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The effect of antibacterial edible film based on sodium caseinate-nanocrystal cellulose containing cells and supernatant of *Lactobacillus reuteri* on quality of kebab

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ABSTRACT

Eible films due to the environmental compability can be a good alternative to packaging made from oil materials. Today, consumers' concerns about the toxicity of artificial preservatives in the food industry have led to the search for natural antioxidants and antimicrobials sources. Ready-to-eat foods such as kebabs are known as the most susceptible foods for the growth of microbes and cause food poisoning. The purpose of this study was to investigate the effect of an antimicrobial film of caseinates-cellulose nanocrystal containing supernatant and *lactobacillus reuteri* (PTCC 1655) on microbial and chemical properties of kebab. Therefore, a sodium caseinate-cellulose nanocrystal was prepared by adding 10^6 CFU/cm^2 *Lactobacillus reutri* PTCC 1655 and germ-free supernatant, and mechanical properties evaluation tests, water vapor permeability, solubility and moisture were evaluated on the prepared films. The films were wrapped on kebab and each and every four days were monitored by microbial tests (total viable count, psychrophile bacteria and mold and yeast), chemical (pH, acidity, thiobarbituric acid and total volatile nitrogen, peroxide index). Among the treatments, the sodium caseinate - nanocellulose film containing *Lactobacillus reuteri* was the most effective treatment in increasing the shelf life of the kebab. As a result, it is suggested to use sodium caseinate-nanocellulose film containing *Lactobacillus reuteri* to increase the kebab shelflife.

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1- Introduction

Meat products are a valuable source of protein and essential amino acids [1]. Among the meat products that are popular in our country, we can mention all kinds of kebabs. Today, all kinds of meat products are used in large quantities in the world, and in Iran, due to mechanization of life and lack of time for cooking, its consumption is increasing day by day [2]. Meat and meat products can be a suitable environment for the growth of pathogenic and dangerous microbes for human health. There are many reports about the transmission and spread of food diseases caused by meat and meat products. Therefore, the safety of meat products is a serious concern for public health [3]. Today, the use of cold above zero (4 degrees Celsius) is one of the meat preservation methods, which reduces the activity of spoilage-producing microorganisms in the product and, as a result, postpones spoilage. Considering the limitation of the time of keeping fresh meat in the cold above 0 degrees Celsius and the importance of maintaining its quality until consumption, researchers are looking for methods to increase the shelf life of meat in a cold and fresh form, and to reach consumers with suitable edible quality [4]. In recent years, the desire to use fresh food that has undergone the least processing has expanded a lot. Because many of the common methods of food processing and storage, especially for fresh food (fresh meat), are not suitable and have adverse effects on the final quality of the product. Using active packaging is a new way to store this type of food. This type of packaging, in addition to having the main deterrent properties of the usual packages (such as the deterrent properties against gases, water vapor and mechanical stresses), by changing the packaging conditions, they improve the safety, shelf life or sensory characteristics of the food, while maintaining the quality of the food. . In recent years, antimicrobial packaging has received attention due to the improvement in the process of food preservation [5]. Their main goal is to maintain quality and ensure safety by inhibiting spoilage and disease-causing microorganisms. Recently, the addition of lactic acid bacteria to biodegradable edible films has been proposed to inhibit pathogenic bacteria [6]. It has been reported that some lactic acid bacteria can limit the growth of various microbes including bacteria, molds and yeasts through the production of organic acids, hydrogen peroxides, enzymes and bacteriocin-like compounds [7]. Also, the lactic acid of bacteria can cause changes in the mechanical and physical properties of the film, which are different depending on the type of bacteria [8]. Sodium caseinate films are odorless, tasteless, have nutritional value, mechanical properties and high resistance against carbon dioxide, oxygen and aroma gases [9]. In antimicrobial packaging, the diffusion of antimicrobial substances from the polymer matrix to the surface of the food is done slowly and over a long period of time, as a result, there will be a high concentration of the antimicrobial substance on the surface of the product for a long time. Antimicrobial substances increase the shelf life of food products by reducing the growth and prolonging the lag phase of microorganisms or destroying microbes [10], so they have been taken into consideration due to the improvement of the food preservation process. The purpose of this research is to prepare new edible films based on the inclusion of Lactobacillus serotrius lactic acid bacteria PTCC 1655 and its supernatant in sodium caseinate-nanocrystalline cellulose film matrix.

2- Materials and methods

2-1- Preparing and multiplying microbial samples

Bacteria*Lactobacillus sereotri*PTCC1655))، *Salmonella enterica*(PTCC 1709) *and Staphylococcus aureus* (PTCC 1189)، They were purchased as lyophilized cultures from the microbial culture collection of Iran Scientific and Industrial Research Organization. For inoculation of lactic acid bacteria in culture medium MRS Broth for*Salmonella enterica* And*Staphylococcus aureus* BHI Broth culture medium was used. In completely sterile conditions, break the microbial ampoule and pour 100 microliters of the culture medium into the ampoule containing the microbe until the lyophilized microbial powder is completely dissolved. The microbial suspension obtained inside 20 cc of MRS Broth culture medium for lactic acid bacteria and BHI Broth culture medium for*Salmonella enterica* And*Staphylococcus aureus* inoculated Also, a linear culture of microbial suspensions was prepared on MRS Agar and BHI Agar culture media respectively. Then all the test tubes and plates were placed in a greenhouse at a temperature of 37 degrees Celsius. The culture medium containing the obtained bacteria was mixed with glycerol in a ratio of 30 to 70 in a Falcon tube as a mother culture in the refrigerator and 2 ml of the culture tube content was kept in a freezer at -80°C.

2-2- Preparation of supernatant without microbial cells

bacteria*Lactobacillus reuteri* In MRS broth medium, it was cultured for 18 hours at 37°C. Then, the supernatant was prepared by centrifuging at 8000 g for 10 minutes at 4°C and a 0.45 µm filter was used to separate the remaining cells [11].

2-3- Preparation of cellulose nanoparticles

20 grams of linen was mixed in 175 ml of 30% sulfuric acid and then placed in a hot water bath with a temperature of 60 degrees Celsius and a shaker for 6 hours. The resulting suspension was diluted with distilled water and centrifuged at 8000 rpm for 15 minutes. Repeat this process 3 times to remove sulfuric acid. After that, the suspension was dialyzed in contact with water for 2 hours and then placed in distilled water overnight until the pH reached 4. Finally, the suspension was treated with ultrasonic and dried with a freeze dryer [12].

