bailii*, *Lactobacillus plantarum*, and *Pseudomonas fluorescens* in comparison with films containing only KS. The obtained results demonstrate the ability of films to act as an antimicrobial hurdle that can contribute to extend the shelf life of food.

Keywords:
Active biopolymeric matrices / Physicochemical properties / *Z. bailii* and *L. plantarum* and *P. fluorescens* growth control

1 Introduction

Self-supporting edible films are one of the emerging technologies used today to optimize food preservation. They are formed from renewable eco-friendly resources, and have proven to be effective and economical [1]. The edible films can delay water vapor transfer and control oxygen and carbon dioxide permeability. Moreover, they can improve organoleptic properties and also contain food additives (*i.e.*: Antioxidants, antimicrobials) providing a highly localized functional effect [2, 3]. These matrices are constituted with proteins or carbohydrates, such as starches, cellulose and derivatives, gums, pectins, and chitosan [4, 5]. Due to their hydrophilic nature and in order to improve the barrier characteristics together with the mechanical and solubility

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Abbreviations: CARV, carvacrol; **CCD,** central composite design; **CFU,** colony forming units; **D,** desirability function value; **DI,** diameter of inhibition; **GLY,** glycerol; **GRAS,** generally recognized as safe; **HPMC,** hydroxypropyl methylcellulose; **KS,** potassium sorbate; **MH,** Mueller–Hinton; **MRS,** de Man, Rogosa, and Sharpe; **PW,** peptone water; **RH,** relative humidity; **RSM,** response surface methodology; **SW,** solubility in water; **TS,** tapioca starch; **WVP,** water vapor permeability; **YI,** yellow index

properties, the use of blends comprising such compounds or their combination with lipids was proposed [6–8]. Recently, Espinel et al. [9] reported that an increase of HPMC content in the formulation of tapioca starch (TS) based films containing potassium sorbate (KS), produced a higher elastic modulus and stress at break while decreasing the YI.

KS is considered to be a generally recognized as safe (GRAS) additive, and in the food industry, it is one of the most commonly used antimicrobials acting against fungi, yeasts, and some bacteria. The addition of KS to the formulation of edible films has been proposed as a strategy to reduce the surface deterioration by microbial contamination [10, 11].

Bakkali et al. [12] reported that CARV and thymol were the major components of the *Origanum compactum* essential oil. According to Ben et al. [13], these phenolic compounds possess extensive and strong antimicrobial activity against Gram-negative bacteria (*Salmonella typhimurium*, *Escherichia coli*, *Enterobacter cloacae*, etc.), and Gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*, etc.). Moreover, carvacrol and thymol also present antifungal ability (*Candida albicans*, *Candida tropicalis*, *Aspergillus niger*, and *Rhodotorula*) [14].

Espinel et al. [9] studied the effect of different amounts of TS, HPMC, and GLY on physical properties of edible films containing KS (0.3 g 100 g^{-1} film forming solution) using a mixture design as a statistical tool. Considering that KS is fundamentally an antimycotic, the addition of CARV can improve the antimicrobial characteristics of the optimized film therein developed, but it must be taken into account that the incorporation of any additive into the film formulation may drastically change its physicochemical and mechanical properties, and consequently the antimicrobial properties might be modified [1]. Accordingly, the objective of the present work was to analyze the effect of the incorporation of KS and CARV on the color, mechanical properties, solubility, WVP, and antimicrobial activity of TS and HPMC based edible films in order to obtain an effective action against yeasts, molds, and bacteria.

2 Materials and methods

2.1 Materials

Food grade TS, (Bernesa S.A., Argentina. Purity: 92–98 g 100 g^{-1}. Amylase content, 19 g 100 g^{-1} according to ISO 6647-1 [15] and amylopectin content, 81 g 100 g^{-1}) with molecular weights of 68×10^6 g/mol (amylopectin) and 0.8×10^6 g/mol (amylose), and HPMC (Methocel K4MCR, the Dow Chemical Company, USA) were used to produce the films. GLY (Sintorgan S.A., Argentina, Code: SIN-086005-01), CARV (Sigma®, USA, Code: W224502), and KS (Sigma®, Code: 359769) were of analytical grade.

2.2 Preparation of edible films

A hydroxypropyl methylcellulose aqueous solution was stirred and heated (approximately at 4°C/min) until dissolution (84°C). A suspension of TS in a KS and GLY water solution was added to the HPMC slurry with stirring until total homogenization. A second heating step (approximately at 4°C/min) was performed on the biopolymers blend with stirring to complete the gelatinization of the starch (93°C). Subsequently, the sample was cooled to 70°C and CARV was added to the KS–TS–HPMC–GLY solution, and the slurry was homogenized using an Ultra Turrax (IKA, Germany) device at 21500 rpm for 2 min. All film forming solutions were centrifuged (Eppendorf AG, Germany) at 25°C in order to remove air bubbles for 5 min at 500 rpm. Finally, 20 g of the slurries were dispensed onto plastic Petri dishes (9 cm in diameter) and dried at 35°C for 24 h. After constitution, the films were removed from the dish and equilibrated at 25°C before testing, for 15 days at 57% relative humidity (RH). For all tested formulations, the TS, HPMC, and GLY final concentrations were maintained at 2.67, 0.67, and 1.7 g 100 g^{-1}, respectively [9]. Contrarily, KS and CARV contents were selected according to the applied experimental design (Section 2.8).

