JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

EVALUATION EFFECT OF ANTIMICROBIAL NANOCELLULOSE FILM COMBINED WITH *LACTOBACILLUS RHAMNOSUS* POSTBIOTICS IN ACTIVE PACKAGING OF MINCED MEAT

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https://doi.org/10.55251/jmbfs.6209

ARTICLE INFO

Received 1. 6. 2022
Revised 15. 1. 2024
Accepted 16. 1. 2024
Published 1. 2. 2024

Regular article

OPEN ACCESS

ABSTRACT

Minced meat (MM) has a high surface-to-volume ratio, which makes it prone to the growth of pathogenic and spoilage bacteria due to increased exposure to oxygen and high nutrient levels. In this study, postbiotics were used as a natural preservative to extend the shelf life of MM. Lyophilized postbiotics from *Lactobacillus rhamnosus* were incorporated into bacterial nanocellulose (BNNC) to create an antimicrobial film for packaging MM. The effectiveness of the postbiotic-embedded BNNC film against *Staphylococcus aureus* was tested using the disk diffusion method and the optimal postbiotic concentrations were determined using the microdilution broth method. Structural changes of the BNNC film after immersion in postbiotics were examined using scanning electron microscopy, and the attachment and chemical nature of the functional groups in the sample were confirmed using FTIR. The antimicrobial efficacy of the postbiotic-embedded BNNC film against *S. aureus* in MM was also tested. The results showed that BNNC with 10% (P_{10}-BNNC) and 4% (P_4-BNNC) postbiotic concentrations were the optimal films, as confirmed by SEM and FTIR. The P-BNNC films significantly reduced the number of *S. aureus* during storage of MM for 9 days at 4°C. In conclusion, BNNC can serve as a suitable carrier for creating antimicrobial films using postbiotics derived from LAB for food packaging applications.

Keywords: Meat, Active Packaging, Food spoilage, *Lactobacillus rhamnosus*, Probiotic, Postbiotic, *Staphylococcus aureus*

INTRODUCTION

One of the vital sources of dietary protein for most people in the world is meat and meat products. Meat is very sensitive to microbial spoilage due to its physical and chemical attributes, such as water activity, pH, and nutrients. The chopping of meat by a meat grinder speeds up the spoilage of decomposed meat, thus making it an appropriate environment for the growth of spoilage and pathogenic microorganisms **(Khanjari *et al.*, 2013)**. The shelf life and bacteriological quality of minced meat (MM) are related to the primary microbial quality, hygiene, temperature during the production process, type of packaging, time, and storage temperature **(Duitschaever *et al.*, 1973)**. Among pathogenic microorganisms, *Staphylococcus aureus* is often found in raw meats, apart from the natural microbial flora of animals and humans **(VORSTER *et al.*, 1994)**. The presence of this bacterium in food is caused by secondary contamination from workers employed in factories and slaughterhouses who directly deal with food or meat, or by contact contamination with animal skin and work instruments during packaging **(Jay *et al.*, 2005)**. The existence of this bacterium in foods produces an enterotoxin, and if food consumption contains 20 ng to 1 µg of enterotoxin, it can result in staphylococcal food poisoning **(Normanno *et al.*, 2007; Shale *et al.*, 2005)**. Active packaging is a new idea for food preservation that has been introduced to address constant variations in consumer demand for food safety with extended shelf life. Generally, active packaging can provide multiple actions that are not present in ordinary packaging systems. Active functions include moisture, oxygen or ethylene absorbents, release of flavorings, ethanol, and antimicrobial activity that may interact directly or indirectly with food. Active packaging is made by combining different functional materials in different forms **(Mousavi Khaneghah *et al.*, 2018; Vilela *et al.*, 2018)**.

Antibacterial packaging is a type of active packaging that controls bacterial growth in food, achieved through the use of antimicrobial agents. Each antimicrobial agent used in this type of packaging has unique attributes and mechanisms of action **(Ghasemi *et al.*, 2015; Mousavi Khaneghah *et al.*, 2018)**. Researchers are currently focused on developing active packaging using renewable sources and biodegradable substances or biopolymers to reduce waste creation. The most common biopolymer sources used for food packaging materials today are polysaccharides, lipids, and proteins. Various polysaccharides, such as cellulose and chitosan, are used in film production for this purpose **(Salgado *et al.*, 2015)**. Cellulose, a polysaccharide based on the glucose monomer unit, is the most abundant biopolymer and is known for being non-toxic and biodegradable, decomposed by bacteria into substances that can be reused in nature. It has excellent mechanical properties, optimal stability, renewability, high molecular weight, and good contact surface with improved bonding properties **(S. D. C. Beristain-Bauza *et al.*, 2017; Curvello *et al.*, 2019; Gholizadeh-Ghaleh Aziz and Almasi, 2018; Rossi *et al.*, 2017; Umaraw and Verma, 2017; Zheng *et al.*, 2013)**. Cellulose-based products are also considered suitable carriers of a vast range of antimicrobials **(Shahmohammadi Jebel and Almasi, 2016)**.