2-4- Preparation of sodium caseinate film-cellulose nanoparticles

Aqueous solution of sodium caseinate was prepared by dissolving 2.5% weight-volume of sodium caseinate powder in 100 cc of distilled water and then stirring continuously for 3 hours at room temperature. Glycerol was added to the solution in an appropriate amount (so that the ratio of glycerol to glycerol/caseinate is equal to 0.21). Cellulose nanocrystals in the amount of 2% of dry weight were dissolved in distilled water using an ultrasonic device and then added to the film solution, in this way the control film solution was prepared. Also in order to prepare a film containing bacteria*Lactobacillus reuteri above solution is prepared again and added to it*bacteria*Lactobacillus reuteri*in the number of Cfu/cm^2 10^6 was added and the supernatant film solution was prepared by adding the supernatant to the solution containing sodium caseinate-nanocellulose. Then the prepared solutions were poured into a mold and dried for 48 hours at 25°C. became The prepared films were placed in a desiccator with a relative humidity of 75% and a temperature of 5°C for a week to be conditioned [8,13].

2-4-1- Film humidity measurement

Pieces of film weighing 50 mlgrams (wet weight) in a 60°C oven for 24 hours, until they reach a constant weight. After the films reached moisture balance, the percentage of film moisture was calculated from equation (1) [8].

$$percentage\ humidity = \frac{water\ weight}{Wet\ sample\ weight} \times 100$$

(1)

2-4-2- Film solubility measurement

The films were cut into 2 x 3 cm dimensions and placed in a 60°C oven for 24 hours. After weighing, these dried films were poured into 80 cc of distilled water and stirred for 1 hour at a speed of 200 rpm. Then the obtained mixture was smoothed by filter paper and dried at 60°C. The solubility percentage was calculated from equation (2) [14].

(2)

$$\frac{The\ film\ in\ the\ initial\ dry\ weight\ of\ the\ material\ is\ immersed\ after\ the\ dry\ weight\ of\ the\ film}{film\ in\ the\ initial\ dry\ material\ weight} \times$$

100= percentage of solubility in water

2-4-3- Evaluation of the mechanical properties of the film (tensile resistance, elongation percentage and elastic modulus)

Film samples with dimensions of 25.4 mm in width and 100 mm in length were cut for one week in a desiccator with a temperature of 5 °C and a relative humidity of 75%. Then, with Santam STM1 histometer made in Iran, tensile strength, percentage of stretchability and elastic modulus were evaluated respectively in terms of megapascals, the amount of increase in length divided by the initial length in terms of percentages and megapascals. The distance between the two jaws of the device was 50 mm and the speed of movement of the jaws was 50 mm/min. Using the D-882 standard approved by ASTM, tensile strength, percentage of elasticity and elastic modulus were obtained from the force curve in terms of deformation. Three repetitions were done for each coating [8].

2-4-4- water vapor permeability of the film

The measurement of water vapor permeability was carried out by the method of Mchugh et al. in 1993, which is a modification of the ESTM-E96 standard. The tested films were stuck on glass vials filled with distilled water and placed in a desiccator. Glass vials with an outer diameter of 2 cm, an inner diameter of 1.2 cm and a height of 4.5 cm were used for the experiment. 10 cc of distilled water was poured into the vials, thus 100% relative humidity was applied. Then, each film sample was cut to the dimensions of the outer diameter of the opening and glued on the opening of the vial smeared with grease and secured on the vial with a plastic washer. The vials along with the film were placed in a desiccator containing supersaturated salt water (supersaturated salt water creates 75% humidity at 5°C) and weight changes were measured every 12 hours to determine the water vapor transfer rate of the films with a 0.0001 scale. , the curve of drawing changes and the slope of each line drawn by line regression (R. 0.999).^{2}=) was calculated. The water vapor transmission rate (WVTR) was obtained by dividing the slope of the drawn line by the film surface.

$$WVTR = \frac{slope\ line}{Surface\ film}$$

(3)

Finally, water vapor permeability was obtained from equation (4) by multiplying the water vapor transfer rate by the film thickness (X) and dividing by the pressure difference between the relative humidity

inside the cells and the relative humidity of the desiccator (ΔP).

$$WFP = \frac{WVTR \times X}{\Delta P} \quad (4)$$

2-4-5- film turbidity

To determine the turbidity, the samples were cut into rectangles and placed in a spectrophotometer cell (Cecil model, made in England). Then, the absorption spectrum of the film samples was obtained at a wavelength of 650 nm and calculated according to equation (5) [15].

$$Turbidity = \frac{(650)attraction}{(mm)\ film\ thickness} \quad (5)$$

2-4-6- Studying the microstructure (morphology) of the film

In order to investigate the effect of adding bacterial lactic acid, supernatant and nanocellulose on the microstructure of the produced films, images with an electron microscope model Philips Xl30 made in the Netherlands Films were prepared from surface and transverse sections. Before photographing the surface section, the films were cut into 5x1 mm and covered with a layer of gold. In preparing the samples for cross-section imaging, first, the samples were broken in liquid nitrogen and then from the opposite side, the broken part was placed on an aluminum base with the help of double-sided adhesive. The bases were coated with gold in a coating machine for 5 minutes [16].

2-5- Preparation of meat product samples

The meat used was procured from the industrial slaughterhouse in the necessary amount and after being transferred to the laboratory environment, it was chopped under sterile conditions. The kebab was prepared according to the instructions. After complete mixing and molding, it was covered with the desired film and kept at a temperature of 4 degrees Celsius. On the meat samples on days 0, 4, 8, 12, 16, 20, microbial tests (counting of cold-loving bacteria, mold and yeast), physical (pH, acidity), chemical (thiobarbituric acid and total volatile nitrogenous bases, peroxide index)) Done.

2-5-1- Total count of the number of microbes

In order to determine the total number of visible bacteria, polycant agar culture medium was used. Then 100 microliters of the prepared sample was spread on the culture medium. If needed (high number of bacteria in a plate) dilution of samples (up to 10 dilution[6]) was done in 0.1% peptone water solution. The cultured plates were counted after 2-3 days in a greenhouse at 30 degrees Celsius using a colony counter [17].

2-5-2- Counting cold-loving bacteria

In order to determine the number of cold-loving bacteria, surface culture was performed on the polytcant agar medium and after 7 days of incubation at 10 degrees Celsius, the colonies were counted using a colony counter [18].

2-5-3- Counting mold and yeast

To count mold and yeast from culture medium*Rose Bengal Chloramphenicol Selective Agar*[1] used. 100 microliters of the prepared sample was spread on the culture medium. Dilution of samples if needed (high number of bacteria in a plate).*It was done in 0.1% peptone water solution. The cultured plates were counted after 3-5 days of incubation at 25°C*[19].

2-5-4- Measurement of peroxide index (PV)

40 grams of minced meat was mixed with 100 ml of chloroform and then filtered with Whatman filter paper. Pour 25 cc of the filtered solution into the beaker to extract fat and put it in the oven until the chloroform evaporates (the difference in the weight of the beaker after the evaporation of the chloroform will represent the weight of the oil) and pour another 25 ml into an Erlenmeyer flask and add 37 ml of acetic acid added to it. One milliliter of saturated potassium iodide was added to the solution. After one minute, 30 milliliters of distilled water and one milliliter of starch solution were added to the solution and titrated with 0.01 normal sodium thiosulfate until the yellow color of the solution disappeared and it turned milky white. The amount of peroxide was calculated from the following equation [20].