2.3 Color evaluation

Color measurements were performed with a colorimeter (Minolta, Japan) using an aperture of 1.5 cm in diameter. The films were rested on a standard white background and the color parameters L^*, a^*, b^* according to the International Commission of Illumination and the *YI* according to the American Society for Testing and Materials, E1925 [16] were determined in at least five different positions for each specimen. Color parameters range from $L^* = 0$ (black) to $L^* = 100$ (white), $-a^*$ (greenness) to $+a^*$ (redness), and $-b^*$ (blueness) to $+b^*$ (yellowness). Calculations were made for the illuminant D65 and 2-degree observer. Three specimens were tested for each sample.

2.4 Mechanical properties of films

The tensile force (N) and extension (mm) profile of the films until the moment of rupture was obtained by using a universal testing machine (Instron, USA). Tested filmstrips (60×6 mm^2) were mounted between pneumatic grips set at 172,369 Pa. The initial grip separation and crosshead speed were 20 mm and 0.8 mm/s, respectively. Force and extension data were used to generate stress (σ, MPa) versus strain (ε) curves. The stress is defined as $\sigma = F/A$, with F as the force (N) and A as the cross sectional area (mm^2) of the specimen; and the strain as $\varepsilon = H/L_0$, with H as the crosshead displacement (extension of the sample) and L_0 as the initial effective length of the sample. In addition, the stress (σ_b) and

the strain at break (ε_b) were registered. Nine specimens were tested for each sample.

2.5 Solubility in water (SW)

The initial percentage of dry matter was determined by drying 2 cm diameter disks in a vacuum oven at 100°C for 24 h. Other disks were cut, weighed, and immersed in 50 mL of distilled water, with periodic stirring, at 25°C for 24 h. The films were taken out by filtration and dried at 100°C for 24 h to determine the final weight of the dry matter. Solubility is reported as the difference between initial and final (after solubilization) dry matter with respect to the initial dry matter. Three specimens were tested for each sample [2].

2.6 Water vapor permeability (WVP)

The water vapor permeability of films was determined gravimetrically at 25°C using a modified American Society for Testing and Materials procedure [17]. The acrylic permeation cell (4.4 cm internal diameter and 3.5 cm deep) contained $CaCl_2$ (0% RH; ≅0 Pa water vapor partial pressure). The film was placed between the cell and its ring shaped cover. An air gap of 1 cm was left between the film and the $CaCl_2$ layer. The covered cell was placed in a chamber (Ibertest, Spain) set at 25°C and 70% RH (≅2300 Pa water vapor partial pressure). After 15 h, a stationary water vapor transmission rate was attained and from that moment, the changes in the weight of the cell (to the nearest 0.01 g) were recorded. The film thickness was measured in triplicate using a thickness gauge (Mitutoyo, Japan) with a precision of 0.001 mm. At least two specimens were tested for each sample.

2.7 Antimicrobial activity of films: Diffusion in solid medium assay

An inoculum of the yeast *Zygosaccharomyces bailii* (NRRL 7256) was prepared using Sabouraud broth (Biokar Diagnostics, France, Code: BK026HA) at 25°C for 24 h. The inoculum of 1 mL (≈6×10^5 Colony Forming Units (CFU)/mL) was then dispensed onto Sabouraud agar (Biokar Diagnostics, Code: BK027HA) plates and distributed evenly on the entire surface. Afterward, the excess was removed. Subsequently, 1 cm diameter disks of the film (without or with antimicrobials, according to the experimental design), were placed in contact with the inoculated agar. The plates were stored at 7°C for 48 h and then incubated at 25°C for 24 h. The antimicrobial effectiveness was determined by the observation of the existence of clear zones due to growth inhibition in the contact area, as well as around the disks, and the measurement of the corresponding diameter of inhibition (DI).

2.8 Experimental designs and statistical analysis

The influence of KS and CARV on the film properties (color, mechanical properties, SW, WVP, and DI) was studied by using a response surface methodology (RSM) procedure, modulating the two selected independent variables (KS and CARV) according to a two-factor, five-level Central Composite Design (CCD). The levels of KS (0.25, 0.28, 0.30, 0.33, and 0.35 g 100 g^{-1} of the film forming solution) and CARV (0.1, 0.33, 0.55, 0.78, and 1.0 g 100 g^{-1} of the film forming solution) were chosen from the results of the preliminary laboratory tests. The different formulations studied are described in Table 1, columns 4 and 5. The films are named in the Section 3 with the corresponding formulation but without making reference to the units.

