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Enhanced viability of *Lactobacillus* spp. via encapsulation in hyaluronan/PVA hybrid electrospun composites for vaginal drug delivery

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Abstract

Background Vaginal dysbiosis, a change in the beneficial vaginal microbiome, leads to a significant depletion in the essential lactobacilli thus increasing the possibility of vaginal infections such as bacterial vaginosis. Probiotics have gained more attention as a means of delivering exogenous lactobacilli but one of the challenges in delivery strategies is maintaining and improving their viability. The objective of this study is to enhance the viability of *Lactobacillus* spp., via encapsulation in hyaluronic acid/polyvinyl alcohol hybrid electrospun nanofibers. Polyvinyl alcohol (PVA) and hyaluronic acid (HA) composite nanofibers integrated with *Lactobacillus* spp. were fabricated by electrospinning. The survival of *Lactobacillus* spp. after its immobilization in electrospun nanofibers with polyvinyl alcohol and hyaluronic acid was evaluated.

Results Scanning electron microscopy indicated larger average diameters in PVA/HA nanofibers with *Lactobacillus* spp. encapsulation ($0.189 \pm 0.041 \mu\text{m}$ to $0.231 \pm 0.061 \mu\text{m}$ between D3 and D4, and $0.177 \pm 0.043 \mu\text{m}$ to $0.212 \pm 0.041 \mu\text{m}$ between D5 and D6) which showed that the nanofibers had the bacterial cells successfully enclosed in them. The viability of the lactic acid bacteria enclosed in the PVA/HA nanofibers was observed to decrease by more than 2-log units.

Conclusion The electrospun nanofiber-based delivery system is promising for the encapsulation and delivery of lactic acid bacteria to the vagina to combat recurrent vaginal infections such as bacterial vaginosis.

Keywords *Lactobacillus* spp., Electrospun fibers, Polyvinyl alcohol, Hyaluronic acid

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Background

Vaginal dysbiosis occurs when there is a disruption in the symbiotic balance of the vaginal microbiome that protects the vagina from the invasion and colonization of external pathogenic organisms [1]. This occurs due to a deficiency in the lactobacilli present in the vagina and an increase in microbial diversity which leads to a series of vaginal infections that are associated with serious sexual and reproductive health outcomes [2]. The standard therapy for these microbial disorders typically involves antibiotics such as metronidazole, cotrimoxazole, and azithromycin. While effective in achieving good therapeutic outcomes, they are still associated with the challenge of adverse effects and reoccurrence after a few months [3]. This problem has heightened interest in the use of exogenous lactobacilli in the form of probiotics or live biotherapeutic products to equilibrate the vaginal microbiota to a state of vagina eubiosis [4].

According to the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), probiotics are defined as living microorganisms that, when administered in adequate amounts, confer a health benefit on the “host” [5]. Most frequently, they are members of the varied *Lactobacillus* genus, which contains many species that have been classified as “qualified presumption of safety” or “generally recognized as safe.” Among the distinct ecological environments of the human body, the vaginal microbiota is the least diverse, where bacteria from the genus *Lactobacillus* predominate in the majority of healthy women (>70%). A healthy vaginal microbiota includes about four to twelve different species. Therefore, using probiotics to reestablish the dominance of lactobacilli in vaginal dysbiosis (e.g., bacterial vaginosis and vaginal candidiasis) is rational and might even be the most justified among the different applications of these probiotics [6].

The use of these probiotics will help to reverse vaginal dysbiosis and provide preventive and curative solutions to improve patient treatment outcomes. For these treatments to be successful, lactic acid bacteria should be integrated into user-friendly delivery systems that will keep the product viable and stable throughout processing, storage, and use [7]. It is also essential that the lactic acid bacteria can adapt to the vaginal environment and remain biologically active at the desired target. For this therapeutic application to be successful, there needs to be an effective delivery system that will ensure the *Lactobacillus* spp. is administered easily and that it remains active and viable from the production stage, through various storage conditions till it gets to the desired site of action.

Encapsulating living bacteria within the right biomaterials is a biotechnological method that can help

to solve many issues associated with drug delivery of probiotics. Encapsulation is the technique of creating a uniform coating around a core of an encapsulated material that is completely positioned within the capsule wall, thereby trapping the product in the matrix [8]. This helps to segregate the live bacterial cells from their immediate environment, providing a physical barrier to shield them while allowing a continuous and controlled release of the organisms [9]. This delivery technique supplies a high probiotic load and eases its adherence, thereby extending the residence time. Probiotics or live bacterial cells can be immobilized or encapsulated using two major techniques which are microencapsulation and electrospinning [10]. In an innovative method called electrospinning, continuous fibers with diameters measured in nanometers are created by applying intense electric fields to polymer solutions [11]. The polymer solution can be infused with various active substances, which will then integrate into the nanofibers through this process [11]. The small size of the nanofibers enables the product to easily access the desired organs and guarantees effective absorption by many cell types. Additionally, it enables the direct and continual delivery of probiotics to the vagina so that adequate bacterial cells can benefit the host. Several bacterial species were successfully incorporated by Zupani et al. [6], into poly (ethylene oxide) nanofibers, which had an average diameter of about 100 nm. Depending on the species, the viability of the embedded lactobacilli varied from 0 to 3 log colony-forming units (CFU)/mg loss [6]. *L. rhamnosus* was successfully nanoencapsulated by Ceylan et al. [12], into sodium alginate and poly (vinyl alcohol)-based nanofibers.

