Nanofiber encapsulation of probiotic cultures via electrospinning: fabrication and quality compliance with ISO/IEC 17043 and ISO 22117 standards

Ahmet KOLUMAN[1,a], Çiğdem AKDUMAN[2,b], Mahmed Sari NJJAR[1,c], Meltem DELİMANLAR[1,d,✉], Ulviye ADAMCI[3,e], Mehmet Kıvanç ALAY[3,f], Mustafa SOYLU[3,g]

[1]Pamukkale University, Faculty of Technology, Department of Biomedical Engineering, Denizli, Türkiye; [2]Pamukkale University, Denizli Vocational School of Technical Sciences, Department of Textile Technology, Denizli, Türkiye; [3]VERITAS Textile, Denizli, Türkiye

[a]ORCID: 0000-0001-5308-8884; [b]ORCID: 0000-0002-6379-6697; [c]ORCID: 0000-0003-2494-1086; [d]ORCID: 0000-0003-4152-7805; [e]ORCID: 0000-0001-6311-5284; [f]ORCID: 0009-0005-8033-9905; [g]ORCID: 0009-0005-5701-263X.

ARTICLE INFO

Article History
Received : 08.06.2024
Accepted : 20.03.2025
DOI: 10.33988/auvfd.1481639

Keywords
Electrospinning
Encapsulation
Nanofibers
Probiotic microorganisms

✉Corresponding author
delimanlarmeltem@gmail.com

How to cite this article: Koluman A, Akduman Ç, Njjar MS, Delimanlar M, Adamcı U, Alay MK, Soylu M (2025): Nanofiber encapsulation of probiotic cultures via electrospinning: fabrication and quality compliance with ISO/IEC 17043 and ISO 22117 standards. Ankara Univ Vet Fak Derg, 72 (3), 323-333. DOI: 10.33988/auvfd.1481639.

ABSTRACT

Probiotics offer numerous health benefits, including inhibiting pathogenic growth, supporting intestinal microbiota, and synthesizing essential biomolecules. However, their viability during storage remains a challenge due to sensitivity to environmental conditions. This study investigates the encapsulation of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* in polyvinyl alcohol (PVA) nanofibers via electrospinning to enhance stability and viability. Near-optimized electrospinning parameters, including solution concentration, voltage, and collector distance, were used to produce nanofibers, which were characterized using Field Emission Scanning Electron Microscopy (FESEM). The results showed non-uniform fiber diameter distributions, with 16 kV producing thicker fibers with an average diameter of 479.11 nm. Homogeneity assessment confirmed uniform probiotic distribution within the nanofibers, with a coefficient of variation of 5.3%. Storage stability tests at 4°C over 15 days were conducted following ISO/IEC 17043 and ISO 22117 standards. The findings demonstrated that encapsulation effectively preserved *L. rhamnosus* viability in 16LR/PVA nanofibers, whereas *L. acidophilus* exhibited reduced viability at both 10 kV and 16 kV.

Introduction

The term "probiotic" originates from Greek, meaning 'for life'. Probiotics, tasked with functions such as preventing the development of pathogenic species, maintaining intestinal flora, improving bowel movements, facilitating mineral absorption, and synthesizing vitamins and antimicrobial substances, positively impact human health when consumed in sufficient quantities (10). Due to the well-known effects of probiotics, there has been an increased demand for both probiotic medications and probiotic foods in recent times. According to a report by the Food and Agriculture Organization, probiotics are defined as living microorganisms that positively influence the health of their host when consumed in sufficient quantities (20). The majority of probiotics belong to the *Lactobacillus* and *Bifidobacterium* genera. However, besides these genera, some cocci, non-lactic acid bacteria, yeasts (*Saccharomyces cerevisiae*, *Saccharomyces boulardii*), and certain other species (EcN, *Sporolactobacillus* spp.) have been observed to exhibit probiotic properties (26). The primary mechanisms that contribute to the positive impact of probiotics on health include the production of inhibitory metabolites such as organic acids, hydrogen peroxide, and bacteriocins, as well as their ability to colonize adhesive regions in the intestines, contribute to pathogen inhibition through

nutrient competition, suppress toxin production, and lower intestinal pH (34). In tissue engineering, various studies have investigated the potential use of probiotics, reporting benefits such as wound healing, protection against ultraviolet radiation, and enhanced innate immunity through topical or systemic applications (10, 31). Wound healing involves processes such as homeostasis, inflammation, proliferation, and tissue remodeling. In the initial stage of homeostasis, platelets are activated, and growth factors, cytokines, and substances present in platelets are released (49). These molecules activate mechanisms such as chemotaxis, cell proliferation, angiogenesis, extracellular matrix accumulation, and tissue remodeling (8). Probiotics are suggested to be effective in wound healing by influencing these mechanisms (49).

For probiotics to be effective, they must remain in sufficient numbers throughout their shelf life and survive harsh environmental conditions to reach the target area. However, since these microorganisms are highly sensitive to their surroundings, they need greater resistance to stay alive. Their ability to maintain metabolic activity is essential for supporting host health. However, many of these microorganisms are sensitive to various environmental factors, including the presence of oxygen, acidity, process temperature, storage temperature, and product processing conditions, all of which can constrain their viability (45). Given that these microorganisms are affected by both process and storage conditions as well as environmental factors, the initial inoculum becomes crucial for realizing the benefits of these microorganisms on the host. Considering the sensitivity of probiotics to environmental conditions and processing parameters during the production of probiotic products, various encapsulation methods have been developed to enable these microorganisms to maintain their metabolic activities and viability for longer durations (49).

In recent years, encapsulation has proven to be a promising method for preserving bacterial cells (15). Various studies have reported different techniques for probiotic encapsulation, such as extrusion, emulsification, and spray drying (4, 16). However, most of these methods involve the use of high temperatures or organic substances that can cause significant cell death in probiotic cells (19). The electrospinning method can be used as an alternative and suitable encapsulation technique for delicate foods and bioactive compounds, thanks to its ability not to damage active agents (22).

The electrospinning method is a simple nanofiber production technique that allows encapsulation both in capsule and fiber forms at the submicron and nanometer scales. It consists of a high-voltage power source, a collector, a syringe pump, and a needle used as a nozzle (37). The electrospinning system has advantages over both traditional encapsulation methods and other nanofiber processes (36), including temperature adaptability, the generation of products with a large surface area, a high surface-to-volume ratio, the use of a wide range of polymers and solutions with various properties, simplicity, cost-effectiveness, and the ability to scale up for industrial production (42). Additionally, the electrospinning method allows the production of materials with desired characteristics and dimensions (micro, submicron, nano) by adjusting polymer properties, system parameters, and environmental conditions (9). To preserve the viability of probiotics, the materials used in the electrospinning method are limited to those that can be successfully drawn in either a water medium or a solution with mild acidity, such as an acetic acid solution. The encapsulation of probiotics is achieved by using a combination of synthetic polymers or synthetic/biopolymers. Many biocompatible synthetic polymers can be directly drawn into ultrafine fibrous matrices. Among these, PVA, polyethylene oxide (PEO), and polyvinylpyrrolidone (PVP) polymers are commonly used. PVA is a hydrophilic polymer with a semi-crystalline structure that is known as Generally Recognized as Safe (GRAS), showing no toxic properties and possessing high thermal and chemical stability (25). Due to its high biocompatibility and cost-effectiveness, PVA is commonly utilized in the electrospinning system (33). Its water-soluble nature facilitates the easy recovery of cells, making it suitable for the encapsulation of probiotics using the electrospinning method (24).

