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Water Sensitivity, Antimicrobial, and Physicochemical Analyses of Edible Films Based on HPMC and/or Chitosan

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Several properties of chitosan films associated or not with hydroxypropylmethylcellulose polymer (HPMC) and HPMC films incorporating or not nisin and/or milk fat were studied. Nisin addition at a level of 250 μg mL^{-1} and likewise chitosan at 1% (w/v) concentration were efficient for total inhibiting *Aspergillus niger* and *Kocuria rhizophila* food deterioration microorganisms. HPMC and chitosan films were transparent, whereas nisin and/or fat incorporation induced a 2-fold lightness parameter increase and, consequently, involved more white films. Measurements of tensile strength, as well as ultimate elongation, showed that chitosan and HPMC initial films were elastic and flexible. High thermal treatments and additive incorporation induced less elastic and more plastic films. Water vapor transmission as far as total water desorption rates suggested that chitosan films were slightly sensitive to water. Water transfer was decreased by <60% as compared with other biopolymer films. Regarding its hydrophobic property, the capacity of fat to improve film water barrier was very limited.

KEYWORDS: Carbohydrate edible packaging; polypeptide nisin; polysaccharide chitosan; active coatings

INTRODUCTION

Increasing consumer demands for high-quality and microbiologically safer foods, together with longer product shelf life, are continuously forcing researchers and the industry to develop new food preservative strategies. Because packaging is an integral part of food, another consumer demand concerns biodegradable and environmentally friendly packaging, rather than polyethylene or polypropylene petrochemical materials, which are not edible and not made from renewable natural resources. To respond to this latter demand, the food industry can employ several biodegradable polymer (proteins, polysaccharides, and lipids) based edible films that could potentially serve as packaging materials. For the past 10 years, many research programs have focused on developing more and more sophisticated edible films and coatings. Among them, polysaccharide polymers such as hydroxypropylmethylcellulose (HPMC) and chitosan have been particularly studied (*1*, *2*, *3*). However, the water sensitivity of such packaging materials, which produces a loss of barrier properties or even a solubilization into foods with high water activities, prevents their industrial applications.

Many attempts have thus been made to incorporate hydrophobic surfactants or lipid compounds, such as fatty acids, into biopolymer matrices as composite films to decrease their moisture permeability. Phan et al. (*4*) worked on arabinoxylan-lipid-based films; whereas sucrose ester emulsifiers stabilize the film structure, lipid incorporation tends to give films having a "two-layer-like" structure, which improves significantly water vapor barrier property. In the same way, Villalobos et al. (*3*) made films with HPMC, sorbitan monostearate, and sucrose palmitate. The water vapor permeability of these films was minimal with a hydrocolloid/surfactant ratio of 0.5. Cross-linking with citric acid was also another way to decrease the affinity of natural polymer toward water (*5*). It consists of the formation of covalent bonds between the chains of polymer; cross-linking induces a decrease in the availability of hydroxyl groups, limiting polysaccharide−water interactions by hydrogen bonding. Cross-linking results also in the water insolubility of films and in an improvement of water vapor barrier properties. Heating and γ-irradiation were investigated by Letendre et al. (*6*) to cross-link calcium caseinate−whey protein isolate edible films. Among the excipients added to the biopolymer-based film formulation before cross-linking was induced, glycerol generated the most important losses of film/water interactions. Poor mechanical resistance of biopolymers was also another negative property attributed to biopolymer-based films, with low elastic parameters and a rigid and breakable behavior (*7*).

Several researchers have investigated different formulations and polymer associations to improve such physicochemical properties. Sothornvit et al. (*8*) have obtained a linear dependence between glycerol, sorbitol, or polyethylene glycol plasticizer concentrations and β-lactoglobulin film elongation. Tanabe et al. (*9*) reported that keratin film is very fragile, and 10−30% (w/v) of chitosan addition allowed a strong and flexible film to be obtained. The casein−gelatin film showed signifi-