The selection and physicochemical characteristics of the biomaterials used for the electrospinning process are crucial, as they determine the efficiency of the encapsulation process as well as the viability and stability of the delivery system [13]. Numerous natural or synthetic polymers and their composites can be used for developing electrospun nanofibers. The most common natural polymers employed in electrospinning and the creation of nanofibers for the delivery of biological products are proteins and polysaccharides [11]. Hyaluronic acid (HA) is a polysaccharide abundantly distributed within the extracellular matrix of living organisms. It exhibits high viscosity at low concentrations, thus it can present a challenge to electrospinning. However, its biocompatibility and water solubility make it a popular choice for biomedical applications [14]. Pliszczak et al. [15] developed a bioadhesive delivery system for encapsulating probiotics and prebiotics, utilizing microparticles composed of pectin and hyaluronic acid, specifically designed for vaginal administration.

Synthetic polymers with hydrophilic properties such as polyethylene oxide (PEO), polyvinylpyrrolidone (PVP), and polyvinyl alcohol (PVA) are generally used in probiotics electrospinning [10, 16, 17]. PVA is a transparent, semicrystalline polymer that dissolves in water and has good mechanical, chemical, and biocompatibility qualities [18]. The aim of this study is to fabricate and characterize electrospun fibers composed of HA/PVA incorporated with *Lactobacillus* spp. to enhance the viability of *Lactobacillus* spp. and prevent vaginal dysbiosis.

Materials and methods

Poly(vinyl alcohol) (MW—30,000–70,000, CAS NO 9002-89-5) and hyaluronic acid (MW: 100,000 and 1,200,000 CAS NO 9067-32-7) were acquired from Shanghai Macklin Biochemical Co., Ltd. *Lactobacillus fermentum* (vaginal epithelial cells from a human) were acquired from the Nigeria Institute of Medical Research, NMIR (Nigeria), Depo-Provera®, Pfizer, NY, USA. Phosphate buffer (pH 4.5) was prepared according to USP XXVII. Acetic acid (MW—60.05, CAS NO 64-19-7) was obtained from Honeywell, Fluka, Germany. Ethanol (46.07, CAS NO 64-17-5) was obtained from Fisher Scientific, UK. Deionized water. De Man–Rogosa–Sharpe (MRS) agar were acquired from Rapid Labs, UK CM-MRSA205. Normal saline was purchased from Fidson Healthcare Plc, Ogun, Nigeria (Batch Number: 1032650). All chemicals used were of analytical grade and used without further purification straight from the vendors.

Fabrication of polyvinyl alcohol and hyaluronan-loaded nanofibers

A 25% w/v polyvinyl alcohol (PVA) solution was prepared by dissolving PVA powder in deionized water and acetic acid (50:50) under constant stirring using a magnetic stirrer at 60 °C for 30 min and then at 80 °C for 1 h. A 0.04 and 0.004% w/v hyaluronic acid (HA) solution was prepared by dissolving HA powder in a normal saline solvent system. The solution was sonicated to ensure thorough mixing and remove any air bubbles. The mixture was left overnight to ensure complete dissolution. Subsequently, the PVA/HA polymer blend solution was stirred at a volume ratio of 99:1 for 6 h at 50 °C in a closed vial to prevent bubble formation and to guarantee homogeneity [19]. The mixture was then kept for 24 h to guarantee homogeneity before being electrospun on an electrospinning machine (Nanolab Instruments NLI Basic (model: PS35-PCL, Selangor, Malaysia)®).

Development of *Lactobacillus fermentum*-loaded nanofibers

Using freshly prepared MRS agar under sterile conditions, a stock culture of *Lactobacillus fermentum* was

prepared by inoculating MRS agar plates following the method described by Ekama et al. [20]. Bacterial cells were subsequently collected from the agar using 5 mL of a 1:2 mixture of skim milk and normal saline. A suspension with a concentration of 150×10^6 CFU/mL was subsequently introduced to the PVA/HA polymer mixture. At room temperature, the mixture was agitated at 600 rpm until a thick and transparent solution formed, which was subsequently electrospun.

Electrospinning process

Nanofibers were fabricated from the prepared polymeric solutions using the NLI electrospinning machine (Model BS-35CL-2-ESD). The electrospinning setup comprised a high-voltage power supply, a syringe pump equipped with Terumo plastic syringes and 18G, 20 mm long needles, and a grounded plate collector. For the electrospinning procedure, optimal conditions included 25 °C temperature, 40% relative humidity (RH), and a flow rate of 1.0 mL/h.