In a study by Amna et al. (3), it was found that *Lactobacillus gasseri* encapsulated within PVA nanofibers remained viable *in vitro* for several months. In another study by Han et al. (27), *E. coli* cells were encapsulated with PEO, glycerol, and dextran using the electrospinning method. The resulting encapsulated fibers were observed to maintain the viability of cells at room temperature for a longer duration compared to free cells. *Lactobacillus paragasseri* K7 was paired with sodium alginate (NaAlg) and PEO polymers, employing a structured electrospinning method with a high electric field and a smooth nozzle (46).

In another study, Mojaveri et al. (38) produced chitosan/PVA nanofibers loaded with inulin-carrying *B. lactis* BB-12 as a probiotic. When compared to pure PVA nanofibers, the hybrid chitosan/PVA nanofibers were noted to provide better protection through intermolecular hydrogen bonding between chitosan and PVA molecules for encapsulated bacteria.

These studies demonstrate that the electrospinning method can be utilized as a tool for producing polymeric fibers containing probiotic bacteria. By using different polymers and electrospinning methods for each bacterial species, the resulting fibers were aimed to have distinct properties (21).

DOI: 10.33988/auvfd.1481639

Ensuring the shelf life and viability of probiotics is crucial for harnessing their health benefits effectively. Probiotics, which are live microorganisms, are delicate and can easily degrade if not stored properly. Factors like temperature, humidity, packaging, and the specific strains of probiotics all play pivotal roles in determining their longevity and effectiveness (48). Refrigeration is often recommended, especially for strains like *Lactobacillus* and *Bifidobacterium*, as they tend to thrive at lower temperatures and can lose potency at room temperature (39). Moreover, the type of packaging used also matters; vacuum-sealed or blister-packed probiotics tend to have longer shelf lives due to reduced exposure to moisture and oxygen. Encapsulation methods, such as electrospinning, provide an additional layer of protection during processing and storage, shielding probiotics from environmental stressors. It's worth noting that the shelf life of probiotics can vary widely, ranging from 1 to 4 years, depending on factors like formulation, storage conditions, and strain specificity (12). By adhering to recommended storage guidelines and choosing high-quality probiotic supplements, consumers can ensure that they receive the maximum health benefits from these beneficial microorganisms (50).

ISO/IEC 17043 (29) is an international standard that outlines the general requirements for the competence of proficiency testing (PT) providers. PT is a critical component of laboratory quality assurance. It is a way of evaluating the performance of laboratories by comparing their results with the results of other laboratories using the same method (29). PT is used to evaluate laboratory proficiency, confirm measurement precision, and pinpoint areas in need of development (5). ISO 22117 (28) expands on the principles of ISO/IEC 17043, concentrating specifically on microbiological testing in food products (28). ISO 22117 covers the preparation and distribution of microbiological samples, emphasizes safety measures, and incorporates specialized statistical methods to ensure the accurate detection and quantification of microorganisms (28).

This study aimed to utilize the electrospinning process for encapsulating *L. acidophilus* and *L. rhamnosus* while examining their response to the process. Validation experiments were conducted in accordance with ISO/IEC 17043 and ISO 22117 standards, specifically focusing on stabilization and homogenization. Production conditions were investigated for *L. rhamnosus and L. acidophilus*, followed by an examination of their viability after encapsulation. Within this scope, the focus is on preserving the viability of probiotics, achieving homogeneous distribution within nanofibers, and investigating the parameters of the directed electrospinning process.

Materials and Methods

Polyvinyl alcohol (PVA) (125,000 MW, 99% hydrolyzed) used in the production of nanofibers was purchased from Sigma-Aldrich Co. (St. Louis, U.S.A.). Distilled water was employed to prepare PVA solutions. For probiotic preparation, *L. rhamnosus* ATCC 7469 and *L. acidophilus* ATCC 4356, Trypticase Soy Broth (TSB) (Oxoid, England), and De Man–Rogosa–Sharpe (MRS) agar (Merck, Darmstadt, Germany) were used.

Preparation of Probiotics: *L. acidophilus* and *L. rhamnosus* strains were used as reference cultures. The strains were transferred into TSB and incubated at 37ºC for 18 hours. After incubation, the cultures were streaked onto MRS agar. Colonies were examined by further incubation at 30ºC for 24 hours to assess colony morphology. The choice of 30°C for further incubation is ideal for lactic acid bacteria, as it closely matches their natural habitat (e.g., fermentation processes). This temperature can enhance the growth and activity of these specific organisms compared to higher temperatures. The relevant ISO standard for incubation at 30 °C is ISO 4833-1 (30). This standard specifies a horizontal method for the enumeration of microorganisms that can grow and form colonies in a solid medium after aerobic incubation at 30 °C. It applies to various products intended for human consumption, animal feed, and environmental samples related to food production and handling. The incubation period specified in this standard is typically 72 hours under aerobic conditions at 30 °C. Subsequently, a single colony was picked, retransferred into TSB, and the enrichment process was repeated. A 5 mL aliquot was taken from the obtained enrichment, centrifuged at 3000 rpm to discard the supernatant, and this step was repeated until a bacterial pellet formed at the bottom. The resulting pellets were diluted with physiological saline solution (0.9% NaCl) until reaching a 0.5 McFarland turbidity. This process rendered the obtained probiotics suitable for the electrospinning process.

Preparation of Electrospinning Solutions: A 15% (w/w) PVA solution was prepared by dissolving 1.5 g of PVA in 8.5 g of distilled water with gentle stirring using a magnetic stirrer at 100°C until fully dissolved, followed by cooling to room temperature under sterile conditions. PVA solutions have been successfully prepared using similar procedures (2, 41). To each PVA solution, 5 mL of *L. rhamnosus* suspension was added, resulting in an *L. rhamnosus*/PVA solution with a final PVA concentration of 10% (w/w). The concentrations used in this study were based on those described by Nagy et al. (40), who applied similar concentrations in their work. The solution was stirred for one hour under the same sterile conditions. Sterility was assessed by measuring total bacterial counts

and conducting swab sampling in the production area, where no bacterial growth was detected. The same procedure was repeated for *L. acidophilus*.