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cantly greater elongation values as compared to films made from gelatin or casein alone. The casein−gelatin (75:25 w/w) formulation cross-linked with transglutaminase showed also the lowest water vapor permeability values (*10*). With regard to reviews by Cha et al. (*11*), Appendini et al. (*12*), and Ozdemir et al. (*13*), the major applications of biopolymers are antimicrobial packaging. This term encompasses any packaging techniques used to control microbial growth in food products. In the case of chitosan, the antimicrobial activity comes from the polymer itself, directly used as a film-forming entity, leading to antimicrobial activity, especially on the food surface (*14*). Chitosan, composed of β-1,4-linked 2-acetamido-2-deoxyglucopyranose and 2-amino-2-deoxyglucopyranose, is a high molecular weight cationic polysaccharide that exhibits antibacterial and antifungal activity as well as film-forming properties. Chitosan has already become a common food ingredient in Japan, and its official approval is currently pending in Europe, where it has achieved a major breakthrough in dietetics as a fat trap involved in the reduction of the absorption of cholesterol by the human body by 20−30% and as a fiber involved in the modulation of the duration of the intestinal transit. As mentioned by Begin et al. (*15*), chitosan antimicrobial activity comes from its positive charges that would interfere with the negatively charged residues of macromolecules on the cell surface, rendering the membrane leaky. Due to its ability to form active edible or biodegradable films (*16*), chitosan coating can be expected to limit contamination on the food surface. When the polymer itself does not exhibit any antimicrobial activity, another possibility is to incorporate antimicrobial agents. Several molecules were studied, including organic acids such as sorbate, citrate, and acetate (*17*), enzymes such as lysozyme (*18*), chelators such as EDTA (*19*), and bacteriocins such as pediocin and lactacin (*11*). Another bacteriocin used is nisin. Produced by *Lactococcus lactis* subsp. *lactis*, nisin is a 34-residue-long Lantibiotic that contains the unusual amino acid residues dehydrobutyrine, dehydroalanine, lanthionine, and β-methyllanthionine. Nisin was proved to be nontoxic and recognized as safe by the U.S. Food and Drug Administration in 1969. It has widely been used in the food industry as a safe and natural preservative (E234). The antimicrobial activity of films containing nisin is due to the bacteriocin release from the film and its diffusion into food.

The main objectives of this study were to assess the potential of chitosan and/or HPMC (incorporating nisin or not) in view of industrial applications. Mechanical resistances were conducted, and water affinities (water sorption isotherms and water vapor transmission rates) were investigated. The antimicrobial activities were tested especially against *Aspergillus niger* and *Kocuria rhizophila* contaminations. These microorganisms were commonly encountered in food deterioration cases. *K. rhizophila*, which is the new name of *Micrococcus luteus*, is very sensitive to nisin. Therefore, it is generally employed to assay its antimicrobial activity.

MATERIALS AND METHODS

Materials. *Chemicals.* HPMC (Culminal 50, Aqualon) and chitosan (deacetylation degree >90%, low viscosity ranged between 14 and 100 cP, France Chitine), polyethylene glycol 400, sodium hydroxide, Tween 80, ethanol, and citric acid (Sigma-Aldrich), sorption isotherm salts (Chimie-Plus), and 75% (w/w) full-fat milk powders (Kievit) were used. Pure nisin (Aplin and Barrett Ltd.) was dissolved in 0.01 M HCl, pH 2, to obtain a final concentration of 250 μg mL^{-1}and stored at 4 °C.

Microorganisms. Micrococcus luteus, which is named at present *Kocuria rhizophila* (ATCC 9341), had been grown at 30 °C in nutritive broth (Difco) for 18−24 h. The 5 day preculture of *Aspergillus niger* (USMA-ISTAB collection, Bordeaux 1 University) was cultured at 30 °C on Sabouraud agar medium (Difco).

Methods. *Film Formation.* HPMC film-forming solutions were prepared using the procedure described by Coma et al. (*20*), by dissolving 3, 6, 9, or 18 parts of HPMC in 200 parts of a 0.01 M HCl solution, 100 parts of absolute ethanol, and 10% (w/w HPMC) of PEG 400. For active film formation, nisin was dissolved in the HCl part. The nisin concentration in the film-forming solution of active films was 250 μg mL^{-1}. Full-fat milk was added at a concentration of 15% (w/w HPMC). After that, the solution was homogenized for 15 min, degassed for 30 s using a vacuum pump, and plated onto polypropylene support to obtain a final solution height of 1 mm. Films had been dried at 60 °C for 2 h and then peeled. For cross-linked materials, 15% (w/w HPMC) of citric acid was added to film-forming solution prior to 2 h of heating at 60 °C. After that, the films were peeled and heated again at 190 °C for 15 min.

Chitosan film-forming solutions of 1 or 2% (w/v) were obtained by dispersing chitosan in a 0.5% (v/v) aqueous 1 M acetic acid solution. The pH was adjusted to 5.4 with 1 or 10 M NaOH. The preparation was filtered through a 5.3 μm membrane (Millipore, VWR) and degassed for 30 s using a vacuum pump. After that, films were plated as before onto polypropylene support and dried at 60 °C for 2 h.

HPMC/chitosan films were obtained by mixing each film-forming solution of HPMC or chitosan in order to formulate a 1.9% HPMC and 0.1% chitosan final concentration solution. After that, films were plated as before onto polypropylene support and dried at 60 °C for 2 h.

For cross-linked HPMC films with surface adsorbing chitosan, cross-linked HPMC films were kept for 2 h in 1% (w/v) chitosan solution. Films were then removed, washed with distilled water, and dried for 20 min at 60 °C.

Film Physical Properties. Film thickness was measured to the nearest 1 μm (Mitutoyo electronic micrometer).

