RESEARCH PAPER

Fabrication and characterization of PVA/WPI nanofibers containing probiotics using electrospinning technique

Zahra Panahi [1], Mohammad Mohsenzadeh [1], Maryam Hashemi [2, 3]*

[1]*Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*
[2]*Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran*
[3]*Departments of Pharmaceutical Biotechnology, School of Pharmacy, Mashhad, University of Medical Sciences, Mashhad, Iran*

ABSTRACT

Objective(s): This study aimed to evaluate the viability of encapsulated probiotics using electrospinning technique. Specifically, the study focused on polyvinyl alcohol-whey protein isolate nanofibers (PVA/WPI) containing *Bifidobacterium bifidum*. These nanofibers have potential applications in active food packaging to improve food safety and extend shelf life, as well as in medical and pharmaceutical fields.

Materials and Methods: PVA/WPI nanofibers were electrospun in varying ratios (ranging from 100:00 to 50:50) and evaluated for their morphology, mechanical properties, FT-IR and DSC characteristics. *B. bifidum* was also encapsulated in the optimized PVA/WPI nanofibers to assess their encapsulation efficiency and viability, and the antimicrobial properties of the nanofibers were determined using the disk diffusion method.

Results: All prepared nanofibers displayed a diameter range of 186.42-612.5 nm, with an inverse relationship between WPI ratio and nanofiber diameter. The PVA/WPI nanofiber with a ratio of 60:40 was found to be the most favorable. DSC analysis showed that adding WPI decreased thermal stability, and the enthalpy of endothermic peaks decreased in nanofibers containing *B. bifidum*. Mechanical evaluation revealed that adding WPI reduced tensile strength and elongation at break, without significant effects from *B. bifidum* (P>0.05). Bacterial encapsulation efficiency was 80.58%. Probiotic nanofibers exhibited antimicrobial properties against Listeria monocytogenes (11.00±0.37 mm) and Escherichia coli (9.71±0.06 mm).

Conclusion: According to the obtained results, the optimized PVA/WPI nanofiber (60:40) contained suitable morphological, mechanical and thermal characteristics with the highest encapsulation efficiency in regards to *B. bifidum* (>80%). Probiotics-containing PVA/WPI nanofibers are a suitable platform for medical applications and food industry packaging due to their antimicrobial properties.

Keywords: *Antimicrobial activity, Bifidobacterium bifidum, Bioactive packaging, Nanofiber, Nanotechnology*

How to cite this article
Panahi Z, Mohsenzadeh M, Hashemi M. Fabrication and characterization of PVA/WPI nanofibers containing probiotics using electrospinning technique. Nanomed J. 2023; 10(3): 216-226. DOI: 10.22038/NMJ.2023.71941.1773

INTRODUCTION

As a type of microorganisms, probiotics are known as live bacteria or yeasts in digestive system [1] with a high rate of nutritional properties that achieved the approval of public knowledge [2]. Probiotics have beneficial effects on host health and are effective in the treatment of many acute diseases such as gastrointestinal infections [3]. They are also used as bio-preservatives in the food industry [4]. As a type of probiotics, *Bifidobacterium* spp. contains high nutritional and functional properties with the ability to prevent the colonization of pathogenic microorganisms in the intestine, facilitating the reduction of diarrhea and constipation, and strengthening the immune system [2]. Probiotics can produce acid by converting sugar into lactic acid and also destroy pathogenic microorganisms through the production of bacteriocins similar to Nisin [5]. The sensitivity of probiotics to harsh conditions, such as processing, storage, transportation and digestive system, can result in their reduction, while the minimum number of required live probiotic at the time of consumption for providing

* *Corresponding author: Email: mohsenzadeh@um.ac.ir*
Note. This manuscript was submitted on April 8, 2023; approved on June 10, 2023

health is 10^6-10^7 CFU/ml [6]. Considering these facts, nowadays new strategies are exerted, such as encapsulation, to protect probiotics against adverse environmental factors [7]. The purpose of encapsulation is to create a micro-environment for the survival of bacteria during processing and storage up to the point of their release in a suitable environment such as the intestine [8]. There are various techniques for completing the encapsulation of probiotics, which include extrusion, spray drying, and electrospinning [9].

Among the many encapsulation approaches, electrospinning is an effective method for the viability and survival of probiotics, which is simple, cheap, and suitable for the production of nanofibers [10]. Nanofibers are usually recognized as fibers with a diameter of less than 100 nm or even less than 500 nm [10]. Electrospinning machines used for the production of nanofibers are composed of various parts including syringe, pump, high voltage source and collector [11], which also require the exertion of diverse compounds such as synthetic polymers polyethylene oxide (PEO) and polyvinyl alcohol), protein (whey protein, zein, gelatin, etc.), polysaccharide (chitosan, sodium alginate, inulin) and lipid [12]. Furthermore, different solutions similar to PVA/sodium alginate [13] and chitosan/PVA/inulin [14] were discovered to be practical for the encapsulation of probiotics through the electrospinning method. One of the applied industrial polymers in the production of nanofibers is Polyvinyl alcohol. PVA is soluble in water and non-toxic with high chemical and thermal stability, which is obtained from the hydrolysis of polyvinyl acetate [15].

Known as one of the main wastes of dairy industry, whey protein exists in the two forms of whey protein concentrate (25-80% protein) and whey protein isolate (more than 80% protein) [16], while containing beta-lactoglobulin and alpha-lactalbumin. These proteins are widely exerted in food industry due to the accommodation of high nutritional properties. Whey protein is soluble in water and biodegradable and is used to produce fibers [17] and edible films [18]. In addition, whey protein can be an effective compound to protect probiotics during storage and processing [19].

