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Investigating the physical and mechanical properties of polylactic acid-nanocellulose nanocomposite film containing *Lactobacillus casei* probiotic bacteria

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ABSTRACT

Nowadays, increasing the viability of probiotics by directly adding them to edible films to prevent their mortality is of great interest. In this study, the survival of *Lactobacillus casei* probiotic bacteria added to polylactic acid-nanocellulose nanocomposite film was investigated. For this purpose, three edible films including polylactic acid, polylactic acid + nanocellulose, polylactic acid + nanocellulose+ *L. casei* (9 log CFU/g) were prepared and the physical and mechanical characteristics of the film , as well as the survival of L. casei bacteria, as well as the survival of *L. casei* bacteria were investigated. The results of the mechanical test showed that the use of probiotic bacteria decreased the tensile strength and elongation at the break of the polylactic acid-nanocellulose film ($P < 0.05$), but the addition of nanocellulose improved the mechanical properties of the polylactic acid film. The results of physical tests including humidity, solubility, and water vapor permeability showed that the addition of probiotic bacteria and nanocellulose improved the physical properties of the film, but the opacity of the films increased ($P<0.05$). Based on the results of the present study, during storage at 4°C, the survival of probiotic bacteria in the nanofilm decreased, so that *Lactobacillus casei* bacteria decreased from 8.25 log CFU/g on the zero-day of the study to 6.12 log CFU/g reached at the end of the study (day 16), but it was within the permissible range (6 log CFU/g). Therefore, adding *L. casei* probiotic to polylactic acid-nanocellulose nanocomposite film can be a suitable carrier at refrigerator temperature.

1. Introduction

Polylactic acid (PLA) is a biodegradable polymer listed by the US Food and Drug Administration.[1]It was placed GRAS and is recognized as safe for all food packaging [1, 2]. PLA, in addition to having favorable characteristics such as high mechanical strength, transparency and resistance against the passage of ultraviolet light, also has disadvantages such as high brittleness, low thermal stability and weak resistance against moisture and oxygen, which is used as a substitute for synthetic polymers in the industry. Packaging is limited [2, 3]. Therefore, in order to improve these properties, modifiers such as nanoparticles are used in its structure. The use of nanoreinforcers and the production of polymer nanocomposites are effective methods that have been popularized to enhance the properties of polymers in recent years. Cellulose (C6H10O5) is one of the most abundant (700 billion tons per year) organic materials on the planet and is widely produced from various sources such as wood, flax, hemp and bacteria. The potential of using cellulose nanofibers as a reinforcement of polymeric materials provides a new path for the production and development of better composites along with the creation of added value. Cellulose nanofibers with a diameter below 100 nm are extracted from the cell walls of various natural sources (such as wood and cotton pulp) by chemical, mechanical or a combination of these treatments. In general, the properties of cellulose nanoparticles, in addition to the raw material, can be influenced by factors such as the preparation method, as well as the degree and state of their homogenization [4, 5]. Cellulose nanofibers have been used as reinforcement in various polymers such as carboxymethyl cellulose [6], chitosan [7], polylactic acid [8] and polylactic acid-chitosan [9], which results in strengthening and improving the physical and mechanical properties of nanocomposites. It has been a production. In addition, the combination of bioactive agents such as probiotics and prebiotics in nanocomposite films improves their performance. This type of nanocomposite films is called "bioactive nanocomposite films" [4, 10]. In this regard, lactic acid bacteria and their metabolites have been introduced as biological preservatives to improve microbial safety and increase the shelf life of food. Lactic acid bacteria are usually considered as food preservative organisms due to the antimicrobial functions caused by the activity of small and heat-resistant peptides called bacteriocins [11, 12]. In addition, the use of edible films containing lactic acid bacteria in food matrix is important in terms of transferring these bacteria to consumers and producing antimicrobial substances or competing with spoilage microorganisms and creating high antimicrobial potential [13, 14]. One of the most common microbial cultures used in the production of various probiotic products can be called probiotic bacteria.*Lactobacillus casei*[2] pointed out [15].*Lactobacillus casei* (*L. casei*) is one of the important probiotics in food products. This bacterium is gram-positive, mesophilic, rod-shaped, microaerophilic, catalase-negative and without spores. Lactic acid produced by*Lactobacillus casei* It is of L+ type and is resistant to vancomycin [16].

