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# Edible Bioactive Fatty Acid−Cellulosic Derivative Composites Used in Food-Packaging Applications

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To develop biodegradable packaging that both acts as a moisture barrier and as antimicrobial activity, nisin and stearic acid were incorporated into a hydroxy propyl methyl cellulose (HPMC) based film. Fifteen percent (w/w HPMC) of stearic acid improved film moisture barrier. However, film mechanical resistance and film antimicrobial activity on *Listeria monocytogenes* and *Staphylococcus aureus* pathogenic strains were both reduced. This lower film inhibitory activity was due to interactions between nisin and stearic acid. The molecular interaction was modeled, and an equation was developed to calculate the nisin concentration needed to be incorporated into the film matrix to obtain a desired residual antimicrobial activity. Because the molecular interactions were pH dependent, the impact of the pH of the film-forming solution on film inhibitory activity was investigated. Adjusting the pH to 3 totally avoided stearic acid and nisin interaction, inducing a high film inhibitory activity.

**KEYWORDS: Carbohydrate edible packaging; polypeptide nisin; fatty acid; electrostatic interactions**

## INTRODUCTION

Active packaging is an innovative food-packaging concept, especially antimicrobial films or coatings, that has been introduced as a response to microbial safety consumer demands. To control undesirable microorganisms on food surfaces, antimicrobial agents can be incorporated into a polymer matrix; bioactivity is based on the release of antimicrobial entities. Several compounds have been proposed for antimicrobial activity in food packaging (*1*), including organic acids (*2*), enzymes such as lysozyme (*3*), and fungicides such as benomyl (*4*) and imazalil (*5*). Another compound that exhibits antimicrobial effects, especially on *Listeria monocytogenes* pathogenic strain, is bacteriocin (*6*−*9*). To control undesirable microorganisms on food surfaces, antimicrobial agents such as nisin can be incorporated in the packaging matrix (*10*, *11*). Moreover, much effort has been made in recent years to develop biodegradable or edible materials. Cellulosic derivatives such as hydroxy propyl methyl cellulose (HPMC) are promising raw materials for edible coatings or films associated with antimicrobial entities. However, cellulosic films are poor water vapor barriers because of the inherent hydrophilicity of polysaccharides. The incorporation of lipid compounds such as fatty acid to a cellulosic ether matrix as a composite film decreases the moisture transfer due to their hydrophobic properties (*10*). To develop bioactive environmentally friendly packaging materials, edible films were obtained from HPMC associated with nisin, in which the bioactivity was based on the release of nisin previously incorporated into the film-forming solution. Such films are preferentially adapted to solid foods such as cheese and meat. This paper reports on the physical properties of HMPC composite films prepared with stearic acid and on the incidence of fatty acid incorporation on the antilisterial activity of films.

## MATERIALS AND METHODS

**Materials.** *Compounds:* HPMC (Culminal 50, Aqualon, Alizay, France), polyethylene glycol 400 (PEG Merck, Fontenay-sous-Bois, France), and stearic acid (4751, Sigma Chemical Co., St. Quentin Fallavier, France), were used.

**Organisms and Maintenance.** *Micrococcus luteus* IP 270 (Institut Pasteur, Paris, France), was grown at 30 °C in nutritive broth (Difco 3178, Detroit, Michigan), whereas *Staphylococcus aureus* IP 58156 and *Listeria monocytogenes* ATCC 15313 were grown in tryptose broth (Difco 62176) at 37 °C in shaken flasks and agitated at 140−160 rpm for 18−24 h.

**Nisin.** Pure nisin (Aplin and Barrett Ltd., Dorset, UK), was dissolved in 0.05 M phosphate buffer (pH 6.1) and stored at 4 °C. Nisin concentration is expressed in international units per milliliter ($IU \cdot mL^{-1}$); 1 $\mu$g corresponds to 40 IU.

**Methods.** *1. Film Preparation. a. Film without Stearic Acid.* Film-forming solutions were prepared using the procedure described by Kamper and Fennema (*12*) and adapted by Coma et al. (*10*), by dissolving 9 parts of HPMC in 200 parts of distilled water, 100 parts of absolute ethanol, and 1 part of PEG 400. Nisin was presolubilized in phosphate buffer (pH 6.1), and the solution was added to a water/alcohol mixture prior to HPMC incorporation. The nisin concentration was adjusted by taking into account the dilution effect. The film-forming

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solution was homogenized, degassed, and poured onto glass plates at a thickness of 1 mm. The plates were heated at 60 °C for 2 h at ambient relative humidity and then cooled, and the films were peeled from the plates.