Up to this date, a very limited number of research has been conducted on the encapsulation of probiotics by electrospinning method with the aim of increasing the viability of probiotics and the antimicrobial properties of probiotics-containing nanofibers. This research has important implications not only for the food industry but also for medical and pharmaceutical applications. Probiotics-containing nanofibers can be used in active food packaging to improve food safety and extend shelf life. Specifically, the study aimed to produce optimized PVA/WPI nanofibers containing *Bifidobacterium bifidum* to enhance its encapsulation efficiency. The research also aimed to investigate the antimicrobial properties of the produced nanofibers against *Listeria monocytogenes* and *Escherichia coli* O157:H7, two important pathogenic bacteria associated with foodborne illnesses.

MATERIALS AND METHODS

Materials

This research implicated the application of Whey protein isolate (WPI) with 90% protein in dry weight (Hilmar™ 9010, Hilmar Ingredients, Hilmar, CA, USA), Poly vinyl alcohol (PVA, Mw = 72,000 Da, Merck, Darmstadt, Germany), Mueller-Hinton (MH) agar (De Man, Rogosa and Sharpe), MRS (De Man, Rogosa and Sharpe) agar and broth (Merck, Darmstadt, Germany). *Bifidobacterium bifidum* (PTCC 1644), *Listeria monocytogenes* (ATCC 7644), *Escherichia coli* O157:H7 (NCTC 12900) were procured from the Food Hygiene Laboratory, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran.

Polymer solutions preparation for electrospinning

Whey protein isolate at 2% (w/v) concentration was dissolved in distilled water under constant stirring for 20 min. Then, the PVA solution was prepared at 7% (w/v) concentration in distilled water under stirring for 30 min at 85 °C [17]. Once the obtained WPI and PVA solutions were mixed at various ratios (PVA:WPI; 100:0, 80:20, 70:30, 60:40, 50:50), each PVA/WPI solution was stirred for 3 h at 25 °C to their complete dissolution.

Characterization of solutions

As the pH value of solutions was measured by a pH meter (pH meter 240, Corning Scientific Products, NY, USA), the rate of viscosity was measured by Brookfield R/S plus rheometer (Brookfield, Middleboro, MA, USA) and also, A VWR Traceable® Expanded Range conductivity meter was used to record the conductivity.

Electrospinning process

Electrospun nanofibres were produced by a

horizontal single-nozzle electrospinning machine (Fanavaran Nano-Meghyas company, Iran). Various weight ratios of PVA/WPI solutions (PVA:WPI; 100:0, 80:20, 70:30, 60:40, 50:50) were loaded into a plastic syringe, which was fitted with a 21-gauge stainless steel needle, to be placed horizontally on a digital controlled syringe pump at a controlled feed rate of 0.5-0.6 mL/h and the voltage of 18-20 kV in the tip-to-collector distance of 20cm.

Scanning electron microscopy (SEM)

The morphology of the nanofibers was investigated through the application of scanning electron microscopy (MIRA3 TESCAN, Brno, Czech). The samples were sputtered with a layer of gold. Image J analysis was exerted to measure the mean diameter of 50 fibers [12].

Thermal characterization of PVA/WPI nanofiber

Differential scanning calorimetry (Mettler-Toledo, Switzerland) was performed under a nitrogen atmosphere at a flow rate of 50 mL/min. Samples were sealed in 40 µL aluminum pans and heated at the temperature range of 10 to 350 °C with a heating rate of 10 °C/min.

Mechanical properties of PVA/WPI nanofiber

Tensile strength (TS) and elongation at break (EB) of nanofibers were tested by Hounsfield H50K-S Testing system regarding the nanofiber membrane at the elongation speed of 10 mm/min. The samples were cut in the measures of 40 × 10 mm at ambient conditions, which were repeated five times for each fiber.

Attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR)

In this section, Fourier transform infrared spectroscopy (FTIR) was exerted to perform the chemical characterization of nanofibers, which was conducted for the cases of PVA powder, whey protein, and PVA/WPI (60:40) nanofiber. Next to preparing the FTIR pills by mixing the sample powder with KBr, the associated Spectra were recorded by a Fourier transform spectrophotometer (Frontier FT-IR, PerKinElmer, USA) at the range of 4000–400 cm^{-1} with the interval of 1 cm^{-1}.

Viability of PVA/WPI-encapsulated Bifidobacterium bifidum

The viability of *B. bifidum* was recorded before and after the application of electrospinning. The *B. bifidum* were inoculated into MRS broth and incubated under anaerobic conditions at 37 °C in MRS broth for 24 h. In addition, the culture solution was centrifuged at 4000×g for 15 min at 4 °C to harvest the cell pellets, which were rinsed three times by sterile water. The 60:40 ratio of PVA/WPI was determined in accordance to its morphology.

Bifidobacterium bifidum (5 MacFarland; 1.5×10^9 CFU/ml) were added into the wall material solution to be continuously mixed on a magnetic stirrer at 25 °C for 1 h [12]. Then, the electrospinning process was performed by applying a high voltage of 18 kV at 25 °C, feed rate of 0.6 mL/h, and tip-to-collector distance of 20 cm. The number of bacterial colonies per milliliter was measured in conformity to the MRS agar plate colony counting method, while the initial feed solution was used as the control. The encapsulation efficiency (EE%) of *B. bifidum* was calculated through the following equation:

$$EE(\%): \log 10\ N\ \times 100/\log 10\ N0$$

where N and N0 represent the viability of cells (CFU/ml) before and after the application of electrospinning, respectively [20].

Antimicrobial activity of PVA/WPI nanofibers with B. bifidum

The antibacterial activity of nanofibers was analyzed against *L. monocytogenes* (Gram positive) and *E. coli* O157:H7 (Gram negative) based on the results of disk diffusion method. For this purpose, bacterial suspension (106 CFU/mL) was spread over the Mueller-Hinton (MH) agar and the nanofibers were cut into 6 mm disks while a blank disk was used as the control. Each nanofiber was placed on micro-organism-cultured agar plates and incubated at 37 °C for 24 h. The diameter of an inhibition zone in the agar plate was observed and measured after 24 h of incubation. The experiments were completed in triplicate to determine the mean values [21].