Therefore, according to the mentioned cases, in this study, the survival of probiotic bacteria and the physical and mechanical properties of the probiotic edible film based on polylactic acid-nanocellulose were investigated.

2- Materials and methods

1-2- Raw materials

In this research, polylactic acid (PLA) granules from Fakior, Germany, cellulose nanofibers (with an average diameter of 35 nm and an average length of 5 micrometers with 99% purity, from Nano Novin Polymer, Iran), MRs broth and MRS agar (from Merck, Germany) and bacterial strain*Lactobacillus casei* (1608 PTCC) Iran's scientific and industrial research center was used in the production of films. Other chemicals used were of laboratory grade purity and were purchased from Merck (Dermadest, Germany).

2-2- Preparation of nanocomposite containing probiotics

Lyophilized ampoules of bacteria*L. casei* It was broken under the biological hood. About 1 gram of lyophilized bacteria per 100 ml was prepared in MRs broth culture medium and incubated at 37°C for 24 hours. Bacterial cells were precipitated by centrifugation at ×9000 for 10 minutes [17].

To prepare pure PLA film, by solution casting method[3] (molding method) was used. For this purpose, a solution of 1% PLA (weight/volume) should be prepared in chloroform (solvent) and stirred with the help of a magnetic stirrer for 8 hours at room temperature until the PLA granules dissolve well. Nano cellulose (0 and 1 percent by weight/volume) was added to the mentioned solution, as well as film samples after bacterial inoculation.*L. casei*(Until reaching the final value of 10 CFU/g^{9}) was poured on a glass plate until the solvent (chloroform) evaporated under a chemical hood at a temperature of 25 degrees Celsius during 48 hours. After this step, the film was separated from the glass mold and placed in a container containing silica gel until use [18].

2-3-Measuring the physical properties of films

3-2-1-Measuring the thickness of films:

A micrometer with an accuracy of 0.01 mm was used to measure the thickness. The thickness was measured

1 -Generally Recognized as Safe
2- *Lactobacillus casei*
3- Solvent Casting Method

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at 5 points of the film and its average value was reported [19].

3-2-2 Measuring the moisture content of films

Film samples with specific weight were placed in glass plates that had already reached a constant weight and were weighed (W1). Then it was dried in the oven at 105 degrees Celsius for 24 hours. The sample with the plate was removed after this period and after cooling in the desiccator, it was weighed again (W2). The moisture content of the films was calculated based on wet weight from Equation 1 [20].

Relationship 1

100×W1/ (W2-W1) = moisture percentage

3-3-2-Evaluation of solubility of films in water

To measure the solubility of the films, 2 cm square pieces of the films were prepared. The dry weight of the samples was obtained by drying them in an oven at 105 degrees Celsius for 24 hours. Then the samples were placed in a 50 ml container of water for 24 hours. Then the film was removed from the water and placed in an oven at 105 degrees Celsius for 24 hours to dry. Finally, the final weight was measured [19].

4-3-2-Permeability against water vapor

In order to measure the permeability of films to water vapor (ASTM E 96-02), first 10 milliliters of distilled water was poured into the permeability measuring cells, and then the glass cells whose surface was sealed by the film and with the help of grease were placed in a desiccator containing silica gel. . Water at a temperature of 25 degrees Celsius creates 100% humidity. The difference in humidity on the two sides of the film at a temperature of 25 degrees centigrade creates a heater pressure difference equal to 337.2 x 103 pascals. Cell weight changes were measured over time using a digital scale with an accuracy of 0.0001 grams. The water vapor transfer rate in terms of (gram)-meter-second was equal to the slope of the resulting lines divided by the cell surface and was obtained from equation 2 [19]. The area of the cells was 0.00287 square meters. From multiplying the water vapor transmission rate (WVTR) by the film thickness (L) and dividing it by the pressure difference on the two sides of the film (AP), the water vapor permeability (WVP) $(10)^{-11}$ $gs^{-1}m^{-1}Pa$) was obtained [19].

Relationship 2

Cell surface (meters) / slope of the line (g/s) = water vapor transfer rate (g^{1-} $Second^{2-}$ Meter)

5-3-2-animosity

To determine the turbidity, the films prepared as pieces with dimensions of cm^2 1 x 4 was cut. Then these parts were placed in the cell of the spectrophotometer and their absorbance was read at a wavelength of 600 nm. Equation 3 was used to determine the turbidity [21].