Dependent variables were fitted to the following second degree polynomial function:

$$\Psi = B_0 + B_1 x_1 + B_2 x_2 + B_{11} x_2^2 + B_{22} x_2^2 + B_{12} x_1 x_2 \quad (1)$$

where, ψ is the generic dependent variable; B_0 is the value of fitted response at the central point of the design, *i.e.* ($x_1 = 0$ and $x_2 = 0$); B_1 and B_2 are the linear regression coefficients; B_{11} and B_{22} are the quadratic regression coefficients; and B_{12} is the cross-product regression coefficient; x_1 and x_2 are the independent variables that should affect ψ.

The analysis of variance (ANOVA) was performed in order to assess the adequacy of the Eq. (1) by calculating the corresponding *p*-value of the lack of fit test and the determination coefficient R^2, as well as to evaluate the significance of the equation coefficients. Moreover, three-dimensional response plots were generated, and the optimal contents of KS and CARV were selected using the desirability function procedure [18]. The Statistica 6.0 software (StatSoft Inc., USA) was used for the statistical treatment of data, the generation, and analysis of the response surfaces, as well as for the optimization procedure.

2.9 Effectiveness of optimized films as barrier to microbial contamination

The antimicrobial hurdle action of films was evaluated using common spoilage agents found in foods [19]: The yeast *Z. bailii* (NRRL 7256), the Gram-positive bacteria *L. plantarum* (ATCC 8014), and the Gram-negative bacteria *P. fluorescens* (ATCC 49838). The inocula were prepared in appropriate broths until an early stationary phase was achieved: *Z. bailii* in Sabouraud broth at 25°C for 24 h, *L. plantarum* in de Man, Rogosa, and Sharpe (MRS, Biokar Diagnostics, Code: BK070GC) broth, and *P. fluorescens* in Mueller–Hinton (MH, Biokar Diagnostics, Code: BK108HA) broth, at 37°C for 18–20 h.

In order to study the protective behavior of the films when they are applied to a high water activity (a_w) and acidic product, Sabouraud, MRS (Biokar Diagnostics, Code:

Table 1. Contents of KS and CARV (original components) of film formulations and the coded values according to Central Composite Design

	Coded values		Original components[a] (g 100 g^{-1})		Responses[b]								
Run	KS x_1	Carv x_2	KS	CARV	L^*	a^*	b^*	YI	ε_b	σ_b (MPa)	SW (g 100 g^{-1})	WVP ($\times 10^9$)[c] (g Pa^{-1} m^{-1} s^{-1})	DI (cm)
1	−2	0	0.25	0.55	89.5 ± 0.6	−1.39 ± 0.04	3.8 ± 0.4	6.7 ± 0.8	0.7 ± 0.1	1.1 ± 0.1	48 ± 1	1.09 ± 0.05	1.78 ± 0.05
2	−1	−1	0.28	0.33	89.4 ± 0.2	−1.49 ± 0.02	3.6 ± 0.1	6.3 ± 0.3	0.5 ± 0.1	1.6 ± 0.2	27 ± 4	1.1 ± 0.01	1.3 ± 0.2
3	−1	1	0.28	0.78	90.3 ± 0.4	−1.43 ± 0.05	4.3 ± 0.3	7.6 ± 0.5	0.72 ± 0.03	0.30 ± 0.04	46 ± 5	1.33 ± 0.03	1.6 ± 0.2
4	0	−2	0.30	0.10	89.5 ± 0.5	−1.32 ± 0.05	4.8 ± 0.7	8.7 ± 0.1	0.58 ± 0.04	3.5 ± 0.4	57 ± 2	1.2 ± 0.2	1.0 ± 0.2
5	0	0	0.30	0.55	90.7 ± 0.4	−1.44 ± 0.03	4.7 ± 0.1	8.2 ± 0.2	0.80 ± 0.03	0.5 ± 0.1	28 ± 9	1.2 ± 0.1	1.7 ± 0.3
6	0	0	0.30	0.55	91.2 ± 0.3	−1.35 ± 0.03	5.0 ± 0.2	8.9 ± 0.5	0.77 ± 0.05	0.47 ± 0.03	29 ± 2	1.28 ± 0.03	2.0 ± 0.4
7	0	0	0.30	0.55	90.6 ± 0.2	−1.46 ± 0.04	4.7 ± 0.3	8.3 ± 0.5	0.75 ± 0.07	0.55 ± 0.04	31 ± 3	1.16 ± 0.03	1.46 ± 0.06
8	0	2	0.30	1.00	90.2 ± 0.6	−1.36 ± 0.06	4.9 ± 0.2	8.8 ± 0.3	0.9 ± 0.2	0.66 ± 0.05	48 ± 1	1.0 ± 0.2	3.95 ± 0.07
9	1	−1	0.33	0.33	90.6 ± 0.6	−1.4 ± 0.1	5.3 ± 0.4	9.4 ± 0.8	0.38 ± 0.04	1.5 ± 0.2	42 ± 5	1.13 ± 0.02	1.2 ± 0.2
10	1	1	0.33	0.78	90.9 ± 0.4	−1.46 ± 0.04	4.8 ± 0.2	8.4 ± 0.4	0.7 ± 0.1	0.26 ± 0.06	63 ± 3	0.99 ± 0.02	1.47 ± 0.06
11	2	0	0.35	0.55	89.7 ± 0.6	−1.48 ± 0.05	4.0 ± 0.3	6.8 ± 0.7	0.6 ± 0.2	0.66 ± 0.04	41 ± 2	1.11 ± 0.05	1.9 ± 0.4

