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Physical and antimicrobial properties of edible films containing *Lactococcus lactis*

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Abstract: In this study, we developed edible films designed to control the growth of *Staphylococcus aureus* in tryptone soya agar. We analyzed three edible film-forming substrates: sodium alginate, sodium carboxymethylcellulose, and collagen. In addition, we evaluated *Lactococcus lactis*, which produces bacteriocin and lactic acid. *Lactococcus lactis*-containing edible films were constructed via tape-casting method. Optical, mechanical, and antimicrobial properties of the edible films were measured to examine the effects of *Lactococcus lactis* on film matrices. Further, we determined the survival of *Lactococcus lactis* after the film-drying process and viability of *Lactococcus lactis* stored for 24 days at 4 °C. Our results indicate that incorporation of *Lactococcus lactis* changed the physical properties of edible films. Films containing *Lactococcus lactis* showed reduced gloss and transparency. There are insignificant modifications were observed in terms of tensile strength and elongation at break. At 4 °C, used to represent a low-temperature environment, the growth of *Staphylococcus aureus* was inhibited for 7 days in edible films populated with *Lactococcus lactis*. The viability of *Lactococcus lactis* was higher in sodium alginate/sodium carboxymethylcellulose films, and was highest in films composed of sodium alginate and methylcellulose. In summary, our study provides a new method for functional food packaging.

Keywords: edible film; *Lactococcus lactis*; antimicrobial activity

1. Introduction:

Petroleum-based packaging shows poor biodegradation, leaves chemical residues in the food products, and creates serious environmental consequences worldwide [1]. Biodegradable and bioactive edible films represent eco-friendly food packaging because they are constructed using natural polymeric ingredients such as sodium

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alginate (SA) [2], sodium carboxymethyl cellulose (CMC) [3], and collagen (Col) [4]. SA, a natural polysaccharide, is mostly extracted from brown algae, but is also found in some bacteria. SA can be used as a thickening, emulsifying, and stabilizing agent in suspensions, gels, films, and textile fibers. Additionally, SA is widely used in flocculation, and in the manufacturing of detergents, paper, foods, and drugs [5]. CMC, an important industrial biopolymer obtained from cellulose, is highly viscous, non-toxic, and non-allergenic [6]. CMC is used in the manufacturing of food, paper, and cosmetics, and in the medical industry [7]. Col is a structural protein and component of the extracellular matrix. Fish-skin collagen possesses good biocompatibility, low antigenicity, and high film-forming ability, and is widely used in edible-protein membranes [8].

A bioactive edible film is defined as a protective coating applied to the surface of a food; this bioactive edible film may contain other functional compounds, such as antioxidants [9], antimicrobial agents [10], prebiotics [11], and probiotics, which increase the functionality of the film [12]. The Food and Agriculture Organization of the United Nations and the World Health Organization define probiotics as active beneficial microorganisms that colonize the human gut and reproductive system, and can provide definite health benefits, thereby playing a beneficial role in the microecological balance of the host [13]. Most probiotics belong to the genera *Lactococcus* and *Bifidobacterium*. Various studies have shown that probiotics decrease lactose intolerance, increase resistance to intestinal invasion by pathogenic bacterial species, stimulate the immune system, and may participate in protection from colon cancer [14], In addition, the membrane components of probiotics has the potential of antitumor activity [15]. The antimicrobial activity of probiotics likely occurs via bacteriocin or by competition in situ. Probiotics can directly or indirectly affect pathogenic bacteria. For these reasons, probiotics are increasingly incorporated into food products. However, incorporating lactic acid bacteria (LAB) into edible films can affect the performance of the films. Romano et al. [16] used methylcellulose as a film-forming substrate and fructo-oligosaccharides (FOS) as prebiotics to prepare *Lactococcus*- and *Bulgaricus*-containing films. Scanning electronic microscopy (SEM) images showed a good incorporation of these microorganisms without affecting the homogeneity of the films. These results indicate that methylcellulose films containing FOS can be used to immobilize LAB, and can potentially be used in the development of functional foods. Sánchez-González et al. [17] used sodium caseinate (NaCas) and methylcellulose as film-forming substrates to evaluate the viability of *Lactococcus acidophilus* and *Lactococcus reuteri* incorporated into these films; they found that viability of these organisms was higher in NaCas films than in methylcellulose films, although bacteriocin production was greatest in the polysaccharide matrix. Additonally, they showed that under refrigeration, these films completely inhibited

the growth of *L. innocua* for a week. Because of their high moisture retention, antimicrobial properties, and high nutritional value, edible films containing LAB are widely used in food preservation to inhibit food spoilage, reduce moisture loss, and prolong the period of food preservation. Concha-Meyer et al. [18] investigated the effect of a coating incorporating *Lactococcus rhamnosus* on the quality of smoked salmon. This film was formulated using two LAB strains and nisin (100 IU/mL). After 28 days, salmon pieces covered with film containing no other inhibitors showed an increase of 2.4 log cycles in the growth of *L. monocytogenes*. However, films containing either one LAB strain, or a combination of both strains and nisin, showed a bacteriostatic effect on the growth of *L. monocytogenes* over a period of 28 days, which exceeds the industrial standard shelf life of smoked salmon. The above results indicate that these films inhibited the growth of *L. monocytogenes* on salmon during refrigerated storage. A gelatin coating incorporating *B. bifidum* was applied to fish to assess the effect of this coating during chilled storage. In that study, López De Lacey et al. [19] found that *B. bifidum* remained viable during storage, and that the growth of H2S-producing microorganisms was reduced in 2 log cycles. A reduction of total viable counts (< 2 log cycles) was obtained at the end of storage. These results indicate that using edible gelatin packaging incorporating bacteria can be employed for fish preservation, especially when combined with other technologies such as a high-pressure treatment.