Nanocelluloses are categorized into three original types: nanocrystalline cellulose, nanofibrillar cellulose, and bacterial nanocellulose (BNNC). Although they have similar chemical compositions, their morphology, particle size, crystallization, dimensions, and some attributes vary due to differences in sources and extraction methods **(Curvello *et al.*, 2019; Phanthong *et al.*, 2018)**. BNNC, generated by acetic acid bacteria such as *Komagataeibacter xylinus* and *Acetobacter hansenii*, has the same molecular formula as plant cellulose and features a complex 3D porous network with excellent purity, a high degree of polymerization (up to 8000), high crystallization (70-80%), and good mechanical resistance, making it a material of considerable interest **(Kuo *et al.*, 2016; Barud *et al.*, 2011)**. Lactobacilli, a diverse genus of gram-positive, anaerobic lactic acid bacteria (LAB), produce various low molecular weight functional activities (such as hydrogen peroxide and carbon dioxide) and high molecular weight bacteriocins during growth or after decomposition. These products are known as cell-free supernatants (CFS) or postbiotics **(Aguilar-Toalá *et al.*, 2018)**. In other words, postbiotics are secondary metabolites that LAB produce during growth and enter the CFS from the microbial suspension **(Gialamas *et al.*, 2010; Koohestani *et al.*, 2018; Moradi, Mardani, *et al.*, 2019)**.

CFS derived from LAB, including *Lactobacillus rhamnosus*, has been recognized as a natural antimicrobial agent. *L. rhamnosus* is a homofermentative bacterium with potential applications for CFS **(S. C. Beristain-Bauza *et al.*, 2016)**. Various strains of *L. rhamnosus* have shown safety and efficacy in preventing acute diarrhea, improving enterotoxigenic diarrhea caused by *Escherichia coli* in piglets, reducing allergies, lowering cholesterol levels, and stimulating the immune system in humans **(Hooshyar *et al.*, 2020; Jorjão *et al.*, 2015)**. These strains have been

identified as potential probiotics due to their resistance to acid and bile, favorable growth characteristics, and ability to adhere to the intestinal epithelial layer. They are commonly used in commercial probiotic products, and their beneficial effects have been extensively studied in clinical trials and human intervention studies **(Segers and Lebeer, 2014)**. CFS enriched with antibacterial metabolites has been found to be an effective antibacterial agent and has been explored for various applications. For example, it has been used to treat allergic airway inflammation in neonatal rat models **(Harb *et al.*, 2013)** and as a cosmetic antioxidant **(Tsai *et al.*, 2013)**. *L. rhamnosus* CFS, which contains diverse and abundant antimicrobial metabolites, can be utilized as an effective antimicrobial agent. Moreover, CFS is considered a natural antimicrobial agent with favorable reception. Postbiotics, which refer to metabolites/CFS or soluble agents secreted by living bacteria or released after bacterial lysis, possess functional properties such as antimicrobial and antioxidant attributes. Although the health effects of postbiotics are not yet fully understood, scientific evidence suggests that they have the potential to positively influence microbiota homeostasis, host metabolic and signaling pathways, and induce specific physiological, immune, neurological, hormonal, regulatory, and metabolic responses **(Aguilar-Toalá *et al.*, 2018)**.

A novel approach in the field of postbiotics involves using them as food additives, potentially replacing the use of live bacteria and their metabolites **(Moradi, Mardani, *et al.*, 2019; Moradi, Tajik, Mardani, *et al.*, 2019)**. Another innovative approach is to incorporate postbiotics into polymer films, which ensures the absence of pathogenic and spoilage organisms since it does not involve any heat process. Pathogenic bacteria such as *S. aureus* and *Salmonella* are major concerns in the transmission of meat products to humans. Packaging strategies offer a viable solution for microbiological control in the context of meat products **(Duitschaever *et al.*, 1973; Eshghinezad *et al.*, 2018; Ghorbanlou *et al.*, 2023)**. The objective of this study was to develop an optimal BNNC film infused with *L. rhamnosus* postbiotics, with the aim of effectively controlling the growth of *S. aureus* and enhancing the shelf life of MM (a specific meat product) during refrigerated storage conditions.

MATERIALS AND METHODS

Preparation of bacterial strains culture

S. aureus ATCC 25923 and *L. rhamnosus* ATCC 7469 were obtained from the Iran Science and Technology Research Organization. Stocks of *S. aureus* and *L. rhamnosus* were prepared in Trypticase Soy Broth (TSB) (Condalab, Madrid, Spain) and de Mann, Rogosa, and Sharpe (MRS) broth (Merk, Darmastadt, Germany), respectively, and then stored at -18 ºC **(Selenius *et al.*, 2018)**. Bacterial suspensions were prepared by culturing in the respective broth and standardized by spectrophotometry at a wavelength of 600 nm to ~8 $\log_{10}$ CFU/mL (optical density: ~ 0.1).