Response values for color parameters (L^*, a^*, b^*, YI), deformation (ε_b) and stress at break (σ_b), solubility in water (SW), water vapour permeability (WVP), and diameter of inhibition (DI).
a) KS and CARV concentrations are expressed as g of antimicrobial per100 g of film forming solution.
b) The responses are reported as mean values ± standard deviations ($n \geq 2$) are reported.
c) Films thickness: (0.15 ± 0.01) mm.

BK089HA), or MH (Biokar Diagnostics, Code: BK048HA) agar of a_w 0.980 and pH 4.5 were formulated. Film disks of 1 cm in diameter were placed on the surface of the formulated agars. Then, a 10 μL culture of *Z. bailii, L. plantarum, or P. fluorescens* containing approximately 6×10^5, 3×10^6, and 2×10^6 CFU/mL, respectively was seeded on the surface of the film disks. The samples were incubated at 25°C for 48 h. Sampling was performed at selected times by taking two disks of each formulation and suspending each one in 1 mL of peptone water (PW) (Biokar Diagnostics, Code: BK018HA). The samples were shaken for 2 min at 2500 rpm with a vortex (IKA Works Inc., USA) and then, serial dilutions were prepared in PW for cell enumeration (log CFU/g of film). *Z. bailii* population was enumerated by surface plating on agar and incubation at 25°C for 5 days. The counts of *L. plantarum* and *P. fluorescens* were performed by pour plating on MRS or MH agar followed by incubation at 37°C for 72 h. All tests were conducted at least in duplicate for the optimized films, for the control system (without antimicrobials), and for the films added only with KS (0.30 g 100 g^{-1}) which were assayed for comparison purposes.

2.10 Contact angle

For the film surface characterization in terms of hydrophobicity and hydrophilicity, contact angle measurements were performed with a tensiometer (Sinterface, Germany) equipped with a charge-coupled device camera and using the sessile drop method. A drop (3 μL) of ultrapure water was deposited with an automatic piston syringe onto the film surface. The software Profile Analysis Tensiometer PAT-1 (Sinterface, Germany) was used for the image analysis in order to measure for 20 s the angle between the film surface and the tangent of the liquid drop at the point of contact with the surface of the film. At least 12 samples of the optimized films and 0.30 g 100 g^{-1} KS films were evaluated.

3 Results and discussion

The coded and uncoded KS and CARV levels on the films as well as the measured responses can be observed in Table 1. The responses were statistically analyzed in relation to their fitting to a quadratic model (Eq. 1). The coefficients of the prediction equation in addition to the adequacy of the regression are reported in Table 2. The responses L^*, b^*, YI, σ_b, and DI were satisfactorily fitted to the proposed polynomial equation with values of R^2 higher than 0.7000 and a non-significant lack of fit ($p > 0.05$). The corresponding response surfaces are shown in Fig. 1.

3.1 Color evaluation

The L^* values were high (89.4–91.2), as illustrated in Table 1, mainly as a consequence of the transparency of the films. An important influence of KS content on L^* was observed since the positive linear and negative quadratic coefficients of the equation were significant ($p < 0.05$). Additionally, the linear and quadratic coefficients of KS were higher than the ones of CARV as shown in Table 2. Figure 1, Panel a shows that L^*

Table 2. Coefficients of the second-degree polynomial function used for the fitting of the dependent variables and ANOVA analysis for the regression

Coefficients[a)]	SW[b)] (g 100 g^{-1})	ε_b	σ_b (MPa)	*YI*	L^*	b^*	a^*	log WVP	DI (cm)
B_o	591.32	−1.86	20.41	−73.12	38.19	−36.83	−2.53	10.62	6.90
Linear									
B_1	−3493.29$^{***c)}$	17.60*	−99.85***	475.88**	315.76**	241.20**	5.63	−10.56	−37.67
B_2	−163.38*	−0.07	−13.02***	29.10*	14.39	14.91	1.15	−0.81**	−1.33
Square									
B_{11}	5874.79***	−33.20*	156.18***	−677.28**	−489.61*	−341.55**	−5.13	12.96	64.35
B_{22}	137.49***	−0.11	7.83***	1.36	−4.83*	0.45	0.40	-0.58**	4.00*
Interaction									
B_{12}	44.34	2.06	4.52	−101.22*	−27.17	−50.83*	−5.39	5.72	−2.22
ANOVA									
Lack of fit (p)	0.0083	0.0278	0.0856	0.1424	0.4490	0.1425	0.5970	0.0556	0.1184
R^2	0.5299	0.5914	0.9938	0.7457	0.8296	0.7401	0.5151	0.6904	0.7047