Statistical analysis

The data are expressed as mean ± standard deviations, and all the analytical results of samples were performed in triplicate. Next to the usage of SPSS Statistics 26.0 software (IBM, Inc., Armonk, NY) for assessing the variance (ANOVA), Dunkan's test was also conducted as the statistical significance level was set at $P<0.05$.

RESULTS AND DISCUSSION

Physicochemical properties of polymer solutions pH analysis

The results of pH analysis for PVA/WPI

Table 1. Physicochemical properties of nanofiber-forming dispersions

Solutions	pH	Conductivity (µS/cm)	Viscosity (Pa/s)
PVA (7%)	5.83±0.12^{b}	231.00±2.00^{f}	0.33±0.03^{a}
WPI (2%)	6.42±0.02^{a}	659.33±1.52^{b}	0.12±0.02^{e}
PVA/WPI (80:20)	5.82±0.02^{b}	569.33±1.52^{f}	0.33±0.00^{a}
PVA/WPI (70:30)	5.83±0.01^{b}	600.67±3.78^{e}	0.24±0.00^{b}
PVA/WPI (60:40)	5.86±0.06^{b}	633.33±2.08^{d}	0.22±0.00^{c}
PVA/WPI (50:50)	5.86±0.11^{b}	653.00±1.00^{c}	0.16±0.01^{d}
PVA/WPI/BB*	5.34±0.19^{c}	703.58±2.14^{a}	0.25±0.01^{b}

The mean ± SD with different lowercase letters within columns are significantly different (P<0.05)
* *Bifidobacterium bifidum* (BB)

solutions are presented in Table 1, which displays the pH value of 7% PVA solution to be 5.83 and the 2% solution of WPI to be 6.42. The pH value was slightly increased subsequent to the (P>0.05) addition of WPI to PVA solution and increasing the proportion of WPI, which could be due to the high initial pH of WPI when compared to that of PVA. Colin-Orozco et al. conducted a study on different ratios of polyethylene oxide-whey protein in 2015. According to their observations, increasing the applied ratio of polyethylene oxide intensified the pH value, which is attributed to the high pH of 9% polyethylene oxide solution (8.16) when compared to the 10% solution of whey protein (7.04) [22]. The probiotic-containing solution had a significantly lower pH than the PVA/WPI solution (60:40) (P<0.05), due to the growth and fermentation of whey protein and the production of lactic acid by the probiotic [23].

Electrical conductivity analysis

In the present study, the electrical conductivity of 2% solution of WPI (659 µS/cm) was significantly higher than that of PVA solution (231 µS/cm) (P<0.05). In conformity to Table 1, the electrical conductivity was intensified subsequent to increasing the ratio of WPI to PVA, which is consistent with the study of Tatlisu et al., in 2019. Electrical conductivity is a critical factor in determining the transfer of charge to the surface of the pendant droplet, which directly impacts the buildup of electrostatic repulsion force. This force is necessary for initiating jetting [24]. The electrical conductivity of a solution can be influenced by the type of solvent, the amount of ionized salts, and the type of solution. The level of electrical conductivity is a crucial factor affecting the diameter and uniformity of fibers. When the electrical conductivity of a solution increases significantly, it leads to a greater transfer of surface charge to the spinning polymer jet. This, in turn, results in a higher electrostatic repulsion force, which induces bending instability and stretching critical for forming submicron fibers [10, 24]. Therefore, an increase in WPI results in heightening the electrical conductivity, which consequently decreases the diameter of produced nanofibers. The obtained results also indicated that the addition of *B. bifidum* to PVA/WPI (60:40) solution increases the electrical conductivity due to the presence of proteins and extracellular ions [25]. In the study conducted by Škrlec et al., 2019, it is reported that the appending of *Lactobacillus plantarum* to polyethylene oxide solution increased the electrical conductivity, which is consistent with the results of our research [26].

Viscosity analysis

Table 1 exhibits the viscosity of solutions, which is another affecting factor on fibers morphology. High viscosity prevents the exit of solutions from jets, while its low values cause the formation of beads and non-uniform fibers [10]. For this reason, choosing the optimal viscosity is quite critical for the production of uniform fibers. In the present study, considering the 0.33-0.12 Pa/s. viscosity

range of prepared solutions, the viscosity of 7% PVA solution (0.33 Pa/s) was significantly higher than the other solutions (P<0.05). Meanwhile, the lowest viscosity was obtained by the 2% solution of WPI (0.12 Pa/s). The addition of *B. bifidum* to PVA/WPI (60:40) solution had no significant effects on its viscosity (P>0.05). However, a reduction was observed in viscosity subsequent to increasing the ratio of WPI, which is consistent with the reports of Keramat et al., 2019 and Tatlisu et al., 2019 [27,17]. The low viscosity of WPI solution can be attributed to the globular structure of whey protein [27]. PVA/WPI (50:50) solution was incapable of spuning due to the domination of surface tension and low viscosity, leading to the formation of non-uniform fibers that held numerous beads in a destroyed state. According to the study of Tatlisu et al., in 2019, the low viscosity of PVA/WP solutions in the ratios of 40:60, 20:80 and 0:100 resulted in the production of fibers with non-uniform and merged structures that seemed to be entirely corrupted [17].

SEM micrographs

The micrographs and diameter distribution histogram of electrospun nanofibers are presented in Fig1. Since the morphology of fibers can be influenced by viscosity and electrical conductivity, creating the right balance between these factors is essential for producing suitable fibers [27]. According to related studies, it is impossible to produce fibers by the solo usage of WPI due to its low viscosity and the same law applies to the case of increasing its proportion as well since it leads to reducing the spinning ability. Moreover, applying a 50:50 ratio of PVA/WPI resulted in non-uniform fibers with numerous beads (Fig.1., E). Therefore, considering these facts and the outcomes of this study and other researches, higher ratios of WPI/PVA were not used for electrospinning [17,28]. PVA/WPI (100:00) nanofiber were observed to be non-uniform with a high diameter and many beads (Fig. 1., A). The addition of WPI to PVA solution caused a reduction in the diameter of produced nanofibers by lowering the rate of viscosity (Fig.1, B, C, D) [17]. As it was mentioned, the diameter and structure of fibers can be affected by the weight ratio of PVA/WPI. The selection of optimized PVA/WPI ratio in this study was based on various parameters such as the absence of beads, proper uniformity, smaller diameter, and lower PVA content. Therefore, the PVA/WPI volume ratio of 60:40 was chosen as the optimized nanofiber due to its smaller diameter, absence of beads, and high uniformity (Fig.1: D). According to the report of Tatlisu et al., in 2019, the diameter of fibers is increased as a result of enlarging the PVA ratio, while an increase in whey protein ratio causes a reduction in the resultants diameter by lowering its viscosity [17].