Polymer solutions were loaded into two 10 mL Terumo® plastic syringes mounted horizontally on the syringe pump. An electric field of +25 kV was applied between the needle and the plate collector using a high-voltage supply connected to the metal needle via an alligator clip. The distance between the collector and the needle tip was maintained at 15 cm.

To recover the final nanofiber sheet, the collector was covered with aluminum foil during electrospinning. Post-electrospinning, the aluminum foil was removed, and the fibers were peeled off. Subsequently, the fibers were air-dried at room temperature (25 ± 1 °C) and 50% RH inside air-tight plastic bags until further characterization.

Surface morphology of the electrospun nanofibers

Scanning electron microscopy (SEM)

The morphological structure of the nanofibers was investigated using scanning electron microscopy (SEM). Portions of fibers with 5×5 mm dimensions were cut out and placed on the SEM stub using carbon conductive tape, sputtered with gold, and imaged using a Hitachi 6600 field emission scanning electron microscope (Tarrytown, NY, USA). Fiber diameters were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The average diameter was calculated from a minimum of 80 fibers per image. The porosity of the nanofibrous membranes was further determined by using ImageJ software to identify the fiber matrix and pores through careful selection of upper and lower threshold values in the binary images, calculating the percentage area by pixel.

Thermal analysis of the electrospun fibers

The fibers' thermal stability was evaluated employing thermogravimetric analysis (TGA) and differential thermal analysis (DTA) (Thermogravimetric analyzer (Shimadzu DSC-60, Kyoto, Japan)). Fiber samples weighing 2 mg were heated in platinum pans under a nitrogen environment at a temperature range of 25–200 °C.

Differential scanning calorimetry

Differential scanning calorimetry (DSC 2/700/14, Mettler Toledo, USA) was used to evaluate the heat flow associated with phase transitions and the physicochemical properties of the nanofibrous membranes. The analysis was conducted over a temperature range of 37–450 °C, with a heating rate of 10 °C/min. Each sample (5 mg) was measured, placed in an aluminum pan, hermetically sealed, and scanned under a nitrogen gas flow rate of 50 mL/min.

Chemical characterization of fabricated electrospun fibers

Fourier transform infrared spectroscopy

The presence of specific functional groups, chemical bonds, and molecular interactions in the nanofibers was determined from the FTIR spectra using an FTIR spectrophotometer (Agilent Cary 630 ATR-FTIR). Spectra between wave numbers 500–4000 cm^{-1} were recorded for the identification of the fibers' absorption bands. Electrospun fiber samples were dried under vacuum at 45 °C before being set on top of a diamond crystal during FTIR measurement. Where necessary, smoothing was used to lower the noise without foregoing any peaks.

X-ray diffraction

The range used for X-ray diffraction examination was 10–80 with a 0.05 step size utilizing a D8 diffractometer (Bruker, Billerica, MA, USA) in the CuK monochromatic radiation. The samples were heated to 600, 1000, 1200, and 1480 °C before being quenched. An examination of the samples was done to recognize post-heat phases and relate potential phase transitions to the detected thermal effects.

Mechanical characterization of the electrospun fibers

Ultimate tensile strength of the electrospun fibers

A Universal Testing Machine (Instron-series 3369®, Norwood, MA, USA) outfitted with a 50 kN load cell was utilized for the assessment of the tensile strength and ductility of the electrospun fiber. The fibers were divided into pieces measuring 50 by 10 mm and evaluated at a humidity level of 60% and a temperature of 20 °C. Then, using a load ranging around 50 N and 50 mm gauge

length, the fibers were secured together and stretched at a rate of 50 mm/min. The tensile strength was measured three times, and a mean reading was determined.

Biological characterization of fabricated electrospun fibers

Viability of *Lactobacilli* spp. before and after the electrospinning process

The viability of the *Lactobacillus* spp. in PVA/HA solutions was evaluated before and after their electrospinning into nanofibers. Initially, 1 mL polymer solution containing the bacterial cells was used to perform a tenfold serial dilution with normal saline, and 10 μL of each dilution were plated onto MRS agar plates. To create anaerobic conditions, Microbiology AnaeroGen® (Thermo Scientific, UK) was activated and placed in a secure plastic container with the agar plates. The plates were then incubated at 37 °C for 48 h. After incubation, the colonies were counted and averaged. These results were expressed as CFU/mL and converted to log CFU/mL.

Subsequently, the viability of bacteria within the nanofibers was determined by dissolving a known cross-sectional area of each of the nanofibers in normal saline to release encapsulated bacterial cells. Bacterial serial dilution and counting followed the same method as described above was done. Bacteria loading in the electrospun nanofibers was compared to initial bacterial cell counts in the polymer solution.