Film surface color was measured using a Minolta Chromameter CR-310. Absolute measurements are displayed as *Lab* tristimulus values (*L*a*b* color space). *L* is the lightness variable, and the chromatic coordinates *a* (from green to blue) and *b* (from yellow to red) were determined by putting films on black or white surfaces.

The mechanical resistance of films, including tensile strength (TS, Pa) and ultimate elongation (UE, % at break point) were determined with a TAXT2 instrument on 10 films previously stored for 7 days at 23 °C and 0% relative humidity (RH). A/TG tensile grips were used. Films, with an area of 12 mm × 65 mm, were uniaxially stretched at a constant velocity of 3 mm min^{-1} with pre- and post-test speeds of 10 mm min^{-1} for a test distance of 250 mm. The probe height was calibrated at 40 mm. The curves of force versus deformation were computer-recorded.

Film Water Sensitivity. The water vapor transmission rate (WVTR) was evaluated using the Association Française de Normalisation (AFNOR) standardized procedure NF ISO 2528 (2001) purchased from AFNOR, La Plaine Saint-Denis Cedex, France. An aluminum cup containing anhydrous $CaCl_2$ desiccant ($P_{H2O\ vapor} = 0$ Pa) was sealed by the test film (50 cm^2 exchange film area). It was placed in a controlled relative humidity and temperature environment (50 ± 5% RH and 23 ± 1 °C, $P_{H2O\ vapor} = 1585$ Pa). The water vapor transmission rate (g m 24 h m^{-2} Pa^{-1}) was determined from the weight increase of the cup over time at the steady state of transfer using the equation

$$\mathrm{WVTR} = \frac{\Delta m \times \text{film thickness} \times 24}{S \times \Delta P}$$

with Δm = the amount of H_2O vapor passing through a film of area S (m^2) for 24 h. Control cups, without the anhydrous $CaCl_2$ desiccant, were analyzed in parallel.

Total water desorption rate (TWDR) measurements were evaluated. TWDR of agar (3% w/w, Difco) coated with the film was determined in a chamber with a controlled humidity and temperature (50 ± 5% RH and 24 ± 1 °C) for 3 days. The slope of the linear part of the desorption curve (mass loss versus time) gives TWDR, expressed as kg_{water} m^{-2} s^{-1}.

Table 1. Physical Properties of Chitosan and HPMC Films Associated with Nisin and/or Full-Fat Milk (FM)

film	thickness (μm)	color[a]			tensile strength (MPa)	ultimate elongation (%)
		a	*b*	*L*		
chitosan 1% (w/v)	54.1 ± 6.5	0.3 ± 0.0b	0.3 ± 0.0*	90.2 ± 0.3*	18 ± 6	61.5 ± 6.6
chitosan 2% (w/v)	97.8 ± 6.6	0.9 ± 1.1*	28.4 ± 1.1*	87.6 ± 0.8*	7 ± 2	3.5 ± 1.3
HPMC 1% (w/w)	12.9 ± 6.6	4.9 ± 0.0*	2.9 ± 0.3*	96.4 ± 0.3*	11 ± 1	29.3 ± 2.5
HPMC 2% (w/w)	25.8 ± 13.2	5.0 ± 0.0*	2.6 ± 0.2*	96.3 ± 0.5*	44 ± 7	33.8 ± 9.1
HPMC 3% (w/w)	20.4 ± 0.9	–0.2 ± 0.0*	2.2 ± 0.3*	96.4 ± 0.1*	60 ± 1	3.3 ± 0.0
HPMC 6% (w/w)	37.2 ± 2.8	–0.2 ± 0.0*	3.2 ± 0.0*	96.4 ± 0.1*	86 ± 6	9.8 ± 2.1
HPMC 1.9% (w/w)–chitosan 0.1% (w/w)	90.1 ± 8.3	0.8 ± 0.1*	6.6 ± 0.6*	95.2 ± 0.5*	7 ± 2	60.9 ± 5
HPMC 2% (w/w) cross-linked–chitosan[b]	102.1 ± 10.3	0.3 ± 0.1*	14.5 ± 0.7*	90.5 ± 0.6*	3 ± 0	11.6 ± 4.2
HPMC 3% (w/w)–nisin 250 mg mL^{-1}	30.5 ± 0.7	–2.0 ± 0.2**	–8.7 ± 0.4**	67.5 ± 1.5**	35 ± 3	3.1 ± 0.7
HPMC 3% (w/w)–fat 15% (w/w HPMC)	39.7 ± 1.6	–1.9 ± 0.1**	–6.4 ± 0.6**	60.4 ± 12.7**	26 ± 4	3.0 ± 0.6
HPMC 3% (w/w)–nisin 250 μg mL^{-1}–fat 15% (w/w HPMC)	41.0 ± 1.8	–1.7 ± 0.2**	–6.2 ± 0.4**	70.7 ± 1.3**	22 ± 3	1.9 ± 0.3

[a] *, chromatic coordinates *a*, *b*, and *L* determined by putting films on a white surface ($a = -3.8$, $b = 5.1$, $L = 97.5$); **, chromatic coordinates *a*, *b*, and *L* determined by putting films on a black surface ($a = -2.6$, $b = 0.2$, $L = 31.3$). [b] Adsorption of chitosan on cross-linked film with citric acid (15% w/w HPMC).