Postbiotics freeze-drying

L. rhamnosus incubated in a CO_2 incubator (Shimaz, Tehran, Iran) in MRS broth at 37±1°C for 48 h. The entire culture was then centrifuged at 8000 × *g* for 10 min at 4 °C using an Orum Tajhiz centrifuge **(S. C. Beristain-Bauza *et al.*, 2016)**. The resulting CFS was filtered through a 0.45 µm filter to remove residual cells and stored in the freezer at -18 ºC for 72 h **(Goyal and Kannan, 2018)**. Freeze-drying, which involved freezing the supernatant at -40 °C, maintaining a pressure of 100 mTorr, and setting a shelf temperature of -80 °C, was performed to concentrate the postbiotics **(Moradi, Tajik, Mardani, *et al.*, 2019)**.

Antimicrobial activity of Postbiotics

To assess the optimal concentrations of postbiotics, a range of numbers was prepared based on similar studies **(S. C. Beristain-Bauza *et al.*, 2016; S. D. C. Beristain-Bauza *et al.*, 2017; Koohestani *et al.*, 2018; Ma *et al.*, 2019; Moradi, Mardani, *et al.*, 2019; Moradi, Tajik, Mardani, *et al.*, 2019)**. The disk diffusion method, as described by Briston Boza 2016, was used to measure the antimicrobial activity of various concentrations of dried frozen postbiotics **(S. C. Beristain-Bauza *et al.*, 2016)**. Target strains were inoculated in TSB and serially diluted to reach a density of approximately 10^8 cells/ml. Then, 0.10 ml of bacterial cells were spread on the agar surface for antimicrobial activity assessment. Circular film sections (diameter 600 mm^2) were placed on the culture surface and plates were incubated at 37 ºC for 24 h. Inhibition zones were measured using a digital caliper (Guanglu, China). Antimicrobial measurements were carried out in triplicate during three independent trials.

Optimal P-BNNC antimicrobial film

Adequate washed membrane of BNNC, produced by *K. xylinus*, was acquired from Nano Novin Polymer (Sari, Iran). The membrane had a crystallite size of 1.87 nm, a crystallinity index of 66.50%, a purity of ≤ 99%, and a diameter of nanofibers of 65 nm. Due to the expansion of P-BNNC (postbiotic-modified nanocellulose) films, the impregnation method was chosen for its high loading valence. A portion of the film (0.60 cm) was immersed in a postbiotic solution at various concentrations and soaked at room temperature (25 ºC) for 20 min without stirring. Coated films were then soaked in distilled water to remove excess postbiotics and incubated at 37±1 ºC for 24 h to investigate their antimicrobial activity.

Antimicrobial activity of P-BNNC film

Antimicrobial discs of films (0.60 cm diameter) were sterilized under a UV lamp for 3 min in a security booth. The disc-covering experiment was used to appraise the antimicrobial activity of P-BNNC film against *S. aureus*. Serial dilutions were created to reach a density of approximately 8 Log_{10} CFU/ml. Then, 0.10 ml of bacterial cells were spread on the agar surface to assess antimicrobial activity. Circular pieces of the film were placed on the surface of the lawn culture of Plate Count Agar and plates were incubated at 37 °C for 24 h. The diameter zone of inhibition (ZOI) were measured **(Razavi *et al.*, 2020)**. Antimicrobial tests were carried out in triplicate during three independent trials.

Scanning electron microscopy of P-BNNC film

The surface morphology and refractive index of the films were determined by scanning electron microscopy (SEM) (Zeiss, Germany) at room temperature **(Ghadetaj *et al.*, 2018)**. SEM images were taken by placing samples on an aluminum SEM disk covered with gold. An acceleration voltage of 10 kV and a magnification of 50 kX were used for each image. To check the profile, samples were observed vertically to the failure surface. Size measurement on SEM images was performed using ImageJ 1.48 software.

Fourier transform infrared spectroscopy of P-BNNC film

Fourier Transform Infrared (FTIR) was used to investigate the factor groups in the bacterial cellulose polymer structure and their interplay effects with postbiotics trapped in the BNNC. This technique was conducted in the range of wave numbers 400-4000 cm^{-1} **(Klemm *et al.*, 2001)**. The spectra of the models were recorded using a Nexus® 670 (USA) and data were processed using Omnic software (Thermo Electron, Madison, WI, USA).

P-BNNC film application on minced meat

To assess the antimicrobial activity of P-BNNC, beef mince was purchased from the local market in Qazvin and transferred to the Qazvin University School of Health under cold and hygienic conditions. The required amount of *S. aureus* suspension was added to the meat in sterile thick nylon bags to achieve a final bacterial population of 5 $\log_{10}$ CFU/g. The mixture was thoroughly mixed using an interscience stomacher at 280 rpm for 5 min. The optimized P-BNNC film (6×6 cm) was placed on both sides of the inoculated MM (4×4×1 cm). Samples were packaged in sterile PET dishes and stored at 4±1 ºC for 9 days. MM samples were prepared with BNNC and without any film as a control. Inoculated samples were counted on days 3, 6, and 9. At each time point, serial dilutions of the samples were prepared in 0.10% distilled water and spread on mannitol salt agar (Merck, Darmstadt, Germany). Plates were incubated at 37±1 ºC for 24 h.