a) 1, KS; 2, CARV. The coefficients are referred to equations fitted using real levels of the variables.
b) SW, Solubility in water; ε_b, Strain at break; σ_b, Stress at break; *YI*, Yellow Index; L^*, a^*, b^*, CIELab color parameters; WVP, water vapor permeability; DI, diameter of inhibition.
c) Significant at: $^*p < 0.1$, $^{**}p < 0.05$, $^{***}p < 0.01$.

presented a maximum value, in agreement with the negative values observed for both quadratic terms. Parameters b^* and *YI* showed similar trends because both parameters expressed the yellow color of the films as depicted in Fig. 1, Panels b and c. The fitted quadratic functions presented a positive and significant KS linear coefficient ($p < 0.05$), a KS negative quadratic term ($p < 0.05$), and a negative interaction term ($p < 0.1$), which denoted an antagonistic effect of the combination of KS and CARV. These characteristics of the equation established the existence of a saddle point for 0.30 KS–0.64 CARV film with b^* and *YI* values of 4.77 and 8.44, respectively. In addition, two zones were denoted for lower values of b^* and *YI*: CARV > 0.78 g 100 g^{-1} and KS > 0.33 g 100 g^{-1}, and CARV < 0.55 g 100 g^{-1} and KS < 0.28 g 100 g^{-1}. With reference to parameter a^* shown in Table 1, it was not significantly affected by the different antimicrobial concentrations, showing a mean value of -1.4 ± 0.4. It had been reported that generally the addition of KS increased b^* and *YI* in cassava starch films, probably due to browning reactions generated by KS degradation [2]. Du et al. [20] stated that the parameters L^* and b^* decreased with increasing concentration of CARV in apple polysaccharides based films.

3.2 Mechanical properties

Parameter σ_b showed an excellent fit to the quadratic model as observed in Table 2. Figure 1, Panel d shows that as the amount of CARV increased, the σ_b drastically decreased. The linear (negative) and quadratic (positive) coefficients of CARV and KS were highly significant ($p < 0.01$), and a minimum value of σ_b (0.16 MPa) was observed for the film 0.31 KS–0.74 CARV. Unfortunately, the ε_b could not be adjusted to the proposed equation. However, as can be seen in Table 1, the deformation increases with an increasing content of CARV. For instance, the film 0.30 KS–1.00 CARV showed an ε_b 52.5% higher than film 0.30 KS–0.10 CARV. This CARV plasticizing effect was also observed by Du et al. [20] in apple puree based films where σ_b significantly decreased (1.9 to 1.6 MPa) and the ε_b increased (20 to 50%) for an antimicrobial content of 1.5 g 100 g^{-1}.

3.3 Solubility in water (SW)

The solubility in water response could not be fitted to Eq. (1) as observed in Table 2. However, it was notorious that 0.28 KS–0.33 CARV and 0.30 KS–0.55 CARV films exhibited a value for SW of ≈30 g 100 g^{-1} as illustrated in Table 1. For different levels of CARV and KS, the SW showed minimum values of 27 g 100 g^{-1} and attained values as high as 63 g 100 g^{-1}. Romero-Bastida et al. [21] observed that banana starch films with cinnamon oil (1.0–1.5 g 100 mL^{-1}) had low solubility values (33–26 g 100 g^{-1}) while the highest solubilities (62–68 g 100 g^{-1}) were observed for formulations containing KS (0.4–0.6 g 100 mL^{-1}). It can be suggested that the interaction between the film matrix components, the amount of each preservative and the resulting film microstructure affected the solubility of the film.

3.4 Water vapor permeability (WVP)

The fit of the WVP data to the proposed equation was not satisfactory as can be seen in Table 2. However, in Table 1 it can be observed that the permeability of