(9) 100 x 0.1 x weight of oil / amount of thiosulfate used = PV

2-5-6- Measurement of thiobarbituric acid (TBA)

TBA was measured by colorimetric method. The amount of 200 mg of the sample was transferred to a 25 ml flask and then made up to volume with 1-butanol. 5 ml of the above mixture was added to dry capped tubes and 5 ml of TBA reagent was added to it (TBA reagent was obtained by dissolving 200 mg of TBA in 100 ml of butanol solvent after filtering). The sealed tubes were placed in a water bath at 95°C for two hours and then cooled to room temperature. Then, the absorbance value (As) at 530 nm wavelength was read against butanol control (Ab). The amount of TBA was calculated based on the following equation [21].

(10) $$TBA = (As - Ab \times 50)/200$$

2-5-7- Measurement of total volatile nitrogen bases (TVB-N)

10 grams of minced meat with 2 grams of magnesium oxide as a catalyst and 300 milliliters of distilled water were poured into the Kjeldahl digestion flask, then a few glass pearls were added to it along with normal octane (as an antifoam). The digestion balloon was connected to the device and heated. A 250 ml Erlenmeyer flask containing 25 ml

[1]- Rose Bengal Chloramphenicol (RBC) selective agar

of 2% boric acid solution with a few drops of methyl red reagent (0.1 methyl red in 100 ml of ethanol) was placed at the end of the device. Methyl red 14 is red in acidic environment and yellow in alkaline environment. Distillation continued until 30 minutes passed from the boiling of the material in the flask or the collection of about 125 ml of distilled liquid in Erlenmeyer. The boric acid solution turns yellow as soon as it is alkalized by distilled volatile nitrogen bases. The titration of this solution with 0.1 normal sulfuric acid continued until the boric acid turned red again. The amount of TVB-N in mg/100 g of quail meat was obtained using the following equation [22].

(11) $(consumption\ of\ sulfuric\ acid\ amount\ x\ 1.4\ x\ 100)/(sample\ weight)$ = TVB-N

2-5-8- Measurement of pH value

5 grams of the prepared sample was mixed with 45 ml of distilled water and the homogenized mixture was used to determine pH by digital pH meter [23].

2-5-9- Measuring the amount of acidity

The acidity of the sample was measured as the amount of 0.1 M NaOH needed to neutralize the acid in the presence of phenolphthalein as a reagent by titration method [24] and was calculated using the following formula.

(12) $TTA = (X\ ml\ NaOH \times 0/009 \times 100)/(g\ Sample)$

6-2- Statistical analysis

In this research, the obtained results were analyzed using a factorial experiment in the form of a completely random design. Average measurement of microbial data was done using Tukey's test and chemical and physical data using Duncan's test at a probability level of 5% by SAS software version 9.1. Graphs were drawn and reported using Excel 2013 software.

3. Results and Discussion

3-1- Changing the physical characteristics of films

3-1-1- percentage of humidity

As Table 1 shows, the moisture content of the prepared films has a significant difference ($p<0.05$). So that the highest amount (0.12±12/28) in F3 film and its lowest value (0.15±10/65) was seen in the movie F1. Presence*Lactobacillus reuteri* And its supernatant in the structure of films caused a significant increase in humidity in them. The results were in conflict with the findings of Ajaq et al. in 2016, they stated that the presence of bacteria in the sodium caseinate film reduced the moisture content. Kanmani et al. reported in 2013 that the presence of bacteria in pullulan film/potato starch and tapioca starch increased the percentage of moisture, but in pure pullulan film it decreased the moisture content [25]. In fact, in order to ensure its survival, lactobacillus increases the ability to hold water in the film, as a result, the moisture percentage of the film increases [8].

3-1-2- Solubility in water

Solubility is an important property in biodegradable films because it determines the rate of release of antioxidant and antimicrobial compounds of the film when it is in contact with the surface of the food [26]. As Table 1 shows, the dissolution percentage of the prepared films has a significant difference ($p<0.05$). The highest dissolution percentage (0.50±91/61) in F3 film and its lowest value (1.51±01/82) was seen in the movie F1. Adding bacteria and supernatant to the film caused a significant increase in the dissolution percentage. Probably, the bacteria present in the film produce hydrophilic compounds and thus increase the solubility of the film. Hosseini and colleagues in 2009 stated that the presence of antimicrobial compounds in the film increases the dissolution percentage [27].

3-1-3- water vapor permeability of the film

Moisture exchange between the food and the environment can cause many problems, including microbial, chemical, and enzymatic spoilage, product weight loss, loss of freshness and freshness, and lumpiness of powdered products. Therefore, the inhibition of polymer films against moisture is very important in terms of food preservation [28]. Souza et al. in 2010 stated that various factors such as solubility coefficient, film matrix continuity, hydrophobicity, diffusion rate, the ability to move polymer chains and the reaction between polymer functional groups have an effect on the water vapor permeability of polymer films [29]. As Table 1 shows, the water vapor permeability of the prepared films has a significant difference ($p<0.05$). So that the highest amount (0.70±19/00) in F3 film and its lowest value is g.mm/kpa.h.m^2 (60/0±10/15) was seen in the movie F1. The obtained results are consistent with the findings of Sanchez et al. in 2013 and 2014 [8,30]. Adding bacteria and

The supernatant to the film has significantly increased the water vapor permeability of the films (P<0.05). Adding bacteria and supernatant to the film causes discontinuities in the film matrix, as a result, the ability to move polymer chains and transfer the mass of water molecules increases. Pruneda et al. reported in 2008 that the addition of antimicrobial compounds to soy protein isolate film resulted in a significant increase in water vapor permeability of the film [31]. On the other hand, Concha-Meyer et al. in 2011 stated that the addition of lactic acid bacteria to sodium caseinate film did not cause significant permeability [32].

3-1-4- Film turbidity

Film opacity is very important because of its direct impact on the appearance of coated products. This film parameter was evaluated at a wavelength of 650 nm and its results are shown in Table 1. The highest amount of turbidity (0.04±1.82) in F2 film and its lowest value nm/mm (0.01±1/11) was seen in the movie F3. Adding supernatant to the film caused a significant increase in turbidity. Also, adding bacteria to the film caused a significant decrease in turbidity (P<0.05). Since moisture affects the appearance of the film, the increase in moisture content can be the reason for the decrease in turbidity [8]. The results obtained were in contrast with the findings of Kanmani et al., 2013, who reported that the addition of lactic acid bacteria in pullulan, pullulan-starch films was reduced due to different film structures [25].