Sorption Isotherms. Aluminum dishes containing about 0.10 g of dried films were weighed to the nearest 0.1 mg. The dishes were stored in sealed glass jars containing saturated salt solutions to give different water activities (a_w): KOH, $KC_2H_3O_2$, K_2CO_3, $CuCl_2$, NaCl, and KCl, respectively, for 0.10, 0.22, 0.44, 0.68, 0.76, and 0.85 a_w at 25 ± 1 °C. The weight of moisture equilibrated samples was determined at the steady state after a minimum of 1 month. A number of sorption isotherm models have been reported in the literature. According to Srinivasa et al. (*21*) and Perez-Alonso et al. (*22*), the Guggenheim−Anderson−deBoer (GAB) model showed a better fit for films compared to other models and is applicable to a wide range of water activity values. Therefore, our experimental moisture sorption isotherm values were averaged and fitted by this GAB model (*1*)

$$X = \frac{w_s c k a_w}{(1 - k a_w)(1 - k a_w + c k a_w)} \quad (1)$$

where X is the weight (g) of sorbed water per 100 g of polymer, a_w is the water activity, w_s is the weight of water (g) in a complete monolayer per 100 g of polymer, c and k are constants related to the heat of sorption. A macro using the GAB equation was elaborated using Excel software. Linear and nonlinear least-squares regression analyses were used to estimate c, k, and w_s. To evaluate the ability of the GAB model to fit experimental data, the coefficients of determination (r^2) were determined using eq 2

$$r^2 = \frac{\sum_i^n (x_{pi} - \bar{x}_i)^2}{\sum_i^n (x_i - \bar{x}_i)^2} \quad (2)$$

where x_i and x_{pi} are experimental and predicted equilibrium moisture contents, respectively, and n is the number of experimental points.

Film Antimicrobial Activity. Anti-A. niger Effectiveness. Dishes containing 5-day cultures of *A. niger* (performed at 30 °C on Sabouraud agar medium) were used to recover fungal spores. This was obtained by pouring 9 mL of sterile physiological water containing 0.1% (v/v) Tween 80 on the agar plate surface. It was followed by a gentle scraping using a sterile rake to try to remove a maximum of spores. After that, the spore suspension was transferred into sterile tubes. The number of spores present in the suspension was measured using a hemocytometer (Malassez) and an optical microscope (Zeiss, magnification ×40). Spore charge or density was expressed as number of spores per milliliter (spores mL^{-1}) and was the mean of at least 10 numerations.

After that, the *A. niger* suspension was serially diluted to fix the number of spores to approximately 100, 1000, or 10000 spores mL^{-1}. Volumes of 0.1 mL from these suspensions were used to inoculate sterile Sabouraud agar medium dishes. These dishes had been dried in a flow hood at room temperature for 30 min (Faster BH 2004, Grosseron).

Finally, the active films were deposited on the surface of the inoculated Sabouraud agar. Dishes were incubated at 30 °C. Visual observations and numerations were performed after different incubation times ranging between 5 and 10 days. Growth controls (inoculated Sabouraud agar dishes without any film deposition) were conducted in parallel to ensure that viable organisms were present. Spores in control plates were counted, and the percentage of inhibition was calculated as follows:

$$\text{inhibition (\%)} = \frac{\text{spore number in control plate} - \text{spore number in assays}}{\text{spore number in control plate}} \times 100$$

Anti-K. rhizophila Effectiveness. An inhibition zone assay was conducted by pouring into a sterile Petri dish exactly 25 mL of nutritive broth in liquid state (50 °C). The nutritive broth was initially prepared by adding 12 g L^{-1} of agar. Also, before pouring, the medium was inoculated with an overnight culture of the test strain at a level of 0.1% (v/v). The Petri dishes were then left at room temperature until total solidification. After that, 5 mm diameter disks were cut from the different test films and placed onto the inoculated agar surface. The dishes had been refrigerated at 4 °C for 45 min3/4 h to allow both antimicrobial molecule desorption from the film and diffusion inside the medium while microorganism growth had been limited. After that, the dishes werehad been incubated at 30 °C for 24 or 48 h. Data are expressed as inhibitor zone diameter (millimeters) and measured to within about 1 mm.

Statistical Treatment. All experiments were replicated at least three times. Treatment means were tested for significant differences using the Student's t test at 95% probability ($p < 0.05$) using software Splus 2000 (Mathsoft, Seattle, WA).

