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Carboxymethyl cellulose based bioactive edible films with *Lactobacillus casei* and fish protein hydrolysates

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Abstract

Nowadays, probiotics have been seriously considered for their potential application in healthy food formulations. The most highlighted concern about probiotics is that the number of probiotic bacteria at the time of consumption may be lower than the required value (10^7 CFU/g). A new approach is the immobilization of probiotic cells in the matrix of edible films. So in this study, edible carboxymethyl cellulose (CMC) probiotic films containing *Lactobacillus casei*, and the silver carp muscle protein hydrolysates (SCMH) prepared by using limited Alcalase hydrolysis, were analyzed and the viability of bacterial cells was determined at 25, 4, and -18°C for 30 days. An alkaline solubilization/acid precipitation method was used to isolate silver carp white muscle proteins. Protein isolate (3%, W/V) was hydrolyzed with 5% E/S ratio (w/w) Alcalase at 50°C and pH 8.0 for 3 min. Briefly, films were prepared by dissolving SCMH and CMC powder (1.5%, w/v) in a ratio of 1:2 in distilled water and *L. casei* was added to a final concentration of 10^8 CFU/mL. Probiotics were counted at intervals of 1, 10, 20, and 30 day. The physical, mechanical [Ultimate tensile strength (UTS) and elongation at break (EB)], thermal and structural properties were determined. XRD patterns of the film samples collected by X-ray diffractometer (XRD) and Fourier transform infrared (FT-IR) spectroscopy of the film samples were recorded. The results indicated that the addition of SCMH significantly ($p<0.05$) improved the *L. casei* viability at all three temperatures. Thickness, moisture absorption, and water vapor permeability (WVP) of the films were not influenced by addition of the probiotic. However, the addition of SCMH negatively affected the film's mechanical properties. The FT-IR analysis confirmed the formation of hydrogen bonds between *L. casei* and the CMC matrix, the XRD and differential scanning calorimetry (DSC) analyses confirmed the plasticizing effect of SCMH on the films. Thus, CMC films containing *L. casei* showed the highest UTS (3.7 MPa) and EB (29.9%). Generally, the results indicated that the SCMH incorporated CMC-based film can be a good carrier for probiotics as bioactive food packaging system with promising potential for shelf life extension of perishable foods.

Keywords: Protein hydrolysates, *L. casei*, Carboxymethyl cellulose, Edible films, Probiotic.

Introduction

Probiotics are increasingly being incorporated into the food products (Ma *et al*., 2019; Pavli *et al*., 2017) due to their ability to maintain the balance of intestinal microbiota, enhance the immune system and reducing the

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risk of gastrointestinal diseases such as inflammatory bowel diseases, diarrhea, and lactose intolerance (Fiocco *et al*., 2020; He *et al*., 2017). The stability and viability of probiotics are influenced by intrinsic food parameters, processing and storage conditions (Fiocco *et al*., 2020; Ebrahimi *et al*., 2018).

Bioactive edible films are thin-layer biopolymers constructed from natural polymeric ingredients that can be used on the surface of products as eco-friendly food packaging (Ma *et al*., 2019; Ebrahimi *et al*., 2018). Apart from their role in food protection (e.g., by reducing the rates of moisture and gas transfer) (da Cruz *et al*., 2007), they may contribute to human health (Espitia *et al*., 2016). Stabilization of probiotics in the matrix of edible films has been used to enhance the cells' viability (Altamirano-Fortoul *et al*., 2012). The production of probiotic edible films by incorporation of various probiotic bacteria into the different biopolymer-based films such as rice protein-shellac composite coating (Wang *et al.*, 2021), polyvinyl alcohol films (Hirsch *et al.*, 2021), bacterial cellulose film (Motalebi Moghanjougi *et al*., 2020), whey protein isolate (WPI) film (Karimi *et al.*, 2020), bacterial cellulose-cashew gum composite film (Oliveira-Alcântara *et al.*, 2020) and alginate films (Alvarez *et al.*, 2021) was the subject of some recent researches in food packaging field.

Hydrocolloids such as proteins and polysaccharides have been used for preparation of edible films. Carboxymethyl cellulose (CMC), a cellulose-derived polysaccharide, is one of those widely used industrial biopolymers in food packaging applications due to its low price, high compatibility, good biodegradability, high transparency, and acceptable mechanical properties (Azarifar *et al*., 2019; Tongdeesoontorn *et al*., 2011; Nie *et al*., 2004). Different bioactive compounds such as indigestible oligosaccharides have been used as prebiotics in the CMC-based probiotic films in order to increase the stability of probiotic bacteria (Zabihollahi *et al*., 2020; Yu *et al*., 2016). However, the potential of protein hydrolysates and peptides, as prebiotics, on growth or activity of beneficial microorganisms in edible film matrix is less studied (Yu *et al*., 2016).

Seafoods are a potential source of functional ingredients such as protein hydrolysates and bioactive peptides (BAP) that could potentially be used for food fortification (Nikoo *et al*., 2020; Sun *et al*., 2020; Xu *et al*., 2019).

Protein hydrolysates and BAP may beneficially modulate the physiological processes in the body and prevent oxidative stress associated with degenerative aging diseases (Wang *et al*., 2013; Kim & Wijesekara, 2013). Therefore, they may be used as promising ingredients for developing functional foods or active packaging due to their multiple health benefits (Sun *et al*., 2020). Silver carp (*Hypophthalmichthys molitrix*) is a major commercially aqua cultured carp species. The global production of silver carp has expanded steadily, rising from 3.8 million tonnes in 2006 to 5.3 million tonnes in 2016 (FAO FishStat). Protein hydrolysates and BAP from silver carp muscle showed various biological functions including antioxidant, anti-thrombotic, antihypertensive, and immunomodulatory activities (Wang *et al*., 2020; Jiang *et al*., 2014).

Lactobacillus casei is a well-known specie of mucosa, the production of antimicrobial Lactobacillus and has been recognized as safe by the US Food and Drug Administration (Arihara, 2006). The beneficial properties of *L. casei* such as resistance to stomach acid and bile salts, the ability to adhere to the cells of the intestinal substances and inhibition of the activity of pathogens have been documented (Rasdhari *et al*., 2008; Mishra & Prasad, 2005). Cold storage or high processing and storage temperatures might adversely affect the cell viability and proliferation in the food industry. Therefore, immobilization of *L. casei* in a matrix of edible films to improve its viability might be beneficial (Fiocco *et al*., 2020). Several studies have investigated the immobilization of *L. casei* in various biopolymer-based films. Mozaffarzogh et al. (2020) prepared probiotic CMC-sodium caseinate films containing *L. casei* for shelf life extension of fresh trout fillets. Khodaei et al.