Fabrication of Probiotic Nanofibers: Based on previous studies (23, 33, 40, 43, 47, 50), the parameters for the electrospinning process were set as follows: a distance of 15 cm between the collector and the needle, applied voltages ranging between 10 and 16 kV, and solution feeding to the system at a rate of 0.8 ml/h using a syringe pump. The experiments were performed at room temperature (25°C) in a fume hood. The electrospinning was carried out using a Nanoliz electrospinning device (Nanoliz, Ankara, Türkiye). Throughout the electrospinning process, nanofibers produced through the application of high voltage to the solutions were gathered on a rotating cylindrical collector covered with sterile aluminum foil. The nanofibers obtained at 10 kV were labeled as 10LA/PVA and 10LR/PVA, while those obtained at 16 kV were labeled as 16LA/PVA and 16LR/PVA. The prepared samples are summarized in Table 1. The samples obtained were stored in a sterile box for further analysis.

Field Emission Scanning Electron Microscopy (FESEM): The morphology of the samples was investigated using a FESEM. Each sample was coated with a thin layer of gold-palladium to provide conductivity (Quorum Q150R). All FESEM images were captured using a SUPRA 40VP microscope (Carl Zeiss, Germany). The magnification used for imaging was 5000X and 10000X. The average fiber diameters of the nanofibers were calculated from these images using the ImageJ program.

Validation and Verification of Probiotic Distribution and Storage Stability:

Homogeneity Assessment of Probiotic Distribution: A homogenization process was conducted to ensure uniform distribution of probiotics within the nanofiber matrices. Following electrospinning, the collected nanofibers were manually separated into smaller, randomized sections and thoroughly mixed to form a composite batch. Representative samples were randomly selected from this batch for further analysis. Homogeneity was assessed by analyzing ten randomly chosen sub-samples from different regions (central, peripheral, and intermediate) of the batch. Probiotic viability within these sub-samples was determined using the plating procedure under standardized conditions to minimize variability. The bacterial counts (CFU/g) were recorded, and the data were subjected to statistical analysis to evaluate distribution uniformity. The coefficient of variation (CV) was calculated using the following formula:

$$CV = \frac{\sigma}{\mu} \times 100 \quad (1)$$

where σ denotes the standard deviation of bacterial counts, whereas μ represents the mean bacterial count across all sub-samples.

Storage Stability and Viability Assessment: The viability of *L. acidophilus* and *L. rhamnosus* in the nanofiber matrices was evaluated under refrigerated storage conditions (4°C) over 15 days. Sampling was performed on days 0, 5, 11, and 15 to monitor bacterial stability. The validation of these experiments followed ISO/IEC 17043 (29) standards, with stabilization and homogenization procedures based on ISO 22117 (28) guidelines. Prior to validation, initial methodological procedures were implemented, referencing standard microbiological protocols to ensure reproducibility and reliability (13). To determine cell viability, a standardized plating procedure was conducted under controlled biosafety conditions within a biosafety cabinet. One gram of the nanofiber sample was accurately weighed and diluted in 9 milliliters of maximum recovery diluent (MRD), serving as the primary dilution for serial dilutions. The serial dilution factor (DF) was determined using the following formula:

$$\mathrm{DF} = \frac{V_{total}}{\mathrm{V_{sample}}} \quad (2)$$

V_{total} represents the total diluted volume, while V_{sample} denotes the initial sample volume. This dilution ensured that bacterial counts were within the quantifiable range. Precise aliquots (100 microliters) from each dilution were plated onto plate count agar using a Drigalski spatula to ensure uniform distribution. The plates were incubated in a controlled chamber at 30°C for 24 hours. If no microbial growth was observed within this

Table 1. Encapsulated probiotics and fabrication parameters.

Sample Coding	Probiotic	Voltage (kV)	Flow Rate (mL/h)	Distance (cm)
10LA/PVA	*L. acidophilus*	10	0.8	15
10LR/PVA	*L. rhamnosus*	10	0.8	15
16LA/PVA	*L. acidophilus*	16	0.8	15
16LR/PVA	*L. rhamnosus*	16	0.8	15

DOI: 10.33988/auvfd.1481639

period, an additional 12-hour incubation was performed to enhance detection. The bacterial count was determined using the formula:

$$\frac{CFU}{g} = \frac{N \times DF}{V_p} \quad (3)$$

Where N denotes the counted colony number, DF represents the dilution factor and V_p corresponds to the plated sample volume.

Results

FESEM Analysis Results: The morphology and diameters of electrospun fibers were investigated by field scanning electron microscopy. The diameters of 100 randomly selected fibers from the FESEM images of probiotic/PVA nanofibers were measured using ImageJ software. Subsequently, the average diameter of the fibers was calculated. This process was applied to all samples. The minimum, maximum, and average diameters of the measured nanofibers are presented in Table 2. The average fiber diameter of 10LA/PVA was 271.04 nm, while the average fiber diameters of 10LR/PVA and 16LR/PVA were 232.16 nm and 479.11 nm, respectively.

Figure 2 shows FESEM images and histogram profile of nanofiber samples. All samples displayed non-uniform diameter distributions. The histogram in Figure 2a illustrates a diameter distribution for 10LA/PVA, centered around 270 nm. For 10LR/PVA, a broader distribution was observed, with notable peaks at approximately 215 nm and 275 nm (Figure 2b). The fiber diameters of 16LR/PVA (Figure 2c) ranged from 228 nm to 800 nm, with the majority falling between 400 nm and 500 nm.

The morphology of *L. acidophilus* and *L. rhamnosus* and their arrangement in electrospun fibers were investigated by scanning electron microscopy. Figure 3 shows the formed fibers for the 16LR/PVA sample in different magnifications. The polymer fibers got thicker by encompassing rod-shaped single or interconnected bacteria.

Figure 4 shows the size of *L. acidophilus* and *L. rhamnosus* embedded in PVA nanofibers.

The FESEM images showed *that L. acidophilus* had a length of 2.35 µm and a width of 0.87 µm, while *L. rhamnosus* had a length ranging between 2.74 and 3.97 µm and a width ranging from 0.74 to 1.79 µm.

Validation and Verification:

***Homogeneity Assessment of Probiotic Distribution*:** Quantitative analysis indicated minimal variance in probiotic counts across the 16LR/PVA sub-samples, confirming a homogeneous distribution. CV for the bacterial counts was calculated at 5.3%, well within the acceptable threshold outlined in ISO/IEC 17043 (29) standards (<7%). Table 3 summarizes the probiotic counts for the tested sub-samples.