In this study, we evaluated three edible film-forming substrates: sodium alginate, sodium carboxymethylcellulose, and collagen. Further, we used *Lactococcus lactis* (*L. lactis;* Lla), which is a LAB strain that produces bacteriocin. *L. lactis*-containing edible films were formed via the tape-casting method. In this work, we aimed to determine the best film-forming substrate for incorporation of *L. lactis*, and how the functionality of edible film is affected by incorporation of LAB.

2. Materials and Methods

2.1. Bacterial strains, culture conditions, and materials

L. lactis ATCC 11454 was acquired in lyophilized form from the CICC (China Center of Industrial Culture Collection, Beijing, China). The activity of *L. lactis ATCC 11454* decreases during storage at -80 °C; therefore, it is necessary to activate these bacteria before using them for procedures.

Liquid MRS culture medium (pH=6.4) was subjected to steam sterilization in an autoclave at 121 °C for 15 min and then cooled to room temperature. Two inoculation loops of *L. lactis* culture, preserved in paraffin, were inoculated into this MRS medium and allowed to incubate for 2-3 cycles at 28 °C to achieve the required viability [12]. Cells were then centrifuged at 4000 rpm for 20 min at 4 °C (Marathon 21K/R, Fisher Scientific, Germany)

and then rinsed thrice with saline solution (that had been sterilized at 121 °C for 15 min) to obtain bacteria for later use [20]. Antimicrobial activity of the films was assessed using the target microorganism *Staphylococcus aureus* (*S. aureus*) ATCC 6538 (China Center of Industrial Culture Collection, Beijing, China).

2.2. Preparation of the bioactive edible films

Sodium alginate (viscosity ≤ 0.02 Pa*s for an aqueous solution of 1 wt% at 20 °C), sodium carboxymethylcellulose, collagen powder extracted from fish scales, and glycerol were purchased from Sigma-Aldrich (Sigma Chemicals, St.-Louis, MO, USA). A SA/CMC solution was prepared by dispersing SA (2 g), CMC (0.3 g), and glycerol (2 g) in 100 mL distilled water under magnetic stirring. A SA/Col solution was prepared by dispersing SA (2 g), Col (4 g), and glycerol (2 g) in 100 mL distilled water under magnetic stirring. The CMC/Col solution for construction of edible film was prepared by dispersing CMC (2 g) and Col (4 g) in 100 mL distilled water under magnetic stirring; this mixture was then plasticized with glycerol (2 g) and solution the pH was adjusted to 7.0 with 2.0 M NaOH. The bacteria collected by centrifugation were then added to the solution at 1.0 g/100 g of film solution. The resulting mixture-hydrosol was stirred in a 30 °C water bath for 30 min to ensure complete mixing; then the mixture-hydrosol was allowed to settle in the water bath for another 30 min to allow for removal of foam. Finally, 25 g of the prepared *L. lactis*-containing mixture was poured onto a glass plate and allowed to dry at 30 °C for 24 h. After formation, the film was removed and equilibrated in a climate-controlled chamber (at 25 °C and 50% relative humidity) for 24 h for later use. The process is shown in Figure 1.

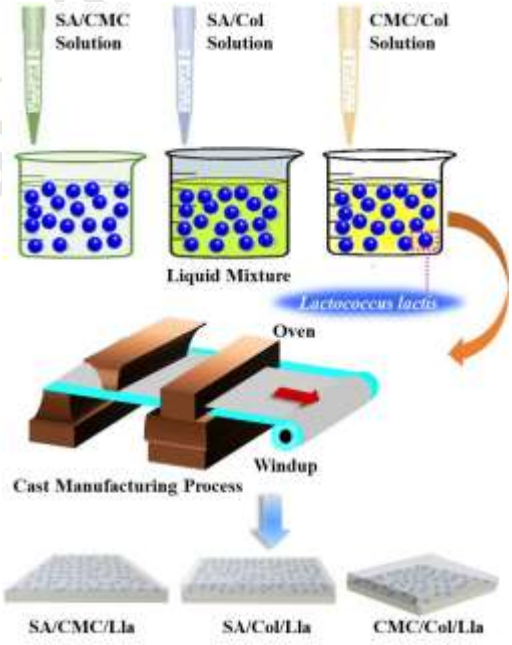

Figure1. Schematic diagram for preparation of the bioactive edible films.