(2020) developed *L. casei* loaded gelatin and low methoxyl pectin based probiotic films and observed the improved cell viability. Orozco-Parra et al. (2020) prepared and characterized bioactive synbiotic edible film based on cassava starch, inulin, and *L. casei*. Pruksarojanakul et al. (2019) developed synbiotic edible film from konjac glucomannan by incorporation of *L. casei* and OraftiGR, and used for coating of bread buns. Dianin et al. (2019) prepared edible films based on WPI and *L. casei* and evaluated the effect of resultant films for shelf life extension of tomato and grape fruits.

So far, immobilization of *L. casei* in the carboxymethyl cellulose edible films incorporated with fish protein hydrolysates has not been investigated. The aim of this study was to investigate the viability of *L. casei*, in CMC-based edible films containing silver carp muscle protein hydrolysates (SCMH) stored at different temperatures (25, 4, and -18°C). The effect of SCMH on the mechanical and thermal properties of the films containing *L. casei* was also evaluated.

Materials and Methods

The strain of *L. casei* PTCC 1608 (as freeze-dried culture) was purchased from Iranian research organization for science and technology (Tehran, Iran). Carboxymethyl cellulose (CMC) was provided by Caragum Parsian (Tehran, Iran, M_w = 250000 Da), and Silver carp (*Hypophthalmichthys molitrix*) was obtained from the local fish market (Urmia, Iran). Alcalase (Protease from *Bacillus licheniformis*, 2.4 L, 2.4 AU/g) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other chemicals used in the research were purchased from Merck (Darmstadt, Germany).

Extraction of fish protein isolates

An alkaline solubilization/acid precipitation method was used to isolate silver carp white muscle proteins (Nikoo *et al*., 2019). Briefly, the pH of the homogenized mince was adjusted to 11 using 1 mol/L sodium hydroxide and continuously stirred using an overhead stirrer (FTDS-11, Sci Finetech Co., Seoul, South Korea) to solubilize proteins. After that, the mixtures were centrifuged at 4000 ×g and 4ºC for 10 min. Soluble proteins were then precipitated at pH 5.5 with the aid of 1 mol/L HCl and then centrifuged at 4000 ×g and 4ºC for 10 min to obtain protein isolate (PI). Finally, the PI paste was dried using a freeze-dryer (SBPE-SUT-02, Iran) and stored at -18°C until further use (<1 month).

Preparation of Silver carp muscle protein hydrolysates (SCMH)

Silver carp muscle PI (3%, w/v) was hydrolyzed with Alcalase at 5% E/S ratio (w/w) at 50°C and pH 8.0 for 3 min. The mixtures were heated in a boiling water bath (~95ºC) for 10 min to stop the reaction and then centrifuged at 4000 ×*g* and 4ºC for 10 min. The supernatants were dried using a freeze-dryer and the powders (SCMH) obtained were kept at -20ºC until used (<1 month) (Nikoo *et al*., 2019). Degree of hydrolysis was determined according to the pH-stat method described by Adler-Nissen (1986). SCMH prepared with Alcalase at 4.67% of degree of hydrolysis was extracted.

Preparation of probiotic cells

The vial of lyophilized culture of bacteria was broken down under sterile condition. About 1 g of lyophilized bacteria per 100 mL of sterilized MRS broth (Merck, Germany) was prepared and incubated at 37°C for 24 h. Cells were collected by centrifugation at 3500 rpm for 15 min at 4°C (Sigma Centrifuge, Osterode am Harz, Germany). The supernatant was drained and the pellet was centrifuged twice by sterile 0.9% NaCl (W/V) to be thoroughly washed. This bacterial suspension was used to prepare the desired inoculum (Ebrahimi *et al*., 2018; De Lacey *et al*., 2012).

In this study, McFarland Standards were used to standardize the approximate number of *L. casei* in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard. Original McFarland Standards (0.5, 1, 2 to 10) were prepared by adding barium chloride and sulfuric acid; the

reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. So, the turbidity of 8 McFarland Standard (approximate bacterial suspension/ml: 24× 10^8 CFU/mL) was used to obtain a *L. casei* suspension of known concentration. The accuracy of the density of bacterial suspension was checked by using a spectrophotometer (UV-Vis, CamSpec, M330, UK) at 650 nm (absorbance: 0.94-0.98). Finally, a bacterium suspension with a concentration of 24× 10^8 CFU/mL was acquired and used in the preparation of the examined films with a final concentration of 10^8 CFU/mL (McFarland, 1907).

Preparation of probiotic films

The method of Ebrahimi et al. (2018) was used to prepare the film solutions with some modifications. Briefly, 1.6 g SCMH was dissolved in 200 mL of distilled water. The resultant mixture was heated to the boiling point for 10 min to eliminate the possible pathogens. Then, 3 g of CMC powder was added into the mixture gradually. The solution was stirred using a magnetic stirrer at 1200 rpm for 2 h at room temperature. After that, Glycerol (50% of CMC weight) was added as a plasticizer and the solution was further stirred at 70°C for 30 min. The temperature of the solution was then allowed to decrease to 37°C and *L. casei* was added to a final concentration of 10^8 CFU/mL. The mixture was kept at 4ºC for 3 h. Afterward, 25 mL of the film-forming solution was poured and casted into sterile plastic plates with a diameter of 8 cm and the plates were dried at 37°C for ~40 h in an oven. The SCMH-loaded probiotic film was coded as a CMC-Pro-Pep sample. Two other CMC films containing probiotic bacteria (CMC-Pro) and SCMH (CMC-Pep) were prepared in the same way as stated. The pure CMC film was considered as a control sample (coded as CMC). The prepared films were stored in zipped bags until use for characterization at three different temperatures (25, 4 and -18°C) within 30 days. Probiotics were counted at intervals of 1, 10, 20, and 30 day.

Physical properties of the films

Thickness

The thickness of the films was randomly determined at 5 points using a digital micrometer (Flower, USA) with a resolution of 0.001 mm (Almasi *et al*., 2020).

Water vapor permeability (WVP)

The WVP was measured by a gravimetric approach using ASTM E96-05 (ASTM, 2005) standard method with minor modifications. Film samples were placed on a cap of glass tubes (diameter of 2 cm and 4.5 cm height) with pores in 7 mm diameter. The tubes were filled with 3 g of anhydrous $CaSO_4$ (RH= 0%), followed by placing them in a desiccator containing saturated K_2SO_4 solution (RH= 97%) at room temperature. The tubes were weighed every 3 h until reaching a fixed weight. The weight changes curves of tubes were plotted as a function of time. After calculating the slope of a line using linear regression, WVP was calculated as:

$$\text{WVP} = \frac{WVTR.X}{P(R_1 - R_2)} \quad (1)$$

where, P is the saturation vapor pressure of water (Pa) at the test temperature (25°C), R_1 is the RH in the desiccator (97%), R_2 is the RH inside the vial (0%), and X is the average thickness of the film samples (m). Under these conditions, the driving force [$P(R_1-R_2)$] was 3115.42 Pa. The water vapor transmission rate (WVTR) was the slope of the linear part of the curve (g/h) divided by the transfer area (7.85 × 10^{-5} m^2).