Table 3. Probiotic counts for the tested sub-samples

Sample ID	log 10 (CFU/g Mean)
Sample 1	6.494
Sample 2	6.489
Sample 3	6.491
Sample 4	6.497
Sample 5	6.490
Sample 6	6.493
Sample 7	6.496
Sample 8	6.487
Sample 9	6.491
Sample 10	6.493
Median	6.492
Standard Deviation	0.281

Storage Stability and Viability Assessment: The viability of *L. rhamnosus* in 16LR/PVA nanofibers produced at 16 kV was preserved from the 5th day onward. However, the electrospinning process at 10 kV had a negative impact on its viability. Similarly, the electrospinning process negatively affected the viability of *L. acidophilus* at both 10 kV and 16 kV. Figure 1 illustrates the stabilization of the 16LR/PVA nanofiber sample.

Table 2. Minimum, maximum, average diameters, and standard deviation of probiotic nanofibers.

Sample	Minimum Diameter (nm)	Maximum Diameter (nm)	Average Diameter (nm)	Standard Deviation
10LA/PVA	132.01	466.44	271.04	54.64
10LR/PVA	119.24	371.56	232.16	50.22
16LR/PVA	227.99	799.93	479.11	127.77

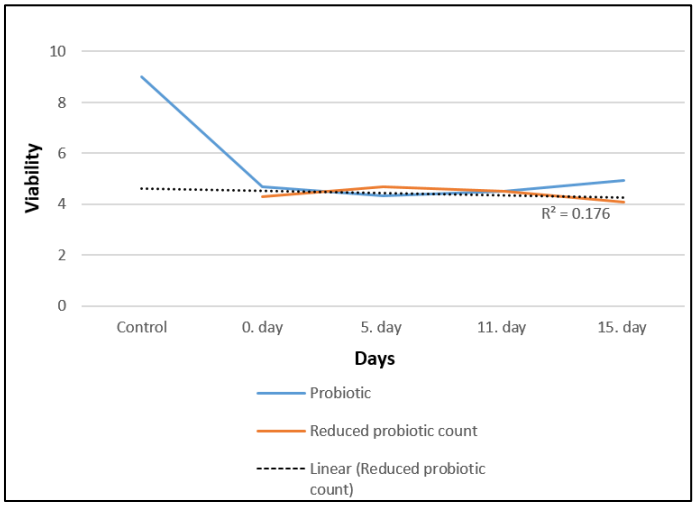

Figure 1. Stabilization of the 16LR/PVA nanofiber sample over 15 days.

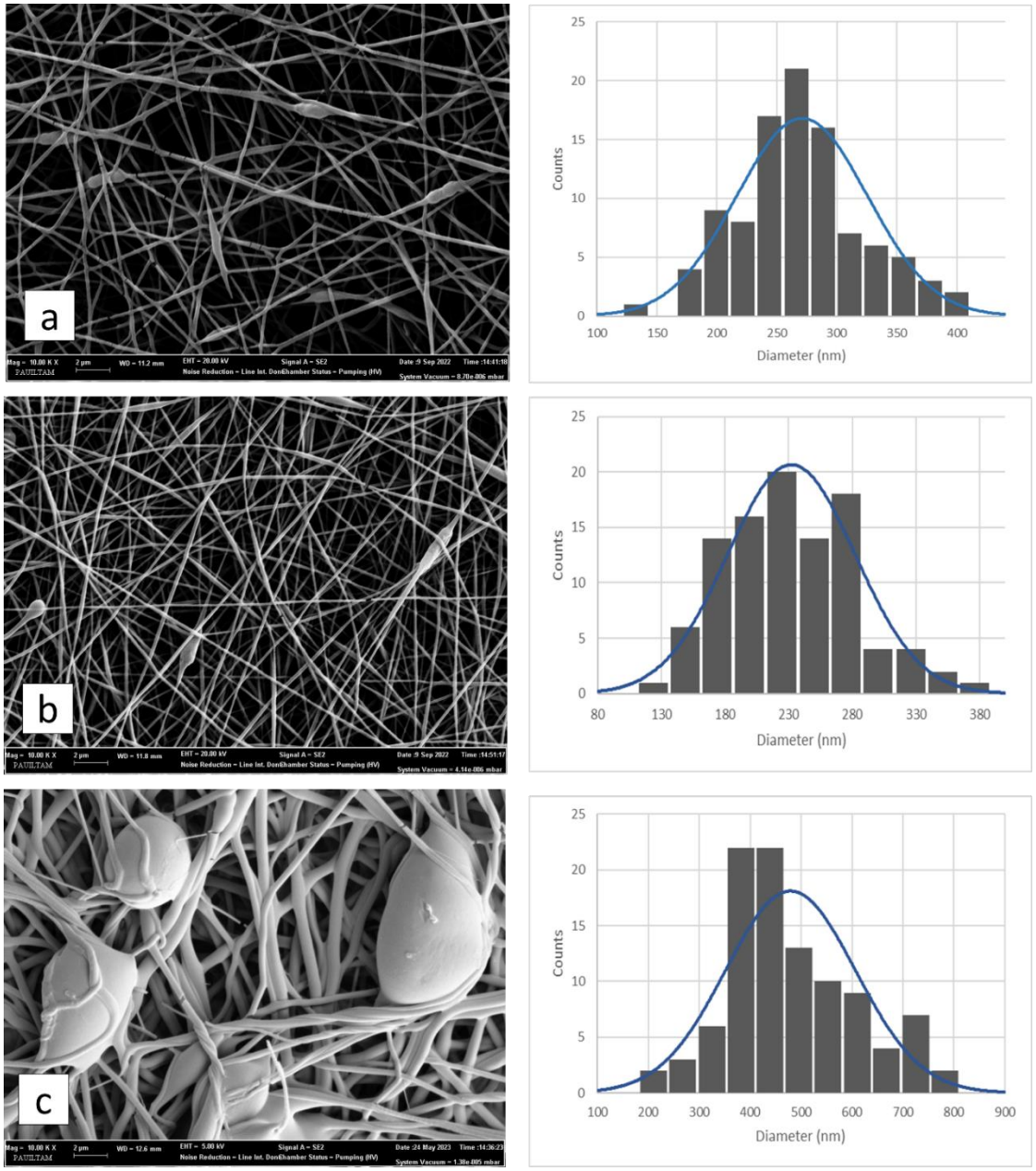

Figure 2. FESEM images 10000X magnification and histogram profile of nanofibers: a) 10LA/PVA, b) 10LR/PVA, c) 16LR/PVA.