Relationship 3

100x absorbance = turbidity

4-2-X-ray diffraction spectrometer (XRD)

In this research, by using CuKa radiation in the range of angle (25) 5 to 50, the X-ray diffraction pattern of the prepared films was recorded. Crystallization percentage was calculated using the Scherer relation by origin software [2].

Relationship 4

$$X\ (\%) = \frac{And}{Ac + Aa} \times 100$$

In this regard, AC is the area of the crystalline area (m^2) and Aa the area of the amorphous region (m^2) Is.

5-2- Measuring the mechanical properties of the film

The amount of tensile strength and elongation to tear was evaluated using a device (Testometric, M350-10CT, England) and according to the ASTM D882 standard. Before the test, the samples were placed in a desiccator containing magnesium nitrate to create relative humidity (50%). Samples with dimensions of 10 cm x 1 cm were prepared and placed between the jaws with a distance of 50 mm and tested with a 50 kg barbell at a speed of 10 mm/s until the breaking point. The test was performed with at least 5 repetitions for each film and the tensile strength values Elongation to breaking point were obtained from the stress-strain curves [19].

6-2- Investigating the survival of probiotic bacteria in composite films

In time intervals of 4 days to 16 days, the films were transferred from the zip cap to Erlenmeyer flasks containing 100 ml of phosphate buffer solution under aseptic conditions and kept in a Shakerdar incubator at a temperature of 37 degrees Celsius for 2 hours until the bacteria had a chance to be released from the film. to have Different dilutions of the whole sample were prepared and after cultured in the form of pore plates in MRS agar culture medium, they were kept in a greenhouse at a temperature of 37 degrees Celsius for 48 hours [22]. Live bacteria cells were selectively counted according to the Subtractive enumeration method (SEM).

2-8 Statistical evaluation

The experiments were conducted in three repetitions and in the form of a completely randomized design. Data analysis was done using SPSS 18 software. Comparison of means was done with Duncan's test (One way Anova) with 5% error level. Graphs were drawn using Microsoft Excel 2013 software.

3. Results and Discussion

1-3-Features of the film

1-1-3-Physical characteristics of the film

Thickness is an important factor in determining the mechanical properties, permeability to water vapor and transparency of the film. According to the results, the lowest thickness values (Table 1) were in PLA film (0.043 mm), by adding nanocellulose and probiotic bacteria to PLA film, the thickness increased

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significantly, and the highest values were in the treatment of PLA + nanocellulose +*L. casei* was observed (0.057 mm) ($P<0.05$). The reason for the increase in thickness after the addition of nanocellulose and probiotic bacteria can be related to the placement of these fillers and the increase of solid matter in different layers of the PLA film matrix [23, 24].

Solubility and moisture content are two important factors of biodegradable films that affect the film's resistance to water, especially in humid environments. The results related to humidity and solubility (Table 1) of different films were consistent. According to the results, the highest values of moisture and solubility were in PLA film (18.51% and 23.02%, respectively) ($P<0.05$). By adding nanocellulose to PLA film, the amount of moisture and solubility decreased ($P<0.05$). Based on studies, the composition of nanocelluloses in PLA matrix reduces moisture absorption due to the creation of hydrogen bonds and electrostatic interactions between cellulose and PLA nanoparticles [7, 24]. But the values of moisture and solubility did not change significantly with the addition of probiotic bacteria ($P < 0.05$). These results are consistent with the results of Salimirad et al.*L.casei*And*Bacillus coagulans* And the combination of these two bacteria had no significant effect on the moisture content and solubility of the films [4]. Also, La Storia et al. (2020) also announced the addition of bacteria*Lactobacillus corvatus* Whey protein film has no significant effect on film moisture [25].