Moisture absorption

In order to uniformize the test conditions, the films specimens (2× 2 cm) were kept in a desiccator containing calcium sulfate (RH=0%) for 24 h. The samples were then weighed (W_0) and mounted in a desiccator containing calcium nitrate saturated solution (RH= 55%) and kept at 25°C. The weight of samples at a specified time was recorded until reaching a fixed weight (W_t). The numerical value of moisture absorption was obtained from the following

equation (Almasi *et al*., 2020; Ghadetaj *et al*., 2018; Angles & Dufresne, 2000).

$$\text{Moisture absorption (\%)} = \frac{Wt - Wo}{Wo} \times 100 \qquad (2)$$

where, W_0 (g) is the initial weight of the sample, and W_t (g) is the weight of the sample after t time.

Color properties

Instrumental color parameters of films were determined using a Hunter lab colorimeter (Minolta CR-400, Japan). Color characteristics were determined using L^* (lightness/brightness), a^* (red to green) and b^* (yellow to blue) parameters. The total color difference (ΔE) was calculated according to the following equation (Almasi *et al*., 2010).

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (3)$$

where, ΔL^*, Δa^*, and Δb^* are the difference between the color of the standard white color plate (L^*= 93.49, a^*= -0.25 and b^*= -0.9) and the film samples.

Mechanical properties

Ultimate tensile strength (UTS) and elongation at break (EB) were determined using a Tensile Analyzer (QTS texture analyzer, CNS Farnell, Essex, UK) according to the ASTM D882-02 standard method (ASTM, 2002). The films were cut in dumbbell shape (8× 0.5 cm) and the distance between the two grips was 50 mm, while the seal velocity was chosen 10 mm/min. The relationship between stress and strain was recorded and UTS and EB were calculated as MPa and percentage, respectively.

X-ray diffraction (XRD) analyses

X-ray diffractometer (X Pert Pro, Panalytical, USA) was used to attain the XRD patterns of the film samples. The analysis was done at room temperature with a diffraction angle (2θ) from 5° to 40°. Cu Kα radiation source (k= 0.154 nm) operating at 40 kV and 40 mA was used for XRD analysis.

Field emission scanning electron microscopy (FE-SEM)

Surface and cross-section morphology of the films were investigated using FE-SEM (SIGMA VP, ZEISS, Germany) at accelerating voltage from 10 to 20 KV. Before observation, the surface of the samples was covered with a thin layer of gold using a direct current sputtering technique (DST1, Nanostructured Coating, Tehran, Iran).

Differential scanning calorimetry (DSC)

Thermal behavior of the films samples (~8 mg) at an approximate velocity of 10°C/min was measured using DSC (Netzsch 200 F3, Germany). The thermal range between -18 to 200°C was used under the constant flow of the nitrogen atmosphere to record the glass transition temperature (T_g) and melting temperature (T_m). An empty pan was used as reference.

Fourier transform infrared spectroscopy (FTIR)

FT-IR spectroscopy (Equinox 55LS 101, Bruker, Germany) of the film samples were recorded over the wavenumber range of 500-3500 cm^{-1} and for sample preparation, the KBr pellet method was used.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 23 (IBM Corporation, Armonk, NY, USA) software. Differences between treatments were determined by Duncan′s mean comparison tests at a 5% significant level. All data were expressed as mean± standard deviation (SD).

Results and Discussion

Survival of *L. casei* in films

The survival of *L. casei* in the produced films (CMC and CMC-Pep) at different temperatures (25, 4 and -18°C) is shown in Table 1. During 30 days of storage at 25, 4 and -18°C, the viable cell numbers significantly ($p<0.05$) decreased in both types of the produced films. Ebrahimi et al. (2018) showed a negative relationship between probiotic survival and time storage. Regarding the effect

of temperature on bacterial viability, a lower viability was found at 25°C than 4°C. Gialamas et al. (2010) attributed the less viability at 4°C to the reduced bacterial metabolism at low temperatures.

There was no significant difference between the viable cell numbers of *L. casei* in two initial film-forming solutions (CMC and CMC-Pep films). However, after 10 days, regardless from the films' types, the highest *L. casei* viability (5.74 log CFU/g in CMC and 7.47 log CFU/g in CMC-Pep films) was related to the refrigerator temperature. The reduction rate of live bacteria at -18°C was higher than that at 25°C in the first 10 days of storage. After this period, the survival of frozen *L. casei* was higher than that at the room temperature. On the last day of storage at -18°C, the countable numbers of *L. casei* in CMC and CMC-Pep films were 4.1 and 6.23 log CFU/g, respectively.

Table 1- Survival of *L. casei* (log CFU/g) in CMC and CMC-Pep film during storage.

Type of films	Temperature (°C)	Time storage (day)			
		1	10	20	30
CMC	25	7.70 ± 0.05^{aA}	5.57 ± 0.2^{bA}	5.26 ± 0.21^{bA}	3.28 ± 0.2^{cA}
	4	7.70 ± 0.05^{aA}	5.74 ± 0.16^{bA}	5.52 ± 0.2^{bA}	4.85 ± 0.13^{cB}
	-18	7.70 ± 0.05^{aA}	5.5 ± 0.12^{bA}	5.38 ± 0.12^{bA}	4.1 ± 0.05^{cC}
CMC-Pep	25	7.79 ± 0.01^{aA}	7.39 ± 0.25^{bB}	6.89 ± 0.11^{cB}	4.91 ± 0.09^{dB}
	4	7.79 ± 0.01^{aA}	7.47 ± 0.13^{bB}	7.22 ± 0.13^{cB}	7.00 ± 0.01^{cD}
	-18	7.79 ± 0.01^{aA}	7.25 ± 0.07^{bB}	6.98 ± 0.02^{cB}	6.23 ± 0.19^{dE}

Data are represented as mean ± standard deviation [different capital and small letters in each column and row (respectively) indicate a significant difference ($p<0.05$) between data].

Călinoiu et al. (2016) stated that initial cold shock from freezing damaged the integrity of bacterial cell membrane. However, after adapting to temperature conditions, depending on the bacterium strain, the survival of the probiotics (especially lactic acid bacteria) would be improved. The viability of *L. casei* in both CMC and CMC-Pep films at the end of storage at 4°C was significantly ($p<0.05$) higher than other temperatures. The type of bacterial strains, water activity, water vapor, and oxygen permeability of film and storage temperature are the main factors that affect the survival of probiotics (Ebrahimi *et al*., 2018; Soukoulis *et al*, 2017; Cruz *et al*., 2009; Vasiljevic & Shah, 2008).