*b. Film with Stearic Acid.* Fifteen percent (w/w HPMC) of stearic acid was added to the film-forming solution prior to film formation. The film-forming solution was heated to 72 °C to dissolve stearic acid, homogenized for 15 min, and then poured as above.

*2. Film Water Affinity. a. Sorption Isotherms.* Aluminum dishes containing ~0.20 g of dried films were weighed to the nearest 0.0001 g. The dishes were stored in sealed glass jars containing saturated salt solutions to give different water activities: $LiCl \cdot H_2O$, $CH_3COOK \cdot 1.5H_2O$, $Mg(Cl)_2$, $K_2CO_3$, $Mg(NO_3)_2 \cdot 6H_2O$, $NaNO_3$, NaCl, KCl, and $KHSO_4$, respectively, for 0.12, 0.22, 0.33, 0.43, 0.53, 0.65, 0.75, 0.85, and 0.97 $a_w$ at 25 ± 1 °C. Weights of moisture-equilibrated samples were determined after all films had been dried at 103 °C for 2 h. The experiment was triplicated, and the experimental moisture sorption isotherm values were averaged and fitted by the Guggenhein−Anderson−DeBoer (GAB) model, usually used to describe water sorption in hydrophilic food products. The GAB model equation (*13*) is

$$x = \frac{x_m c k a_w}{(1 - k a_w)(1 - k a_w + c k a_w)}$$

with $x$ = moisture content (g of water/g of dried film), $a_w$ = water activity, $x_m$ = monolayer moisture content (g of water/g of dried film), $c$ = constant related to thermal effect, and $k$ = GAB coefficient.

*b. Water Vapor Transmission Rate (WVTR).* The WVTR of edible films was evaluated using the AFNOR standardized procedure (NF ISO 2528, 2001). An aluminum cup containing anhydrous $CaCl_2$ desiccant was sealed by the test film (50 $cm^2$ exchange film area) with paraffin wax at 50 °C. It was placed in an environment of controlled relative humidity (RH) and temperature (50 ± 5% RH and 23 ± 1 °C). The WVTR ($g \cdot m^{-2} \cdot day^{-1}$) for a given film thickness and 760 mmHg as atmospheric pressure was determined from the weight increase of the cup over time at the steady state of transfer using the equation

$$\mathrm{WVTR} = \frac{\Delta m \times 24}{\Delta t \times S}$$

with $\Delta m$ = amount of $H_2O$ vapor passing through a film of area $S$ ($m^2$) during time $\Delta t$ (h). Control cups, without the anhydrous $CaCl_2$ desiccant, were conducted in parallel. All tests were performed in triplicate.

*c. Goniometer.* A water droplet was deposited on the film surface, which was chosen to be totally smooth with a roughness coefficient close to zero. The $\theta$ angle at the water/film interface was measured to the nearest degree as soon as the water droplet was deposited (Krüss instrument). $\theta$ varies from 0° for hydrophilic to 90° for hydrophobic film nature. Five measurements on each film were performed at random positions.

*3. Film Characterization. a. Film Thickness.* Film thickness was measured to the nearest 1 $\mu$m (Mitutoyo electronic micrometer). Ten measurements were performed at random positions.

*b. Film Color.* Film surface color was performed using a Minolta Chromameter CR-310. The instrument used a wide-area illumination and a 0° viewing angle, with a 50 mm diameter measuring area. Absolute measurements are displayed as *Lab* tristimulus values (*L*a*b* color space). Contrary to the *Yxy*, the *Lab* color system was preferred because it closely represents human sensitivity. *L* is the lightness variable. *a* and *b* are the chromatic coordinates. Ten measurements were performed at random positions, and *L*, *a* (from green to blue), and *b* (from yellow to red) were determined by putting films on a black surface. Black support was used to better characterize film whiteness induced by stearic acid addition.

*c. Mechanical Properties.* The mechanical resistance of films, including tensile strength (TS, Pa), ultimate elongation (UE, percent at break point), and Young's modulus (*Y*, Pa), were performed (Amadel Lhomargy instrument) according to AFNOR NF ISO 527-3 (1995) on 10 films previously stored for 7 days at 23 ± 1 °C and 50 ± 5% RH. Films, with an area of 25 mm × 60 mm, were uniaxially stretched at a constant velocity of 3 mm/min. Curves representing force versus deformation were computer-recorded.