The encapsulation of *B. bifidum* was performed by the PVA/WPI volume ratio of 60:40. This addition resulted in increasing the diameter of nanofibers and intensifying the formation of beads, while indicating the successful encapsulation of bacteria (Fig.1., F). These observations were consistent with the results of Ceylan et al., 2018, and Yilmaz et al., 2020 [29,30].

Thermal analysis

The application of Differential Scanning Calorimetry (DSC) test can help in better understanding the thermal stability of structural components. Fig. 2 displays three endothermic peaks in the structure of each sample. Consistent with the results of other reports, all the samples containing WPI showed an endothermic peak in the range of 60-70 °C that was associated with the denaturation temperature of beta-lactoglobulin (the main fraction of whey protein) [31, 32]. The second endothermic peak, observed in the temperature range of 170-200 °C, was related to the melting temperature of nanofibers and faced a reduction after the addition of WPI. The third endothermic peak in the samples structure was attributed to the elimination of acetate group and the main chain decomposition of PVA [33]. In this peak, increasing the ratio of PVA volume intensified the enthalpy of the samples and by decreasing this ratio, the enthalpy was reduced as well. The inclusion of WPI to PVA caused a significant decrease in the thermal stability of all the samples, which was proved by the recorded decrease in the denaturation temperature of every sample. In regards to the *B. bofidum*-containing nanofibers, the observed reduction in the enthalpy of all endothermic peaks indicated the induced decrease in the required thermal energy for the thermal degradation of nanofibers in the presence of bacteria. In conformity to the report of Yilmaz et al., in 2020, a decrease in the weight loss and heat of reaction throughout the PVA/sodium alginate nanofiber is a sign for the successful integration of probiotic cells into the

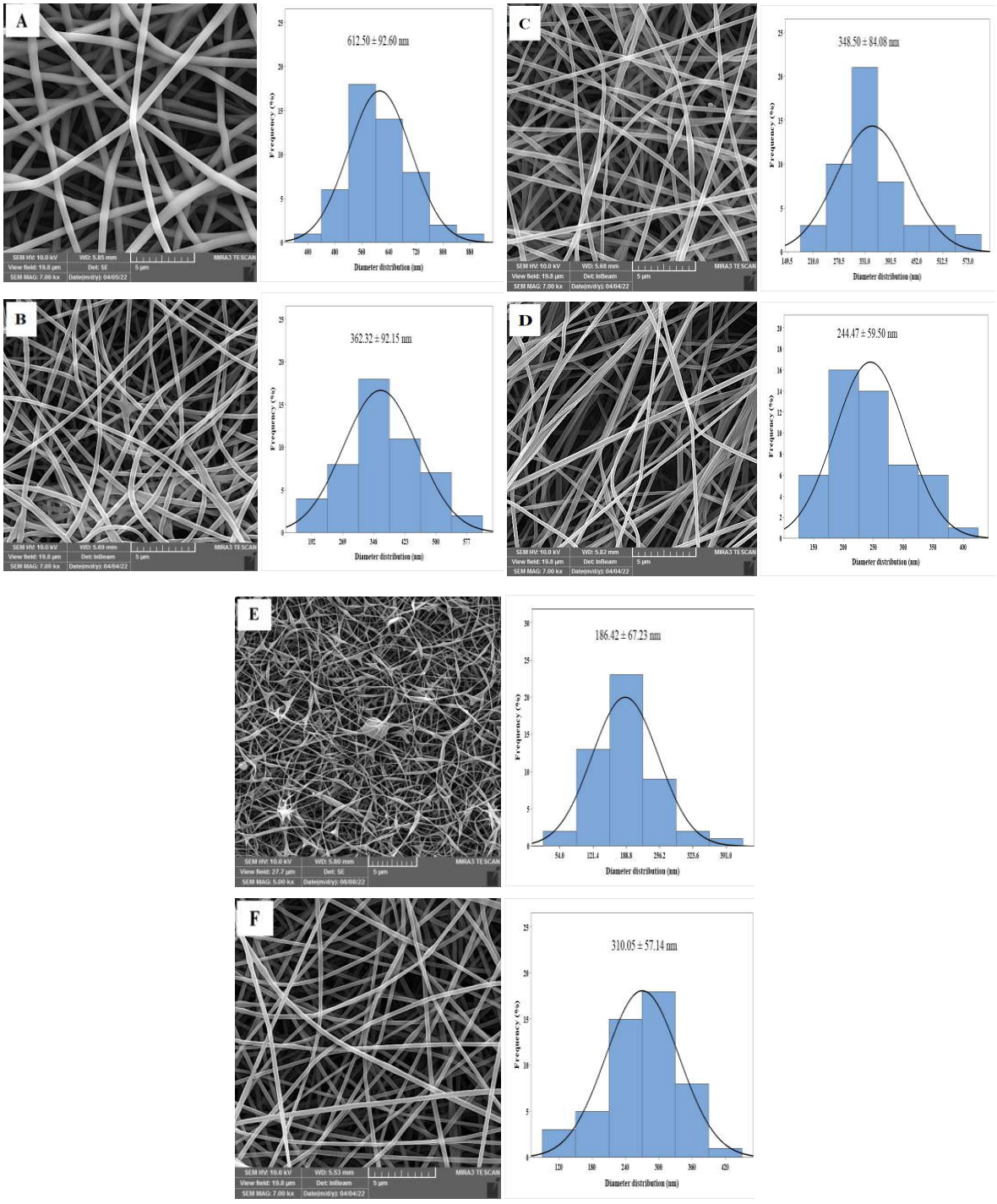

Fig 1. SEM images and diameter distribution of the (A) PVA/WPI (100:00), (B) PVA/WPI (80:20), (C) PVA/WPI (70:30), (D) PVA/WPI (60:40), (E) PVA/WPI (50:50), and (F) PVA/WPI/BB nanofibers

nanofibers [30].