2.3. Enumeration of L. lactis

Preserving the bioactivity of *L. lactis* in edible films is critical for the bacteriostatic and food-preserving effects of this organism [21]. Therefore, we performed counts of viable *L. lactis* cells in *L. lactis*-containing films. Using the method of López De Lacey et al. [19] 1 g of *L. lactis*-containing edible film was added to 9 mL of sterile saline solution under constant stirring to ensure complete dissolution. The resulting solution was then sequentially diluted multiple times with sterile saline. For each dilution, portions of the diluted solution were coated onto MRS agar in culture dishes. The culture dishes were then placed into a bacterial incubator for anaerobic incubation at 28 °C for 24 h to allow for colony growth. The bacterial colonies on the MRS agar plates were then counted. The total bacterial count was expressed as log colony-forming units per gram (log CFU/g) [22]. The survival rate of *L. lactis* throughout the drying process of the film was calculated using the following equation:

$$\text{Viability}(\%) = \frac{N}{N_0} \times 100 \quad (1)$$

where N_0, N represent the number of viable bacteria before and after the film drying process.

2.4. Mechanical and optical properties

An electronic tensile tester was used to characterize the mechanical properties [23]. Before performing this measurement, edible films were equilibrated under a relative humidity of 50% for 24 h. Then, the films were cut into 80 × 10 mm strips, which were then immobilized using jigs. We evaluated at least eight samples per each film formulation.

Tensile strength (TS) was calculated using the following formula:

$$TS = \frac{F}{L \times W} \quad (2)$$

where F is the maximum tensile force (N) when the film breaks, L is the average thickness of the film (mm), and W is the width of the film sample (mm).

Elongation at break (E) was calculated using the following formula:

$$E(\%) = \frac{L_1 - L_0}{L_0} \times 100 \quad (3)$$

where E is the elongation (%), L_0 is the length of a film sample before testing, and L_1 is the length of the film sample when it breaks.

An electronic digital Vernier caliper (0.01 mm accuracy) (Guilin Guanglu Measuring Instrument Co. Ltd., China) was used to measure the thickness of the films; each film sample was measured in triplicate [24].

The color characteristics of edible films were assessed with a color-difference meter using three parallel samples for each condition; each sample was measured in triplicate [25]. A standard plate was used as control film

(L*=88.45, a*=0.60, b*=0.90). The total color difference of the synthetic biofilm was calculated using the following formula:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad [4]$$

2.5. Water vapor permeability

The wet cup method was adapted in this study [26]. In a moisture permeable cup, allochroic silica gel was added until its level reached approximately 5 mm from the mouth of the cup; then, the mouth was sealed with edible film, and total mass was measured. The cup was then placed into an artificial climate chamber (set at 75% relative humidity and 25 °C) to allow the film to equilibrate. Weight measurements were performed every 8 h for 3 consecutive days, using three parallel samples for each type of edible film. WVP was calculated using the following equation:

$$WVP = \frac{m \times L}{A \times t \times \Delta P} \quad [5]$$

where, m is the mass (g) of water that permeates throughout the film; L is the thickness (m) of the film; A is the film area (m^2) through which the water permeates; (t) is the water permeation time (t); and ΔP is the water-vapor pressure difference (Pa) across the film.

2.6. Moisture content

Moisture content (MC) of the edible films was measured using the method of Kurek et al. [27] with slight modifications. Each film sample was cut into 2 × 2 cm specimens; these specimens were then weighed and placed into an oven at 105 °C for 24 h. MC values were calculated using the following equation:

$$MC = \frac{W_i - W_f}{W_i} \times 100 \quad [6]$$

where W_i is the weight of the initial sample, and W_f is the weight of the dried sample.

2.7. Swelling test

Film samples were cut into 2 × 2 cm squares; these squares were then immersed in 0.1-M NaCl aqueous solution for 30 min and 25 °C [28]. The squares were then removed from this aqueous solution and placed between two pieces of filter paper for 5 min to remove excess surface water. Swelling ratio (Sw) was calculated using the following equation:

$$Sw(\%) = \frac{M_f - M_i}{M_i} \times 100 \quad [7]$$

where M_f is the weight of the swollen sample and M_i is the weight of the dried sample.

2.8. Determination of total soluble matter

Film samples were cut into 2 × 2 cm squares, dried in an oven set at 105 °C for 24 h, and then weighed to determine the weight of initial dry matter (M_i). Each dried square was immersed in a 100-mL Erlenmeyer flask containing 20 mL of distilled water. These flasks were then agitated in a shaker at 25 °C for 24 h at 200 rpmd [29]. The remaining (undissolved) contents of each sample were removed from the Erlenmeyer flask, and dried in an oven set at 105 °C for 24 h, and weighed to determine M_f. Total soluble matter (TMS) was calculated using the following formula:

$$TMS(\%) = \frac{M_i - M_f}{M_i} \times 100 \quad [8]$$

2.9. Surface morphology and Fourier-transform infrared spectroscopy [FT-IR]

Images obtained via SEM were used to analyze the microstructure of edible films. Surfaces of the film samples were gold sputtered using ion-plating equipment for 20-30 min under vacuum to produce a layer with a thickness of ~10 μm. Then, the samples were observed with a scanning electron microscope (ESEM XL30 FEI; Hillsboro, Oregon, USA) at 20 kV. Attenuated total reflection (ATR)-FTIR measurements were conducted on a Nicolet 6700 FTIR spectrometer (Thermo-Fisher Scientific Co., Ltd., MA, USA) at 4000 to 650 cm^{-1}, using a resolution of 4 cm^{-1} and 32 scans [30].