Shelf-life evaluation

For this purpose, samples of MM were prepared without inoculation, with or without the film, using a similar method as described in the previous section. The samples were then stored at 4±1 °C and evaluated for microbiological and chemical analysis on days 3, 6, and 9.

Microbial assessment

To initiate the analysis, an aseptic mixture of 10 g of the sample was prepared using a stomacher at designated time intervals. A decimal dilution (1:10) was subsequently made in distilled water, resulting in a concentration of 0.10%. The diluted sample was cultured on Mannitol Salt Agar (Merck, Darmstadt, Germany) and incubated at 37±1 °C for 24 hours to ascertain the presence of *S. aureus*.

Chemical evaluation

To evaluate the pH level, a 10 g sample was thoroughly mixed with 90 mL of sterile 0.10% distilled water using a stomacher. The resulting solution's pH was measured at room temperature using a calibrated pH meter.

Data analysis

Experimental results from microbial tests on MM are expressed as the mean and standard deviation (SD) of three repeated measurements. The Colony Forming Units (CFUs) in all experiments are converted to their logarithmic values before statistical analysis. Experimental data are analyzed using a two-way ANOVA with Bonferroni correction post hoc tests. Statistical tests are performed using R

software version 4.0.5. A significance level of $p \leq 0.05$ is considered for all data comparisons.

RESULTS AND DISCUSSION

Table 2 presents the observed values for each solution. Based on the ZOI test results, solutions with 10% (P_{10}-BNNC) and 4% (P_4-BNNC) postbiotic concentrations in BNNC were chosen as optimal compositions for subsequent tests (Table 1). SEM images in Figure 1 reveal good uniformity, well-structured porosity, and random orientation of nanocellulose fibers in BNNC. The FTIR spectrum of the unmodified nanocellulose film is illustrated in Figure 2.

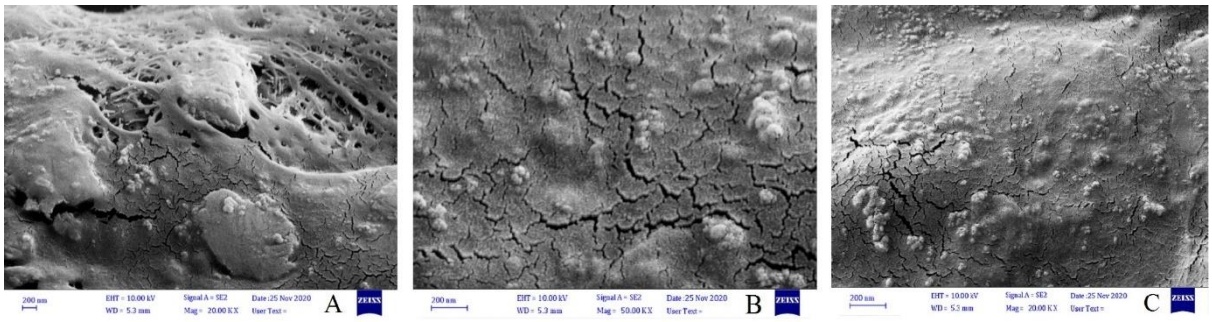

Figure 1 SEM images of empty nanocellulose (A) and nanocellulose containing postbiotic by conventional immersion method [4% (B) and 10% (C) concentration].