Mechanical properties

The mechanical properties of nanofibers can provide useful data on the uniformity of their physical structure and durability. The tensile strength and Elastic modulus of nanofibers are illustrated in Table 2. Tensile strength is the maximum tensile stress that a nanofiber can withstand while maintaining its structure [34].

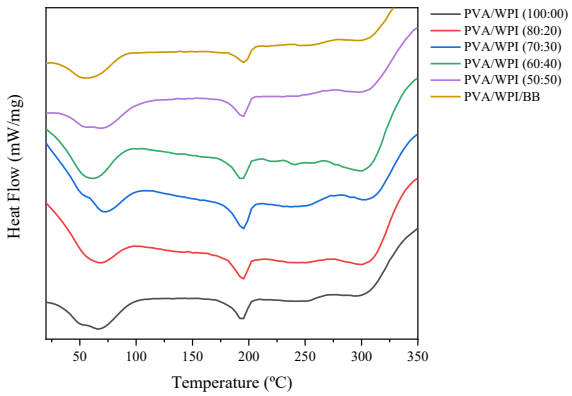

Fig. 2. DSC thermograms of PVA/WPI (100:00, 80:20, 70:30, 60:40, 50:50), and PVA/WPI/BB nanofibers

Considering how PVA/WPI (100:00) nanofiber showed the highest tensile strength, this factor of composite nanofibers were increased after the addition of PVA to WPI and increasing the volume ratio of PVA. According to the outcomes, the elastic modulus was significantly increased as a result of adding PVA to WPI ($p < 0.05$), which was more evident in higher ratios of PVA volume. However, although the tensile strength was slightly decreased with the addition of *B. bifidum*, yet it had no significant effects on the tensile strength of nanofiber ($P>0.05$). Being consistent with the results of this study, the report of Gialamas et al., in 2010, claimed the ineffectiveness of probiotic cells on the mechanical properties of sodium-caseinate film [35]. Furthermore, the study of Ghalehjooghi et al., in 2023, observed that the addition of probiotics to gelatin-sodium alginate fibers had no significant effects on tensile strength [36], while in another study, the addition of probiotics to starch-pullulan films caused a decrease in the tensile strength of prepared edible films [37]. In general, the usage of probiotics in polymer matrices can reduce mechanical resistance by weakening the intermolecular attractions of adjacent polymeric chaines and increasing the flexibility of nanofibers [38].

Elongation at break indicates the percentage of induced changes in initial length of materials. Elongation at break in PVA/WPI (100:00) nanofibers was higher than that of the other samples. The structural density, molecular mobility of chains, and flexibility of other composite nanofibers were decreased, which caused a reduction in their elongation at break. According to the work of Kurt et al. (2017), intermolecular interactions in the structure of composite fibers can reduce the free space of biopolymer chains and cause a decrease in the Elongation at break of samples [39]. In addition, Leuangsukrerk et al. (2014) reported the higher fragility of whey protein fibers when compared to the fibers of carbohydrates. Therefore, the presence of WPI in the structure of composite nanofibers is considered as a reducing factor for the elongation at break of nanofibers [40]. As a similar observation to the study of Ghalehjooghi et al., in 2023, the addition of *B. bifidum* increased the rate of elongation at break, which was not statistically significant ($P > 0.05$) [36].

ATR-FTIR analysis

The FTIR spectroscopy outcomes of PVA powder, WPI powder and PVA:WPI 60:40 nanofiber are presented in Fig. 3. This analytical method can determine the induced changes in chemical structure of materials and identify the characteristic functional groups of polymers.

Table 2. Mechanical properties of PVA/WPI (100:00, 80:20, 70:30, 60:40, 50:50), and PVA/WPI/BB nanofibers

Treatment	Elastic modulus(Mpa)	Tensil strength(Mpa)	Elongation at break(%)
PVA/WPI (100:00)	9.70±7.67^{a}	4.85±3.4^{a}	44.0±0.10^{a}
PVA/WPI (80:20)	7.28±5.93^{bc}	1.93±2.3^{b}	41.0±0.04^{a}
PVA/WPI (70:30)	6.85±2.12^{c}	1.26±1.0^{c}	14.0±0.02^{b}
PVA/WPI (60:40)	4.85±3.22^{d}	1.10±3.1^{c}	15.0±0.02^{b}
PVA/WPI (50:50)	4.24±2.31^{d}	0.83±1.6^{d}	6.0±0.05^{c}
PVA/WPI/BB	4.94±0.83^{d}	1.00±2.7^{c}	16.0±0.09^{b}

The mean ± SD with different lowercase letters within columns are significantly different ($P<0.05$)