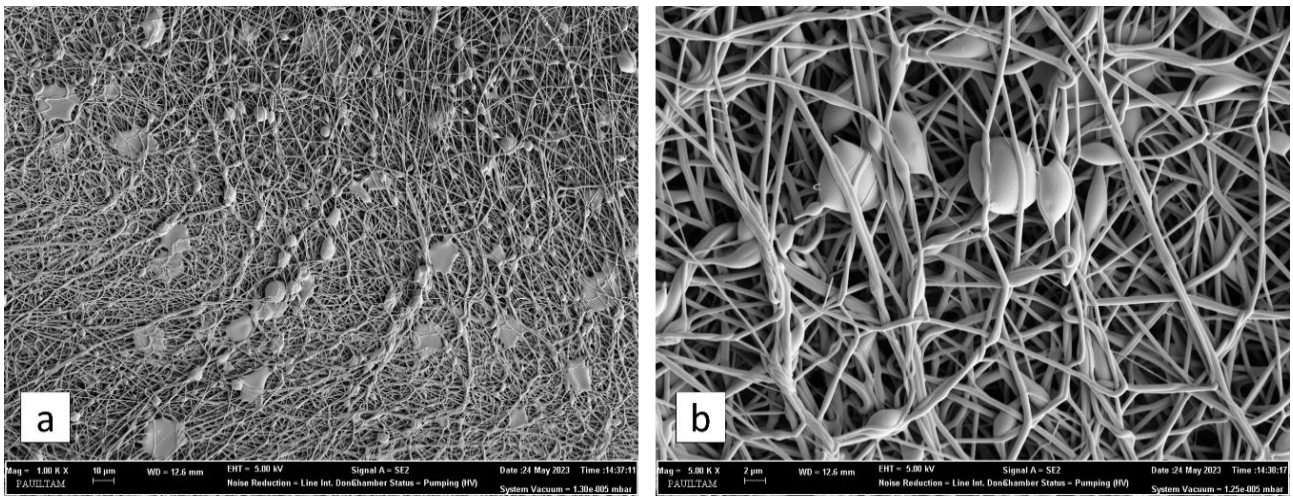

Figure 3. FESEM images of 16LR/PVA nanofibers at different magnifications. a) 1000X b) 5000X

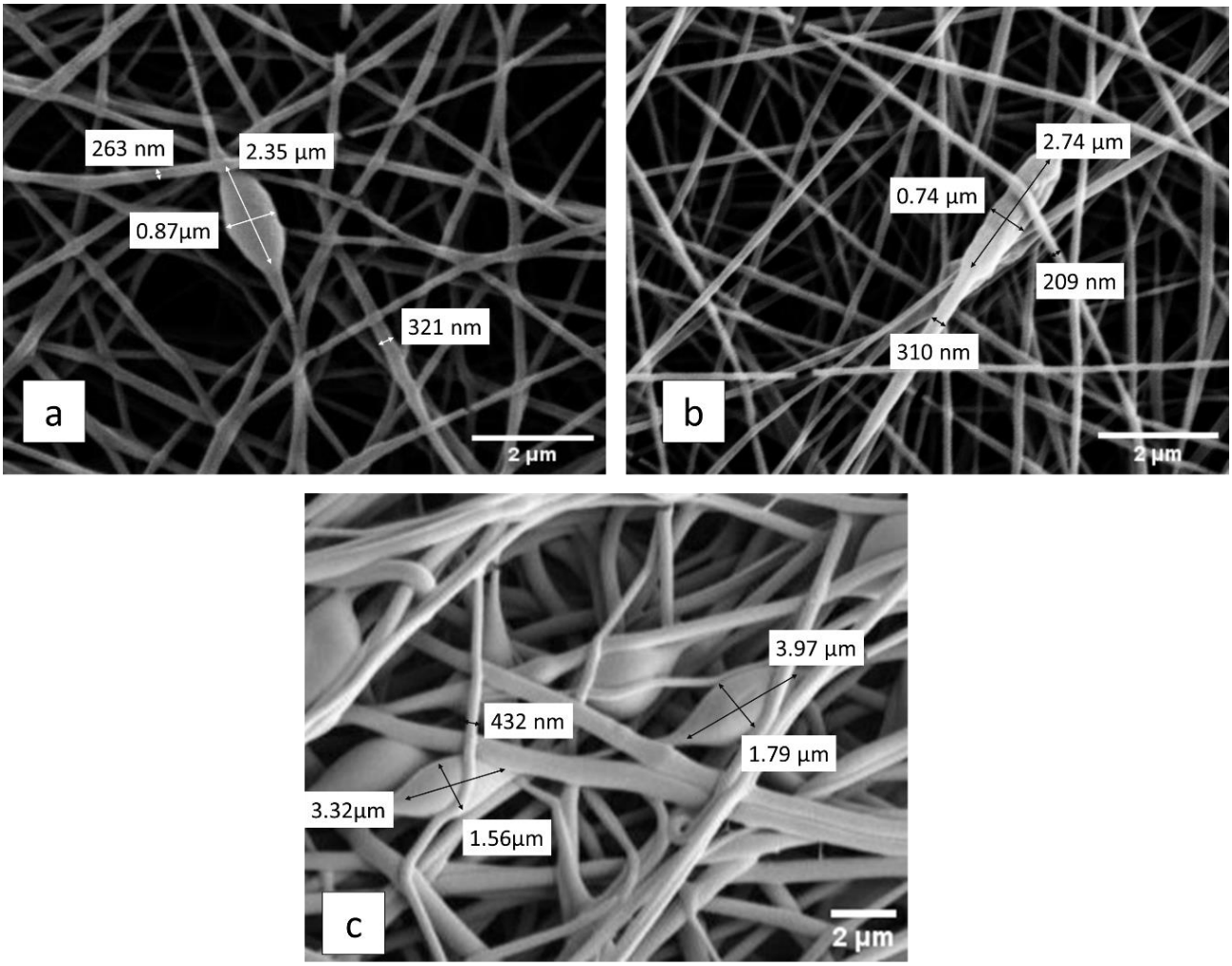

Figure 4. FESEM image of *L. acidophilus* and *L. rhamnosus* embedded in PVA nanofibers with thickness data: a) 10LA/PVA, b) 10LR/PVA, c) 16LR/PVA.

DOI: 10.33988/auvfd.1481639

Discussion and Conclusion

Considering the literature reviews, PVA polymer is generally recognized as safe (GRAS), showing non-toxic properties, high thermal and chemical stability, and is a semi-crystalline, hydrophilic polymer (14). Due to its high biocompatibility and low cost, it is frequently used in electrospinning systems. Therefore, PVA polymer was preferred for the production of nanofibers containing probiotics in this study.

Previous research (17, 37, 51) indicates that developing practical applications for electrospun nanofibers necessitates a complete understanding of the electrospinning parameters, since the structural morphology and diameter of the electrospun nanofiber will have an impact on the finished product. There are numerous parameters that can potentially influence the electrospinning process. Therefore, examining all of them within a single study is almost impossible. However, certain parameters can be kept constant during experiments. To conduct the experiments under controlled environmental conditions, ambient factors such as temperature and humidity are maintained consistently (51). The electrospinning was carried out in a fume hood. The continuous ventilation provided constant humidity during the process owing to the same inlet air. The relative humidity was around 55%, and the temperature was 25°C.