Table 1- The effect of *Lactobacillus reuteri* bacteria and its supernatant on the physical properties of the film

Treatment	M%	S%	WVP (g.mm/kpa.h.m^2)	O (nm/mm)
F1	10.65±0.15 ^{C}	82.01±1.51 ^{C}	15.10±0.60 ^{C}	1.40±0.03 ^{A}
F2	11.30±0.10 ^{B}	84.04±0.75 ^{B}	16.50±0.50 ^{B}	1.82±0.04 ^{B}
F3	12.28±0.12 ^{A}	91.61±0.50 ^{A}	19.00±0.70 ^{A}	1.11±0.01 ^{C}

F1 sodium caseinate film - nanocellulose, F2 sodium caseinate film - nanocellulose - supernatant, F3 sodium caseinate film - nanocellulose - lactobacillus rheuteri. ; M moisture percentage, ; S solubility, ; WVP permeability to water vapor, ; O turbidity. Similar common letters in each column indicate no significant difference in data (P<0.05).

3-1-5- Studying the microstructure (morphology) of the film

Examining the microstructure of biopolymers can be an important factor in understanding the behavior and properties of biopolymers [33]. The images from the electron microscope taken from the surface of the films provide us with useful information about the biopolymer substrate. The reaction between the components and the changes that occur during the drying of the film have an effect on the final microstructure of the film. The study of the microstructure of the film gives information about the arrangement of the components of the film, which can be used to better interpret the chemical and microbial evaluation. The F1 film has a more uniform surface than the F2 and F3 films. Electron microscope images related to the cross-section of sodium caseinate-nanocellulose-supernatant film and sodium caseinate-nanocellulose-*Lactobacillus reuteri* They show that the supernatant and the bacteria reacted with the film components and replaced the previous bonds, and were well embedded in the structure of both films (Figure 1). Randazzo et al. in 2016 stated that if an antimicrobial compound is well placed in the film structure, its diffusion rate decreases and its concentration remains at a high level during storage [34].

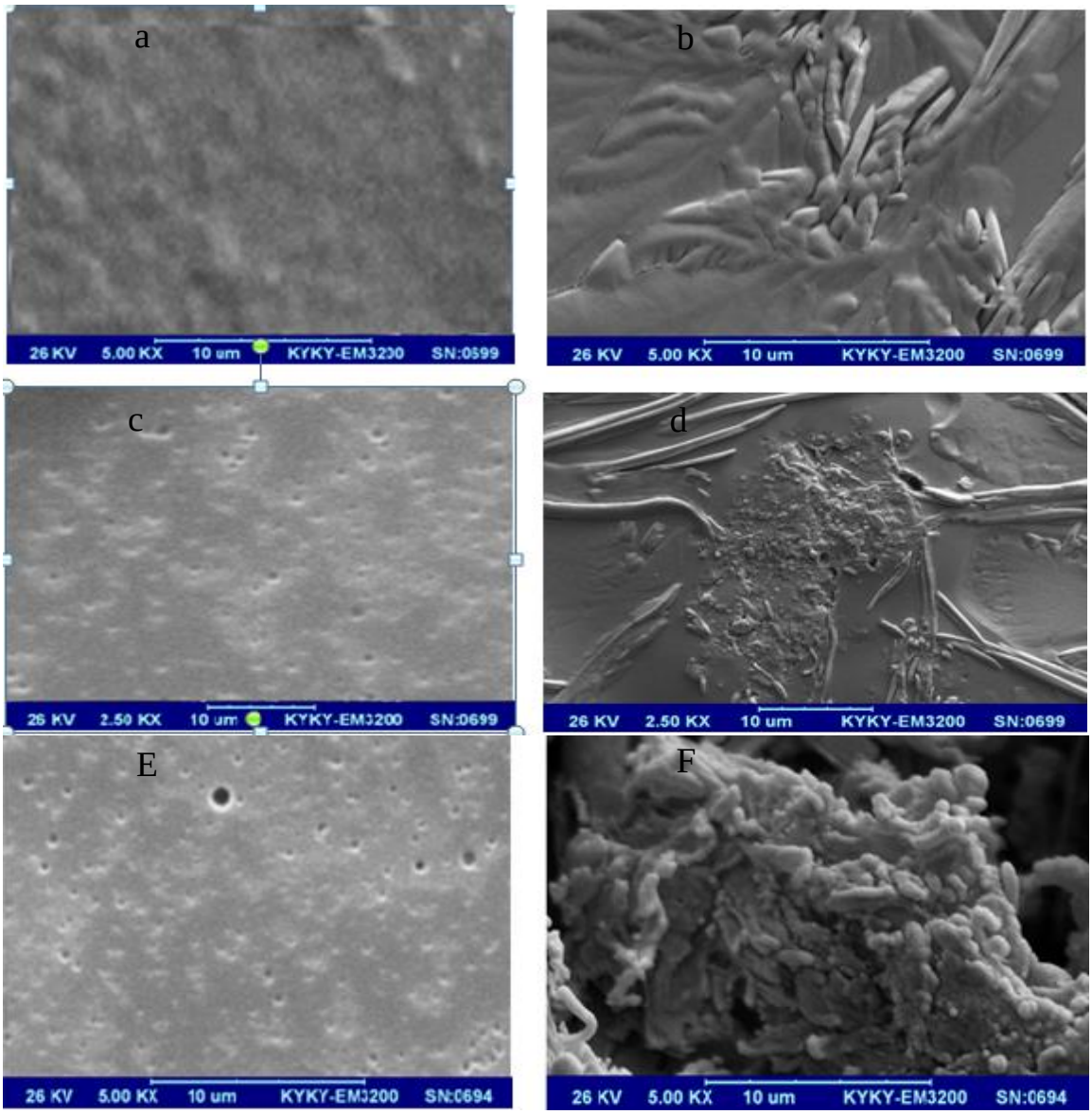

Figure 1 Electron microscope images.
Surface image (a) and cross-section (b) of sodium caseinate-nanocellulose film, surface image (p) and cross-section (t) of sodium caseinate-nanocellulose-supernatant film, surface image (c) and cross-section (c) of sodium caseinate film - Nanocellulose - *Lactobacillus reuteri*

3-1-6- evaluation of the mechanical properties of the film (tensile resistance, elongation percentage and elastic modulus)

Usually, the mechanical properties of polymer films are measured with three parameters: tensile strength (TS), elongation percentage (E) and modulus of elasticity (EM). TS: Maximum tensile stress without the film undergoing permanent strain. E: the ratio of elongation to the initial length of the film, EM: this parameter expresses the hardness of the film. Addition of supernatant to the film caused a significant decrease in tensile strength. The addition of supernatant did not significantly change the elastic modulus (Table 2). Adding the supernatant to the film caused a significant increase in the stretchability percentage. Adding bacteria to the film caused a significant decrease ($P<0.05$) in tensile strength and elastic modulus. The percentage of stretchability increased significantly in the presence of bacteria. Our results were identical to the findings of Sanchez-Gonzalez et al in 2014 [8]. But the results contradicted the findings of Gialamas et al., who stated that spraying and inoculation*Lactobacillus saki* 1189 LQC to sodium caseinate film did not change the tensile strength [35]. The mechanical properties of films are influenced by the interconnection of polymer chains in the film matrix. Probably, the

addition of supernatant and the inoculation of bacteria cause a change in the cohesive structure of the polymer matrix in images (c) and (c). As a result, the links of the polymer network and then the mechanical resistance are reduced. On the other hand, the addition of bacteria in the film structure has increased the percentage of moisture. Moisture acts as a plasticizer and reduces the tensile strength, elastic modulus and increases the percentage of elasticity.