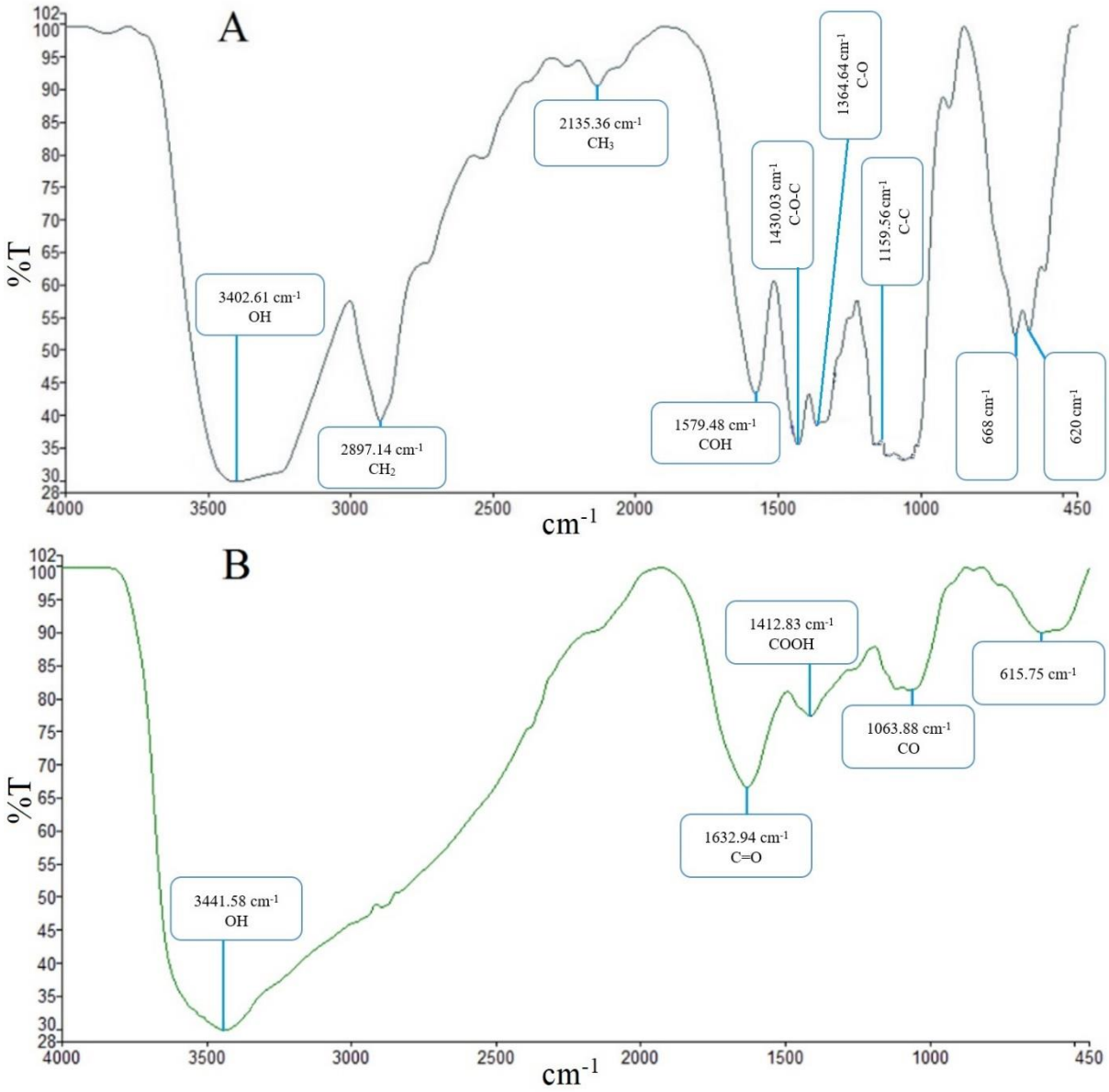

Figure 2 Characteristics of FTIR samples. Image of empty nanocellulose (A) and postbiotic nanocellulose treated by conventional immersion method (B).

Table 1 Determining the optimal concentration of postbiotics.

Treatment	1	2	3	4	5	6	7	8	9	10	11
Concentration (%w/w)	4	6	8	10	12	16	18	20	30	35	40
Impregnation Time (min)	15	15	15	15	15	15	15	15	15	15	15
ZOI (mm)	8.80[a]	8.60[a]	8.60[a]	11.40[a]	11.40[a]	11.40[a]	11.80[a]	12.50[a]	16.90[b]	14.40[b]	14.80[b]

Legend: a. MIC; b. MBC

Table 2 Results of measuring the zone of inhibition of different concentrations of postbiotics by disk diffusion method.

Treatment	Concentration (%w/w)	ZOI (mm)	Treatment	Concentration (%w/w)	ZOI (mm)	Treatment	Concentration (%w/w)	ZOI (mm)
1	4	8.80	**12**	10	11.60	**23**	20	12.40
2	4	8.90	**13**	12	11.40	**24**	20	12.70
3	4	8.70	**14**	12	11.50	**25**	30	16.60
4	6	8.60	**15**	12	11.20	**26**	30	17
5	6	8.40	**16**	16	11.40	**27**	30	17.20
6	6	8.50	**17**	16	11.70	**28**	35	14.10
7	8	8.80	**18**	16	11.30	**29**	35	14.50
8	8	8.60	**19**	18	12	**30**	35	14.70
9	8	8.50	**20**	18	11.70	**31**	40	14.40
10	10	11.30	**21**	18	11.90	**32**	40	14.80
11	10	11.20	**22**	20	12.30	**33**	40	15.20

Legend: ZOI. zone of inhibition

Table 3 The efficacy of PBNNC film of antibacterial activity value of minced meat during storage at 4 °C for 9 days.