At first the electrospinning process of polymers was optimized without bacteria, and the collected nanofibers were investigated by FESEM. Probiotics containing polymer solutions were prepared at the determined optimal concentration. Koski et al. (33) observed that low solution concentrations resulted in the electrospinning of beaded fibers. Therefore, it was hypothesized that a concentration range of 8% to 12% would promote the formation of stable, bead-free fibers with circular cross-sections. According to Rwei and Huang (43), a 10% solution concentration is in the range that yields an acceptable electrospinning process. Similar results have been reported in the literature (1, 40). Thus, for this study, the polymer concentration was set as 10% (w/w).

Applied voltage exerts two significant effects on fiber diameter. Firstly, increasing the voltage enhances the electric field strength, leading to a greater electrostatic stretching force that accelerates the jet within the electric field, thereby promoting the formation of thinner fibers. Secondly, since charge transport in the electrospinning process is solely conducted by the polymer flow (50), an increase in voltage would result in more surface charges on the jet. This, in turn, increases the mass flow rate from the needle tip to the collector, causing the solution to be drawn more rapidly from the needle tip and potentially increasing the fiber diameter. The interplay of these two effects determines the final fiber diameter. Consequently, increasing the applied voltage may decrease, increase (7, 35), or have no effect (6, 32) on the fiber diameter.

The histogram (Figure 2a) indicated a non-uniform diameter distribution centered at approximately 270 nm for sample 10LA/PVA. As can be seen in Table 2, the average fiber diameter of 10LA/PVA was found to be 271.04 nm. 10LR/PVA displayed a broader range of data, with notably higher frequencies observed around 215 nm and 275 nm compared to other measurements (Figure 2b). The diameter distribution of 16LR/PVA was non-uniform (Figure 2c), with fiber diameters ranging from 228 nm to 800 nm. The majority of the nanofibers had diameters between 400 nm and 500 nm. As can be seen in Table 2, the average fiber diameters of 10LR/PVA and 16LR/PVA were found to be 232.16 and 479.11 nm, respectively. Even though there is no clear correlation, it may be concluded that the diameter of the fibers increased with the increase of the voltage. The increase in the diameter of the *L. rhamnosus* samples could be explained with the model proposed by Ziabari et al. (51). The change in fiber diameter as a function of voltage is dramatically influenced by spinning distance. At a short distance, the electric field is a high and dominant factor. Whereas, at long distances where the electric field is low, the effect of the mass of the solution would be a determining factor according to which fiber diameter increased with applied voltage.

The use of 5-20 cm for spinning distance was reported in the literature (22). Short distances are suitable for highly evaporative solvents, whereas it results in wet coagulated fibers for nonvolatile solvents due to insufficient evaporation time. Since water was used as a solvent for PVA in this study, short spinning distances were not expected to be favorable for dry fiber formation. Afterwards, this was proved by experimental observations, and 15 cm was considered as the effective spinning distance. This can also be supported by the results of Nagy et al. (40).

When investigating probiotic encapsulation, it is critical to take into account the complex impact of electrospinning parameters. To be more precise, the voltage and concentration have a substantial impact on the nanofibers' properties (17, 50). Nonetheless, there is remarkable variation in the impact of voltage on probiotic encapsulation amongst research investigations (18). According to some research findings, voltage may not always affect probiotics' encapsulation efficiency even while it has a discernible effect on the characteristics of nanofibers (31). This complex association calls for a closer look at the complex interactions between the particular behavior of the probiotics throughout the encapsulation process and the electrospinning parameters. The necessity of high voltage in electrospinning for nanofiber production juxtaposes concerns regarding its

potential deleterious effects on probiotics. Škrlec et al. (47) explored the impact of voltage on the viability of *L. plantarum* cells, revealing optimal viability at 15 kV (0.81 log reduction compared to theoretical loading). However, viability decreased with increased voltage (2.03 log reduction at 20 kV) or decreased voltage (1.30 log reduction at 10 kV). Additionally, electrospinning at 10 kV exhibited lower efficiency than at 15 kV or 20 kV, resulting in reduced fiber production rates. Conversely, Feng et al. (23) demonstrated that elevating the applied voltage from 10 to 16 kV did not significantly alter the viability of *L. plantarum* cells, with loaded cells maintaining high viability levels even under heightened voltage conditions.

In this study, *L. rhamnosus* maintained its viability at 16 kV, and a decreased viability was observed at 10 kV. The electrospinning process had a negative impact on *L. acidophilus* samples both at 10 and 16 kV. Additionally, electrospinning at 10 kV was less efficient than that at 16 kV and resulted in lower nanofiber production per unit time, similar to Škrlec et al.'s results (47). The primary concern for medical applications lies in the biological activity of probiotics within the nanofibers, which was assessed after their dissolution. The impact of the electrospinning process on bacterial viability was investigated over a 15-day period at 4°C. As can be seen in Figure 1, the probiotic viability of 16LR/PVA nanofibers obtained at 16 kV was preserved from day 5 onwards. The viability of *L. acidophilus* and *L. rhamnosus* was reduced from day 1 after the electrospinning process was carried out. The homogeneity test results confirm that the probiotic nanofiber batches produced via the described electrospinning process demonstrate a consistent distribution of probiotics. This ensures the reliability of further analyses and verifies compliance with ISO/IEC 17043 (28) standards.

The morphology of probiotics and their arrangement in electrospun fibers was also investigated by scanning electron microscopy. As can be seen in Figure 3, FESEM showed that the polymer fibers got thicker by encompassing rod-shaped single or interconnected bacteria. The polymer coating was formed around bacteria as a result of the electrospinning process. Similar findings have been reported by Nagy et al. (40) and Ceylan et al. (11), who observed an increase in fiber diameter associated with probiotic incorporation. The probiotics aligned along the nanofibers, consistent with the observations of Salalha et al. (44). Their findings revealed that bacteria, which were initially scattered randomly in a polymer solution, tend to position themselves within the Taylor cone during electrospinning, largely following the streamlines. This alignment remains consistent throughout the jet creation process, eventually becoming entrenched in the formed nanofibers.

Observations from the FESEM images indicate that *L. rhamnosus* in sample 16LR/PVA tends to aggregate, forming small clusters. The sizes of bacteria can be seen in Figure 4, which is similar to that of their original form (typical size of *L. acidophilus*: width ~ 0.6 - 0.9 µm, length: ~ 1.5-6.0 µm; typical size of *L. rhamnosus*: width ~ 0.8 - 1.0 µm, length: ~ 2.0 - 4.0 µm). The FESEM images showed that *L. acidophilus* had a length of 2.35 µm and a width of 0.87 µm (Figure 4a). While *L. rhamnosus* had a length ranging between 2.74 and 3.97 µm and a width ranging from 0.74 to 1.79 µm (Figures 4b, 4c).