Table 2- The effect of *Lactobacillus reuteri* bacteria and its supernatant on the mechanical properties of the film

Treatment	AND%	TS(MPa)%	EM(MPa)%
F1	10.16±0.31 C	15.40±0.35 A	323±23 A
F2	11.96±0.24 B	12.69±0.56 C	294±24 A
F3	12.82±0.19 A	13.96±0.71 B	245±15 B

F1 sodium caseinate –nanocellulose film, F2 sodium caseinate -nanocellulose-supernatant film, F3 sodium caseinate -nanocellulose-*Lactobacillus reuteri*, E percentage of stretch, TS tensile strength, EM modulus of elasticity.

Similar common letters in each column indicate no significant difference in data ($P<0.05$).

3-2- The effect of investigated variables on the characteristics of kebab

3-2-1- Changes in total microbial load (TVC)

Changes in total microbial load of different treatments of kebab samples during storage time are shown in Figure 2. The amount of total microbial load of different treatments on day 0 was in the range of (Log CFU/gr) 1.4-2.4. The trend of increasing the total number of microbes during 20 days of storage in the kebab samples prepared with different treatments is different from each other. As can be seen, from the zero day to the end of the maintenance period, the TVC value of the F1 treatment and the control treatment increased steeply. In F2 treatments, until the fourth day of storage, the number of bacteria increased steeper. After that, the slope of increasing the number of bacteria decreased. So, on the 20th day of storage, the number of bacteria in F2 treatments reached (Log CFU/gr) 7.9. In the F3 treatment, the microbial population increased with a very gentle slope until day 8. But from the 8th to the 12th day, the microbial population increased with a steeper slope, and after that it was again in a gentle slope. The lowest amount of total count was observed in F3 treatment. So that on the 20th day, the maximum number of microbes reached 5.7 logarithmic cycles. The maximum recommended limit for TVC of fresh meat is (Log CFU/gr) 7 [36]. Control treatment on day 4, F1 treatment on day 8, F2 treatment on day 12, and F3 treatment on day 13 of maintenance were within the recommended range. In total, the use of sodium caseinate-nanocellulose edible film with bacteria*Lactobacillus reuteri* And its supernatant has reduced the total microbial load. Zargar et al., in 2013, the effect of sodium caseinate edible coating on the edible quality of rainbow trout (*Oncorhynchus mykiss*) investigated during storage at refrigerator temperature, the results showed that the coating of sodium caseinate in the last days of the storage period significantly reduced the microbial load, especially mesophilic aerobic bacteria (on day 20 in the control and coated treatment, respectively, 1±1.9 and 0.07±7.7) ($P<0.05$) during storage in the refrigerator [37]. In 2016, Rangebrian et al investigated the effect of sodium caseinate active nanocomposite film and coating containing cinnamon essence in increasing the shelf life of chicken breast fillet. The samples packed with nanocomposite film containing cinnamon essential oil were in the acceptable range for human consumption until the 12th day. But the control and coated samples were beyond the permissible limit [38].

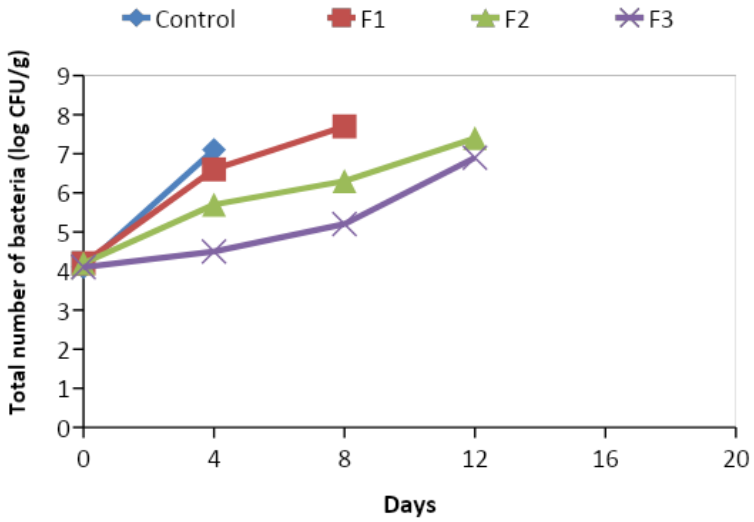

Figure 2- The effect of the examined variables on the total number of bacteria
Control, roast without coating; F1 roast - sodium caseinate – nanocellulose film, F2 roast - sodium caseinate – nanocellulose – supernatant film, F3 roast - sodium caseinate - nanocellulose - *Lactobacillus reuteri* film.

3-2-2- Changes in the number of cold-loving bacteria

Cold-loving bacteria, gram-negative bacteria such as*Pseudomonas, Altromonas, Schwanella, Flavobacterium* are [39]. Cryophilic bacteria are the most important group of spoilage microorganisms in products kept at low temperatures. One of the important features of cold-loving bacteria is having strong proteolytic and lipolytic enzymes and their rapid reproduction in a short time [40]. The growth of cold-loving microorganisms depends on the temperature of the environment, and as the temperature decreases, the growth rate becomes progressively slower [41]. Changes in the amount of cold-loving bacterial load are shown in Figure 3. The initial number of cryophilic bacteria of different treatments was in the range (Log CFU/gr) of 3.4-3.8. The allowable bacterial load for aerobic cold-loving bacteria (Log CFU/gr) is also reported to be 7 [42]. The control treatment and F1 on day 8, F2 on day 12, and F3 on day 14 were within the recommended range. In 2014, Fernandez-Pan et al. applied oregano and clove essential oils in whey protein isolate coating and obtained similar results [43]. In 2016, Rangebrian et al investigated the effect of sodium caseinate active nanocomposite film and coating containing cinnamon essence in increasing the shelf life of chicken breast fillet. The results showed that there was a significant increase in the amount of cryophilic bacteria in the samples packed with cover compared to the samples packed with film ($P<0.05$) [38].