Safety evaluation using an animal model

Twenty female adult treatment naive rats in good physical condition, weighing between 160–170 g, were used in this experiment. Before commencing the study, the rats spent 8 days getting used to their new habitat, which was kept at a constant 29 °C, relative humidity of 40%, and a 12-h light/dark cycle. The management and utilization of experimental animals adhered to the National Institutes of Health's guidelines. The evaluations complied with the institutional guidelines endorsed by the Health Research and Ethical Committee. The results of the in vivo studies were presented according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) documentation standards. Five days before the administration of the electrospun fiber, all rats had a subcutaneous injection of medroxyprogesterone acetate (2 mg/kg/body weight) to synchronize their hormones. Four groups of experimental animals were randomly assigned, each with three rats, and intravaginal dosages of the nanofibers D1, D3, and D4 were supplied daily utilizing a sterile feeding needle with a ball-point end made of stainless steel. Daily observations of the external features of the vagina were made; if any of the following conditions were present, they were noted: difficulty inoculating vaginal contraction, excessive animal screams, spasms, redness, and burning. There

was no anesthetic utilized at any point in the procedure, and on day 14, carbon dioxide gas was used to humanely euthanize the rats. For histological evaluation, the tissues of the vagina and rectum were surgically removed and placed in sterile sample vials that contained 10% formalin solution. The excised sections were examined under a Leica DM 750 microscope and captured with an ICC HD 50 camera. Observations were carried out utilizing magnifications of $\times 100$ and $\times 20$.

Statistical analysis

The results were presented as the mean and standard deviation of experimentally derived values for each variable.

Results

Surface morphology of the electrospun fibers

Figure 1 presents a comparison of the SEM (scanning electron microscopy) micrographs of PVA/HA nanofibers and bacteria-loaded PVA/HA nanofibers. ImageJ software (version 1.80, National Institutes of Health, USA) was used to quantify the average diameter and pore size distribution of the electrospun nanofibers from the SEM images.

The D1 fibers displayed an even distribution with an average diameter of $0.211 \pm 0.053 \mu\text{m}$. D2 fibers, slightly larger at $0.271 \pm 0.057 \mu\text{m}$, showed a similar pattern. D3 fibers, thin and closely intertwined, had a mean diameter of $0.189 \pm 0.041 \mu\text{m}$. D4 fibers, closely interwoven, had an average diameter of $0.231 \pm 0.061 \mu\text{m}$. D5 fibers, tightly interwoven with a near-uniform surface, measured $0.177 \pm 0.043 \mu\text{m}$. D6 fibers, tightly packed with tiny

bead formations, had a diameter of $0.212 \pm 0.041 \mu\text{m}$. The porosity measurements of nanofibers D1–D6 varied significantly. D3 had the lowest porosity at $20.55 \pm 2.0\%$, followed by D4 at $20.44 \pm 1.2\%$, then D5 at $22.79 \pm 0.6\%$, and D6 at $22.57 \pm 1.1\%$. D1 exhibited a porosity of $27.8 \pm 1.7\%$, while D2 was the most porous with a porosity of $29.14 \pm 1.8\%$.

Thermal analysis of the electrospun fibers

The thermal properties of the electrospun fibers were assessed using thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTA), with weight loss and derivative curves presented in Fig. 2A and B, illustrating percentage weight loss as a function of temperature.

The TGA analysis indicated an initial weight loss of 12.61% in D1 and 13.60% in D2 around 100–200 °C, resulting in higher final residual masses of 21.53% in D1, 23.03% in D2, and 12.22% in D6 at 700 °C. Formulations with higher molecular weight HA (1,200,000 Da) in D3, D4, D5, and D6 showed greater major weight loss rates (84.83–93.18%) between 300 and 500 °C, compared to those with lower molecular weight HA (100,000 Da) in D1 and D2, which exhibited major weight loss rates of 63.37–65.86% in the same temperature range.

DTA profiles of the electrospun nanofibers as shown in Fig. 2B revealed that samples containing low molecular weight HA (D1 and D2) demonstrated primary decomposition occurring at temperatures between 300 and 320 °C, whereas samples containing high molecular weight HA (D3–D6) revealed enhanced thermal stability, with the primary decomposition occurring at higher temperatures