RESULTS AND DISCUSSION

Film Physical Properties. *Film thickness* depended very much on the film nature and composition (**Table 1**). A relationship between the film thickness and the film-forming polymer or additives content could be observed: the higher the percentage of chitosan or HMPC was, the higher the film thickness was, and in the same way, the level of additive incorporation tends to increase film thickness. An average increasing film thickness order could then be obtained: HPMC films < composite HPMC films < chitosan films < cross-linked films. Such thickness differences were also measured in previous works dealing with similar films, where a 2-fold increase of film thickness was obtained when 15% (w/w HPMC) stearic

acid was added to 3% (w/w) HPMC film (*7*). Moreover, standard errors were small, which suggested good film homogeneity, except for cross-linked films, for which high-temperature treatment seemed to deteriorate film and enhance its heterogeneity.

Film Color and Transparency. The tristimuli *a*, *b*, and *L* values were measured (**Table 1**). Although *a* and *b* values were significantly different in some cases ($p < 0.05$), these chromatic parameters were not relevant because films became white. Whereas HPMC and chitosan films were totally transparent, nisin and fat incorporation induced a 2-fold *L* parameter increase, which indicated that films were whiter. However, this white color development was limited because films kept a sufficient transparency, which is essential for some consumers eager to see foods through the packaging before any purchase. Yang et al. (*23*) obtained similar results with stearic−palmitic acid addition to gellan films. The film opacity increased as the lipid concentration increased. This phenomenon is probably due to light scattering from lipid droplets, distributed throughout the film network after formation.

Film Mechanical Properties. The suitable use of such biopolymer films, that is, their capacity in protecting food integrity, was investigated. Tensile strength (TS) and ultimate elongation (UE) were measured, and the results are summarized in **Table 1**. For chitosan, increasing biopolymer content resulted in reducing film mechanical resistance and film elastic deformation and flexibility, suggesting that chitosan addition made a film stiffer. According to these first results, only 1% (w/v) chitosan film was kept for further analysis.

For HPMC films, depending on the biopolymer content, the trends for UE values were the opposite of those of TS. High HMPC concentrations result in TS value increase and UE value decrease, suggesting less elastic and more plastic HPMC films (**Table 1**). These properties are largely dependent on the distribution and intensity of inter- and intramolecular interactions. In this case, increasing HPMC molecules intensified such interactions and therefore induced very strong behavior with low possibilities of movements. From 1 to 6% (w/w) HPMC, TS and UE increased 8-fold and decreased 3-fold, respectively. According to these second results, only 2 and 3% (w/w) HPMC films, with intermediate responses, were kept for further analysis. In addition, chitosan−HPMC association gave more fragile and elastic films, compared to 2% HPMC films. This result suggested that chitosan addition gave to HPMC film more elastic properties, as mentioned by Tanabe et al. (*9*), who made similar observations when chitosan was added to keratin film. For these authors, this phenomenon was particularly true for low chitosan content addition [<5% (w/w) of keratin]. The 2% cross-linked HPMC film presented a dramatically reduced film resistance compared to the non-cross-linked 2% HPMC film. TS and UE decreased up to 93 and 65%, respectively. This result was not so surprising. Cross-linking uses very high thermal treatment for covalent bond formation. This induced a negative impact on mechanical resistance (*5*). Rioux et al. (*24*) proposed that the loss of film resistance resulted from the high heterogeneity of spacing between cross-links, leading to the appearance of new breaking points. On the contrary, when enzymes, such as transglutaminase, and lower temperatures (of about 50 °C) were used to cross-link, greater elongation values were obtained (*10*).

In nisin or fat addition incorporation cases, TS was not significantly affected, whereas UE was dramatically reduced (10-fold reduction) (**Table 1**). Cha et al. (*25*) reported that resistance of films with antimicrobial agents (nisin, lysozyme, or grapefruit seed extract) was weaker than that of films without antimicrobial compounds. Loss in mechanical resistance may be attributed to the breaking up of the film network caused by the addition of antimicrobial agents. The negative effect of the lipids on mechanical properties may also have resulted from the partial replacement of the polymer by lipids in film matrix, creating discontinuities within the HPMC networks, favoring film disruption (*26*).

Film Water Sensitivity. One of the most important drawbacks of biopolymer-based films is their high water sensitivity. In food applications, the level of such water sensitivity may influence the film applicability's. Three methods are commonly employed to study water/film interactions: (i) the transmission rate and (ii) the sorption isotherms when water in the vapor state is investigated and (iii) the desorption rate for water in the liquid state. Depending on the surrounding air, Kim et al. (*27*) have mentioned that sorption isotherms are a useful way to estimate film barrier and other properties. Also, the first step of WVTR corresponds to the sorption of water molecules on the specific hydrophilic groups of the amorphous phase. Afterward, water permeability depends on the swelling capacity of the polymer. Strong water vapor sorption consequently induces high WVTR. In the case of the desorption rate experiment, intimate contact between film and agar broth was set to create conditions close to those of food applications.