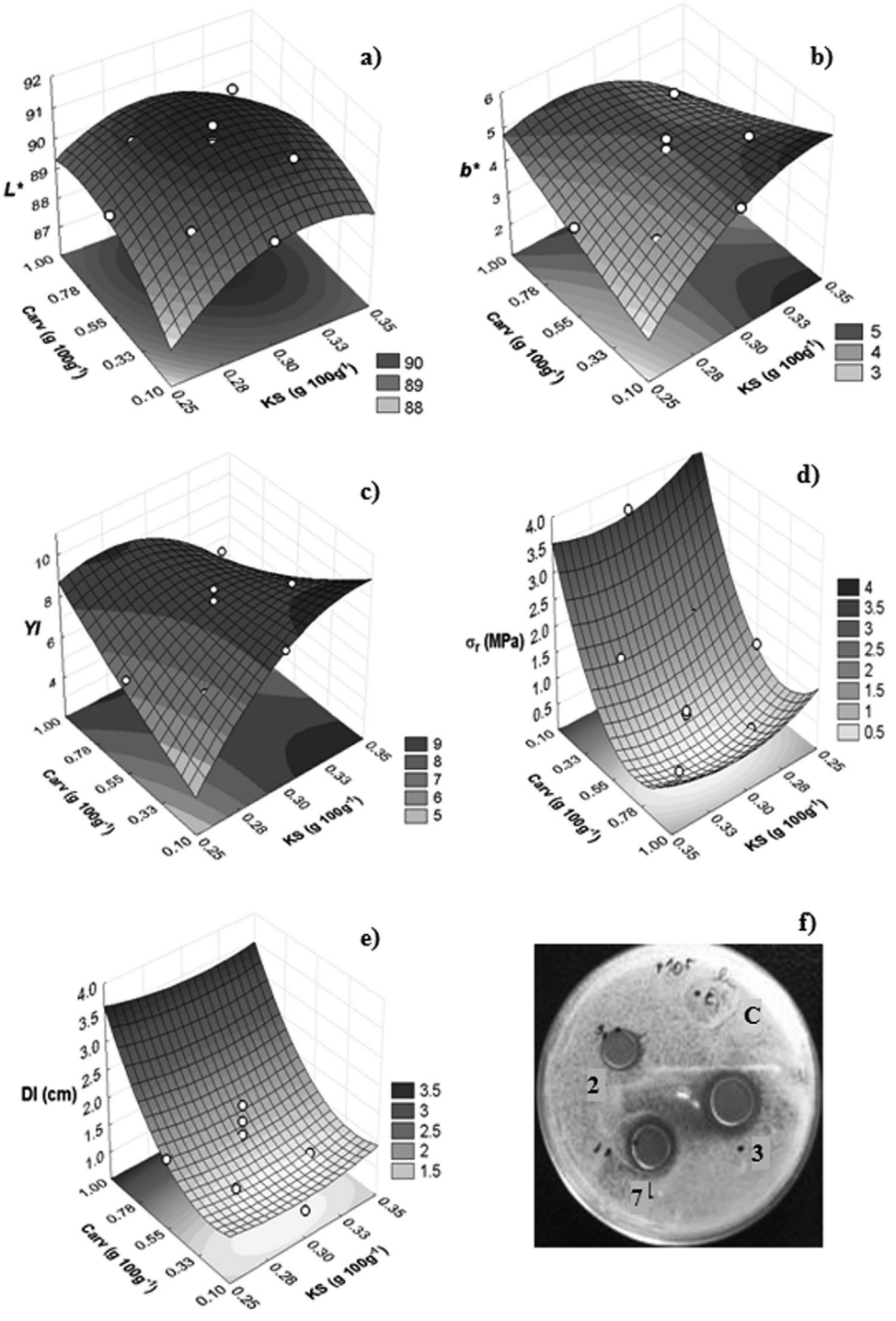

Figure 1. Response surfaces for the analysis of the effect of KS and CARV contents on: a) L^*, b) b^*, c) Yellow index (*YI*), d) stress at break (σ_b), e) diameter of inhibition (DI). On panel f) it can be observed the diameter of inhibition (DI) images for 0.28 g 100 g^{-1} KS −0.33 g 100 g^{-1} CARV (2), 0.28 g 100 g^{-1} KS −0.78 g 100 g^{-1} CARV (3), 0.30 g 100 g^{-1} KS −0.55 g 100 g^{-1} CARV (7), and Control (C) formulations.

the films took similar values of around $(1.1 \pm 0.1) \times 10^{-9}\,g^{-1}\,m^{-1}\,s^{-1}\,Pa^{-1}$), which are in agreement with those reported by Romero-Bastida et al. [21] for banana starch films with cinnamon oil ($0.51–1.36 \times 10^{-9}\,g^{-1}\,m^{-1}\,s^{-1}\,Pa^{-1}$) and with the WVP obtained for other polysaccharide based films [2, 22].

3.5 Diffusion in solid medium assay

The DI values from 1.0 cm (only contact zone) to 3.95 cm demonstrated that the KS and CARV diffusion allowed the inhibition of *Z. bailii*, as illustrated in Table 1. This parameter was well-adjusted to the proposed polynomial model as

observed in Table 2 and as can be seen in the corresponding response surface shown in Fig. 1e. It was determined that only the positive quadratic coefficient of CARV was significant ($p < 0.1$). Therefore, the DI values rose as the CARV concentration increased, reaching the highest DI for the 0.30 KS–1.00 CARV film. The concentration of KS, in the tested range, did not have a significant impact on the DI observed. It is important to remark that, additionally, a control system (a film without antimicrobials) was evaluated as illustrated in Fig. 1, Panel f and it did not exert inhibition of yeast growth, allowing its development even in the contact zone. Manohar et al. [23] observed, using an in vitro assay, the inhibition of the yeast *C. albicans* in the presence of oregano essential oil, where CARV was one of its main components. Such antimicrobial activity was attributed to the inhibition of the formation of germ tubes [23] as well as to the disruption of membrane integrity due to the formation of channels that increased its permeability, permitting the leakage of potassium ions, and other cytoplasmic components [14].