3.10. Viability and antimicrobial properties of edible films

The viability of *L. lactis* in different edible films was analyzed after storage at 4 °C for 24 days. Briefly, the films were stored in a microbial incubator using a low-temperature environment (4 °C); the films were removed from the incubator every 3 days. Next, 1 g of each *L. lactis*-containing edible film was placed in a sterile plastic bag containing 9 mL of tryptone phosphate water and homogenized for 2 min in a Stomacher blender. Serial dilutions were made and then poured onto MRS agar. These plates were incubated for 24 h at 28 °C to allow for growth of *L. lactis* colonies. The colonies were then counted, and viable bacterial count was expressed as log colony-forming units per gram (log CFU/g).

Kristo's [31] method was used to measure the antibacterial properties of edible films containing different levels of *L. lactis*. First, we added 20 g of tryptone soya agar (TSA) culture medium to culture dishes. After the culture medium solidified, the surface was inoculated with solutions of *S. aureus*. Next, *S. aureus*, thusly inoculated onto TSA medium, was covered by the different edible films [containing or not containing *L. lactis*] cut to the same diameter as that of the Petri dishes. Next, these TSA plates were placed into a constant-temperature incubator at 4 °C. The inocula were examined, and colonies of *L. lactis* on TSA plates were counted, immediately

after the inoculation and periodically during the incubation period. The TSA culture medium was removed aseptically from each culture dish, placed into a sterile bag containing 80 mL of tryptone phosphate water, and homogenized using a homogenizer for 2 min. Then, 1 mL of the homogenized solution was transferred into 9 mL of sterile saline under constant stirring. Each resulting mixture was serially diluted multiple times. Each time, portions of the diluted mixture were coated onto TSA agar plates, which were then placed into an incubator and allowed to incubate at 28 °C for 24 h; this was followed by counting of the bacterial colonies. All assessments were performed in triplicate.

2.11. Statistical analysis

All data are expressed as mean ± standard deviation. Analysis of variance [ANOVA] and significance testing were performed using Duncan in statistical analysis software SPSS 24, and mapping was performed with Origin 2018.

3. Results and discussion

3.1. Survival of L. lactis

Composition of the edible film and film-forming procedure are critically important to bacterial survival post-processing. In this study, some of the *L. lactis* cells did not survive the film drying process. The viable counts of *L. lactis* in film-forming solution and edible films were obtained using a serial dilution method; the results are displayed in Figure 2. The sub-lethal effects of the air-drying step were strongly dependent on the type of plasticized substrate [21]. The addition of SA/CMC provided the highest protection to the incorporated *L. lactis* cells, allowing for retention of 5.01% of the initial number of living *L. lactis* cells. SA/Col films and CMC/Col films provided moderate protection to the incorporated *L. lactis* cells, indicating that SA/CMC film was the best substrate in terms of protecting *L. lactis* during the film drying process. Additionally, this may be due to the presence of polysaccharide, although further investigation is required to fully understand the mechanisms involved in this protective activity of SA/CMC. The viability of living cells during heating is affected by multiple factors [32]. The viability of *L. lactis* in edible-film solutions may not solely depend on film base material, but also on nutritional conditions, initial bacterial count, environmental pH, and fluid osmotic pressure [33].

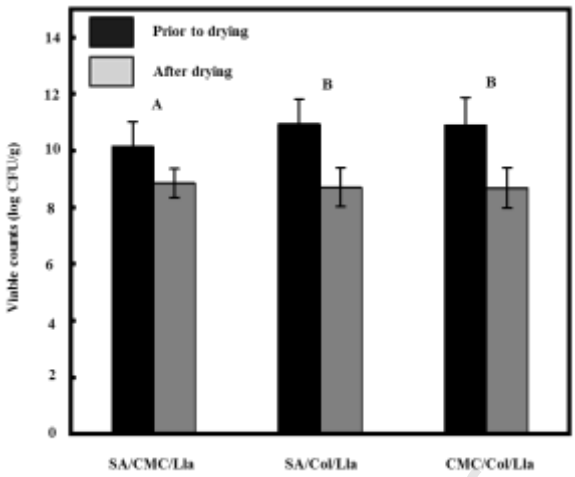

Figure 2. Survival of *Lactococcus lactis* through out air drying at 30 °C for 48 h. Data followed by different capital letters are significantly different ($p<0.05$) when comparing survival rate of *Lactococcus lactis.*

3.2. Morphology of edible films

The microstructure of edible films is an important factor that controls diffusion of antibacterial molecules [34]. The surface morphology of edible films constructed in this study was analyzed via SEM images (Figure 4). Further, it indicated that *L. lactis* have the ability to form edible films at 30 °C. When we compared edible films containing and not containing *L. lactis* (Figure 4a, b, and c), we found that incorporation of *L. lactis* (Figure 4d, e, f) did not change the morphology of the cross-sectional surface of edible films. The cross-sectional surfaces of films containing *L. lactis* showed smooth surfaces with no cracks [35]. Conversely, CMC/Col films containing *L. lactis* showed poor distribution of *L. lactis* over the edible-film area. This indicates that *L. lactis* cells were poorly incorporated into these protein films and agrees with results shown in previous studies [36]. When *L. lactis* was incorporated into edible films, we observed small bumps, corresponding to *L. lactis* aggregates, on the cross-sectional surface (Figure 4h, i, g). Both images of the cross-sectional surface show *L. lactis* aggregates breaking the smooth surface of the edible films. CMC/Col/Lla films showed rougher cross-sectional surface than that of SA/Col/Lla films, and the structure of SA/CMC/Lla films was irregular, uniform, and tightening. These results indicate that among the film compositions evaluated in this study, SA/CMC substrate performed the best in preserving the viability of incorporated *L. lactis*, and can, therefore, be used to improve the antibacterial properties of edible films.