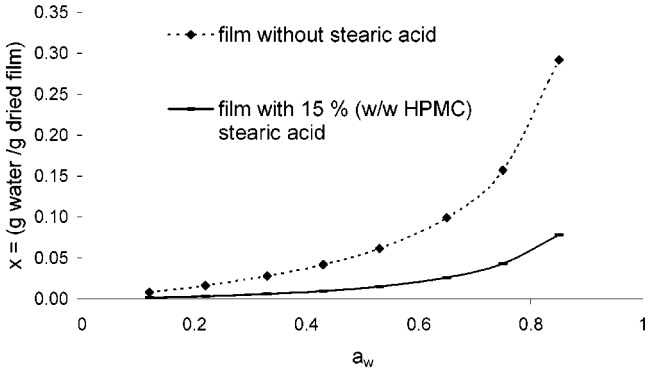


**Figure 1.** Water sorption isotherms of free fatty acid and 15% stearic acid (w/w HPMC) films. Experimental moisture sorption isotherm values (means of three experiments) were averaged and fitted by the GAB model.

*d. Film Roughness and Film Air Permeance.* The Bendtsen tester (Lorentzen and Wettre) was used to measure film air permeance and roughness, according to AFNOR standardized procedures, respectively NF Q03-076 (1986) and NF Q03-049 (1972). The roughness is defined as the air volume (mL) which, under a defined differential pressure (2 bar), flows out per time unit ($mL \cdot min^{-1}$) between the surface of the film and a surface reference. Otherwise, air permeance through a 10 $cm^2$ film area is done in $\mu m^3 \cdot \mu m^{-2} \cdot Pa^{-1} \cdot s^{-1}$. Ten measurements were performed at random positions.

*4. Antimicrobial Effectiveness of Edible Films.* An inhibition zone assay was conducted by inoculating the agar (12 g/L) tryptose broth medium with an overnight culture of the test strain (*10*). For free nisin tests, 70 $\mu$L of the nisin solution was poured into wells (5−6 mm diameter) previously cut into agar medium. For films, 5 mm diameter disks were cut from different test films and placed on appropriately inoculated agar in Petri dishes. Dishes were then refrigerated at 4 °C for 4 h and incubated at 30 °C for 24/48 h. Data, triplicated, are expressed as inhibitor zone diameter (mm) and measured at the nearest 1 mm.

*5. Nisin−Stearic Acid Interaction.* Different stearic acid percentages were mixed with several nisin concentrations in phosphate buffer (pH 6.1) (*14*). The emulsion was stirred at 500 rpm for 10 min at 80 °C, cooled, and filtered through a 0.22 $\mu$m filter (Millex GV, Millipore) before being analyzed. Residual nisin was quantified using a BCA protein microassay kit at 562 nm (Pierce, Rockford, IL). The content of hydrophobic compound added to 40 mL nisin solutions was expressed in percentage of HPMC (w/w). The experiment was triplicated. The percentage of fixed nisin was calculated as follows:

$$\text{Fixed nisin (\%)} = \frac{\text{initial nisin} - \text{residual nisin}}{\text{initial nisin}} \times 100$$

## RESULTS AND DISCUSSION

The hydrophilic nature of the HPMC matrix explained the poor water vapor barrier of such cellulose derivative based films. Previous studies showed that stearic acid could be used to reduce the film WVTR. Fifteen percent of stearic acid (w/w HPMC) was selected by taking into account that >15% of $C_{18:0}$ induced high film opacity and nonhomogeneous structure (*10*, *11*). Moreover, a previous study (*10*), supported by Dean and Zottola works (*6*), showed that high lipid concentration decreased bacteriocin antimicrobial activity.

Film thickness was first checked. Stearic acid incorporation weakly increased film thickness, but the difference was not statistically significant ($p < 0.05$). The average film thickness was ~30−40 $\mu$m.