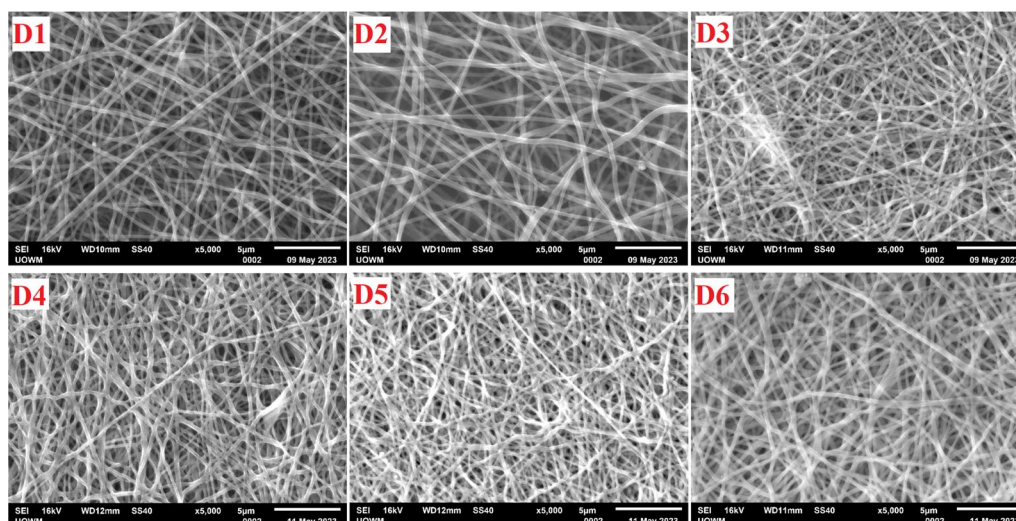


Fig. 1 Scanning electron micrographs of the PVA/HA electrospun nanofibers. (D1—25%w/v PVA:0.004% HA 100 kDa: *Lactobacillus* spp., D2—25%w/v PVA:0.04% HA 100 kDa: *Lactobacillus* spp., D3—25%w/v PVA:0.04% HA 1200 kDa, D4—25%w/v PVA:0.04% HA 100 kDa: *Lactobacillus* spp., D5—25%w/v PVA:0.004% HA 100 kDa, and D6—25%w/v PVA:0.004% HA 100 kDa: *Lactobacillus* spp.)

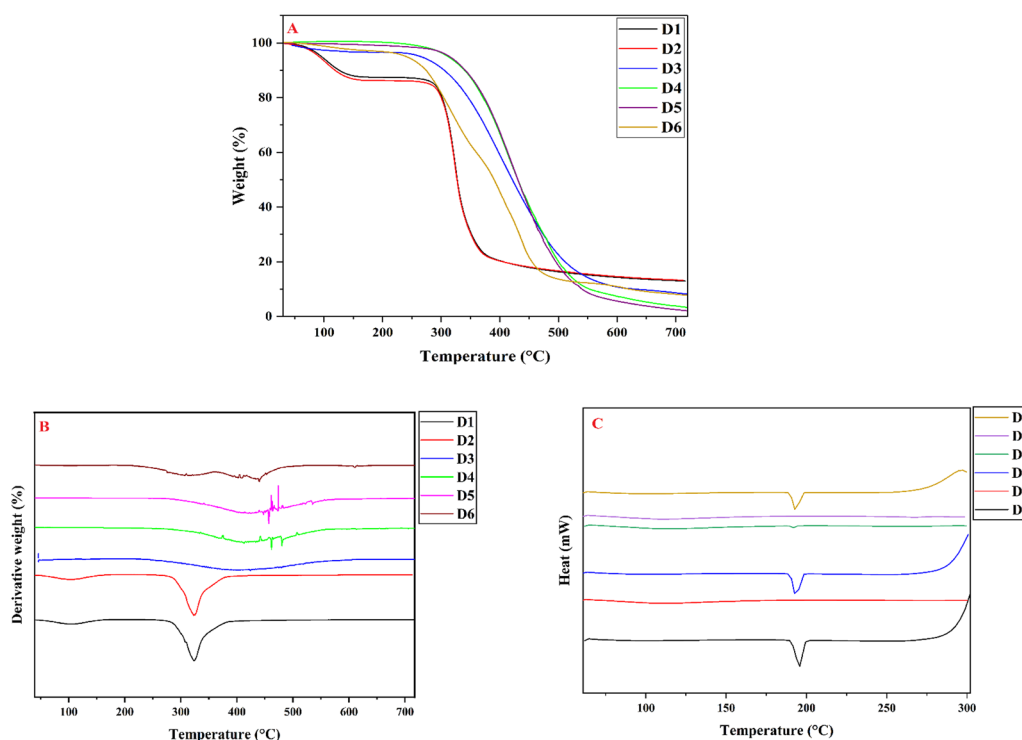


Fig. 2 Thermal characterization of the electrospun fibers using **A** TGA, **B** DTA, and **C** DSC

between 400 and 450 °C. The introduction of lactobacilli cells (D4 and D6) resulted in intricate multi-stage breakdown profiles, with D6 exhibiting prominent peaks at temperatures ranging from 300 to 350 °C and 400 to 450 °C. D5 and D6, despite having a lower percentage of HA (0.004%), maintained consistently high decomposition temperatures.

Chemical characterization of the electrospun fibers

The FTIR spectra for the formulations are depicted in Fig. 3A, highlighting major peaks at 3400, 2900, 1750,

1250, 1100, and 800 cm^{-1} , consistently observed across all formulations.

The XRD patterns as shown in Fig. 3B were analyzed to assess the crystallinity of the nanofibers. Evaluation of the diffractograms across all formulations revealed that HA was completely amorphous with no crystalline peaks observed.