The results showed that the addition of SCMH to the films significantly caused less bacterial death in the films over the storage time. The remaining bacterial count in the CMC film at the end of 30 days was lower than 10^7 CFU/g at all temperatures. However, at 4 °C, it was significantly ($p<0.05$) higher compared to those stored at 25 and -18°C. Besides, *L. casei* at 4°C in CMC samples from the initial value of 7.70 log CFU/g reached to 4.85 log CFU/g at the end of storage time (showed decreasing approximately 3 log of CFU). In contrast, the countable number of *L. casei* cells in CMC-Pep film at refrigerated storage conditions remained above 10^7 CFU/g throughout the 30 days with ~1 log CFU /g decrease in viability.

The results of this study were similar to those of Yu *et al*. (2016), proving that whey peptide extract accelerated probiotics reproduction. Additionally, Settier-Ramírez et al. (2020) showed that the inclusion of casein hydrolysates in PVOH/Protein based films resulted in higher survival of *L. lactis subsp. Lactis*. Soukoulis et al. (2017) stated that the film composition significantly affected the viability of probiotics and biopolymer chemistry was a critical factor in bacterial stability. Furthermore, the enhanced *L. casei* viability with the addition of SCMH could be due to providing a micronutrient source (Soukoulis *et al*., 2017; Charalapopoulos *et al*., 2003), maintenance of suitable water activity (Zabihollahi *et al*., 2020; Soukoulis *et al*., 2017), scavenging of free radical and preventing lipid autoxidation (Soukoulis *et al*., 2017; Peng *et al*., 2010). The mentioned factors

were implicated in maintaining *L. casei* cell to keep its physical structure (Soukoulis *et al*., 2017). Similar results have been reported for the effect of other prebiotics such as inulin (Orozco-Parra *et al*., 2020; Zabihollahi *et al*., 2020), polydextrose (Karimi *et al*., 2020), oligofructose and inulin (Alvarez *et al*., 2021) on the viability improvement of probiotic bacteria within edible films matrix.

Physicochemical properties of the films

The results related to different physicochemical properties of the produced films are presented in Table 2. Thickness is an essential factor affecting the mechanical properties, transparency, and WVP of the films (Ebrahimi *et al*., 2018; Ghanbarzadeh & Almasi, 2011). The results showed that adding probiotics had little effect on the thickness, probably due to the good interaction of *L. casei* with the CMC polymer (Ye *et al*., 2018). However, the addition of SCMH significantly ($p<0.05$) increased this index. Zabihollahi *et al*. (2020) reported that the incorporation of inulin in the CMC-based probiotic films significantly elevated film thickness, because of changing the dry matter content. The lowest value (72 µm) was for the CMC film. This amount reached 72.95, 102, and 102.5 µm in the CMC-Pro, CMC-Pep, and CMC-Pro-Pep films, respectively. These results contradicted the data obtained by Ebrahimi et al. (2018), who observed that the addition of probiotics caused a significant increase in the thickness of the film and it reached from 40.3 µm in the control films to 50 µm in the films containing *L. casei*. Khodaei et al. (2020) reported that adding probiotics to the low methoxyl pectin films did not have meaningful effect on the film thickness.

Evaluation of the moisture absorption of the produced films showed that by adding SCMH, their moisture absorption increased significantly ($p<0.05$). The addition of *L. casei* also partially increased the moisture absorption, however, this increase was not significant and could be ignored. The highest observed value was 13.8% in the CMC-Pro-Pep film. The moisture absorption increased with the enhancement of available OH groups in the polymer. The hygroscopic nature of film composition was also involved in the value of moisture absorption index of the films (Ghanbari *et al*., 2018). The addition of SCMH apart from enhancing OH groups, due to their high hygroscopicity, could raise the moisture absorption. SCMH has polar carboxyl and amine groups, enabling it to attach to water molecules by hydrogen bonds (Shavandi *et al*., 2019; Nuanmano *et al*., 2015). Moreover, the interrupting effect of the additives and their prevention from interconnected network formation in CMC matrix, causes to increase the free reactive –OH and –COOH groups of CMC which leads to increase the moisture absorption capacity of probiotic films.

The water vapor barrier properties of films can be measured by the WVP index. To prevent moisture exchange between the environment and the food product, the packaging materials must have lowest permeability to water vapor as much as possible. Generally, various factors, such as the type of compounds and degree of interaction between them, the film thickness, solubility and permeability of water vapor molecules in film matrix affect the amount of WVP (Nuanmano *et al*, 2015; Kanmani, & Lim, 2013). Adding *L. casei* to the film did not make a significant change in WVP amounts. These results were similar to those of Gialamas *et al*. (2010), who showed that the addition of *L. sakei* to sodium caseinate film had no impact on the WVP. Unlike the *L. casei*, the effect of the SCMH was more noticeable. The numerical value of WVP increased from 1.12 in the CMC film to 1.68 ($\times 10^{-7}$ g/m.h.Pa) in the CMC-Pep film, attributable to the plasticizing effect of the low molecular weight SCMH. As mentioned, the presence of SCMH resulted in increased hydrophilic groups in the film structure and subsequently, the presence of more water molecules in thus leads to increase the WVP of films (Nuanmano *et al*, 2015; Kanmani, & Lim, 2013). Nuanmano et al. (2015) confirmed that gelatin hydrolysate could enhance the WVP values of fish myofibrillar protein film and the higher degree of hydrolyses led to a more upward trend because of increasing content of

hydrophilic groups. Dianin et al. (2019) reported that the incorporation of *L. casei* increased the WVP and water solubility of WPI film. Mozaffarzogh et al. (2020) observed profound effect of different probiotic bacteria on the weakening of water barrier properties of CMC-sodium caseinate films. They stated that the probiotic microbial cells probably exist in the polymeric matrix as discontinuous particles and therefore increase the chain mobility of the polymers that leads to increase WVP and moisture absorption of films.

Table 2- Physicochemical properties of produced films

Film samples	Thickness (µm)	Moisture absorption (%)	WVP ($\times 10^{-7}$ g/m.h.Pa)
CMC	72± 2.64^{a}	10.60± 0.6^{a}	1.12± 0.05^{a}
CMC-Pro	72.95± 3.69^{a}	11.24± 1.35^{a}	1.26± 0.09^{a}
CMC-Pep	102.00± 2.6^{b}	13.00± 0.04^{b}	1.68± 0.07^{b}
CMC-Pro-Pep	102.5± 3.5^{b}	13.80± 1.12^{b}	1.98± 0.10^{c}

Different letters in each column indicate a significant difference ($p<0.05$) between data