The *sorption isotherms* were carried out at 24 °C and 50% RH, which correspond to conditions encountered during food storage. The results are summarized in **Figure 1** and **Table 2**. For all films, the coefficient of determination was up to 0.97, so we can consider that experimental data are well fitted by the GAB model.

Before $a_w = 0.6$, the moisture content of the films increased slowly. After $a_w = 0.6$, small increases in humidity led to large mass gains, suggesting a swelling phenomenon as water activity increased (*28*).

The value of the monolayer (w_s) is of particular interest, as it indicates the amount of water that is strongly adsorbed to specific sites and is considered as the optimum value at which film is most stable. Our results showed for all biopolymer films a value of 4 g/100 g, which is close to those obtained by Velazquez et al. (*29*) for methylcellulose and ethylcellulose films and by Villalobos et al. (*3*) for HPMC films (2.7−5.1 g/100 g). The constant *c* related to water/substrate interaction energy was higher for low HPMC concentrations, so 2% HPMC films were more hydrophilic and water molecules were adsorbed with less energy in the active site (*3*). On the other hand, chitosan and HPMC−nisin−fat films presented the lowest values of *c* and the highest values of *k*. The values for chitosan were similar to those obtained by Srinivasa et al. (*21*) for chitosan films ($c = 0.08$ and $k = 0.835$).

The form of the curves was similar to those observed elsewhere for biopolymer-based films. This type of isotherm is characteristic of molecules such as sugars and proteins (*30*). In the domain of high a_w, where differences were more significant, an order in increasing water sensitivity could be established: cross-linked film < HPMC and HPMC−chitosan < chitosan < composite HPMC films with nisin. The lower percentage of sorbed water was thus obtained for cross-linked membrane, attributable to a real reduction of free OH of HPMC by covalent reaction with carboxylic acid groups of citric acid. The capacity of chitosan polymer to bind moisture was higher than that of HPMC, whereas chitosan addition into the HPMC matrix resulted in a limitation of film/water interactions. Differences obtained could be due to modifications in HPMC/HPMC intra-

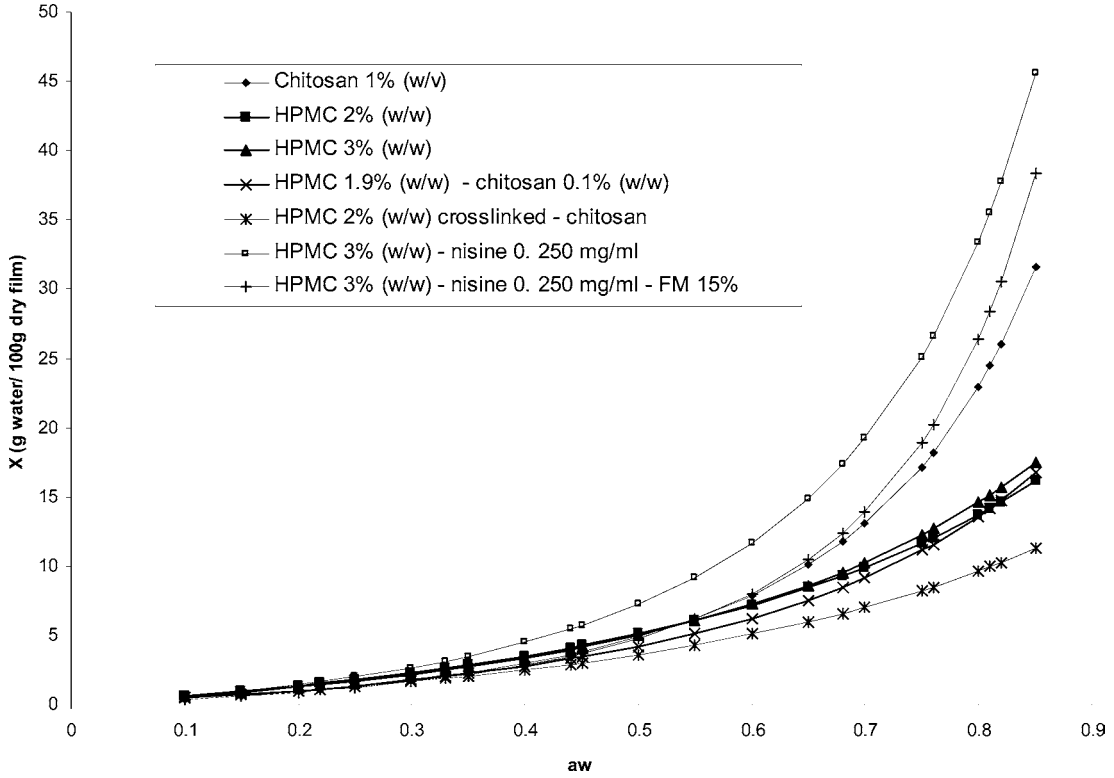

Figure 1. Water sorption isotherms of HPMC and chitosan films performed at 24 °C and 50% RH. Experimental moisture sorption isotherm values were averaged and fitted by the GAB model.