There was a single major peak that occurred at a 2θ value of around 20° which was attributed to PVA and was consistent across formulations D1, D5, and D6, which featured the lowest HA content (0.004% HA)

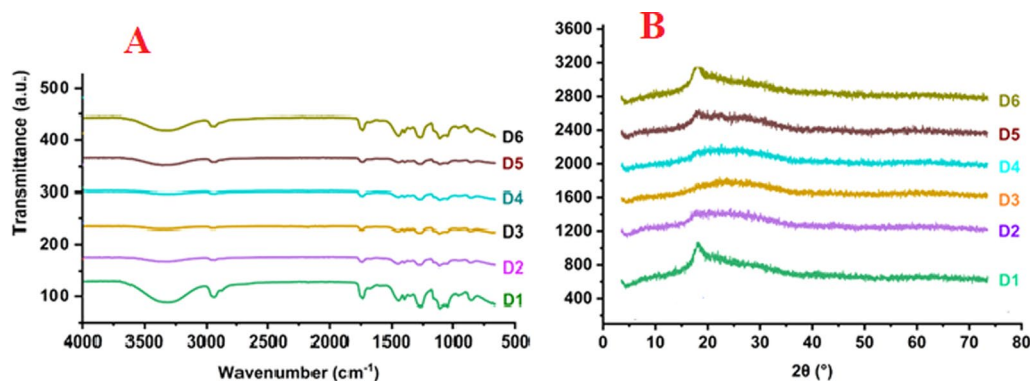


Fig. 3 Chemical characterization of the electrospun fibers using **A** FTIR and **B** XRD

and introduced some level of crystallinity to these formulations.

Mechanical characterization of the electrospun fibers

After measuring the ultimate tensile strength (UTS) of the electrospun fibers, D1 exhibited a UTS of 0.0607 MPa. D2 and D3 had UTS values of 0.0640 and 0.1175 MPa, respectively (Fig. 4A). D4 and D5 showed UTS values of 0.0529 and 0.1572 MPa, respectively, while D6 had a UTS of 0.1697 MPa.

In terms of ductility, the fibers exhibited varying percentages: D1 showed the highest ductility at 41%, followed by D4 at 36%, D2 at 34.25%, D3 at 32.74%, D5 at 26.75%, and D6 at 24.64%.

Biological characterization of the electrospun fibers

Viability of *Lactobacilli* spp. before and after electrospinning process

To ensure the efficacy of *Lactobacillus* spp. within the electrospun fibers, it was crucial to evaluate their viability. This ensures that a viable number of colony-forming units remains after electrospinning and subsequent storage at 25 ± 0.5 °C. This is particularly important as *Lactobacillus* spp. produce lactic acid in the vagina, contributing to their intended therapeutic action.

For formulation, D2, the average number of CFU/mL in the polymer solution was 1.45×10^8 , and the average number of CFU/mL in a portion of the nanofiber weighing 0.035 g was 1.44×10^5 . For formulation D4, the average amount of *Lactobacillus* spp. present in the polymer solution was 1.51×10^8 CFU/mL, whereas a 0.042 g

portion of the nanofiber contained 1.53×10^5 CFU/mL on average (Table 1).

Safety assessment in an animal model

The safety assessment evaluated signs of vaginal irritation following administration of the fiber samples. The histological section of the control group's vaginal tissue displayed normal histology with lamina propria (LP), blood vessels (BVs), lymphocytes (LMs), and non-cornified stratified squamous epithelium (EP) (Fig. 5A and B). Tissues exposed to D1 exhibited estrogen-induced stratification and cornification of the hypertrophied epithelium (Fig. 5C and D).

Figure 5E and F depicts tissues treated with D3 which displayed normal vaginal tissue histology. Mice treated with D4 (Fig. 5G and H) exhibited a standard vaginal structure with lamina propria (LP) containing blood vessels (BVs) and non-cornified stratified squamous epithelium (EP) featuring a permeable layer which was maintained by day 14.

Table 1 Viability of *L. fermentum* in polymer solutions and electrospun nanofibers

	<i>L. fermentum</i> colony count AVERAGE CFU/mL	<i>L. fermentum</i> colony count Log CFU/mL
D2 (Electrospun fiber)	1.44×10^5	5.16 ± 0.021
D2 (Polymer solution)	1.45×10^8	8.16 ± 0.004
D4 (Electrospun fiber)	1.53×10^5	5.18 ± 0.012
D4 (Polymer solution)	1.51×10^8	8.17 ± 0.017