Mechanical and optical properties

The color of the film can be affected by its constituents (Shahrampour *et al*, 2020). The color parameters of the produced films are shown in Table 3. There was no visual difference between the probiotic and non-probiotic films. The results of the colorimetric analysis also revealed that probiotics had no significant effect on the lightness (L^*) of the films, but significant changes were made by adding SCMH i.e. lightness decreased. Apart from films' components and the nature of polymer, the thickness of the films can change the color parameters (Liu and Han, 2005). Addition of SCMH resulted in higher thickness of the films, which in turn causes lower L^* values (Shahrampour *et al*, 2020). The L^* values ranged from 84.21 (CMC-Pro-Pep film) to 89.38 (CMC film). The SCMH was green in color and its addition to the film compositions resulted in the film turning from colorless to green. The highest tendency for green (a^*= -9) was in CMC-Pep and CMC-Pro-Pep films. Although the produced films were somewhat yellowish, the trend was higher in the SCMH-loaded films. Piermaria et al. (2015) stated that when the difference of ΔE was over 3, the color difference between the films was detectable by the naked eyes. The ΔE in the SCMH-containing films was significantly ($p<0.05$) higher than that of the CMC films and the color difference between these two categories of films was clearly detectable. Similarly, previous studies showed that the immobilization of probiotics in the edible films did not cause significant changes in color parameters including L^*, a^*, b^*, and ΔE values (Shahrampour *et al*, 2020). Moreover, in agreement with our results on the effect of SCMH prebiotics on color parameters, Orozco-Parra et al. (2020) and Zabihollahi et al. (2020) reported that the addition of inulin even at lower concentrations, caused an increase in the ΔE values of CMC and cassava starch-based probiotic films respectively.

Table 3- Mechanical and color properties of produced films

Film samples	UTS (MPa)	EB (%)	L^*	a^*	b^*	ΔE
CMC	2.548± 0.5^{a}	17.54± 0.48^{a}	89.38± 0.18^{a}	-1.46± 0.02^{a}	4.22± 0.02^{a}	6.078± 0.07^{a}
CMC-Pro	3.679± 0.9^{a}	29.91± 0.81^{b}	89.25± 0.29^{a}	-1.70± 0.01^{b}	3.42± 0.001^{b}	5.69± 0.19^{b}
CMC-Pep	2.186± 0.6^{a}	17.05± 0.02^{a}	84.42± 0.31^{b}	-9± 0.02^{c}	7.46± 0.05^{c}	14.69± 0.20^{c}
CMC-Pro-Pep	3.138± 0.2^{a}	21.58± 1.13^{c}	84.21± 0.001^{b}	-9± 0.001^{c}	6.91± 0.08^{d}	14.55± 0.009^{c}

Different letters in each column indicate a significant difference ($p<0.05$) between data

Mechanical properties show the film's durability and ability to preserve food integrity. The effect of adding SCMH was different from that of the probiotic microorganism. The

addition of SCMH reduced both the UTS and EB indices; however, in the presence of *L.casei*, both factors increased, possibly because of plasticizing effect of SCMH in the films which reduces the intra- and intermolecular interactions between CMC polymer chains. As a result, the mobility of the polymer chains increased, which could reduce both UTS and EB values (Mandal and Chakrabarty, 2019; Nuanmano *et al*, 2015). Previous studies reported that mechanical properties were reduced with the incorporation of fish protein hydrolysates into the edible films (Hasanzati Rostami *et al*., 2017; Hoque *et al*., 2011; Giménez *et al*., 2009). The reducing of mechanical stiffness by incorporation of other prebiotics such as inulin (Zabihollahi *et al*., 2020) and polydextrose (Karimi *et al*., 2020) to the biopolymer-based films formulations, has also been approved.

As mentioned, probiotic bacteria improved both UTS and EB, probably due to good interaction between the bacteria and the film components (Ye *et al*, 2018). The UTS value increased from 2.548 MPa in the CMC film to 3.679 MPa in the CMC-Pro film. The amount of EB in these two films also increased after the addition of *L. casei* and reached from 17.54% to 29.91%. The results of this study contradicted those of Ebrahimi et al. (2018), showing that probiotics interfered with the film cohesiveness and had a negative effect on the mechanical properties. However, Piermaria et al. (2015) reported similar results. They revealed that the subjoining of microorganisms (yeast and lactic acid bacteria) in glycerol-plasticized kefiran films enhanced the UTS, attributable to the positive effect of microorganisms on reducing the plasticizing properties of glycerol. Khodaei et al. (2020) incorporated three different probiotics (*Lactobacillus plantarum*, *L. casei*, and *Saccharomyces boulardii*) into the two different biopolymer films (gelatin and low methoxyl pectin), separately. They achieved more interesting results indicating that mechanical properties of probiotic films strongly depends on the type of both biopolymer and bacteria. For example, *L. plantarum* improved the UTS of low methoxyl film but decreased the UTS of gelatin film. According to their results, the type of microorganism and biopolymer determine the effect on mechanical properties of probiotic films and this should be investigated case by case.

Fourier transform infrared spectroscopy (FTIR)

Fig. 1 shows the FTIR spectra of the CMC-based probiotic films. The pure CMC films exhibited specified peaks at 3436, 2926, 2151, 1614, 1418, 1054, 922, 855, and 719 cm^{-1}. The peaks at 3436 and 2926 cm^{-1} were related to the stretching vibration of –OH and C-H groups, respectively. Besides, the asymmetric and symmetric vibrations of –COOH groups of CMC were assigned by the peaks appeared in 1614 and 1418 cm^{-1}, respectively. The peak at 1054 cm^{-1} could be attributed to the C-O-C stretching vibrations of the polysaccharide skeleton. Furthermore, the peaks observed in the region of 700-950 cm^{-1} indicated the bending vibrations of C-H groups (Akhtar *et al*., 2018).

The incorporation of probiotic bacterium caused some changes in the spectra of the CMC film. The most important changes were the shifting of the peaks at 2926 and 1054 cm^{-1} to lower wavenumbers (2915 and 1040 cm^{-1}, respectively) and disappearing of –COOH symmetric vibration peaks at 1418 cm^{-1}. Moreover, the addition of SCMH caused a change in the FTIR spectrum of the CMC film. The decrease in the intensity of 3436 cm^{-1} peak, assigned to –OH groups, was the main observable change in the spectrum of the SCMH-loaded CMC film. In the film containing both of probiotics and SCMH, the effect of bacteria on FTIR spectrum was more than SCMH. The changes observed in the bands corresponding to –COOH and –OH groups by addition of *L.casei* and SCMH, respectively, confirmed the possible interactions (hydrogen bonds) between CMC, SCMH and probiotic bacteria, which was in agreement with the results of Zabihollahi et al. (2020), who reported the formation of hydrogen bonds

between inulin, *L. plantarum* probiotic and CMC matrix.