Table 2. Parameter Values of the GAB Model Fitted to Various Films in the Water Activity Range of 0.10–0.85 ($n = 6$)

	c	k	ws	r^2
chitosan 1% (w/v)	0.010	0.828	3.99	0.98
HPMC 2% (w/w)	0.023	0.562	4.00	0.98
HPMC 3% (w/w)	0.019	0.611	3.99	0.99
HPMC 1.9% (w/w)–chitosan 0.1% (w/w)	0.014	0.669	4.00	0.97
HPMC 2% (w/w) cross-linked–chitosan	0.017	0.551	4.00	0.99
HPMC 3% (w/w)–nisin 250 μg mL^{-1}	0.015	0.821	4.00	0.99
HPMC 3% (w/w)–nisin 250 μg mL^{-1}–15% (w/w HPMC) fat milk	0.009	0.876	3.99	0.98

and interchain interactions and organization by chitosan capacity in intercalating. Composite films made from nisin and/or milk fat addition prior to film formation exhibited the highest moisture-binding capacity. Concerning nisin, a small decrease of film hydrophilic property could be the result of the additional hydrophilic groups (−OH and −COOH) introduced by this bacteriocin. As far as fat is concerned, the results obtained were all the more unexpected. However, it is necessary to know that HPMC films incorporating fat were very hard to elaborate because of the difficulties in fat solubility and homogenization.

Water vapor transmission rates were then conducted, and the corresponding results are presented in **Table 3**. Water permeation through films was the sum of three phenomena: (i) sorption already investigated with isotherms, (ii) water diffusion inside the polymer, and (iii) water desorption. The WVTR was a way to get further information both on the film network and on its impact on water transfer. Measurements of WVTR for both HPMC plus nisin and HPMC plus nisin and fat were in total accordance with water sorption isotherms. Measurements of WVTR for films made from HPMC with or without chitosan were similar and not statistically different ($p < 0.05$). When isotherms showed a limited water affinity for cross-linked films, results of WVTR did not show any improvement in the water barrier. This was in accordance with previous studies conducted on the moisture transfer through cross-linked films, which suggested a weak improvement of moisture barrier properties with the chemical modification extent (*31*). According to McHugh et al. (*32*), the structuring of polymer inside film matrix significantly affects the water vapor transfer property. Differences in WVTR values were reported by Chambi et al. (*10*), who introduced cross-linkages. When cross-linking induced more flexible films with increases in elongation properties, the chain mobility was modified and the water diffusion coefficient was increased. Our results, which were opposite those reported by these authors, canceled that hypothesis. However, for these same authors, the new cross-linked conformation acquired, with probably large pores and an open network, may favor the water diffusion. The most important reduction in water permeability was obtained for chitosan film. The water vapor transmission rate was, for this film, a 5−14 times better water barrier compared to the other biopolymer packaging.

Therefore, further experiments were also conducted to confirm moisture barrier properties in conditions close to food applications. An agar gel, used as a high water activity food model, was coated with biopolymer films. *The total water desorption rate* (TWDR) was then determined by evaluating the water loss during the chosen storage conditions. The results are presented in **Table 3**. The TWDR values obtained from our experiments [$(\sim 2-5) \times 10^{-6}$ kg m^{-2} s^{-1}] were comparable to those determined by Desobry et al. (*33*) in the same conditions. The small differences could be due to the experimental arrangement; in fact, the authors put their samples in a chamber with circulating air, which increased water evaporation on the gel surface. TWDR increased as the percentage of HPMC increased. For composite films, TWDR corroborated the results obtained everywhere else using isotherms or WVTR methods. Chitosan appeared, thus, to be efficient in reducing water

Table 3. Water Affinity of HPMC and Chitosan Films

film	WVTR[a] (g m^{-2} 24 h Pa^{-1} m) × 10^6	TWDR (kg m^{-2} s^{-1}) × 10^6
chitosan 1% (w/v)	1.2 ± 0.1 a	2.0
HPMC 2% (w/w)	5.5 ± 1.0 b	2.5
HPMC 3% (w/w)	4.9 ± 0.2 b	5.7
HPMC 1.9% (w/w)–chitosan 0.1% (w/w)	4.1 ± 0.9 b	1.9
HPMC 2%(w/w) cross-linked–chitosan[b]	9.7 ± 0.4 c	
HPMC 3% (w/w)–nisin 250 μg mL^{-1}	9.6 ± 0.4 c	5.6
HPMC 3% (w/w)–nisin 250 μg mL^{-1}–fat milk 15% (w/w HPMC)	13.7 ± 0.1 d	5.5

[a] Different letters indicate significantly different groups determined by Student's *t* test ($p < 0.05$). [b] Adsorption of chitosan on cross-linked film with citric acid (15% w/w HPMC).