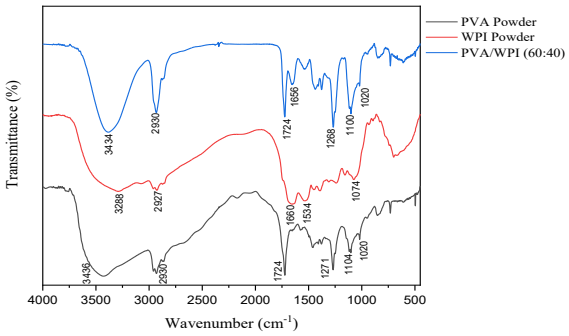

Fig. 3. FTIR spectra of PVA powder, WPI powder, and PVA/WPI (60:40) nanofiber

Certain changes will be visible in FTIR spectra upon the occurrence of molecular interactions between the constituents of polymers, which includes shifts and alterations in the width and intensity of peaks that can be attributed to the miscibility of polymers [41]. The observed broad peak of PVA-containing nanofibers in the range of 3400 cm^{-1} was ascribed to the stretching vibration mode of hydroxyl groups (-OH), while the increased intensity of the peak at the range of 3400 cm^{-1} in PVA/WPI (60:40) nanofiber was caused by the presence of WPI. The peaks corresponding to the wave-numbers of 2930, 1724, 1104 and 1020 cm^{-1} in the PVA samples were assumedly associated with the symmetrical- asymmetrical vibrations of C-H and C=O stretching of carbonyl group, C-O as the stretching vibrations of acetate group in the PVA, and C-C-C stretching vibrations, respectively [33, 42]. The WPI-containing samples displayed peaks in correspondence to the wavenumbers of 1660 and 1534 cm^{-1} (WPI powder), 1656 cm^{-1} (PVA/WPI 60:40 nanofiber), which were allocated to the amide I and amide II of WPI, respectively [33]. According to the data of PVA/WPI (60:40) nanofiber in Fig. 3, the presence of WPI caused a shifting in the peak of 3436 cm^{-1} wavenumber throughout the PVA that may be due to the induced structural changes in the matrix of composite nanofiber components [43]. Therefore, the FTIR results indicated the compatibility and favorable interaction of PVA/WPI in the composite nanofiber.

Viability of B. bifidum

Table 3 exhibits the encapsulation efficiency of probiotic to be 80.58%, which also presents the viability list of *B. bifidum* before and after encapsulation. Although the viability of *B. bifidum* in the feeding solution in prior to encapsulation was 8.86 CFU/ml, yet it reached 7.14 CFU/ml after the process in PVA/WPI nanofibers and was decreased for about 1.5 log. According to an investigation on the viability of encapsulated *B. bifidum* in PVA-chitosan fibers, it was observed to be 9.47 before the encapsulation process and seemed to be reduced after electrospinning by about 1 log to reach 8.42 CFU/ml; these outcomes were in agreement with the results of this study [14]. Apparently, the implication of a high voltage and the changes in osmotic environment as a result of solvent evaporation throughout the electrospinning process caused a reduction in the viability of *B. bifidum* [44]. Ma et al., 2021, reported the difference in viability of *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casei*, as well as the reduction of bacteria after electrospinning in the range of (0.2-1.5 log). The difference in viability of varying probiotics can be due to the production of extracellular polysaccharides and the structures of cell surface such as cell membrane fatty acids, S layer proteins and lipoteichoic acid, which causes significant differences in surface hydrophobicity [12].

Antibacterial activity

The antimicrobial properties of produced nanofibers were evaluated through the disk diffusion method. The data of Table 4 confirms the lack of any inhibition zones in the WPI solution, PVA solution, and PVA/WPI (60:40) nanofiber, which is consistent with the studies of other researchers. The performed investigations on the antimicrobial property of PVA [45] and WPI [46] indicated the incapability of these compounds in displaying antimicrobial properties against Gram-positive and Gram-negative pathogenic bacteria, including *Staphylococcus aureus* and *E. coli*. The diameter size of inhibition zones in free cells of

Table 3. Viability of *B. bifidum* before and after applying electrospinning

Treatment	Before exposure (log CFU/ml)	After exposure (log CFU/ml)	Viability rate (%)
PVA/WPI/BB nanofiber	8.86±0.21	7.14±0.56	80.58±4.08

Table 4. Antibacterial activity of PVA powder (7%), WPI powder (2%), PVA/WPI (60:40), and PVA/WPI/BB nanofiber, and free cells of *B. Bifidum*

Treatment	*L. monocytogenes* (mm)	*E. coli* O157:H7 (mm)
PVA powder (7%)	-	-
WPI Powder (2%)	-	-
PVA/WPI nanofiber (60:40)	-	-
PVA/WPI/BB nanofiber	11.00±0.37^{b}	9.71±0.06^{b}
B. bifidum (Free cells)	13.06±1.02^{a}	12.03±0.56^{a}

The mean ± SD with different lowercase letters within columns are significantly different (P<0.05)

B. bifidum was higher than those encapsulated in nanofibers, which may be due to their control over the release of bacteria from nanofibers [47]. Also, the results showed the antimicrobial properties of *B. bifidum* and PVA/WPI/BB nanofibers against *L. monocytogenes* and *E. coli* O157:H7. According to certain reports, the antimicrobial properties of *B. bifidum* against pathogenic bacteria is caused by the production of various compounds such as lactates, acetates, diacetyl, hydrogen peroxide, and bacteriocins [5, 48]. Lim and Shin (2020) reported that *Bifidobacterium* strains have antibacterial effects against various pathogens, and some of them inhibit multidrug-resistant pathogens. Also, substances such as lipoprotein A, which are found in the outer layer of the bifidobacterial cell walls, can inhibit pathogens [49].

CONCLUSION

This study aimed to produce PVA-WPI nanofibers with varying ratios using the electrospinning method. The SEM results showed that increasing the volume ratio of WPI resulted in a reduction in the diameter of nanofibers due to the induced decrease in viscosity and increase in electrical conductivity. The evaluation of thermal and mechanical properties revealed that an increased WPI ratio led to decreased thermal stability and tensile strength. Uniform and beadless nanofibers with an average diameter of 244.47±59.50 nm in a PVA/WPI ratio of 60:40 were identified as optimal based on SEM results. *Bifidobacterium bifidum* was successfully encapsulated in the optimized PVA-WPI (60:40) nanofiber, with an encapsulation efficiency of 80.58%. The PVA/WPI nanofibers containing *B. bifidum* demonstrated effective antimicrobial properties against pathogenic bacteria such as *L. monocytogenes* and *E. coli* O157:H7, indicating their potential use in the pharmaceutical, medical, and food industries for producing biodegradable and active packaging with antimicrobial properties. Further research is required to improve the controlled release of probiotics from nanofibers, increase encapsulation efficiency, and enhance probiotic stability.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support by Ferdowsi University of Mashhad (FUM) (Grant number: 3/55379) and Mashhad University of Medical Sciences.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