**1. Film Water Affinity.** *a. Water Sorption Isotherms.* **Figure 1** shows experimental data and sorption isotherm curves

**Table 1.** Water Affinity, Characterization, Mechanical Properties, and Air Barrier of Free Fatty Acid and 15% (w/w HPMC) Stearic Acid Films[a]

| | film without stearic acid | film with 15% stearic acid |
|---|---|---|
| thickness ($\mu$m) | 26 ± 12 | 44 ± 9 |
| WVTR ($g \cdot m^{-2} \cdot day^{-1}$) | 397 ± 24 | 161 ± 32 |
| contact angle (deg) | 49 ± 7 | 82 ± 3 |
| color | | |
| *a* | –0.17 ± 0.03 | 0.41 ± 0.01 |
| *b* | –0.75 ± 0.01 | 1.99 ± 0.23 |
| *L* | 42.78 ± 0.19 | 55.13 ± 2.43 |
| mechanical properties | | |
| UE (%) | 6.63 ± 1.28 | 2.14 ± 0.33 |
| *Y* (Pa) | 191 ± 61 | 110 ± 24 |
| TS (Pa) | 336 ± 63 | 114 ± 38 |
| air permeance ($\mu m^3 \cdot \mu m^{-2} \cdot Pa^{-1} \cdot s^{-1}$) | 0.05 ± 0.02 | 0.04 ± 0.01 |
| roughness ($mL \cdot min^{-1}$) | 6 ± 2 | inside side: 4 ± 3<br>evaporation side: 657 ± 101 |

[a] Values, followed by their standard deviations, are at least means of three experiments. Treatment means were separated using Student's *t* test ($p < 0.05$).

predicted by the GAB model for free fatty acid and 15% stearic acid (w/w HPMC) edible films. For all $a_w$ investigated, lipid−cellulosic films showed a lower moisture content than free lipid films due to the higher hydrophobicity of the fatty acid. Water molecule sorption decreased when the stearic acid/HPMC ratio increased. These results are in accordance with several works done on the sorption mechanism of, especially, methyl and ethyl cellulose edible films (*15*, *16*).

*b. Water Vapor Transmission Rate.* The influence of hydrophobic compound incorporation on film water vapor barrier properties was further investigated by determining the films' WVTR at 23 ± 1 °C and 50 ± 5% RH. These conditions were chosen because they reproduced an environment commonly encountered during food storage under commercial conditions. As expected, cellulosic films without hydrophobic compounds have poor moisture barrier properties (**Table 1**). However, the incorporation of 15% (w/w HPMC) of stearic acid allowed a decrease of WVTR of ∼60%, due to the apolar nature of stearic acid, which decreases the moisture affinity of films.

*c. Goniometer.* A surface tension measurement method was also used to investigate the impact of stearic acid incorporation on film−water wettability. Completely smooth film sides with a roughness close to zero were used for this study. **Table 1** shows that stearic acid incorporation increased the contact angle of ∼40%.

By and large, results from the three experiments presented above showed that stearic acid introduction into the film matrix decreased film hygroscopicity. Film hydrophilicity is due to hydroxyl groups in cellulose ethers (*17*). Stearic acid hydrophobic molecules decreased the moisture affinity of films. This is in accordance with some previous papers, which state that the rate of transmission of water through films decreases with the lipid content (*18*, *19*). Five percent stearic acid incorporation decreases by ∼40% the WVTR (*18*). This behavior is explained by the increase of the film hydrophobicity and the lower chain mobility of fatty acids, which reduces the WVTR. Similarly, fatty acid/cellulose based films reduced moisture transfer (*13*, *21*) and addition of 15% stearic−palmitic acid improved the film water vapor barrier by 13% (*20*).

**2. Film Characterization.** *a. Film Color.* Color was performed, and *L*, *a*, and *b* parameters were measured. Composite film, incorporating stearic acid, showed a lightness (*L*) higher by ∼30% than free fatty acid film. Noncomposite films were colorless and transparent. Although their values were significantly different ($p < 0.05$), the *a* and *b* parameters were not relevant because stearic acid impact on film color was especially a white coloration development. This latter increased by increasing stearic acid content. Other researchers reported similar effects. Yang and Paulson (*20*) incorporated stearic−palmitic acids into gellan films and showed that the opacity increased as concentration of lipids increased. Similarly, Debeaufort et al. (*18*) observed that solid fat content increased film whitishness. The increased film opacity is probably due to light scattering from lipid droplets, which were distributed throughout the polymer network after the film formed.