Figure 3. The photographs of edible films (a) without *Lactococcus lactis*, (b) added with *Lactococcus lactis*.

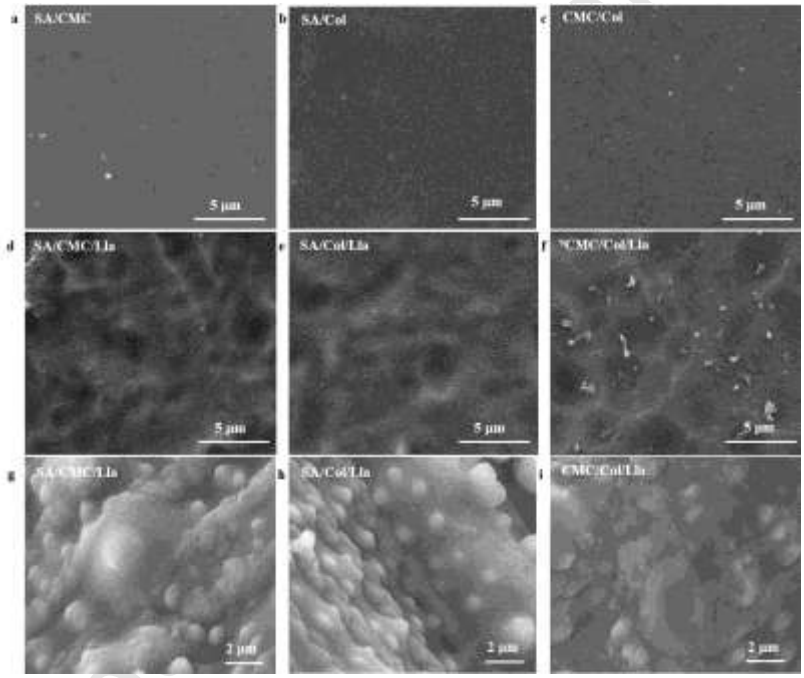

Figure 4. SEM images of edible films (a-c) without *Lactococcus lactis*, (d-e) edible films added with *Lactococcus lactis*, and (g-f) the cross-section of edible films added with *Lactococcus lactis*.

3.3. Optical properties of edible films

Given that the directly affect the appearance of packaged products maintenance of the *L. lactis* in edible films, it is necessary to measure the optical properties, including the color and transparency of the films. The color of edible films evaluated in this study changed with increasing numbers of bacterial cells (Table 1). Overall, the presence of *L. lactis* only slightly affected the color of the edible films, and the increase was not significant ($p>0.05$); this result agrees with those of Kanmani [37]. We found no significant differences in the L* of edible films; however, CMC/Col/Lla and SA/Col/Lla films showed the highest ($p<0.05$) scores for green and yellow components (a* and b*). In terms of color difference (ΔE*), SA/CMC film showed the lowest, and SA/Col/Lla film showed the highest,

color divergence from those of control films. With respect to tone, all the films incorporating proteins or protein hydrolysates showed similar hue values; thus, opacity and color differences can primarily be attributed to the *L. lactis* and type of film-based. Film luster is related to gloss, with smoother surfaces providing a higher degree of luster [38]. This occurs primarily because the volume of microbial cells in the film differs from that of the film-forming material, which generates a continuous, irregular layer during drying. Further, this results in a rough film surface that increases optical refraction, decreases film transparency, and changes the surface color of the film. Nevertheless, these changes in film appearance, induced by *L. lactis* cells, were very minor from the practical view point.

Table 1. Effect of the incorporation of *Lactococcus lactis* on colour characteristics of biopolymer films.

Edible Film	**L***	**a***	**b***	**ᐞE**
control	88.45±1.82^{a}	0.60±0.47^{d}	0.90±0.77^{f}	
SA/CMC	87.63±0.82^{ab}	1.24±0.16^{c}	3.85±0.16^{d}	3.13±0.33^{f}
SA/CMC/Lla	85.06±0.82^{c}	1.44±0.24^{ab}	4.72±0.17^{b}	5.18±0.27^{c}
SA/Col	87.76±1.22^{ab}	1.32±0.09^{bc}	4.24±0.07^{c}	3.49±0.39^{e}
SA/Col/Lla	86.06±1.67^{bc}	1.56±0.12^{a}	6.61±0.20^{a}	6.26±0.25^{a}
CMC/Col	85.81±1.14^{bc}	1.44±0.19^{ab}	3.52±1.51^{e}	3.81±0.49^{d}
CMC/Col/Lla	84.24±0.47^{c}	1.56±0.24^{a}	4.38±0.11^{c}	5.55±0.36^{b}

a–f Different letter between rows indicate significantly different values ($p<0.05$) according to Duncan's post hoc means comparison test. Data are presented as mean ± SD (n = 3).