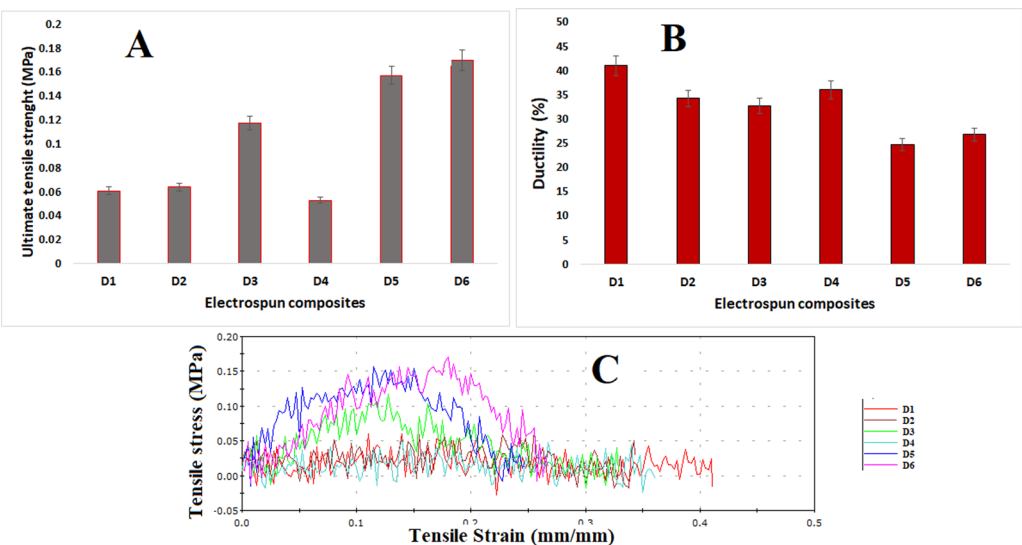


Fig. 4 **A** Ultimate tensile strength of electrospun composites, **B** ductility (%) of electrospun fibers, and **C** tensile strain versus tensile stress of electrospun fibers D1–D6

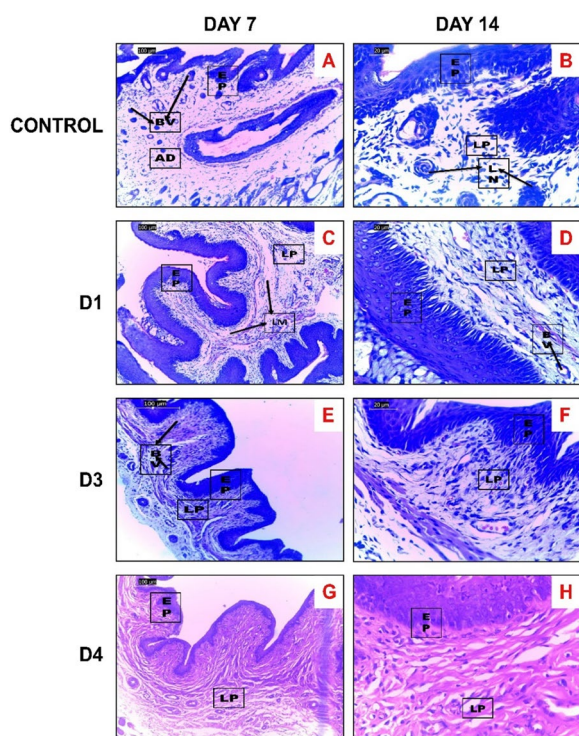


Fig. 5 Photomicrographs (H and E $\times 100$) of the histology slides of a piece of vaginal tissue treated with (A and B) control/no treatment, (C and D) electrospun fiber D1, (E and F) electrospun fiber D3, and (G and H) electrospun fibers D4. KEY: lamina propria (LP), blood vessels (BVs), lymphocytes (LMs), squamous epithelium (EP), and adventitia (AD)

Discussion

Vaginal infections in reproductive-aged women such as bacterial vaginosis and vulvovaginal candidiasis are an indication of vaginal dysbiosis in which suboptimal levels of lactobacilli activity will increase diverse communities of anaerobes and other microbes. This problem has deepened interest in the use of exogenous lactobacilli in the form of probiotics or live biotherapeutic products to return the vaginal microbiota to a state of vaginal eubiosis [4].

Probiotics are thought to have anti-adhesion capabilities for BV in addition to their ability to produce bacteriocins, including hydrogen peroxide, and host immunomodulatory effects by competing with other pathogens to the binding site on the vaginal epithelium [21]. Probiotics, such as *Lactobacillus* spp., have also been shown to reduce pathogen adhesion to the surface epithelium by producing biosurfactants with anti-biofilm characteristics [22].

Although it is still not advised as a treatment for BV, probiotic supplementation appears to be effective for treating BV, regardless of the use of antibiotics concurrently [21]. This will help provide solutions that can

prevent or cure these infections and improve patient therapeutic outcomes [23]. There is a need for the lactic acid bacteria to be integrated into patient-friendly delivery systems that will maintain the stability and viability of the product throughout processing, storage, and use [7].

Electrospinning is a viable technique for incorporating probiotics into nanofibers, enabling simultaneous drying of the bacteria and creation of a solid delivery system in one step. This method offers significant advantages over other encapsulation techniques such as microencapsulation and lyophilization [6].

When developing electrospun fibers for probiotic encapsulation, the choice of polymers is crucial for maintaining probiotic viability. A hybrid electrospun fiber matrix was created using polyvinyl alcohol (PVA) and hyaluronic acid (HA). Natural polymers like HA have poor electrospinnability due to their semicrystalline/crystalline properties and intricate secondary/tertiary structures. Therefore, successful electrospinning requires the aid of synthetic polymers like PVA, which have good spinnability. Selecting an appropriate binary system can significantly enhance probiotic survivability under stress conditions [24].