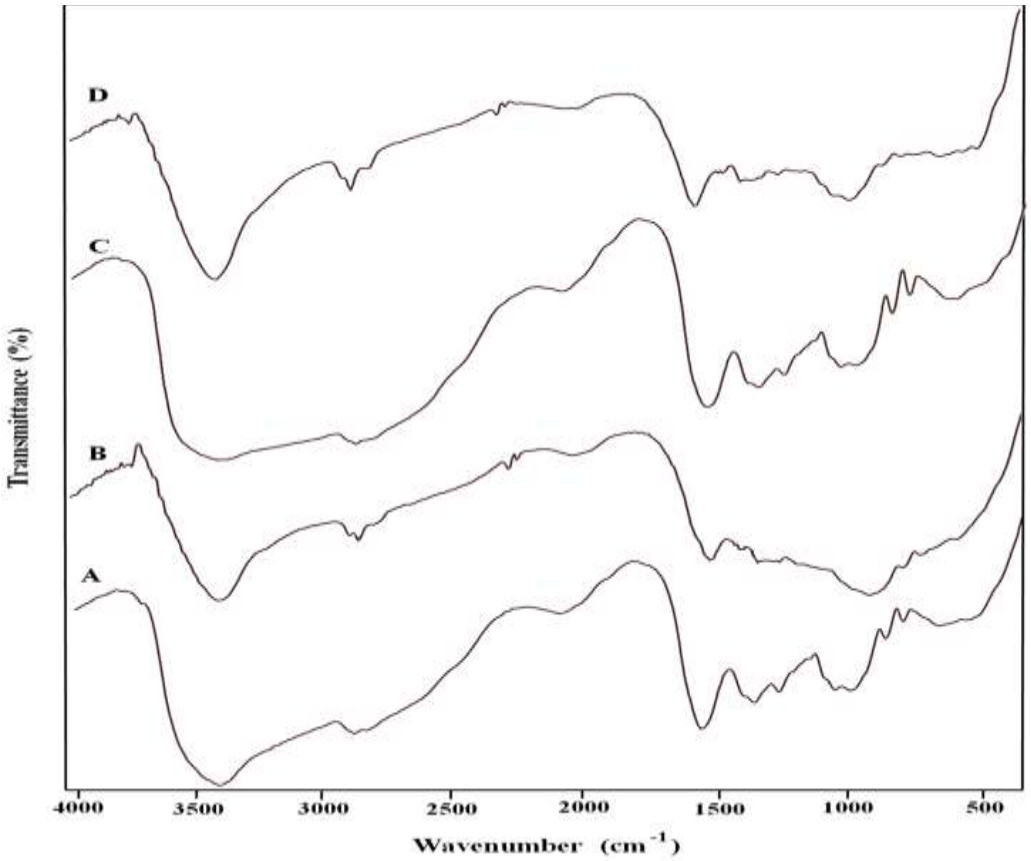

Fig. 1. FT-IR spectra of probiotic active films; (A: pure CMC film, B: *L. casei* loaded CMC film, C: SCMH loaded CMC film and D: *L. casei* and SCMH loaded CMC film).

X-ray diffraction (XRD) analysis

The XRD analysis was carried out in order to study the crystalline structure of the films. Fig. 2 shows the XRD patterns of pure and probiotic CMC films. As shown in this figure, the pure CMC film exhibited two specific peaks at 2θ of 7.8° and 20.5°, indicating the semi-crystalline structure of the CMC film. These results were in line with the previously reported studies on the CMC films (Zabihollahi *et al*., 2020; Dai *et al*., 2018). According to Fig. 2, the incorporation of probiotic bacteria had no significant effect on crystalline structure of the CMC film. However, with the addition of SCMH, the intensity of peaks, particularly the peak at 7.8°, decreased. This observation approved the plasticizing effect of low-molecular weight SCMH chains in the CMC matrix, leading to the decrease in the compactness of CMC matrix and thus the decrease of the crystalline domains in its structure. Arfat et al. (2014) reported similar results for fish protein isolate incorporated to the fish skin's gelatin films. As shown in Fig. 2, by simultaneous incorporation of *L. casei* and SCMH, the semi-crystalline structure of the CMC was preserved and probiotic bacteria decreased the negative effect of SCMH on structure of the CMC film. Karimi et al. (2020) reported similar results for the effect of *L. plantarum* and polydextrose as probiotic and prebiotic agents, respectively, on the semi-crystalline structure of the WPI films. Mozaffarzogh et al. (2020) prepared CMC-sodium caseinate probiotic films containing various probiotic bacteria including *Lactobacillus acidophilus*, *L. reuteri*, *L. casei*, *L. rhamnosus*, and *Bifidobacterium bifidum*. They observed that none of them had any effect on the crystallinity of composite film.

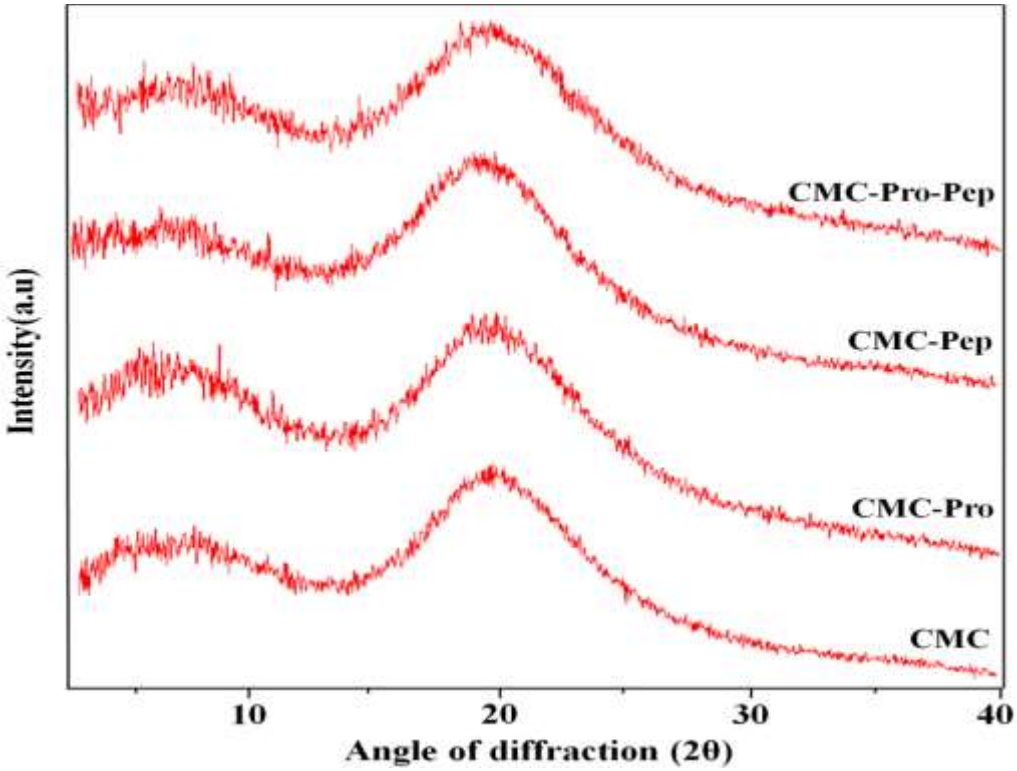

Fig. 2. XRD patterns of probiotic active films; (CMC: pure CMC film, CMC-Pro: *L. casei* loaded film, CMC-Pep: SCMH loaded film and CMC-Pro-Pep: *L. casei* and SCMH loaded film).