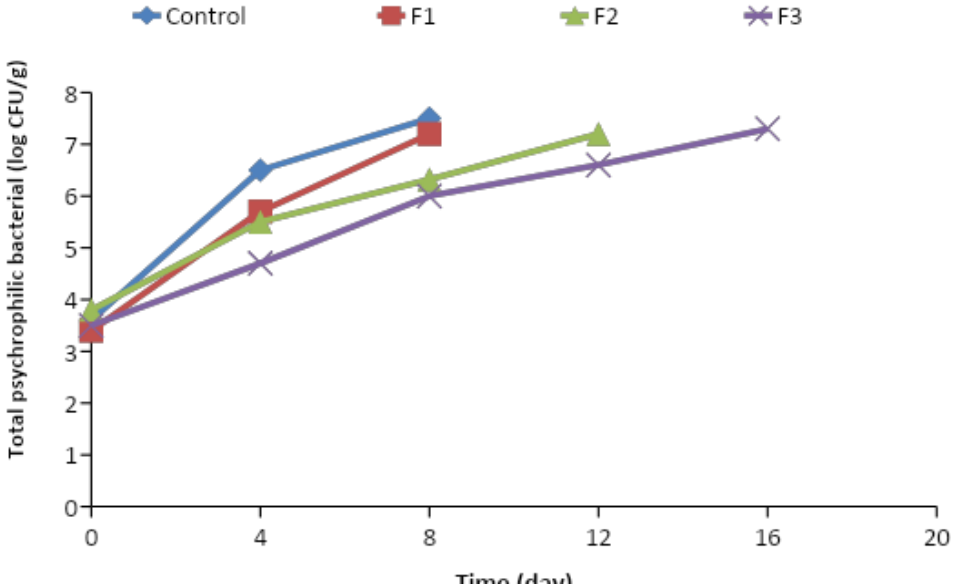

Figure 3- The effect of the examined variables on total psychrophilic bacteria.

Control roast without coating. F1 roast - sodium caseinate – nanocellulose film, F2 roast - sodium caseinate - nanocellulose – supernatant film, F3 roast - sodium caseinate - nanocellulose - *Lactobacillus reuteri* film.

3-2-3- Mold and yeast changes

As seen in Figure 4, the trend of increasing the number of mold and yeast during 20 days of storage in the kebab samples prepared with different treatments is different from each other. The initial number of mold and yeast in different treatments was in the range of (Log CFU/gr) 2.8-2.4. From day zero to the end of the storage period, the number of mold and yeast in the control treatment increased steeper. The number of mold and yeast in treatment F1 and F2 increased steeply until day 4 and from day 4 until the end of the storage period with a gentle slope, and at the end of the period in treatment F1 and F2, it was (Log CFU/gr) 6.4 and 9/5 arrived. The growth of mold and yeast in F3 treatment was slow so that at the end of the storage period it reached (Log CFU/gr) 5.3.

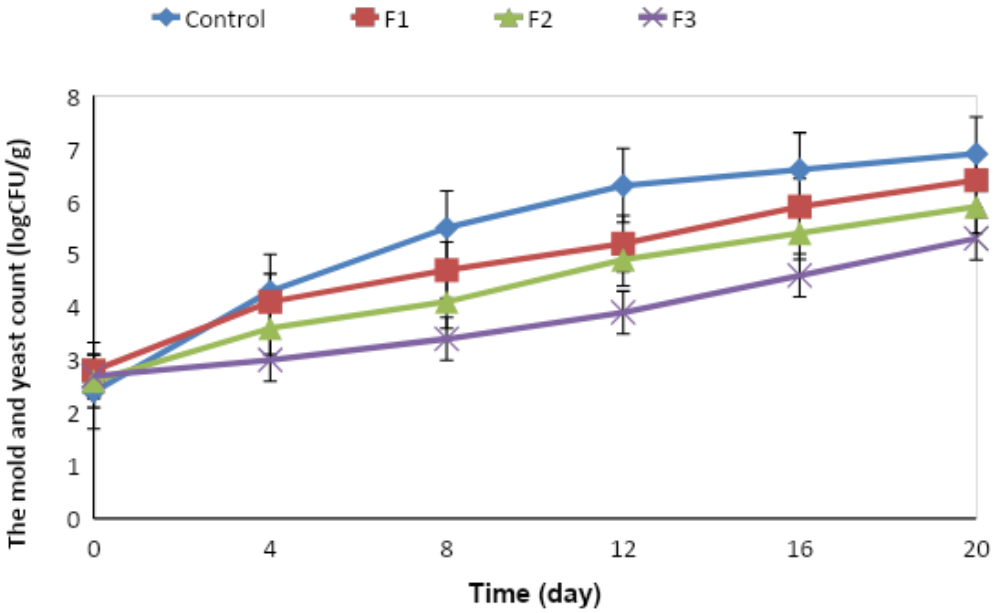

Figure 4- The effect of the examined variables on mold and yeast.

Control roast without coating. F1 roast - sodium caseinate – nanocellulose film, F2 roast - sodium caseinate - nanocellulose – supernatant film, F3 roast - sodium caseinate film - nanocellulose - *Lactobacillus reuteri* film.

3-2-4- changes in peroxide index (PV)

The peroxide index is used to measure hydroperoxides, which are the primary product of fat oxidation and polyunsaturated fatty acids. Because peroxides are tasteless and odorless compounds, they cannot be detected by consumers. But these compounds cause the formation of secondary compounds such as aldehydes and ketones, which cause oxidation acceleration [44]. Table 3 shows the amount of peroxide number in the kebab samples prepared during 20 days of storage at 4°C. The amount of peroxide on day zero is in the range of 0.476-0.493 oli meq/kg. With the increase of storage days, this amount had an increasing trend and differences in the amount of peroxide were observed between different treatments. From the fourth day of storage, the increasing trend became more intense and reached its highest level on the twelfth day in all treatments. The control treatment on the twelfth day has the highest amount of peroxide (meq/kg oli^{A,a}020/0±2.162) was among the treatments and treatment F3 had the lowest amount of peroxide (meq/kg oli^{A,d}006/0±was 1/080). From the 12th day to the end of the storage period, the peroxide index of all treatments suddenly decreased. The increase in peroxide could be due to the faster rate of formation of peroxides during days 0 to 12 of storage compared to the decomposition of peroxides into secondary oxidation products [45]. Sudden reduction of hydroperoxides may be due to secondary oxidation reactions and production of carbonyls and volatile compounds [44]. A significant difference was observed between the amount of peroxide during the storage period in different treatments ($P<0.05$), which can be a reason for preventing the passage of oxygen by the casein coating, these results are similar to the results of Atares et al. The oil sample covered with aluminum foil and the oil covered with sodium caseinate film were consistent in the delay of oxidative spoilage compared to oil without protection [46]. In 2013, Zargar et al. investigated the effect of

sodium caseinate coating on the quality of colored salmon.*Oncorhynchus mykiss*) they paid. The results showed that the amount of peroxide increased in both the control treatment and the casein-coated fish, but this increase was more intense in the control treatment and reached its highest level on the 16th day (1.1±1/16) receipt; Then its decrease was seen on day 20 [37].