3.4. Mechanical properties of edible films

Edible films are widely used in food and medical packaging. These films can extend the shelf life of foods either by acting as a barrier to gases and volatile compounds, or by controlling water permeability. Because edible films can constitute the main body of food packaging, TS and E are key indicators used to measure the applicability of edible films. TS represents the resistance of the film to elongation or its stretching capacity, while E is a measure of the stiffness of the film. In this study, incorporation of *L. lactis* did not alter the thickness of edible films, which

was approximately 0.050 ± 0.05 mm. Odila Pereira et al. [36] have shown similar results for edible films embedded with bacteria. Conversely, the addition of *L. lactis* adversely affected the TS and E of *L. lactis*-containing edible films (Table 2), where SA/CMC films were mechanically more resistant to fracture and more stretchable than those containing Col. On the other hand, the mechanical properties of both kinds of edible films presented similar trends when *L. lactis* were incorporated into the material, with the addition of bacteria, the edible films TS and E decreased, while the *L. lactis* had no significant effect on the TS and E of the edible films ($p>0.05$) (Table 2). During film formation, bacterial cells interrupt the inter-macromolecular interactions in film solutions, which reduces cohesive forces in the polymer network, causing loss of mechanical resistance. The effects are not significant because the number of added cells introduced in each case is relatively low.

WVP is one of the most important factors in food packaging because WVP can affect water exchange between the food and its environment, possibly impacting the quality and safety of the food [39]. Table 2 shows the WVP of different types of edible films. Edible films containing Col showed lower WVP values compared to edible films containing polysaccharide. In general, sodium alginate can be used to improve barrier properties of edible membranes. Conversely, WVP increased when *L. lactis* was incorporated into films. The WVP value of CMC/SA film increased from 2.86×10^{-11} gm/m^2 s Pa to 3.23×10^{-11} gm/m^2 s Pa; similar phenomena were observed with other edible films evaluated in this study. Although WVP values generally showed an increasing trend, there was no significant difference in these values ($p>0.05$). The lowest WVP values were recorded for SA/Col and CMC/Col films having a low residual moisture content, indicating that a combination of water affinity and reduced water mobility may affect WVP. This may also have occurred because Col can reduce intermolecular spacing by hydrogen bonding with film-forming agents, thereby impeding water mobility and causing lowered WVP [17]. Further, the addition of *L. lactis* can change the spatial structure of the molecules, destroy intermolecular interactions, and enlarge the intermolecular space, thereby directly increasing WVP [40].

Table 2. Effect of the incorporation *Lactococcus lactis* on thickness, water vapour permeability, and mechanical properties of the films.

Edible film	Thickness(mm)	WVP(10^{-11} g m/m^2 s Pa)	TS(MPa)	E(%)
SA/CMC	0.045±0.03^{a}	2.86±0.34^{a}	21.82±3.16^{a}	25.17±2.7^{a}
SA/CMC/Lla	0.051±0.02^{a}	3.23±1.22^{ab}	17.27±1.76^{abc}	22.56±3.91^{ab}
SA/Col	0.051±0.04^{a}	1.74±0.48^{ab}	17.38±1.79^{ab}	19.51±3.89^{abc}
SA/Col/Lla	0.056±0.04^{a}	2.02±0.12^{b}	14.96±2.46^{bc}	18.17±1.40^{c}

CMC/Col	0.053±0.06[a]	1.52±0.05[b]	13.99±1.33[bc]	15.64±2.63[c]
CMC/Col/Lla	0.055±0.07[a]	1.63±0.16[b]	12.56±0.71[c]	14.96±1.57[c]

a–c Different letter between rows indicate significantly different values ($p<0.05$) according to Duncan's post hoc means comparison test. Data are presented as mean ± SD (n = 3).

3.5. Physical properties of edible films

The MC of edible films is key to providing an environment suitable for prolonging the viability of *L. lactis*. MC is also crucial to the stability of edible films. The MC, Sw, and TMS values of edible films evaluated in our study are shown in Figure 5. Our results indicate that the MC of the edible films was between 15.58 ± 1.10 and 23.57 ± 1.16%, and that film-forming materials significant affected the moisture content ($p<0.05$). Furthermore, incorporation of *L. lactis* increased the water content of the films, but did not significantly affect moisture content; these findings are similar to those of Ebrahimi et al. [41], and may be attributed to the fact that probiotic cells contain a small amount of water. The Sw of our edible films was between 24.32 ± 1.89 and 51.54 ± 1.56%. Incorporation of *L. lactis* decreased the relative content of hydrophilic substances in the films [42], which in turn reduced Sw of the films. Polysaccharides are known to have high water absorption and high moisture retention [43]; expectedly, SA/CMC-containing edible films showed the highest Sw. Conversely, incorporation of *L. lactis* decreased the TMS values of edible films (Figure 5c). The TMS value of SA/CMC film differed significantly from those of SA/Col and CMC/Col films, indicating that collagen-containing films had better water solubility during short-term soaking than films not containing collagen. Although incorporation of *L. lactis* affected the MC, Sw, and TMS values, it was not the main factor contributing to changes in these properties. Changes in MC, Sw, and TMS values were mainly attributed to differences in the properties of different materials used to form edible films.