According to the results, the highest values of permeability to water vapor (Table 1) were in PLA film (10^{-11} gs^{-1}m^{-1}Well 1/60). By adding nanocellulose to PLA film, the permeability to water vapor decreased (10^{-11} gs^{-1}m^{-1}Pa 1/52) ($P>0.05$). The reason for this is the high degree of crystallinity of nanocellulose. which acts as a nucleating agent in the polymer matrix. The presence of cellulose nanoparticles in the polymer matrix creates a longer path for water molecules to pass through. The increase in polymer crystallinity due to the presence of cellulose nanoparticles increases cohesion and density between polymer chains and reduces the free spaces between them, which is another reason for reducing the permeability of nanocomposite biofilms [26، 27]. These results are consistent with the results of Mirabolghasemi (2021) in relation to water vapor permeability values of PLA nanocomposite biofilm reinforced with cellulose nanocrystal [26]. The values of permeability to water vapor also decreased with the addition of probiotic bacteria, and the lowest values were in the PLA+ nanocellulose+ treatment.*L. casei*observed (10^{-11} gs^{-1}m^{-1}Pa 1/48) ($P>0.05$). According to the study of Souza et al. (2010), the permeability of edible films to water vapor and oxygen depends on various factors such as thickness, chemical bonds between polymer and water molecules, the size of the holes in the film structure, and the degree of flexibility of the polymer chain [28]. It seems that the probiotic bacteria present in the film matrix reduce the degree of flexibility in the polymer chain [13].

Transparency is a very important feature for films that are used as a coating or packaging for food. According to the results, the lowest turbidity values (Table 1) were found in PLA film (0.96) ($P < 0.05$) and with the addition of nanocellulose and probiotic bacteria to PLA film, the turbidity level increased and the highest values were in the treatment of PLA + nanocellulose +*L. casei* observed (1/21) ($P<0.05$). The addition of nanocellulose to PLA film blocked the passage of light through the film network and thus reduced the transparency. In addition, adding bacterial cells to the nanocomposite film can affect the light passing through the film by increasing light scattering [29]. Similar results were reported by Bekhit et al. (2018), who stated that probiotic bacteria, with different refractive index, reduce film transparency due to increased light scattering [30]. Increasing the opacity of films may reduce consumer demand, but the reduction of transparency by films can be an advantage in the food packaging industry, because it reduces the passage of visible and ultraviolet light through the film, and thus reduces unwanted chemical reactions such as oxidation. Lipids and nutritional value decrease [2، 31].

Table 1. Physical properties of PLA/nano cellulose (NC) composite films containing *L. casei*

Film	Thickness (mm)	Moisture (%)	Solubility (%)	WVP (10^{-11} gs^{-1}m^{-1}well)	Turbidity
PLA	0.043± 0.001 ^{b}	18.51± 0.50^{a}	23.02± 0.50^{a}	1.60± 0.02^{a}	0.96± 0.02^{c}
PLA + NC	0.050± 0.001 ^{a}	17.04± 0.19^{b}	20.42± 0.56^{b}	1.52± 0.03^{b}	1.05± 0.03^{b}
PLA + NC+ *L. casei*	0.057± 0.002 ^{a}	16.55± 0.49^{b}	19.44± 0.60^{b}	1.48± 0.01^{c}	1.21± 0.03^{a}

Different letters in the each column show a significant difference between treatments at $p<0.05$.

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Data are shown as mean value± standard deviation.

2-1-3-Mechanical properties of the film

As can be seen (Table 2), by adding nanocellulose to PLA film, the tensile strength has increased (P<0.05). In general, the mechanical performance of composites, especially bio-nanocomposites, depends on factors such as compatibility between polymer matrix and reinforcement, stress transfer to reinforcement, volume fraction of reinforcement, aspect ratio of reinforcement, orientation of reinforcement and crystallinity of the matrix [32]. The improvement of the mechanical properties of bio-nanocomposites by adding cellulose nanocrystals to the PLA matrix can be attributed to the high degree of crystallization and good mechanical properties of this nanoparticle. Increasing the crystallinity of the polymer matrix leads to the improvement of the tensile modulus and strength of nanocomposites [26، 33]. These results are consistent with the results of Mirabolghasemi (2021) in relation to the tensile strength values of biofilm PLA nanocomposites reinforced with cellulose nanocrystal [26]. The results related to the maximum tension before the breaking point were opposite to the results related to the tensile strength, the addition of nanocellulose caused a decrease in the maximum tension before the breaking point (P<0.05), which indicates an increase in the strength of the film after adding It is nanocellulose. But by adding probiotic bacteria to PLA film, the tensile strength and maximum tensile strength decreased before the breaking point. The reason for the reduction of the mentioned characteristics after the addition of probiotic bacteria is due to the placement of microbial cells inside the nanocomposite matrix and subsequently the reduction of cohesive bonds in the film structure [4].