Field emission scanning electron microscopy (FE-SEM)

Fig. 3 depicts the FE-SEM images of the cross-section of the CMC films. All films exhibited a dense and pores-free cross section, indicating the compact microstructure of the CMC films. The only change was the increase of the projections after incorporation of bacterial cells and SCMH which is common for the heterogeneous films. Ebrahimi et al. (2018) reported similar results for the probiotics-incorporated CMC films. The surface images of films are shown in Fig. 4. The pure CMC film exhibited a smooth and homogenous surface without any cracks and projections, in agreement with results of Akhtar et al. (2018) reported for the pure CMC film having glycerol plasticizer. However, after incorporation of *L. casei*, some cracks were observed on the surface of the CMC-Pro film. The addition of SCMH caused an increase in discontinuity in the film surface. Moreover, the phase separation and aggregation phenomena were observed in the CMC-Pep film, confirming that the SCMH could be accumulated without uniform distribution in the CMC matrix. This observation is in accordance with XRD results. Moreover, the decrease of tensile strength and increase of WVP of CMC films after the incorporation of SCMH could be explained by this observation in FE-SEM analysis. Orozco-Parra et al. (2020) reported that inulin incorporation increases the discontinuity of cassava starch film microstructure. However, Karimi et al. (2020) observed more compact and uniform structure of WPI film after polydextrose incorporation indicating that polydextrose acted as a filler of the interspaces in the WPI network. Therefore, the effect of prebiotics on the microstructure of edible films depends on the nature of compounds and their miscibility with biopolymer matrix. The simultaneous incorporation of probiotic bacteria and SCMH prevented the aggregation of SCMH, but the cracks were observable in the CMC-Pro-Pep sample. In general, the presence of bacterial cells diminished the adverse effect of SCMH on the structural properties of the CMC films.

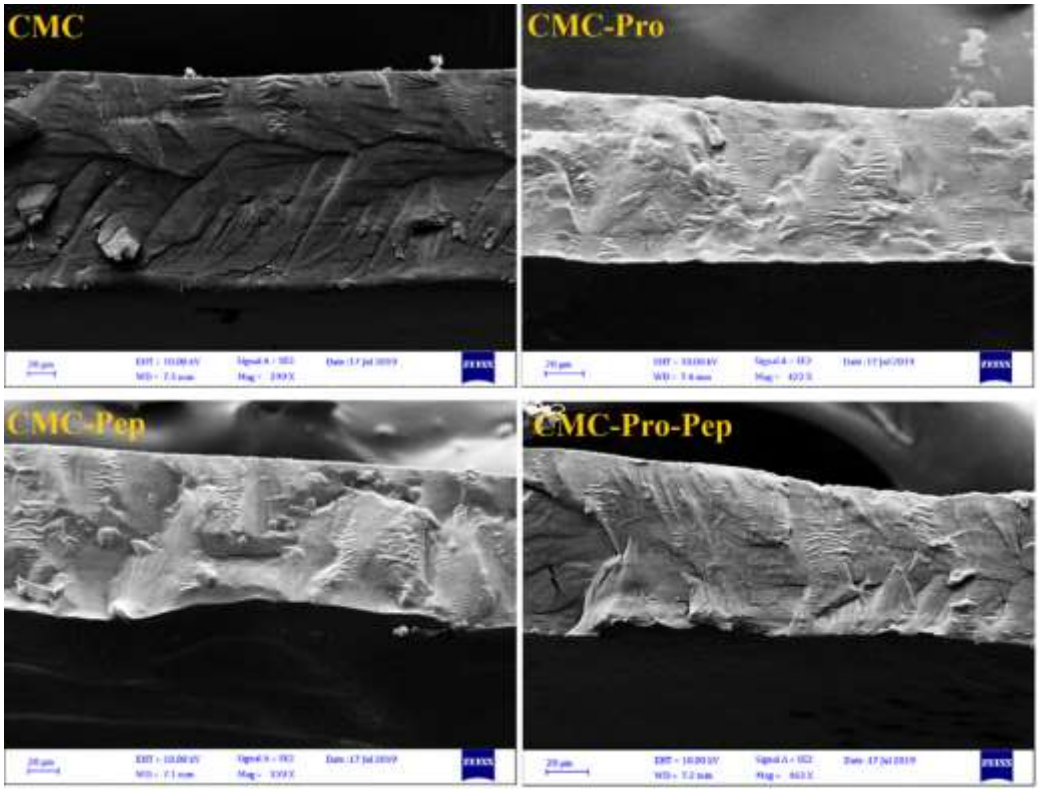

Fig. 3. FE-SEM images of cross-section of probiotic active films; (CMC: pure CMC film, CMC-Pro: *L. casei* loaded film, CMC-Pep: SCMH loaded film and CMC-Pro-Pep: *L. casei* and SCMH loaded film).

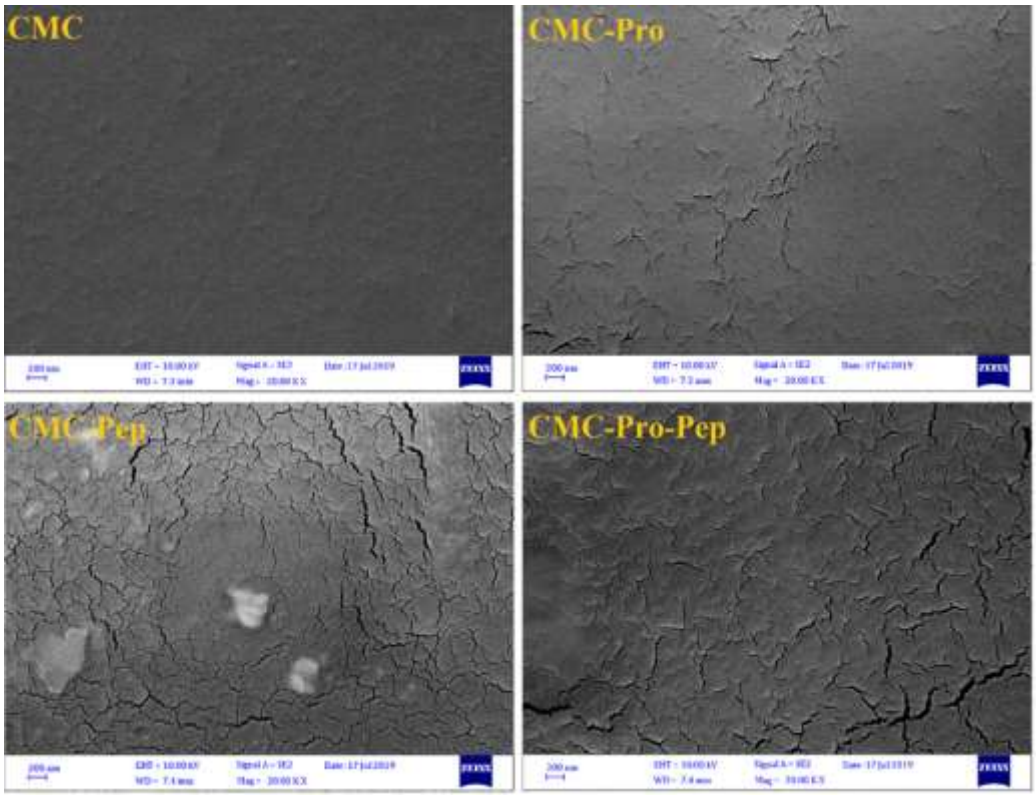

Fig. 4. FE-SEM images of surface of probiotic active films; (CMC: pure CMC film, CMC-Pro: *L. casei* loaded film, CMC-Pep: SCMH loaded film and CMC-Pro-Pep: *L. casei* and SCMH loaded film).