Treatment	Control (M±SD)	BNNC(M±SD)	P_4-BNNC (M±SD)	P_{10}-BNNC (M±SD)
3	0.07±5.55	0.08±5.92	0.03±3.56	0.01±3.49
6	0.07±6.23	0.15±6.89	0.02±3.48	ND
9	0.09±7.05	0.13±7.95	ND	ND

Legend: The interaction of time, treatment and their main effects is significant based on the mixed effects modeling method (P<0.001), M±SD. Mean±Standard Deviation; ND. Not Detect

Table 4 The effectiveness of PBNNC film of pH value of minced meat during storage at 4 °C for 9 days.

Treatment	Control (M±SD)	BNNC(M±SD)	P_4-BNNC (M±SD)	P_{10}-BNNC (M±SD)
0	5.90 ± 0.02	5.88 ± 0.10	5.91 ± 0.10	5.84 ± 0.15
3	6.30 ± 0.16	6.47 ± 0.09	5.54 ± 0.09	5.46 ± 0.04
6	7.05 ± 0.11	7.05 ± 0.10	5.24 ± 0.05	5.03 ± 0.14
9	7.38 ± 0.3	7.67 ± 0.09	5.72 ± 0.10	5.79 ± 0.17

Legend: In each line, the presence of non-common letters indicates a significant difference (p <0.05). Results are reported as mean standard deviation.

Postbiotics and P-BNNC film's Antimicrobial Ability

The antimicrobial activity of *L. rhamnosus* postbiotics is concentration-dependent. The diameter of the ZOI was measured for postbiotic concentrations of 30%, 10%, and 4%, resulting in ZOI values of 16.90 mm, 11.40 mm, and 8.80 mm, respectively. LAB possess several key components that contribute to their antibacterial efficacy. These components include lactic acid, bacteriocins, hydrogen peroxide, pyrrolo[1,2-a]pyrazine-1,4-dione, bacteriocin-like substances, and benzoic acid. These components are secreted by LAB during bacterial growth. The antibacterial efficacy of LAB is linked to the combined action of these components **(Şanlıbaba and Güçer, 2015)**. The antibacterial activity of postbiotics in the film can be influenced by various factors. Saadatzadeh et al. reported that the antimicrobial and antioxidant activity of lyophilized *Lactobacillus casei* extract was higher than that of fresh probiotic extract due to the removal of moisture and an increase in the concentration of lactic acid **(Saadatzadeh *et al.*, 2013)**. Therefore, increasing the concentration of postbiotic can lead to an enhancement of the inhibition zone by increasing the concentration of antimicrobial compounds.

P-BNNC films exhibited significant inhibitory activity against gram-positive bacteria in vitro. *L. salivarius*, *L. casei* 431, and *L. acidophilus* LA-5 showed low ZOI (15-24 mm) in controlling *Listeria* strains, according to **Moradi, Mardani, *et al.*, 2019**. The BNNC film containing 5% ZnO by weight (dry base BNNC) showed moderate antimicrobial activity against *S. aureus*, with a ZOI of 11.80 mm **(Shahmohammadi Jebel and Almasi, 2016)**. *L. acidophilus* and *L. salivarius* postbiotics at a concentration of 10% exhibited ZOI of 19.60 mm and 23.70 mm, respectively, against *Listeria monocytogenes*. The antimicrobial activity of *L. acidophilus* is not related to the presence of bacteriocin, while the antimicrobial activity of *L. salivarius* is attributed to bacteriocins, organic acids, and bacteriocin-like substances **(Moradi, Mardani, *et al.*, 2019)**. **Malheiros *et al.*, 2018** reported that the antimicrobial peptides present in *L. sakei* postbiotics, tested using the disk diffusion method, had a ZOI of 30 mm. Several factors can affect the antibacterial activity of postbiotics in the film. For instance, oxygen metabolites and hydrogen peroxide components may lose their antibacterial activity during lyophilization, as reported by **(Rodríguez *et al.*, 1997)**. Therefore, the antibacterial activity of lyophilized postbiotics may depend on the freeze-drying agents used.

The antimicrobial activity of *L. rhamnosus* postbiotics appears to be primarily attributed to bacteriocins and organic acids, as the conditioned fermentation supernatant (CFS) remains active even when not neutralized **(Hsiu *et al.*, 2016)**. This finding aligns with similar studies conducted on *L. casei* 431**(Mirnejad *et al.*, 2013)** and *L. salivarius* **(Moradi, Mardani, *et al.*, 2019)**. Therefore, it can be deduced that the effectiveness of the postbiotics is more influenced by the concentration of the active components rather than the duration of saturation, as the hydration capacity of BNNC limits further solution absorption after a certain period.