Table 3- Peroxide index (meq/kg oil) in different kebab treatments during storage time at refrigerator temperature

	Storage time (day)					
Treatment	0	4	8	12	16	20
Control	0.493±0.020 But	1.077±0.040 Yes	1.648±0.010 And	2.162±0.020 Aa	2.117±0.010 Not	2.065±0.008 That
F1	0.476±0.007 But	1.007±0.025 Eb	1.501±0.010 Db	1.909±0.030 Ab	1.869±0.030 Bb	1.710±0.021 Cb
F2	0.484±0.010 But	0.822±0.027 Ec	1.372±0.007 Cc	1.608±0.010 Ac	1.492±0.012 Bc	1.223±0.023 Dc
F3	0.482±0.010 But	0.606±0.012 Ed	0.951±0.010 Cd	1.080±0.006 Ad	0.998±0.020 Bd	0.699±0.013 Dd

Control roast without coating. F1 roast with sodium caseinate – nanocellulose film, F2 roast with sodium caseinate - nanocellulose – supernatant film, F3 roast with sodium caseinate - nanocellulose - *Lactobacillus reuteri* film. Similar uppercase and lowercase letters respectively indicate the absence of significant data differences in each row and column (P<0.05).

3-2-5- Changes in thiobarbituric acid reaction compounds (TBARs)

TBA is used to evaluate fat oxidation. When the conversion rate of hydroperoxides into secondary products exceeds their formation rate, the amount of hydroperoxides decreases. In this theory, primary products in the initial stages and secondary products in the final stages constitute the majority of compounds of the oxidation process. In the second stage of autoxidation, when hydroperoxides are oxidized to aldehyde and ketone, malondialdehyde is formed. Secondary oxidation products cause unpleasant taste and smell in the product [44]. The table shows the amount of TBA in the kebab samples prepared during 20 days of storage at 4°C. The amount of TBA on day zero was in the range of (0.26-0.17 MgMDA/kg). With the increase of storage days, this amount had an increasing trend in all treatments. The threshold limit of malondialdehyde, which leads to the production of unpleasant aroma and flavor in food, is 2 mg/kg [17], none of the treatments crossed this limit. The control treatment on the 20th day has the highest value (^{A,a}۰۷ /۰±1.65) and treatment F3 the lowest amount (^{A,c}۰۶/۰±0.70) which can confirm the effect of sodium caseinate coating in preventing fat oxidation in kebab samples. The results were consistent with Zargar et al.'s research in 2013 [37]. Also, these findings were consistent with the results of Caprilio et al. in 2009 regarding the reduction of TBA in 15 g pieces of cooked turkey meat wrapped with sodium caseinate film compared to uncoated pieces [47].

Table 4- TBARs index (MgMDA/kg) in different kebab treatments during storage time at refrigerator temperature

	Storage time (day)					
Treatment	0	4	8	12	16	20
Control	0.26±0.06 But	0.70±0.03 Yes	0.92±0.03 And	1.06±0.06 That	1.33±0.02 Not	1.65±0.07 Aa
F1	0.25±0.03 Yes	0.45±0.02 Db	0.64±0.02 Db	0.84±0.05 Bb	0.88±0.02 Bb	0.95±0.03 Ab
F2	0.20±0.02 Fab	0.38±0.03 Ec	0.51±0.04 Dc	0.69±0.03 Cc	0.77±0.02 Bc	0.89±0.03 Ab
F3	0.17±0.01 Fb	0.25±0.03 Ed	0.33±0.02 Dd	0.43±0.04 Cd	0.55±0.04 Bd	0.70±0.06 And

Control roast without coating. F1 roast with sodium caseinate – nanocellulose film, F2 roast with sodium caseinate - nanocellulose – supernatant film, F3 roast with sodium caseinate - nanocellulose - *Lactobacillus reuteri* film. Similar uppercase and lowercase letters respectively indicate the absence of significant data differences in each row and column (P<0.05).

3-2-6- Changes in total volatile nitrogen bases (TVB-N)

The increase in TVB-N content is dependent on bacterial spoilage and endogenous activity [48,49], as volatile bases are produced by the separation of amines from amino acids by microbial enzymes [50]. The measurement of volatile nitrogenous bases is an indicator for detecting the freshness of meat products, which includes a wide range of volatile compounds such as ammonia, methylamine, dimethylamine, and the like, which are produced by microbial activities [39]. The amount of 25 mg of TVB-N per 100 grams of product is the highest acceptable level for human consumption. According to this suggested limit, in our study, control treatment on day 8, F1 treatment on day 16, F2 treatment on day 20 exceeded the limit, but F3 treatment did not exceed the limit until the end of the maintenance period (Table 5). The results were consistent with the findings of Zolfaghari et al. 2010 [51]. In 2016, Rangebrian et al investigated the effect

of sodium caseinate active nanocomposite film and coating containing cinnamon essential oil in increasing the shelf life of chicken fillet [38] and reached similar results. The increase in TVB-N content is dependent on bacterial spoilage and endogenous activity [48,49], as volatile bases are produced by the separation of amines from amino acids by microbial enzymes [50]. The reason for the increase in the amount of pernitrogenous bases during storage is spoilage caused by the growth and activity of microorganisms as well as autolysis (self-digestion) enzymes [52].

Table 5- TVB-N in different kebab treatments during storage time at refrigerator temperature

	Storage time (day)					
Treatment	0	4	8	12	16	20
Control	8.62±0.22 ^{But}	22.36±0.32 ^{Yes}	28.83±0.55 ^{And}	30.22±0.21 ^{That}	33.90±0.62 ^{Not}	37.66±0.35 ^{Aa}
F1	8.68±0.13 ^{But}	14.54±0.42 ^{Eb}	20.76±0.72 ^{Db}	22.99±0.32 ^{Cb}	28.53±0.52 ^{Bb}	30.67±0.34 ^{Ab}
F2	8.74±0.12 ^{But}	12.34±0.31 ^{Ec}	15.24±0.43 ^{Dc}	19.26±0.41 ^{Cc}	22.52±0.71 ^{Bc}	26.78±0.32 ^{And}
F3	8.66±0.21 ^{But}	11.21±0.20 ^{Ed}	14.97±0.61 ^{Dc}	16.77±0.42 ^{Cd}	21.11±0.41 ^{Bd}	23.61±0.09 ^{Ad}

Control roast without coating. F1 roast with sodium caseinate – nanocellulose film, F2 roast with sodium caseinate - nanocellulose – supernatant film, F3 roast with sodium caseinate - nanocellulose - *Lactobacillus reuteri* film.
Similar uppercase and lowercase letters respectively indicate the absence of significant data differences in each row and column ($P<0.05$).

3-2-7- pH changes

pH is one of the important indicators of meat quality evaluation [53]. The changes in pH value calculated in different kebab treatments when stored at 4 degrees Celsius are given in Table 6. According to the table, the initial pH value of all treatments was in the range of 6.06-6.02. In all treatments, except for control, the pH value decreased until the 8th day of storage and increased with the passage of time until the 20th day. The pH of the control treatment increased faster than the other treatments from day 0 to the last day of storage. The highest pH in the control treatment was observed on the 20th day of 7.28 and the lowest pH in the F3 treatment was observed on the eighth day of 5.42. The decrease in pH and then its increase during storage was consistent with the results of Ranjbarian et al. (2016) on the effect of sodium caseinate active nanocomposite film and coating containing cinnamon essence in increasing the shelf life of chicken breast fillet [38]. Also, the effect of sodium alginate coating containing different antioxidants on the quality and shelf life of fish was consistent with the results of Song et al. in 2011 [54]. The formation of inorganic acids, including lactic acid, at the beginning of the storage period, which is due to the decomposition of glycogen, causes a decrease in pH, prevents the growth and activity of microorganisms [55]. With the passage of time, the concentration of ammonium compounds and alkaline molecules increased, which provides a suitable environment for the growth and activity of microorganisms and also causes enzyme activity [56].