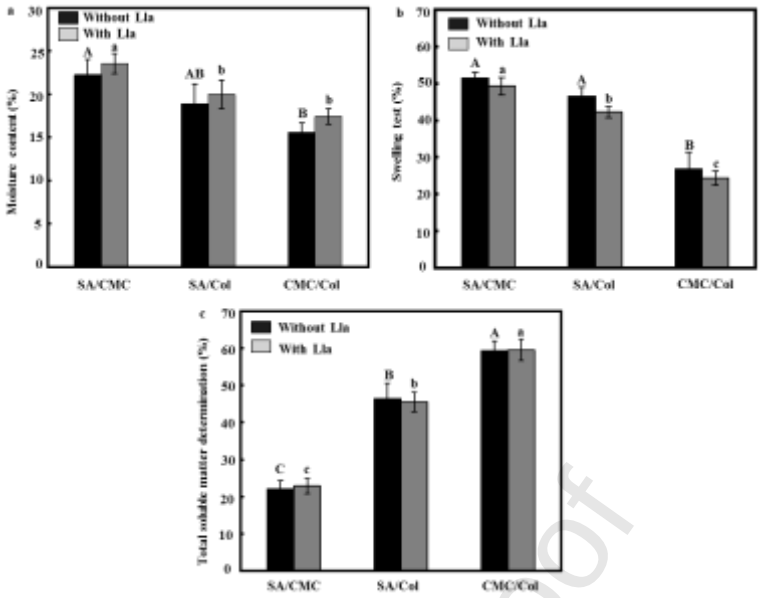

Figure 5. Moisture content (a), swelling test (b) and total soluble matter determination (c) of different edible films. Data followed by different capital letters are significantly different ($p<0.05$) when comparing MC, Sw, and TMS of edible film added not *Lactococcus lactis*. Data followed by different lowercase letter are significantly different ($p<0.05$) when comparing MC, Sw, and TMS of edible film added *Lactococcus lactis*.

3.6. FT-IR analysis

FT-IR measurements were used to evaluate how incorporating *L. lactis* affected intermolecular forces in SA/CMC, SA/CMC/Lla, SA/Col, SA/Col/Lla, CMC/Col, and CMC/Col/Lla films. FT-IR spectra of edible film samples (Figure 6a, b, and c) show that chemical structure remained unchanged after the addition of *L. lactis*. In SA and CMC, which are polysaccharides containing numerous glucose residues, an -OH stretching peak was observed at approximately 3295 cm^{-1} (Figure 6). An absorption peak was observed near 2928 cm^{-1}, indicating the presence of CH stretch attached to O. The asymmetric and symmetric stretching of carboxylate vibrations appeared at 1619 and 1420 cm^{-1}, respectively [44]. An absorption peak near 1610 cm^{-1} was attributed to presence of C=O stretching bonds, and an absorption peak near 1400 cm^{-1} indicated the presence of 3,6 glycosidic bridge. A C-4 absorption peak appeared at 1070 cm^{-1} [45], and a C-O-C absorption peak at 900 cm^{-1}, which agrees with the presence of galactose in polysaccharides [46]. As for previous pure SA, CMC films, the IR spectra of both edible film samples which containing probiotics are also very similar and bear testimony to the excellent chemical properties of them were maintained [12]. In addition, this indirectly indicates that these edible films can effectively incorporate *L. lactis*.

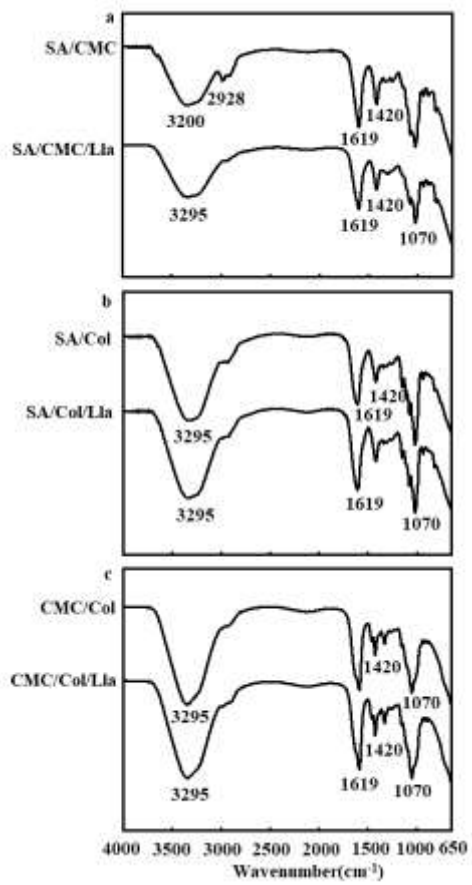

Figure 6. FT-IR spectra of (a) SA/CMC and CMC/CMC/Lla; (b) SA/Col and SA/Col/Lla; (c) CMC/Col, and CMC/Col/Lla films.