3.6 Optimization of KS-CARV film formulation

In order to select a formulation that can optimize the film desirable characteristics, i.e., mechanical strength, colorless, and appropriate bioavailability of antimicrobials, the desirability function (D) method was used [18]. The parameters used were σ_b, b^*, and DI, which fitted adequately to the proposed model considered. Table 3 summarizes the optimization criteria for each selected response. According to this statistical method, the optimized film is constituted by 0.30 g 100 g^{-1} KS and 0.50 g 100 g^{-1} CARV. The predicted responses from Eq. (1) and the corresponding D values (0.79 to 1.00) observed in Table 3 indicate that the optimized formulation met the individual requirements. In addition, the simultaneous optimization for the three responses led to a $D = 0.89$, value that confirmed the adequacy of the recommended formulation. As can be observed in Table 1, runs 5, 6, and 7 showed a similar formulation to the optimized system. It is important to mention that the former films showed SW values ranging from 28 to 31 g 100 g^{-1}, which are some of the lowest values observed.

3.7 Effectiveness of optimized films as barrier to microbial contamination

With the aim to further investigate the potential protective effect of films, the antimicrobial barrier test was performed against *Z. bailii*, *L. plantarum*, *and P. fluorescens* as target microorganisms. According to the previous optimization, the 0.30KS-0.50CARV films were tested. The control system and the films added only with 0.30 g 100 g^{-1} KS were also evaluated for comparative purposes.

With regard to *Z. bailii*, results are illustrated in Fig. 2, Panel a and show that the control system allowed the yeast to grow from 5 to 9 log CFU/g after storing at 25°C for 48 h. The 0.30 KS films moderately inhibited the microorganism development, without modification of the cell count along 24 h, reaching a 7 log CFU/g yeast population after 48 h of storage. The additional incorporation of 0.50 g 100 g^{-1} CARV produced the total yeast inhibition (count <100 CFU/g) after 24 h of storage. Ben et al. [13] reported a good performance of CARV against *Saccharomyces cerevisiae*; they observed a minimum inhibitory concentration of 0.25 g/L, lower than those of eugenol and menthol, according to the results obtained by means of a dilution assay in a liquid medium. This result was related to the appropriate hydrophobicity of CARV, which allowed it to be accumulated in the cell membrane, disturbing the permeability to certain ions and metabolites, and ultimately leading to cell death.

When the films were tested against *L. plantarum* as seen in Fig. 2, Panel b, it was observed that the cell count had no significant changes during the studied period for the control and the 0.30 KS films (bacteriostatic effect). It had been reported that KS does not exert an important antimicrobial action against the genus *Lactobacillus*, due to the fact that some species are able to metabolize sorbates [19, 24]. The films 0.30 KS–0.50 CARV showed a high inhibitory effect from 2 h of storage, reducing the cell count to <100 UFC/g. According to Si et al. [25], diverse in vitro sensitivity to CARV had been reported for different *Lactobacilli*. For example, *L. plantarum* from pig intestine was informed as more CARV tolerant than *L. acidophilus*.

Table 3. Optimization of edible film responses

	Observed response				
Parameter[a)]	Minimum	Maximum	Optimization criteria	Predicted response[b)]	D[c)]
σ_b (MPa)	0.26	3.5	>0.5	0.47	0.88
b^*	3.63	5.26	<5.0	4.74	1.0
DI (cm)	1.0	3.9	>1.50	1.50	0.79

a) σ_b, stress at break; b^*, yellow CIELab color parameter; DI, diameter of inhibition.
b) Predicted response evaluated from the corresponding second order equation for the optimal formulation.
c) D, individual desirability function value.