Table 6- pH value in different kebab treatments during storage time at refrigerator temperature

	Storage time (day)				
Treatment	0	4	8	12	16
Control	6.06±0.05 ^{But}	6.27±0.03 ^{Yes}	6.49±0.13 ^{And}	6.66±0.05 ^{That}	6.97±0.10 ^{Not}
F1	6.02±0.02 ^{EDa}	5.99±0.03 ^{Eb}	6.17±0.07 ^{Db}	6.31±0.06 ^{Cb}	6.47±0.03 ^{Bb}
F2	6.03±0.03 ^{That}	5.89±0.05 ^{Dbc}	5.74±0.09 ^{Ec}	5.94±0.11 ^{DCc}	6.13±0.08 ^{Bc}
F3	6.04±0.03 ^{ABa}	5.82±0.12 ^{Cc}	5.42±0.09 ^{Ed}	5.68±0.06 ^{Dd}	5.92±0.07 ^{BCd}

Control roast without coating. F1 roast with sodium caseinate – nanocellulose film, F2 roast with sodium caseinate - nanocellulose – supernatant film, F3 roast with sodium caseinate - nanocellulose - *Lactobacillus reuteri* film.
Similar uppercase and lowercase letters respectively indicate the absence of significant data differences in each row and column ($P<0.05$).

3-2-8- Acidity changes

The changes of acidity in different treatments of kebabs when stored at 4°C are shown in Table 7. According to the table, the initial acidity value of all treatments is equal to 0.01±0.37% was lactic acid. The acidity of the control treatment decreased from day 0 to the end of the storage period, the acidity of the F2 and F3 treatments increased until the eighth day, and decreased from the 8th day to the end of the storage period. The acidity of the F1 treatment increased until the fourth day, then decreased over time. The highest amount of acidity in F3 treatment

on the eighth day was 0.02±0.93 and its lowest value in the control treatment on the 20th day of storage was 0.03±0.08 was observed. In an experiment in 2000, the effect of bacteria*Lactococcus lactis* And*Lactobacillus plantarum* Sensory and microbial characteristics of minced goat meat were investigated. The amount of acidity in the samples treated with lactic acid bacteria was higher than the control sample [24].

Table 7- Acidity value in different kebab treatments during storage time at refrigerator temperature

	Storage time (day)					
Treatment	0	4	8	12	16	20
Control	0.37±0.01 Aa	0.34±0.01 Not	0.31±0.02 Not	0.18±0.02 That	0.11±0.01 And	0.08±0.03 Yes
F1	0.37±0.01 Not	0.43±0.03 Ab	0.36±0.01 Bb	0.32±0.01 Cb	0.27±0.02 Db	0.22±0.01 Eb
F2	0.37±0.01 That	0.54±0.01 Bc	0.75±0.03 And	0.53 ±0.01 Bc	0.36±0.02 Cc	0.32±0.01 Eb
F3	0.37±0.01 But	0.65±0.02 Cd	0.93±0.02 Ad	0.75±0.03 Bd	0.56±0.02 Dd	0.41±0.02 Ed

Control roast without coating. F1 roast with sodium caseinate – nanocellulose film, F2 roast with sodium caseinate - nanocellulose – supernatant film, F3 roast with sodium caseinate - nanocellulose - *Lactobacillus reuteri* film.
Similar uppercase and lowercase letters respectively indicate the absence of significant data differences in each row and column ($P<0.05$).

4- General conclusion

Sodium caseinate film is a good carrier for lactic acid bacteria. The compounds added to the films increased the water vapor permeability of the films. Movies containing*Lactobacillus reuteri* and the supernatant of anti-*Staphylococcus* And*Salmonella* They had significant Based on the results of microbial (TVC) and chemical (TVB-N) tests, the control treatment lasted 4 days. Coating with sodium-caseinate-nanocellulose film, sodium-caseinate-nanocellulose-supernatant film increased the shelf life up to 8 and 12 days, respectively. sodium caseinate film - nanocellulose -*Lactobacillus reuteri*The longest shelf life was up to the 13th day.

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6- Resources

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اثر فیلم خوراکی ضدباکتری کازئینات سدیم- نانوکریستال سلولز حاوی سلول و سوپرناتانت باکتری *لاکتوباسیلوس رئوتری* بر خواص کیفی کباب

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چکیده

فیلم‌های خوراکی به دلیل سازگار بودن با محیط زیست می‌توانند جایگزین مناسبی برای بسته‌بندی‌های حاصل از مشتقات نفتی باشند. امروزه نگرانی مصرف‌کنندگان در مورد سمیت مواد نگهدارنده مصنوعی صنعت غذا را مجبور به یافتن منابع طبیعی آنتی‌اکسیدانی و ضدمیکروبی کرده است. غذاهای آماده مصرف مانند کباب به عنوان مستعدترین موادغذایی برای رشد میکروب‌ها و شایع‌ترین عامل مسمومیت‌های غذایی شناخته شده‌اند. هدف از این تحقیق، بررسی اثر فیلم خوراکی ضدباکتری سدیم کازئینات -نانوکریستال سلولز حاوی سلول و سوپرناتانت باکتری *لاکتوباسیلوس رئوتری PTCC 1655* بر خصوصیات میکروبی و شیمیایی، کباب می‌باشد. بدین منظور، فیلم سدیم کازئینات-نانوکریستال سلولز با افزودن 10^6 Cfu/cm^2 باکتری *لاکتوباسیلوس رئوتری PTCC 1655* و سوپرناتانت فاقد میکروب تهیه و بر روی فیلم‌های تهیه شده آزمون‌های ارزیابی خصوصیات مکانیکی، نفوذپذیری بخار آب، حلالیت و رطوبت بررسی گردید. فیلم‌های تهیه شده، بر روی کباب پیچیده شدند و هر چهار روز نمونه‌برداری انجام و آزمون‌های میکروبی (شمارش کلی میکروارگانیسم‌های قابل رویت، باکتری‌های سرمادوست و شمارش کپک و مخمر)، شیمیایی (pH، اسیدیته، اسید تیوباربیتوریک و بازهای نیتروژنی فرار، شاخص پراکسید) انجام شد. در بین تیمارهای مورد آزمون فیلم سدیم کازئینات-نانوسلولز محتوی *لاکتوباسیلوس رئوتری* موثرترین تیمار در افزایش ماندگاری نمونه‌ی کباب تیمار شده بود. از این رو، پیشنهاد می‌شود از فیلم سدیم کازئینات-نانوسلولز حاوی *لاکتوباسیلوس رئوتری* برای افزایش ماندگاری کباب استفاده شود.