Table 4. Effect of Different Chitosan Films on *A. niger* Previously Inoculated on Sabouraud Agar Medium at Different Inoculation Rates from 100 to 10000 Spores and Incubated 5 Days at 30 °C[a]

film	inoculation rate (spores per Petri dish)	control plates (spores mL^{-1})	inhibition (%)
chitosan 1% (w/v)	100	8738 ± 4	100 ± 0
HPMC 1.9% (w/w)–chitosan 0.1% (w/w)	10000	551833 ± 6	100 ± 0
HPMC 2% (w/w) cross-linked–chitosan[b]	1000	7620 ± 3	98 ± 0

[a] Values represent the means and standard deviations from at least three experiments. [b] Adsorption of chitosan on cross-linked film with citric acid (15% w/w HPMC).

permeability. Degrees of water transfer were decreased by >60% as compared with films without chitosan.

Film Antimicrobial Activity. Chitosan incorporation into the film matrix and chitosan adsorption onto the film surface were two ways that have been tested to obtain antimicrobial films. *Anti-A. niger activity* data are presented in **Table 4**. Control plates (without chitosan) had increasing spores amounts after 5 days of incubation as the inoculation rate increased from 100 to 10000 spores. No relationship between initial (inoculation rate) and final (after incubation) spore amounts was observed. Very low standard deviations were also observed, which indicates a good repeatability. Whatever the film was, *A. niger* was completely inhibited, indicating that both methodologies for chitosan film formation offered a great advantage in preventing *A. niger* growth. Chitosan adsorption on spore surface induced probably a reduction in nutriment exchange, which caused microorganism death. Planscencia-Jatomea et al. (*34*) obtained a strong inhibition of *A. niger* spore germination (73%) on Czapeck agar medium supplemented with 30 g L^{-1} of chitosan. Using scanning electron microscopy, these authors showed that chitosan produced spore aggregation and morphological anomalies affecting germ tube emergence. When chitosan was used as an edible film, Jeon et al. (*14*) obtained a significant decrease in oxygen exchange, which could induce a low oxygen availability, leading in our case to a limitation of aerobic *A. niger* spore germination. Previous studies gave similar results, and total inhibition of *A. niger* growth was obtained (*35*). In this previous study, the effect of chitosan on the physiology of the fungal strain was investigated by epifluorescence analysis on spores after staining with acridin orange. According to McFeters et al. (*36*), the outcome of the acridin orange staining reaction can predict physiological activity, and it is possible to distinguish a lag and a stationary phase microorganism on the basis of color. When chitosan was incorporated, 100% green cell percentage was observed, indicating that the spores were in a stationary phase, with a high amount of DNA instead of RNA, which suggested a very low metabolic activity, probably due in our case to chitosan inhibitory activity.

For HPMC films incorporating nisin, *anti-K. rhizophila activity* was measured by the inhibition zone assay, and the data are presented in **Table 5**. Only one nisin concentration was tested in films with or without fat. Whereas nisin-free film did not exhibit any antimicrobial activity, films incorporating 250 μg mL^{-1} nisin showed a great activity. However, a 3-fold reduction of their antimicrobial activity was observed when 15% fat was added to HPMC before film formation. In a previous study, similar results were obtained when stearic acid was added to HPMC film: the increasing stearic acid content of a film resulted in a decrease of antimicrobial activity of the film against tested bacteria (*20*). Jung et al. (*37*) reported similar effects on nisin activity in systems containing high fat levels and specified in their conditions that nisin activity decreased by about 88% in the presence of 13% fat. Dean et al. (*38*), who used nisin to inhibit *L. monocytogenes* in ice cream, reported that nisin exhibited a greater effect in 3% than in 10% fat ice cream.

Table 5. Effect of Different HPMC Films (Diameter = 5 mm) on *K. rhizophila* Previously Inoculated on Nutrient Agar Medium (0.1% v/v) and Incubated for 24 h at 30 °C[a]

film	growth inhibition diameter (mm)
HPMC 3% (w/w)	0 ± 0
HPMC 3% (w/w)–nisin 250 μg mL^{-1}	14.4 ± 0.7
HPMC 3% (w/w)–FM[b] 15% (w/w HPMC)	0 ± 0
HPMC 3% (w/w)–nisin 250 μg mL^{-1}–FM[b] 15% (w/w HPMC)	4.9 ± 1.9

[a] Values represent the means and standard deviations from at least three experiments. [b] Full-fat milk.

In conclusion, this study proved that film formulation and formation had a significant impact on the physical, chemical, and biological properties of films. Interestingly, chitosan film associated or not with HPMC seemed to give satisfying results for both physicochemical and antimicrobial properties. Further experiments with more chitosan/HPMC part variations should be conducted to analyze their impact on film properties. Electron microscopy analysis will also be used to provide a better understanding of the phenomena underlying the barrier properties observed.

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