3.7. Viability of L. lactis during storage of edible films

The viability of *L. lactis* in different edible films stored for 24 days under refrigeration is shown in Figure 7. The viability of *L. lactis* in SA/CMC film was greater than that in collagen-containing films. A significant reduction in the initial population of *L. lactis* was observed during the first week of storage, indicating that this bacterial strain is sensitive to stress during storage. At the end of storage period, the SA/CMC-containing edible film was the most effective at maintaining the viability of *L. lactis*, and provided the highest protection as shown by the bacterial count of 5.7 ± 0.41 log CFU/g of live *L. lactis* cells. Among the three film-forming substrates, polysaccharide was more beneficial for the survival of *L. lactis*, which agrees with results reported in other studies [21]. During the storage period, the substrate used to form the edible film can determine bacterial survival. However, extrinsic factors, such as water activity, temperature, and presence of oxygen, can adversely influence the viability of encapsulated probiotic living cells [32]. Although mechanisms accounting for probiotic stability in edible films during storage are not fully delineated, factors such as steric solute hindrance, translational diffusion of oxygen in matrices, nutrient

content, free-radical scavenging agents, and interactions with polar head groups of membrane phospholipids via hydrogen bonding, likely play roles in the stability of probiotics in edible films [47, 48].

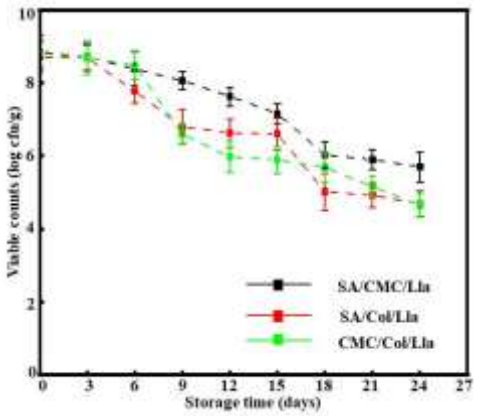

Figure 7. Survival of *Lactococcus lactis* in different films throughout storage time at 4 °C for 24 days.

3.8. Antimicrobial activity of edible films

Antibacterial activity of *L. lactis*-containing edible films was evaluated using TSA culture medium and storage at 4 °C for 7 days; TSA culture medium without the covering of *L. lactis*-containing edible film was used as control. SA/CMC, SA/Col, and CMC/Col films not containing LAB were used to assess the effect of film materials on antibacterial properties of the film. Counts of *S. aureus* are shown in Figure 8. Expectedly, probiotic-free films did not exhibit antibacterial activity and did not reduce the growth of *S. aureus*, which was recorded as 9.27 ± 0.10 to 10.57 ± 0.21 log CFU/g. This occurred because these films provided nutrients for *S. aureus* growth, and those containing polysaccharide provided an especially nutrient-rich environment for the growth of *S. aureus* [21]. In contrast, films incorporating *L. lactis* showed significant antibacterial activity ($p<0.05$). *S. aureus* population decreased from 9.31 ± 0.13 to 7.23 ± 0.22 log CFU/g at the end of storage period used to assess antimicrobial activity. After 3 storage days, the best results were obtained with SA/CMC/Lla edible films; however, no differences were observed among the *L. lactis*-containing films at the end of short storage period. This result is similar to that of Sánchez-González et al. [49], who showed that addition of probiotic bacteria to films markedly inhibited the growth of *S. aureus*. It was indicated that with increased storage time, the number of viable *S. aureus* cells tended to increase, and the film based on collagen have the more obvious change. This likely occurred because substrates used to form edible films are the main factors determining antimicrobial activity of the films, and because polysaccharides provide a very suitable environment for the metabolism of probiotic cells. Additionally, some of *L. lactis* cells in the films died after long storage periods, leading to a decrease in the concentration of bacteriocin in the films [13].

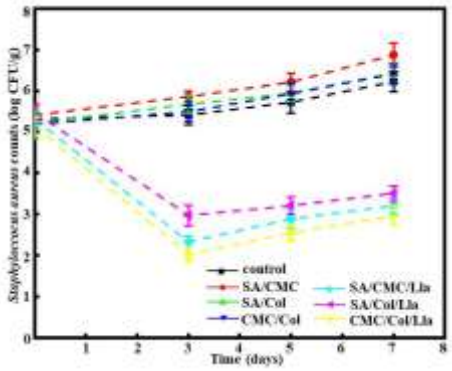

Figure 8. Effect of bioactive films on the growth of *Staphyloccocus aureus* on TSA medium stored at 4 ℃.

4. Conclusions

Our results indicate that the addition of *L. lactis* did not significantly change the thickness, and physical and mechanical properties, of edible films, but did alter the color and luster of the films. Due to the films undergoing significant changes in different composition, where both the water vapor permeability, Swelling, Total soluble matter, tensile strength and elongation at break had the significantly changes. Although the viability of *L. lactis* decreased during film drying, the population of *L. lactis* remained constant during 20 days in a low-temperature environment. SA/CMC edible film offered the greatest stability during storage, as shown by high numbers of viable cells of *L. lactis*. Edible films incorporating *L. lactis* showed robust antimicrobial activity against *S. aureus*. The best results were obtained with CMC/SA films, which showed a greater production of bacteriocin than that in other films. This may have occurred because polysaccharide-containing substrate was more suitable for the survival of probiotic cells. Overall, SA/CMC film was the best edible film for supporting the viability and antimicrobial activity of *L. lactis*. Our present study describes a new method for functional food packaging.

Acknowledgments

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