Scanning electron microscopy

Figure 1 displays SEM images of BNNC, revealing uniformity, a well-structured porosity, and random orientation of nanocellulose fibers. The dimensions and pores within the fibrous film matrix are influenced by various factors, including bacterial strain and culture conditions employed in nanocellulose production **(Wan *et al.*, 2011)**. Figure 1 also demonstrates the incorporation of postbiotics into BNNC, resulting in changes in the porosity structure of the layer and the formation of a cohesive network with a consistently homogeneous surface in the BNNC membrane. This matrix is insoluble in aqueous solutions but interacts readily with water molecules. This attribute enables the structure to accommodate hydrophilic materials, leading to greater compatibility and enhanced uniformity. Exploiting this property, bacterial postbiotics can permeate the matrix and contribute to the creation of a homogeneous structure **(Shahmohammadi Jebel and Almasi, 2016)**. In a morphological study, a starch cellulose layer matrix coated with alizarin was examined using SEM, demonstrating successful embedding of alizarin within the cellulose structure **(Moradi, Tajik, Almasi, *et al.*, 2019)**.

Fourier transform infrared spectroscopy:

FTIR analysis was performed on both BNNC films and *L. rhamnosus* postbiotic-modified nanocellulose films using the conventional immersion method at optimal concentrations of 4% (P_4-BNNC) and 10% (P_{10}-BNNC). As shown in Figure 1, SEM images of BNNC indicate good uniformity, a well-structured porosity, and random orientation of nanocellulose fibers. Figure 2 shows the FTIR spectrum of the nanocellulose film before modification (Figure 2A) with stretching vibrations of the hydroxyl (OH) group at 3402 cm^{-1}, symmetric and asymmetric stretching of methylene (CH_2) and methyl (CH_3) groups at 2897 cm^{-1} and 2135 cm^{-1}, respectively. The band at 1579 cm^{-1} can be attributed to stretching vibrations of the C-O-H bond and vibrations of water molecules trapped in the nanocellulose film. The transitions observed at 1430 cm^{-1}, 1364 cm^{-1}, and 1159 cm^{-1} may be due to bending vibrations of the methylene group, C-O-C, C-C, and C-O ester bonds, respectively. A broad and overlapping band at 1052 cm^{-1} confirms the presence of pyranose rings associated with the structure of nanocellulose polysaccharides. In the spectrum of the typical P-BNNC film (Figure 2B), a slight shift in the OH peak is observed compared to the BNNC film spectrum before and after mixing with postbiotics (from 3402 to 3441 cm^{-1}), which may be due to the formation of hydrogen bonds leading to interactions between nanocellulose and bioactive compounds. Compared to spectrum A, spectrum B shows a significant change in the stretching vibrations of C=O (located at the peak of 1579 cm^{-1} in the BNNC spectrum) to a wavenumber of 1632 cm^{-1} in the P-BNNC film. The stretching vibration associated with COOH at 1412 cm^{-1} indicates an interaction between carboxylic acid groups in postbiotics. The wavenumber at 1063 cm^{-1} may be attributed to stretching vibrations of the CO bond. Additionally, the wide bands observed in the range of 1063 to 615 cm^{-1} may be related to sugar rings derived

from prepared postbiotics **(Garside and Wyeth, 2003)**. Another study reported that the presence of hydroxyl groups in the structure of glucose as a cellulose monomer and hydrogen bonding with bioactive compounds could explain the change in the OH peak **(Moradi, Tajik, Almasi, *et al.*, 2019)**.

Antibacterial activity of P-BNNC film in minced meat

S. aureus produces a range of enterotoxins, many of which are pathogenic **(Azizkhani and Tooryan, 2019)**. LAB postbiotics are a novel approach to combat the potential threat of bacteria in food. CFS probiotic microorganisms are a suitable alternative for creating antimicrobial packaging in food **(S. C. Beristain-Bauza *et al.*, 2016)**. However, the effectiveness of postbiotics in food models is limited by the complex structure of food, as shown by previous research **(Hartmann *et al.*, 2011)**. The results of statistical analysis demonstrate the effectiveness of postbiotics-wrapped BNNC film against *S. aureus* in MM, as shown in Table 3. No survival or increase of *S. aureus* (~2 Log_{10}) was observed in unpacked and BNNC packed MM samples within 9 days of storage. This improvement is due to the hydration of the nanocellulose polymer matrix, which creates a suitable substrate for pathogen transport and activity. P_{10}-BNNC films, however, exhibited a significant decrease (~5 Log_{10}) which reached an undetectable level (~10 Log_{10} CFU/g) at the end of the storage period. The bacterial populations of *S. aureus* on the ninth day increased from 5 to 7.05±0.09 Log_{10} and 5 to 7.95±0.13 Log_{10} in unpacked MM and BNNC packaged MM samples, respectively. The nanocellulose polymer matrix is hydrated, providing a suitable substrate for pathogen transport and activity. P_4-BNNC and P_{10}-BNNC films exhibited a significant decrease on days 3, 6, and 9 (approximately 1.40, 1.50, and 5 Log_{10}) and (approximately 1.50, 5, and 5 Log_{10}), respectively. At the end of the storage time, bacterial populations were undetectable (less than 10 Log_{10}). On day 3, the highest decrease in the number of bacteria was observed in P_4-BNNC and P_{10}-BNNC. On day 6, there was a significant difference between all samples. On day 9, there was no notable difference between P_4-BNNC and P_{10}-BNNC, but there was a significant difference between the control and nanoparticle treatment ($p < 0.05$). In a similar study, fresh beef wrapped in whey protein film containing *L. sakei* CFS (18 mg/mL) reduced *L. monocytogenes* by 1.40 Log_{10} CFU/g after 5 days of storage at 5 ºC **(S. D. C. Beristain-Bauza *et al.*, 2017)**. In another study, alginate/collagen film containing a 12% concentration of *Lactococcus lactic* postbiotic reduced *S. aureus* population by 2.80 Log_{10} **(Ma *et al.*, 2019)**. Numerous factors such as the type and adaptability of the film and substance **(Almasi *et al.*, 2018)**, the type of food, and simulator can affect the resistance and release of antimicrobial factors from the polymer matrix. The release of postbiotics from BNNC to MM began immediately after meat packaging and continued significantly until the ninth day. Due to its unique three-dimensional structures, BNNC plays a key role in the rapid release of postbiotics from the film. BNNC hydration and postbiotic solubility in foods with high water activity, such as meat, may be considered as major reasons for the rapid spread of postbiotics in meat immediately after packing in meat. Lack of hydration in any film used for food can prevent the release of antibacterial agents from the film into food **(Sánchez González *et al.*, 2011)**.