*b. Mechanical Properties.* The influence of stearic acid incorporation on film mechanical properties was investigated. The suitable use of such edible composite packaging strongly depends on these properties. **Table 1** shows the tensile strength, the Young modulus, and the ultimate elongation for homogeneous and composite films prepared with 15% of fatty acid. The TS, *Y*, and UE show decreases of ∼66%, ∼42%, and ∼68%, respectively, when fatty acid was added. A decrease in mechanical resistance was obtained, associated with a decrease in film elasticity and extensibility. In accordance with Gontard et al. (*22*) and Yung and Paulson (*20*), the resistance of film decreased with lipid addition. For 15% stearic−palmitic acid addition, the authors reported that TS decreased by 34% (*20*). Conversely, Debeaufort et al. (*16*) observed that solid fat contents incorporated into methyl cellulose films did not affect the films' mechanical properties. However, glycerol monostearate was used, and such surfactant is well-known for its plasticizer effect (*23*). The negative effects of the lipids on mechanical properties may have resulted from the partial replacement of the polymer by the lipids in the film matrix. Lipid globules create discontinuities within the HPMC network, which favor the disruption of the film and thus decrease mechanical properties.

*c. Film Air Permeance and Roughness.* Air permeance was studied for all films. Whatever the composition of the films, high air barriers were observed. No statistical difference ($p < 0.05$) was calculated. **Table 1** shows also the influence of lipid addition on film roughness. Both film faces were completely smooth for free fatty acid films, whereas stearic acid based films presented differences depending on the side of the film analyzed. The inside face, in contact with the drying−casting support was smooth, with a roughness coefficient very close to zero, and was not statistically different from free fatty acid film roughness. In contrast, the evaporation side showed a coefficient of 657 $mL \cdot min^{-1}$. This roughness difference between the faces was due to the casting procedure used to form films. Debeaufort and Voilley (*23*) studied the impact of different drying parameters on methyl cellulose film formation and characterization. The rapid migration of solvent induced lipid globule migration, coalescence, and aggregated droplets, which created a heterogeneous film structure and discontinuities in lipid distribution. This could explain, in the case of fatty acid films, the roughness of the evaporation side, taking into account that the casting support flattened a film face, which will become completely smooth. Similarly, Yang and Paulson (*20*) showed increased surface irregularity with the addition of lipids.

**3. Film Antimicrobial Properties and Nisin—Stearic Acid Interactions.** The antimicrobial effectiveness of active films based on 15% of stearic acid (w/w HPMC) as hydrophobic compound and $5 \times 10^4$ $IU \cdot mL^{-1}$ of nisin as antimicrobial

**Table 2.** Inhibition Effectiveness of Nisin Incorporated into either Free Fatty Acid or 15% Stearic Acid Films[a]

| | nisin incorporated into film without stearic acid | nisin incorporated into stearic acid film |
|---|---|---|
| *L. monocytogenes* | 7 ± 1 | 4 ± 1 |
| *S. aureus* | 5 ± 0 | 3 ± 1 |

[a] Values correspond to inhibition zone diameters. Data, followed by their standard deviations, are means of three experiments. Treatment means were separated using Student's *t* test ($p < 0.05$).

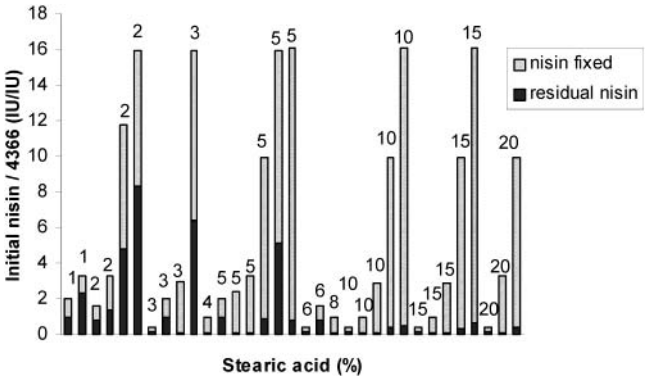


**Figure 2.** Nisin–stearic acid interaction in phosphate buffer (pH 6.1) solutions. Initial (added to the solution) and residual nisin (free) were measured by BCA protein microassay. The total histogram area represents initial nisin. All nisin concentration values were divided by 4366. Data are means of three experiments.