In conclusion, this study explored the potential of electrospinning for encapsulating probiotic microorganisms, namely *L. rhamnosus* and *L. acidophilus*. The findings revealed a differential effect on probiotic viability. Electrospinning negatively impacted the viability of *L. acidophilus*, but *L. rhamnosus* encapsulated within 16LR/PVA nanofibers exhibited sustained viability from day 5 onwards. These results demonstrate the potential of electrospinning as a method for preserving the viability of certain probiotic strains. This approach holds promise for industrial-scale production of probiotics with enhanced stability and efficacy.

However, limitations were identified in the efficiency of electrospinning *L. acidophilus*. Further research is warranted to optimize the homogenization and encapsulation process parameters specifically for this probiotic strain. Additionally, broader investigations are needed to optimize parameters for encapsulating a wider range of probiotic species and maintaining their viability for extended periods. Future studies could explore the impact of different factors on the success of this technique:

Microorganism Selection*:* The selection of probiotic strains for encapsulation is crucial. This study focused on *L. rhamnosus* and *L. acidophilus*, but exploring strains with varying morphologies, stress tolerance, and surface properties could yield valuable insights. Strains exhibiting greater inherent robustness during electrospinning would be ideal candidates.

Polymer Selection: Polyvinyl alcohol (PVA) was used in this study, but other polymers with tailored properties for probiotic encapsulation should be investigated. Biocompatible and biodegradable polymers with controllable degradation rates could be explored.

Process Optimization: Optimizing homogenization and electrospinning parameters is critical. Future studies could explore variables such as needle size, flow rate, and the distance between the collector and the needle to improve encapsulation efficiency and minimize stress on the probiotics.

Storage Conditions: The long-term viability of encapsulated probiotics is directly affected by storage conditions. Future studies should investigate the impact of temperature, light exposure, and humidity on the encapsulated probiotics' viability and functionality.

By addressing these limitations and expanding the research scope, electrospinning can be established as a robust and versatile technique for the development of novel and effective probiotic delivery systems. Exploring the factors mentioned above can lead to the development of optimized protocols for encapsulating a diverse range of probiotic strains with enhanced viability and functionality. This holds the potential to revolutionize the production and delivery of probiotics for various applications, including functional foods, dietary supplements, and even targeted drug delivery systems.

Financial Support

This work was supported by the project numbered 2022HZDP011 by the scientific research project unit of Pamukkale University - Scientific Research Projects Coordinatorship.

Ethical Statement

Ethics committee approval is not required for this study.

Conflicts of Interest

The authors declared that there is no conflict of interest.

Author Contributions

All authors have contributed equally to all aspects of this study, including the conception, methodology, data analysis, and manuscript preparation.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available in the article.

References

1. **Agarwal S, Greiner A** (2011): *On the way to clean and safe electrospinning—green electrospinning: emulsion and suspension electrospinning.* Polym Adv Technol, **22**, 372–378.
2. **Akduman Ç, Morsümbül S, Kumbasar EPA** (2019): *The removal of reactive red 141 from wastewater: a study of dye adsorption capability of water-stable electrospun polyvinyl alcohol nanofibers.* Autex Research Journal, **21**, 20–31.
3. **Amna T, Hassan MS, Pandeya DR, et al** (2013): *Classy non-wovens based on animate l. Gasseri-inanimate poly(vinyl alcohol): upstream application in food engineering.* Appl Microbiol Biotechnol, **97**, 4523–4531.
4. **Anekella K, Orsat V** (2013): *Optimization of microencapsulation of probiotics in raspberry juice by spray drying.* LWT- Food Sci Technol, **50**, 17–24.
5. **Atkins P, Stainback L** (2022): *What does your proficiency testing (PT) prove? a look at inorganic analyses, proficiency tests, and contamination and error*. Spectroscopy, **Part I**, 9–14.
6. **Ayutsede J, Gandhi M, Sukigara S, et al** (2005): *Regeneration of Bombyx mori silk by electrospinning. Part 3: characterization of electrospun nonwoven mat.* Polymer, **46**, 1625–1634.
7. **Baker SC, Atkin N, Gunning PA, et al** (2006): *Characterisation of electrospun polystyrene scaffolds for three-dimensional in vitro biological studies.* Biomaterials, **27**, 3136–3146.
8. **Barrientos S, Stojadinović O, Golinko MS, et al** (2008): *Perspective Article: Growth factors and cytokines in wound healing.* Wound Rep Reg, **16**, 585–601.
9. **Bhushani JA, Anandharamakrishnan C** (2014): *Electrospinning and electrospraying techniques: potential food based applications.* Trends Food Sci Technol, **38**, 21–33.
10. **Caramia G, Atzei A, Fanos V** (2008): *Probiotics and the skin.* Clin Dermatol, **26**, 4–11.
11. **Ceylan Z, Meral R, Karakaş CY, et al** (2018): *A novel strategy for probiotic bacteria: ensuring microbial stability of fish fillets using characterized probiotic bacteria-loaded nanofibers.* Innov Food Sci Emerg Technol, **48**, 212–218.
12. **Champagne CP, Gardner NJ** (2008): *Effect of storage in a fruit drink on subsequent survival of probiotic lactobacilli to gastro-intestinal stresses.* Food Res Int, **41**, 539–543.
13. **Champagne CP, Ross RP, Saarela M** (2011): *Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices.* Int J Food Microbiol, **149**, 185–193.
14. **Choo K, Ching YC, Chuah CH, et al** (2016): *Preparation and characterization of polyvinyl alcohol-chitosan composite films reinforced with cellulose nanofiber.* Materials, **9**, 644.
15. **Coghetto CC, Brinques GB, Ayub MAZ** (2016): *Probiotics production and alternative encapsulation methodologies to improve their viabilities under adverse environmental conditions.* Int J Food Sci Nutr**, 67**, 929–943.
16. **De Mandal S, Hati S** (2016): Microencapsulation of bacterial cells by emulsion technique for probiotic application. 273-279. In: EC Opara (Ed), Cell Microencapsulation: Methods and Protocols. Humana Press, New York.
17. **Deitzel JM, Kleinmeyer JD, Harris D, et al** (2001): *The effect of processing variables on the morphology of electrospun nanofibers and textiles.* Polym, **42**, 261–272.
18. **Deng L, Zhang H** (2020): *Recent advances in probiotics encapsulation by electrospinning.* ES Food Agrofor, **2**, 3-12.
19. **Eratte D, McKnight S, Gengenbach TR, et al** (2015): *Co-encapsulation and characterisation of omega-3 fatty acids and probiotic bacteria in whey protein isolate–gum Arabic complex coacervates.* J Funct Foods, **19**, 882–892.
20. **FAO** (2006): Probiotics in food: Health and nutritional properties and guidelines for evaluation. Food and Agriculture Organization of the United Nations, Rome.
21. **Feng K, Huangfu L, Liu C, et al** (2023): *Electrospinning and electrospraying: emerging techniques for probiotic stabilization and application.* Polymer, **15**, 2402.