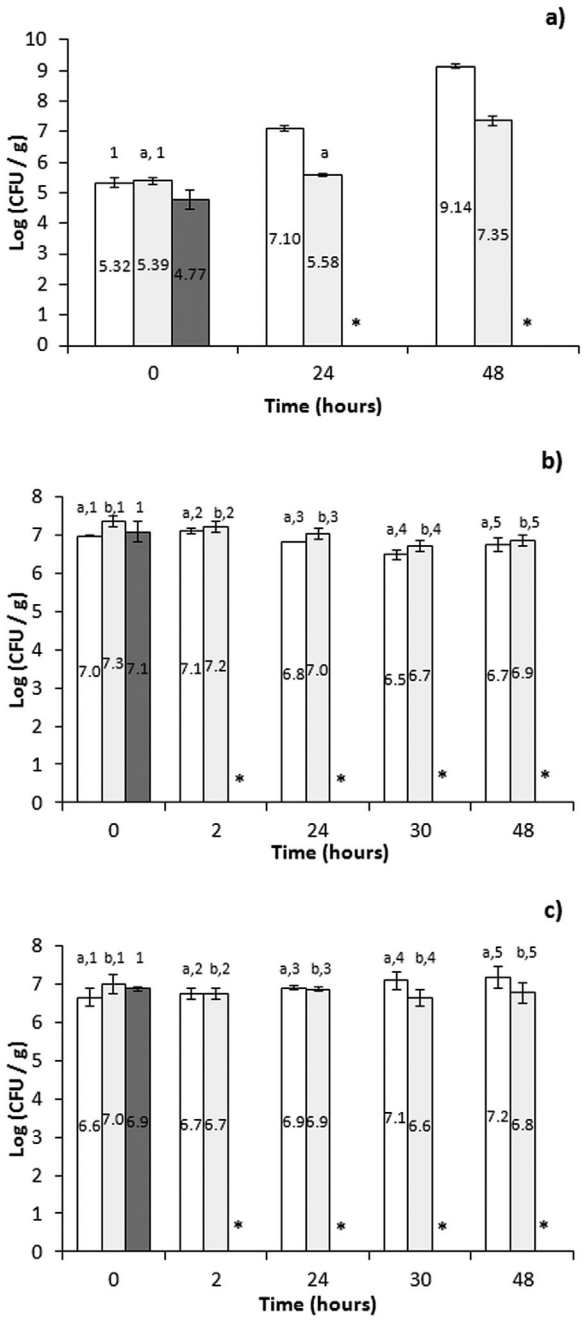

Figure 2. Antimicrobial barrier assay of TS–HPMC–GLY films containing KS and CARV against a) *Z. bailii*, b) *L. plantarum*, and c) *P. fluorescens*. Results are expressed as the log of the colony forming units per gram of film (Log CFU g). White bars: Control, light gray bars: 0.30 g 100 g^{-1} KS, gray bars: 0.30 g 100 g^{-1} KS −0.50 g 100 g^{-1} CARV. *: Indicates cell count <100 CFU/g for 0.30 g 100 g^{-1} KS −0.50 g 100 g^{-1} CARV formulation.

Finally, for *P. fluorescens* observed in Fig. 2, Panel c, a similar behavior to the one reported for *L. plantarum* was registered. According to Arrieta et al. [26], CARV is capable of altering the outer membrane of Gram-negative bacteria, resulting in the disruption of the proton driving force, the flow of electrons. and the active transport. Du et al. [20], applying the inhibition zone test, detected that *E. coli* O157: H7 grew normally at 35°C on agar in contact with apple polysaccharides based films without CARV. By contrast, growth was not observed for films containing 0.75–1.00 g 100 g^{-1} CARV.

3.8 Contact angle

The magnitude of the contact angle is influenced by the relative magnitude of cohesive molecular forces within water drop and adhesive molecular forces between the liquid and the solid [27]. The contact angle can vary between 0 and 180° and a high value is indicative of the surface hydrophobicity, which means high interfacial tension [28]. A contact angle of $(28 \pm 1)°$ for 0.30 KS film and of $(43 \pm 5)°$ for 0.30 KS–0.50 CARV film was then determined. These results could be attributed to the CARV hydrophobic character. Therefore, films containing CARV suffered an interfacial tension increase, and are accordingly, expected to have higher resistance to surface water adsorption than films formulated only with KS. This trend remarks the possibility of overcoming the limitations of the hygroscopicity of TS–HPMC matrices while improving antimicrobial capacity.

4 Conclusions

A RSM procedure was successfully applied to analyze the effect of KS and CARV addition on properties of TS–HPMC–GLY based films. It was established that antimicrobials affected the film yellowness, tensile resistance, and SW, but did not have any influence on WVP. An optimized film formulation containing 0.30 g 100 g^{-1} KS and 0.50 g 100 g^{-1} CARV showed a remarkably improved antimicrobial action against *Z. bailii*, *L. plantarum*, and *P. fluorescens*. These results prove the usefulness of TS in combination with HPMC as macromolecules for the development of edible films with active properties by means of the addition of KS and CARV. These novel materials could be applied, taking into account the properties and the flavor of CARV, as protective coatings for sausages, cheeses, or as separating film (interleaves) between chilled or frozen burgers or sliced meats or cheese, thereby helping to optimize food preservation by means of an emerging and eco-friendly active material.

5 Novelty statement

The obtained results demonstrate the ability of films to act as an antimicrobial hurdle that can contribute to extending the shelf life of food. These novel materials can be applied, taking into account the properties and the flavor of CARV as

protective coatings for sausages, cheeses, or as separating film (interleaves) between chilled or frozen burgers or sliced meats or cheeses, thereby helping to optimize food preservation by means of an emerging and eco-friendly active material.

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