molecule was measured by the inhibition zone assay (**Table 2**). A $5 \times 10^4$ IU·mL$^{-1}$ nisin concentration was used, according to a previous study that determined a nisin minimum inhibitory concentration of $\sim 10^3-10^4$ IU·mL$^{-1}$ (*10*). The inhibitory activity of stearic acid films was lower than that of pure cellulosic matrix. Fifteen percent stearic acid addition decreased the film inhibitory activity by about 70 and 40% for *L. monocytogenes* and *S. aureus*, respectively. In previous studies (*10*, *14*), this phenomenon was especially explained by electrostatic interactions between the cationic nisin and the anionic fatty acid, which decreases nisin desorption from the film. At pH 6.1, stearic acid was preferentially negatively charged, whereas nisin was preferentially positively charged (*24*−*26*). Nisin antimicrobial activity is an initially bacteriocin binding to the target membrane of sensitive bacteria through electrostatic interactions between the positively charged nisin molecule and the anionic phospholipids of the lipid bilayer. "Barrel-stave" pores completely dissipate $\Delta\psi$ and $\Delta$pH and induce bacterial death. Much more, a stearic acid−methyl stearate comparative study showed a nisin−stearic acid interaction, absent in the nisin−methyl stearate case (*14*).

To further investigate nisin−stearic interactions, complementary experiments were conducted by mixing different stearic contents (0−20%) with different nisin concentrations ($0-10^5$ IU·mL$^{-1}$) in phosphate buffer (pH 6.1) at 80 °C to form a microemulsion. Initial nisin and residual nisin are presented in **Figure 2**. For clarity, the nisin concentrations (**z**) were expressed as

$$z = (\text{nisin concn})/(4366)$$

where 4366 IU·mL$^{-1}$ corresponds to the nisin content fixed by 1% of stearic acid (*y*).

According to the data of Figure 2, nisin−stearic acid interaction was observed whatever the concentrations. Increasing stearic acid percentages decrease residual nisin.

**Table 3.** Influence of pH of Film-Forming Solution on Inhibitory Activity of Composite Films [15% Stearic Acid (w/w HPMC)][a]

| | pH | inhibition zone diameter[b] (mm) |
|---|---|---|
| reference sample | 8 | 12 ± 1 a |
| assay | 7 | 7 ± 2 b |
| | 5 | 8 ± 0 c |
| | 3 | 12 ± 2 d |

[a] Values correspond to inhibition zone diameters, evaluated on *M. luteus*. Data, followed by their standard deviations, are means of three experiments. Treatment means were separated using Student's *t* test ($p < 0.05$). [b] "a" and "d" were statistically identical but meaningfully different from "b" and "c".

Residual nisin content depends on either initial nisin concentration or stearic acid percentage. To model the nisin−stearic acid interaction, data were treated with Statgraphic program 4.1 (Sigma-plus). A multifactor linear regression method was used with 95% confidence interval. Analysis of the effects of factors *y*, *z*, and their interactions *yy*, *zz*, and *yz* showed that factors were statistically significant (different from zero) except for the factor *zz*, which was at the limit of significance. A model was established giving the following equation:

$$\text{residual nisin} = 0.615 - 0.247y + 0.323z + 0.013y^2 - 0.027yz + 0.006z^2$$

An $R^2$ coefficient >80% was obtained for the model. Despite the weak *R* coefficient value, the model gave a good estimation of fixed nisin. The model was confirmed experimentally, and a $10^5$ IU·mL$^{-1}$ initial nisin concentration was calculated to be incorporated into 15% stearic acid film to observe residual nisin of $5 \times 10^3$ IU·mL$^{-1}$. Further experiments would be conducted to optimize the model and other regressions, especially polynomial ones would be tested to improve the $R^2$.

**4. Influence of Film pH on Edible Film Antimicrobial Activity.** Because nisin and stearic acid charges are pH dependent, the influence of the molecular charges and, especially, the influence of the pH of the film-forming solution on antimicrobial activity were investigated using inhibition zone assays on *M. luteus* as reference strain.

Data (**Table 3**) showed that a decrease of film pH induced an increasing film antimicrobial activity. Similar inhibition zone diameters from composite film and pure cellulosic film were obtained for a pH adjusted to 3, and no antimicrobial activity was observed from a solution of 0.01 M HCl (pH 2−3). Therefore, antimicrobial activity of composite film seemed to be due to only an improvement of nisin desorption from films. Taking into account that pH 3 is lower than nisin's p*I* and stearic acid's p$K_a$, protonated nisin and stearic acid were favored, which removes nisin−stearic acid interaction.

**5. Perspectives.** To identify applications of such films, mechanical properties would be further investigated as a function of water activity. The impact of film pH on film physical properties would be tested. Nisin desorption from the cellulosic film and bacteriocin diffusion in food model are currently being studied.

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