Table 2. Mechanical properties of PLA/nano cellulose (NC) composite films containing *L. casei*

Film	Tensile strength (MPa)	Elongation at break (%)
PLA	43.26± 0.74^{b}	2.96± 0.03^{a}
PLA + NC	48.34± 0.47^{a}	2.71± 0.03^{b}
PLA + NC+ *L. casei*	42.39± 0.58^{b}	2.43± 0.09^{c}

Different letters in the each column show a significant difference between treatments at p<0.05.
Data are shown as mean value± standard deviation

3-1-3- X-ray diffraction test (XRD)

According to the X-ray diffraction test (Chart 1) for different films, it was found that all films have their highest peak at the angle^{about}Show ϴ2=12. As can be seen, the pure PLA film showed an amorphous nature, so PLA can be considered a semi-crystalline polymer. After adding nanocellulose, the intensity of the peak appeared in^{about}12 increased, which indicates an increase in the crystallinity of the PLA film after adding NC [2]. But with the addition of probiotic bacteria, no noticeable change in the intensity of the peaks was observed. These results are consistent with the results of Dai et al. (2018) who reported that the XRD pattern of the film based on hydroxymethyl cellulose of lonjac flour containing*Lactobacillus paracasei* There was no significant difference with the control group [34]. Also, similar results were reported by Mozaffarzogh et al. (2020), who stated that probiotic bacteria had no effect on the XRD pattern of the film [13].

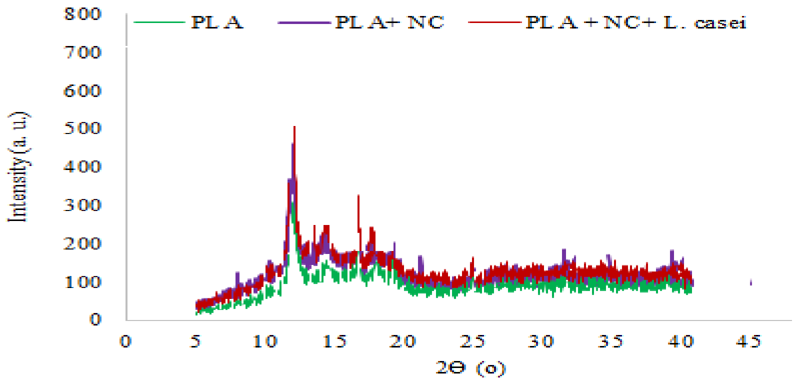

Figure 1: X-ray diffraction of different films.

2-3-Survival of probiotic bacteria in nanocomposite film

The main condition to guarantee the effectiveness of probiotic active films is to ensure the survival and performance of probiotic microorganisms included in the matrix of biopolymers [25]. Based on the results of the present study (Chart 2), with increasing storage time, the survival of probiotic bacteria in the film decreased. So that the number of bacteria*L. casei*From 8.25 log CFU/g on the zero day of the study to 6.12 log CFU/g at the end of the study (day 16). According to the law approved by the Food and Agriculture Organization of the United Nations (FAO) (2018), the number of probiotic bacteria on the surface of the film should be at least 6 log CFU/g [35], which in this study had the allowed amount until the end of the storage period. In fact, during the storage period, probiotic bacteria decreased by 2 log CFU/g. Therefore, it seems that PLA-nanocellulose film can be a good carrier for live probiotic cells. Recent studies have shown that the number of probiotic bacteria in protein and polysaccharide-based films decreases by 1-2 log CFU/g during storage at refrigerator temperature [4، 13، 31].