REFERENCES

1. Oak SJ, Jha R. The effects of probiotics in lactose intolerance: a systematic review. Crit Rev Food Sci Nutr. 2019;59(11):1675-1683.
2. Shu G, He Y, Chen L, Song Y, Meng J, Chen H. Microencapsulation of Bifidobacterium bifidum BB01 by xanthan–chitosan: Preparation and its stability in pure milk. Artif Cells Nanomed Biotechnol. 2018;46(sup1):588-596.
3. Fujiya M, Ueno N, Kohgo Y. Probiotic treatments for induction and maintenance of remission in inflammatory bowel diseases: a meta-analysis of randomized controlled trials. Clin J Gastroenterol. 2014;7(1):1-3.
4. Mirhadi Zadi, T, Mohsenzadeh, M, Ghahramani Seno, MM. Molecular Isolation, Probiotic Property, and Bacteriocin Production of Enterococcus faecium (TM81) and Lactobacillus curvatus (TM51) with Anti-Listerial Activity in Native Dairy Products of Iran. Iran J Chem Chem Eng. 2021; 40(4):1346-1363.
5. Rahimifard N, Moghni M. Naseri. Evaluation and

comparison of three antimicrobial activity methods using bifidobacteria bifidum and bifidobacteria infantis as probiotic bacteria against salmonella enterica serotype enteritidis. J Bacteriol Mycol. 2016;2(3):61-64.
6. Afzaal M, Saeed F, Ateeq H, Ahmed A, Ahmad A, Tufail T, et al. Encapsulation of Bifidobacterium bifidum by internal gelation method to access the viability in cheddar cheese and under simulated gastrointestinal conditions. Food Sci Nutr. 2020;8(6):2739-2747.
7. Liu H, Gong J, Chabot D, Miller SS, Cui SW, Zhong F, et al. Improved survival of Lactobacillus zeae LB1 in a spray dried alginate-protein matrix. Food Hydrocoll. 2018;78:100-108.
8. Chávarri M, Marañón I, Villarán MC. Encapsulation technology to protect probiotic bacteria.InProbiotics 2012. IntechOpen.
9. Çanga EM, Dudak FC. Improved digestive stability of probiotics encapsulated within poly (vinyl alcohol)/ cellulose acetate hybrid fibers. Carbohydr Polym. 2021;264:117990.
10. Fahami A, Fathi M. Fabrication and characterization of novel nanofibers from cress seed mucilage for food applications. J Appl Polym Sci. 2018;135(6):45811.
11. Ramakrishna S. An introduction to electrospinning and nanofibers. World Scientific. 2005.
12. Ma J, Xu C, Yu H, Feng Z, Yu W, Gu L, et al. Electro-encapsulation of probiotics in gum Arabic-pullulan blend nanofibres using electrospinning technology. Food Hydrocoll. 2021;111:106381.
13. Feng K, Huang RM, Wu RQ, Wei YS, Zong MH, Linhardt RJ, et al. A novel route for double-layered encapsulation of probiotics with improved viability under adverse conditions. Food chem. 2020;310:125977.
14. Mojaveri SJ, Hosseini SF, Gharsallaoui A. Viability improvement of Bifidobacterium animalis Bb12 by encapsulation in chitosan/poly (vinyl alcohol) hybrid electrospun fiber mats. Carbohydr Polym. 2020;241: 116278.
15. Santos C, Silva CJ, Büttel Z, Guimarães R, Pereira SB, Tamagnini P, et al. Preparation and characterization of polysaccharides/PVA blend nanofibrous membranes by electrospinning method. Carbohydr polym. 2014;99:584-592.
16. Badr K, Ahmed ZS, El-Gamal M. Assessment of antimicrobial activity of whey protein films incorporated with biocide plant-based essential oils. J Appl Sci Res. 2013;9(4):2811-2818.
17. Tatlisu NB, Yilmaz MT, Arici M. Fabrication and characterization of thymol-loaded nanofiber mats as a novel antimould surface material for coating cheese surface. Food Packag Shelf Life. 2019;21:100347.
18. Azevedo VM, Dias MV, de Siqueira Elias HH, Fukushima KL, Silva EK, Carneiro JD, et al. Effect of whey protein isolate films incorporated with montmorillonite and citric acid on the preservation of fresh-cut apples. Food Res Int. 2018; 107:306-313.
19. Cordeiro BF, Oliveira ER, Da Silva SH, Savassi BM, Acurcio LB, Lemos L, et al. Whey protein isolate-supplemented beverage, fermented by Lactobacillus casei BL23 and Propionibacterium freudenreichii 138, in the prevention of mucositis in mice. Front Microbiol. 2018;9:2035.
20. Duman D, Karadag A. Inulin added electrospun composite nanofibres by electrospinning for the encapsulation of probiotics: characterisation and assessment of viability during storage and simulated gastrointestinal digestion. Int J of Food Sci Technol. 2021;56(2):927-935.
21. Wang WB, Clapper JC. Antibacterial Activity of Electrospun Polyacrylonitrile Copper Nanoparticle Nanofibers on Antibiotic Resistant Pathogens and Methicillin Resistant Staphylococcus aureus (MRSA). Nanomaterials. 2022; 12(13):2139.
22. Colin-Orozco J, Zapata-Torres M, Rodriguez-Gattorno G, Pedroza-Islas R. Properties of poly (ethylene oxide)/whey protein isolate nanofibers prepared by electrospinning. Food Biophys. 2015;10(2):134-144.
23. Sharma P, Trivedi N, Gat Y. Development of functional fermented whey–oat-based product using probiotic bacteria. 3 Biotech. 2017;7(4):1-8.
24. Lugo A. C. V. Understanding the Role of Poly (ethylene oxide) in the Electrospinning of Whey Protein Isolate Fibers (Doctoral dissertation, University of Guelph). 2012.
25. Diep E, Schiffman JD. Encapsulating bacteria in alginate-based electrospun nanofibers. Biomater Sci. 2021;9(12): 4364-4373.
26. Škrlec K, Zupančič Š, Mihevc SP, Kocbek P, Kristl J, Berlec A. Development of electrospun nanofibers that enable high loading and long-term viability of probiotics. Eur J Pharm Biopharm. 2019;136:108-119.
27. Keramat M, Esteghlal S, Safari J, Golmakani MT, Khalesi M. Fabrication of electrospun persian Gum/Poly (Vinyl Alcohol) and whey protein Isolate/Poly (vinyl alcohol) nanofibers incorporated with Oliveria decumbens Vent. essential oil. Nanosci Nanotechnol. 2019;9(3):371-380.
28. Vega-Lugo AC, Lim LT. Effects of poly (ethylene oxide) and pH on the electrospinning of whey protein isolate. J Polym Sci B Polym Phys. 2012;50(16):1188-1197.
29. Ceylan Z, Meral R, Karakaş CY, Dertli E, Yilmaz MT. A novel strategy for probiotic bacteria: Ensuring microbial stability of fish fillets using characterized probiotic bacteria-loaded nanofibers. Innov Food Sci Emerg Technol. 2018;48:212-218.
30. Yilmaz MT, Taylan O, Karakas CY, Dertli E. An alternative way to encapsulate probiotics within electrospun alginate nanofibers as monitored under simulated gastrointestinal conditions and in kefir. Carbohydr Polym. 2020;244: 116447.
31. Dissanayake M, Vasiljevic T. Functional properties of whey proteins affected by heat treatment and hydrodynamic high-pressure shearing. J Dairy Sci. 2009;92(4):1387-1397.
32. Patel MT, Kilara A, Huffman LM, Hewitt SA, Houlihan AV. Studies on whey protein concentrates. 1. Compositional and thermal properties. J Dairy Sci. 1990;73(6):1439-1449.
33. Ucpinar Durmaz B, Aytac A. Effects of Polyol-Based Plasticizer Types and Concentration on the Properties of Polyvinyl Alcohol and Casein Blend Films. J Polym Environ. 2021;29(1):313-322.
34. Sun Y, Liu Z, Zhang L, Wang X, Li L. Effects of plasticizer type and concentration on rheological, physico-mechanical and structural properties of chitosan/zein film. Int J Biol Macromol. 2020;143:334-340.
35. Gialamas H, Zinoviadou KG, Biliaderis CG, Koutsoumanis KP. Development of a novel bioactive packaging based on the incorporation of Lactobacillus sakei into sodium-caseinate films for controlling Listeria monocytogenes in foods. Food Res Int. 2010;43(10):2402-2408.
36. Ghalehjooghi HD, Tajik H, Shahbazi Y. Development and characterization of active packaging nanofiber mats based on gelatinsodium alginate containing probiotic microorganisms to improve the shelf-life and safety quality of silver carp fillets. Int J Food Microbiol. 2023;384: 109984.
37. Kanmani P, Lim ST. Development and characterization of novel probiotic-residing pullulan/starch edible films. Food