Differential scanning calorimetry (DSC)

Table 4 shows the results of DSC analysis of the CMC films. The T_g value of the CMC film increased from 41.2 to 42°C after the incorporation of *L. casei*. However, SCMH addition decreased T_g value. Karimi et al. (2020) reported similar results for the effect of polydextrose on thermal properties of WPI films. The SCMH-loaded film had the lowest melting point and increased by incorporation of probiotic cells. Similar to other results, the presence of bacteria decreased the adverse effect of SCMH on the thermal properties of the CMC film. As shown in table 4, the CMC-Pro-Pep sample had the highest T_m (112.4°C). The reduction of plasticizing effect of SCMH, due to the tight entrapment of bacterial cells in the CMC matrix, and the formation of new interactions after incorporation of probiotics were the reasons of this observation. In spite of forming new bands (approved by FT-IR test), the plasticizing effect of SCMH was higher, resulting in weakened thermal properties of the CMC films. The decrease of T_g and T_m of the CMC films after the incorporation of inulin as prebiotic agent was reported by Zabihollahi et al. (2020).

Table 4- Thermal properties (glass transition temperature (T_g) and melting temperature (T_m)) of CMC-based probiotic film.

Sample	T_g (°C)	T_m (°C)
CMC	41.2	109.0
CMC-Pro	42.0	109.6
CMC-Pep	38.4	108.0
CMC-Pro-Pep	41.5	112.4

Conclusions

The CMC-based probiotic films were successfully developed and characterized. Based on the results, the incorporation of probiotic cells showed no significant effects on the water barrier properties of CMC-based films. The FT-IR, FE-SEM, DSC and XRD results revealed the formation of interactions between CMC matrix and SCMH, indicating their good compatibility. In addition, the thermal properties, and crystallinity of *L. casei* and SCMH incorporated CMC-based probiotic films were acceptable. The viability of probiotic bacteria in the CMC-based films was improved through the addition of SCMH. In conclusion, the SCMH incorporated CMC-based film can be a good carrier for probiotics as a bioactive food packaging system. The as prepared films may be applied as coatings or wrappings to a variety of foods including meat products, fruits and vegetables, cheese and butter, and bakery products, providing them with potential health benefits to the consumers, besides being potentially able to inhibit the growth of spoilage microorganisms on food surface, thus increasing food shelf life. Further studies are required to investigate the effects of developing probiotic films on the shelf-life extension of real food systems.

Conflicts of interest

The authors certify that they have no conflict of interests with respect to this manuscript.

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فیلم خوراکی زیست فعال برپایه کربوکسی متیل سلولز حاوی لاکتوباسیلوس کازئی و پروتئین هیدرولیز شده ماهی

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چکیده

به‌دلیل تمایل به غذاهای طبیعی و سالم، بازار غذاهای فراسودمند به سرعت در حال رشد است. در این میان، پروبیوتیک‌ها به دلیل توانایی بالقوه آن‌ها، در فرمولاسیون‌های غذایی سالم، به‌طور جدی مورد توجه قرار گرفته‌اند. بیشترین نگرانی در مورد پروبیوتیک‌ها این است که ممکن است تعداد باکتری‌های پروبیوتیک در زمان مصرف کمتر از مقدار مورد نیاز (10^7 CFU/g) باشد. بنابراین در این مطالعه، فیلم‌های خوراکی پروبیوتیک کربوکسی متیل‌سلولز (CMC) حاوی *لاکتوباسیلوس کازئی* و پروتئین هیدرولیز شده عضله ماهی کپور نقره‌ای (SCMH)، تهیه شد و زنده‌مانی سلول‌های باکتری در طول ۳۰ روز نگهداری (در فواصل زمانی ۱، ۱۰، ۲۰ و ۳۰ روز) در دماهای ۲۵، ۴ و ۱۸- درجه سانتی‌گراد بررسی گردید. جهت استخراج پروتئین از روش انحلال قلیایی/ ترسیب اسیدی استفاده شد. ایزوله پروتئین استخراجی به‌وسیله آنزیم آلکالاز (۵٪ وزنی/ وزنی) در دمای ۵۰°C و pH= ۸ به مدت ۳ دقیقه هیدرولیز گردید. فیلم‌ها با انحلال SCMH و CMC با نسبت ۲:۱ در آب مقطر، تهیه شدند و *لاکتوباسیلوس کازئی* با غلظت 10^8 CFU/mL به فیلم‌ها اضافه شد. خصوصیات رنگی، فیزیکی، استحکام کششی نهایی (UTS) و ازدیاد طول در نقطه شکست (EB) فیلم‌ها بررسی شد. الگوهای ساختاری نمونه‌های فیلم با پراش‌سنج اشعه X در دمای اتاق با زاویه پراش (۲θ) از ۵ تا ۴۰ درجه به‌دست آمد. طیف‌سنجی FT-IR فیلم‌ها در طول موج $3500-500\ cm^{-1}$ ثبت شد. نتایج آنالیز FT-IR، XRD و DSC، حاکی از شکل‌گیری پیوند هیدروژنی بین *لاکتوباسیلوس کازئی* و ماتریس فیلم و همچنین اثر پلاستی‌سایزری SCMH بودند. به‌طوری که فیلم CMC خالص حاوی باکتری، بالاترین خصوصیات مکانیکی (EB=۲۹/۹٪، UTS=۳/۷ MPa) را داشت. افزودن SCMH به فیلم‌ها، به‌طور قابل توجهی ($p<0.05$) زنده‌مانی *لاکتوباسیلوس کازئی* را در همه دماها افزایش داد و توانست در پایان دوره نگهداری در دمای ۴°C مقدار آن را در حد ۰/۰۱ ±۷ log CFU/g نگه دارد.

واژه‌های کلیدی: پروتئین هیدرولیز شده، *لاکتوباسیلوس کازئی*، کربوکسی متیل‌سلولز، فیلم خوراکی، پروبیوتیک.

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