22. **Feng K, Wen P, Yang H, et al** (2017): *Enhancement of the antimicrobial activity of cinnamon essential oil-loaded electrospun nanofilm by the incorporation of lysozyme.* RSC Adv, **7**, 1572–1580.
23. **Feng K, Zhai M, Zhang Y, et al** (2018): *Improved viability and thermal stability of the probiotics encapsulated in a novel electrospun fiber mat.* J Agric Food Chem, **66**, 10890–10897.
24. **Fung W, Yuen K, Liong M** (2011): *Agrowaste-Based Nanofibers as a Probiotic Encapsulant: fabrication and characterization.* J Agric Food Chem, **59**, 8140–8147.
25. **Gaaz TS, Sulong AB, Akhtar MN, et al** (2015): *Properties and applications of polyvinyl alcohol, halloysite nanotubes and their nanocomposites.* Molecules, **20**, 22833–22847.
26. **Goktepe I, Juneja VK, Ahmedna M** (2005): Probiotics in food safety and human health. 1st edn. CRC Press, Boca Raton.
27. **Han J, Liang C, Cui Y, et al** (2018): *Encapsulating microorganisms inside electrospun microfibers as a living material enables room-temperature storage of microorganisms.* ACS Appl Mater Interfaces, **10**, 38799–38806.
28. **ISO 22117** (2017): ISO. Available at https://www.iso.org/standard/67052.html. (Accessed May 5, 2024).
29. **ISO/IEC 17043** (2023): ISO. Available at https://www.iso.org/standard/80864.html. (Accessed May 5, 2024).
30. **ISO 4833-1** (2013): ISO. Available at https://www.iso.org/standard/53728.html. (Accessed May 5, 2024).
31. **Khan MA, Hussain Z, Ali S, et al** (2019): *Fabrication of electrospun probiotic functionalized nanocomposite scaffolds for infection control and dermal burn healing in a mice model.* ACS Biomater Sci Eng, **5**, 6109-6116.
32. **Kidoaki S, Kwon IK, Matsuda T** (2005): *Mesoscopic spatial designs of nano- and microfiber meshes for tissue-engineering matrix and scaffold based on newly devised multilayering and mixing electrospinning techniques.* Biomaterials, **26**, 37–46.
33. **Koski A, Yim K, Shivkumar S** (2004): *Effect of molecular weight on fibrous PVA produced by electrospinning.* Mater Lett, **58**, 493–497.
34. **Kumar M, Mohania D, Poddar D, et al** (2009): *A probiotic fermented milk prepared by mixed culture reduces pathogen shedding and alleviates disease signs in rats challenged with pathogens.* Int J Probiotics Prebiotics, **4**, 211–218.
35. **Li Q, Jia Z, Yang Y, et al** (2008): *Preparation and properties of poly (vinyl alcohol) nanofibers by electrospinning.* J Polym Eng. **28**, 87-100.
36. **Liu H, Cui SW, Chen M, et al** (2019): *Protective approaches and mechanisms of microencapsulation to the survival of probiotic bacteria during processing, storage and gastrointestinal digestion: a review.* Crit Rev Food Sci Nutr, **59**, 2863–2878.
37. **Mitchell GR, Mohan SD, Davis FJ, et al** (2015): Electrospinning: Principles, Practice and Possibilities. The Royal Society of Chemistry, Cambridge.
38. **Mojaveri SJ, Hosseini SF, Gharsallaoui A** (2020): *Viability improvement of Bifidobacterium animalis Bb12 by encapsulation in chitosan/poly(vinyl alcohol) hybrid electrospun fiber mats.* Carbohydr Polym, **241**, 116278.
39. **Mortazavian AM, Ehsani MR, Mousavi M, et al** (2007): *Effect of refrigerated storage temperature on the viability of probiotic microorganisms in yogurt.* Int J Dairy Technol, **60**, 123–127.
40. **Nagy Z, Wagner I, Suhajda Á, et al** (2014): *Nanofibrous solid dosage form of living bacteria prepared by electrospinning.* Express Polym Lett, **8**, 352–361.
41. **Njjar MS, Akduman Ç, Koluman A** (2023): *Antibakteriyel, kanama durdurucu ve yaralanma tespit sistemi içeren askeri operasyon kıyafeti*. Savunma Bilim Derg, **2**, 424–453.
42. **Ricaurte L, Quintanilla-Carvajal MX** (2019): *Use of electrospinning technique to produce nanofibres for food industries: a perspective from regulations to characterisations.* Trends Food Sci Technol, **85**, 92–106.
43. **Rwei S, Huang C** (2012): *Electrospinning PVA solution-rheology and morphology analyses.* Fibers Polym, **13**, 44–50.
44. **Salalha W, Dror Y, Khalfin RL, et al** (2004): *Single-walled carbon nanotubes embedded in oriented polymeric nanofibers by electrospinning.* Langmuir, **20**, 9852–9855.
45. **Sanz Y** (2007): *Ecological and functional implications of the acid-adaptation ability of Bifidobacterium: a way of selecting improved probiotic strains.* Int Dairy J, **17**, 1284–1289.
46. **Simonič M, Slapničar Š, Trček J, et al** (2023): *Probiotic Lactobacillus paragasseri K7 nanofiber encapsulation using nozzle-free electrospinning.* Appl Biochem Biotechnol, **195**, 6768–6789.
47. **Škrlec K, Zupančič Š, Mihevc SP, et al** (2019): *Development of electrospun nanofibers that enable high loading and long-term viability of probiotics.* Eur J Pharm Biopharm, **136**, 108–119.
48. **Soukoulis C, Singh P, MacNaughtan W, et al** (2016): *Compositional and physicochemical factors governing the viability of Lactobacillus rhamnosus GG embedded in starch-protein based edible films.* Food Hydrocoll, **52**, 876–887.
49. **Tsiouris CG, Tsiouri MG** (2017): *Human microflora, probiotics and wound healing.* Wound Med, **19**, 33–38.
50. **Zavišić G, Ristić S, Petković B, et al** (2023): *Microbiological quality of probiotic products.* Arhiv Za Farmaciju, **73**, 17–34.
51. **Ziabari M, Mottaghitalab V, Haghi AK** (2010): *A new approach for optimization of electrospun nanofiber formation process.* Korean J Chem Eng, **27**, 340–354.