Shelf life of P-BNNC film wrapped minced meat

Table 4 shows an increase in pH levels in the BNNC and control groups, while the pH of MM packed with P_{10}-BNNC and P_4-BNNC film decreased significantly during the storage period due to the presence of lactic acid. The postbiotics from *L. rhamnosus* are resistant to both acidic and alkaline pH, but their antimicrobial activity is limited by a bacteriocin-like substance that is sensitive to heat. A similar study by Bauza (2016) showed that the reduction in antimicrobial activity of *L. rhamnosus* free fluid was related to the heat sensitivity of the bacteriocin-like substance **(S. C. Beristain-Bauza *et al.*, 2016)**. **Léonard *et al.* (2015)** reported a 30% reduction in the inhibition of *L. innocua* after heat treatment (100 °C for 1 h) of *L. lactis* supernatant. Due to the type and value of metabolites in the CFS, it can be used as a natural antibacterial with good acceptance. In a study by **S. C. Beristain-Bauza *et al.*, (2016)**, the antibacterial activity of *L. rhamnosus* against *S. aureus*, *E. coli*, *S. typhimurium*, and *L. monocytogenes* was attributed to a bacteriocin-like substance and lactic acid. However, when the supernatant was neutralized (pH 6.50), antimicrobial activity focused only on the bacteriocin-like substance. It has also been observed that other antimicrobial compounds are effective in controlling spoilage bacteria and increasing the microbial shelf life of meat. The mechanism of postbiotic action by LAB and their antimicrobial mechanisms involves changes in cell membranes and the presence of organic acids, interference in genetic mechanisms, and the production of all types of proteins due to the strong effects of organic acids. The formation of organic acids after permeability in the cytoplasmic membrane enters cells, and all types of protons derived from organic acids cause changes in intracellular acidity. The cell attempts to maintain intracellular pH by consuming high ATP and expelling these protons, resulting in loss of cell energy and pathogen's ability to continue living and being active **(Tajik *et al.*, 2014)**. Previous reports suggest that the antimicrobial activity of *L. rhamnosus* postbiotic is largely related to organic acids (because CFS has not been neutralized) and, at the same time, the presence of bacteriocins is significant **(Hsiu *et al.*, 2016)**. Our findings are consistent with the results of similar studies on postbiotic *L. casei 431* and *L. salivarius* **(Mirnejad *et al.*, 2013; Moradi, Mardani, *et al.*, 2019)**.

CONCLUSIONS

In the current study, we optimized a new antimicrobial film made of bacterial and postbiotic nano cellulose, which had an optimized effect on *S. aureus* in vitro on MM. *S. aureus* is one of the natural microbial flora of humans and animals and is commonly found in raw meat. Due to the lack of heat treatment during the preparation and packaging of MM, packaging appears to be a good choice for microbiological control of this type of food. The concentration of postbiotics and the saturation of BNNC in postbiotics directly affect the antimicrobial activity of BNNC films. Additionally, the suitability of the BNNC polymer matrix for receiving LAB-derived postbiotics was demonstrated by FTIR and SEM analysis, which revealed the three-dimensional structure of nanofibers. The solubility of postbiotics on the in vitro meat surface was immediately hydrated by BNNC after packing the meat, leading to the rapid release of postbiotics from BNNC into the food. This release rate appears to be ideal for short-term foods such as MM, providing a means to control pathogen growth and warranting further investigation.

Acknowledgment: We would like to express our gratitude to the Vice Chancellor for Research at Qazvin University of Medical Sciences and the Qazvin Standard Organization for their cooperation in conducting our experiments.

Competing interests: The authors declare that they have no competing interests.

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