chem. 2013;141(2):1041-1049.
38. Oliveira-Alcântara AV, Abreu AA, Gonçalves C, Fuciños P, Cerqueira MA, Gama FM, et al. Bacterial cellulose/cashew gum films as probiotic carriers. LWT-Food Sci Technol. 2020;130:109699.
39. Kurt A, Toker OS, Tornuk F. Effect of xanthan and locust bean gum synergistic interaction on characteristics of biodegradable edible film. Int J Biol Macromol. 2017;102:1035-1044.
40. Leuangsukrerk M, Phupoksakul T, Tananuwong K, Borompichaichartkul C, Janjarasskul T. Properties of konjac glucomannan–whey protein isolate blend films. LWT-Food Sci Technol. 2014;59(1):94-100.
41. Marvdashti LM, Koocheki A, Yavarmanesh M. Alyssum homolocarpum seed gum-polyvinyl alcohol biodegradable composite film: Physicochemical, mechanical, thermal and barrier properties. Carbohydr Polym. 2017;155:280-293.
42. Durmaz BU, Aytac A. Development and characterization of poly (vinyl alcohol) and casein blend films. Polym Int. 2019; 68(6):1140-1145.
43. Aloui H, Baraket K, Sendon R, Silva AS, Khwaldia K. Development and characterization of novel composite glycerol-plasticized films based on sodium caseinate and lipid fraction of tomato pomace by-product. Int J Biol Macromol. 2019; 139:128-138.
44. Fung WY, Yuen KH, Liong MT. Agrowaste-based nanofibers as a probiotic encapsulant: fabrication and characterization. J Agric Food Chem. 2011; 59(15):8140-8147.
45. Hu D, Wang L. Physical and antibacterial properties of polyvinyl alcohol films reinforced with quaternized cellulose. J Appl Polym Sci. 2016;133(25).
46. Chollakup R, Pongburoos S, Boonsong W, Khanoonkon N, Kongsin K, Sothornvit R, Sukyai P, et al. Antioxidant and antibacterial activities of cassava starch and whey protein blend films containing rambutan peel extract and cinnamon oil for active packaging. LWT-Food Sci Technol. 2020;130:109573.
47. Wahbi W, Siam R, Kegere J, El-Mehalmey WA, Mamdouh W. Novel inulin electrospun composite nanofibers: prebiotic and antibacterial activities. ACS omega. 2020; 5(6):3006-3015.
48. Gibson GR, Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J Appl Bacteriol. 1994;77(4):412-420.
49. Lim H. J., Shin H. S. Antimicrobial and immunomodulatory effects of Bifidobacterium strains: A review. J Microbiol Biotechnol. 2020;1793-1800.