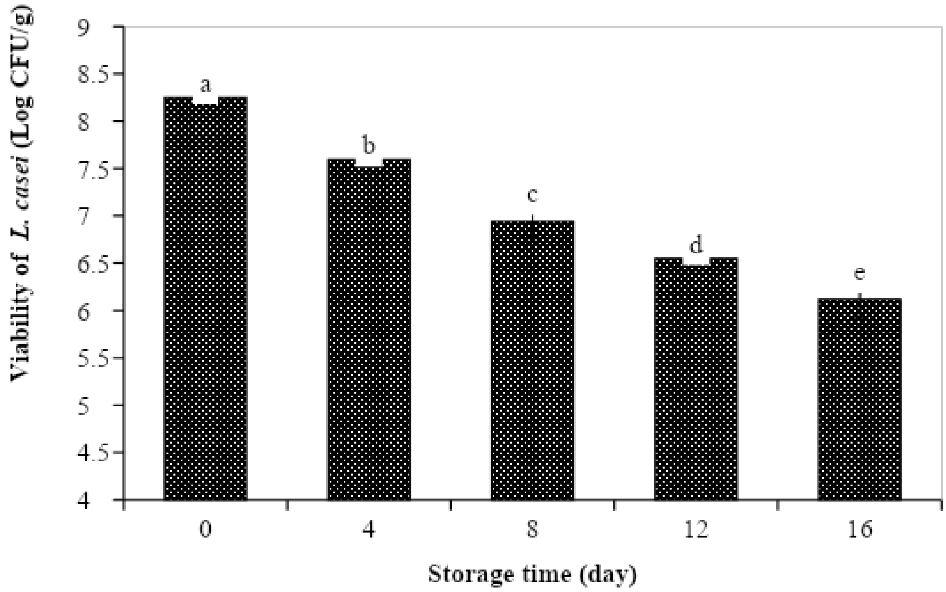

Figure 2: Viability of *Lactobacillus case*i in the nano composite films during storage at 4 aboutC.

Different letters in the each day show a significant difference between treatments at.

4 - Conclusion

In the present study, edible PLA/nanocellulose composite nanofilm containing bacteria*L. casei* Stored at a temperature of 4 degrees Celsius until the end of the 16-day storage period, it had the permitted amount of probiotic bacteria. A significant increase in thickness, transparency and a significant decrease in tensile strength, moisture and solubility were observed in probiotic nanocomposites compared to nanofilm. In general, PLA/nanocellulose composite nano film can be used as a suitable carrier for probiotic bacteria*L. casei* in food packaging at refrigerator temperature. Future studies can be done with the aim of investigating the effect of adding probiotics to edible film on the sensory characteristics in order to use them in food packaging.

5- Resources

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بررسی ویژگی‌های فیزیکی و مکانیکی فیلم نانوکامپوزیتی پلی‌لاکتیک اسید-نانوسلولز حاوی باکتری پروبیوتیک *لاکتوباسیلوس کازئی*

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مسئول مکاتبات: *

چکیده

امروزه بالا بردن زنده مانی پروبیوتیک‌ها از طریق افزودن مستقیم آنها به فیلم‌های خوراکی برای جلوگیری از مرگ و میر آنها، بسیار مورد توجه است. در این مطالعه، بقای باکتری پروبیوتیک *لاکتوباسیلوس کازئی* اضافه شده به فیلم نانوکامپوزیتی پلی‌لاکتیک اسید-نانو سلولز در یخچال بررسی شد. بدین منظور ۳ فیلم خوراکی شامل، پلی‌لاکتیک اسید، پلی-لاکتیک اسید + نانوسلولز، پلی‌لاکتیک اسید+ نانو سلولز+*لاکتوباسیلوس کازئی* (log CFU/g ۹) تهیه و ویژگی‌های فیزیکی و مکانیکی فیلم‌ها و همچنین بقای باکتری *لاکتوباسیلوس کازئی* بررسی شد. نتایج آزمون مکانیکی نشان داد استفاده از باکتری پروبیوتیک سبب کاهش مقاومت کششی و ازدیاد طول تا نقطه شکست، فیلم پلی‌لاکتیک اسید- نانوسلولز شد (P<۰/۰۵) اما افزودن نانوسلولز سبب بهبود ویژگی‌های مکانیکی فیلم پلی‌لاکتیک اسید شد. نتایج حاصل از آزمون‌های فیزیکی شامل رطوبت، حلالیت، نفوذپذیری به بخار آب نشان داد افزودن باکتری پروبیوتیک و نانوسلولز سبب بهبود خواص فیزیکی فیلم شد، اما کدورت فیلم‌ها افزایش یافت (P<۰/۰۵). براساس نتایج مطالعه حاضر، با افزایش زمان نگهداری در دمای ٤ درجه سانتی گراد، بقای باکتری‌های پروبیوتیک در نانوفیلم کاهش یافت به طوریکه، باکتری‌های لاکتوباسیلوس کازئی از log CFU/g ۸/۲۵ در روز صفر مطالعه به log CFU/g ٦/۱۲ در پایان مطالعه (روز ١٦) رسید، اما از محدوده مجاز (٦ log CFU/g) برخوردار بود. بنابراین، افزودن پروبیوتیک *لاکتوباسیلوس کازئی* به فیلم نانوکامپوزیتی پلی‌لاکتیک اسید-نانو سلولز، می‌تواند حامل مناسب آن در دمای یخچال باشد.