For instance, studies have shown that the viability of probiotics encapsulated in synthetic polymer fibers (such as PEO) decreased by more than 2-log units after storage at 25 °C for 7 days [25]. However, incorporating disaccharides (such as sucrose and trehalose) into the fiber mat reduced viability loss during the electrospinning and storage process. This improvement is attributed to the amorphous nature of the disaccharides in the fibers and their interactions with the probiotic cells.

A natural linear hydrophilic polymer with promising biocompatibility, hyaluronic acid (HA), has been widely used as the framework for loading and delivering specialized active ingredients. According to Xiao et al. [26], HA hydrogel can increase the survivability of *L. rhamnosus* during short-term storage. This was attributed to the protective quality, energy supply, and nutritional supply of the HA-based delivery system.

The biological effects of HA are significantly influenced by its molecular weight [27]. Lower molecular weights have reduced cohesion compared to higher molecular weights, potentially facilitating deeper penetration into the vaginal epithelium and enhancing probiotic colonization in mucosal layers. On the other hand, higher molecular weight HAs form a more cohesive gel that adheres to the vaginal surface longer, promoting prolonged contact between probiotics and mucosal surfaces.

Furthermore, the molecular weight and concentration of HA also affect parameters such as viscosity and surface tension which are critical for the electrospinning process of the polymer solution. Therefore, we utilized different

molecular weights and concentrations to evaluate their impact on both *Lactobacillus* spp. viability and the electrospinnability of PVA/HA solutions.

PVA was selected due to its mucoadhesive properties, biocompatibility, and hydrophilic nature. It creates a strong oxygen barrier that secures bacterial bioactivity when it is in a dry form. PVA is also widely acknowledged as a safe (GRAS) substance, similar to lactobacilli, which validates its safe use as a nanocarrier for vaginal probiotic delivery [28].

Electrospun fibers' distinctive shape and microstructure are crucial for the delivery of vaginal drugs. The use of PVA resulted in the creation of spider-net-like structures to varying degrees within the nanofiber mat. This has been shown to enhance the strength of the PVA's nanofiber mat, increasing its resistance to deformation [29].

The research findings showed that as the concentration of hyaluronic acid (HA) increased, the diameter of nanofibers also increased. For instance, nanofibers in group D2, which had a higher HA concentration, were thicker compared to those in D1 with a lower HA concentration. This trend was similarly observed between D4 and D6. The increase in nanofiber diameter correlated with higher viscosity of the polymer solution as more HA was added. This increased fiber diameter can help to create more room for lactobacilli encapsulation.

Interestingly, when comparing different molecular weights of HA at the same concentrations, there was only a slight increase in average diameter (0.001 μm between D1 and D6) and a minimal decrease (0.04 μm between D2 and D4).

Additionally, the data indicated larger average diameters in PVA/HA nanofibers with *Lactobacillus* spp. encapsulation (0.189 $\pm$ 0.041 μm to 0.231 $\pm$ 0.061 μm between D3 and D4, and 0.177 $\pm$ 0.043 μm to 0.212 $\pm$ 0.041 μm between D5 and D6). This suggests that the bacteria were successfully encased in the nanofibers.

Another critical property of electrospun fibers that significantly influence cellular responses is their porosity [30]. For instance, Hofmann et al. [31] showed that the connections between pores play a major role in how pore size distribution affects cell adhesion and enhances intracellular interactions.

Porosity refers to the volume of empty spaces within a nanofiber mat. The distribution of pore sizes is crucial in biomedical scaffolds, influencing cell access and drug release [32]. An increase in hyaluronic acid (HA) concentration generally leads to higher porosity, observed between D5 and D3, and D6 and D4, except for D1 and D2. Porosity decreases significantly with higher molecular weight HA. This occurs because higher molecular weight results in a more interconnected fiber network

that is tightly packed. Comparing blank nanofibers with lactobacillus-loaded nanofibers shows decreased porosity between D3–D4 and D5–D6 pairs.

Porosity and pore size in electrospun fibers depend on fiber diameter and packing density [33]. Fiber density also affects porosity, based on how fibers pack in three-dimensional space. At lower molecular weights (100 kDa), where packing density is low, porosity depends on fiber diameter. Conversely, at higher molecular weights (1200 kDa) with higher packing density, porosity decreases with increasing fiber diameter, indicating less dependency on fiber diameter.

The mechanical characteristics of electrospun fibers are crucial in determining their suitability for various applications [34]. To analyze the tensile strength of electrospun fibers, the stress–strain relationship was examined. Figure 4 depicts this relationship, revealing that at low molecular weights, the ultimate tensile strength (UTS) of the nanofibers showed a slight increase, while ductility decreased with increasing HA concentration, as seen in the D1–D2 pair. Conversely, at high molecular weights of HA, the tensile strength decreased, and ductility increased in a concentration-dependent manner, as observed in the D5–D3 and D6–D4 pairs. Nikbakht et al. [35] also noted a concentration-dependent reduction in tensile strength upon adding HA to their electrospun scaffold.

It was observed that at low HA concentrations, tensile strength increased while % ductility decreased with increasing molecular weight. On the other hand, at higher HA concentrations, tensile strength decreased while percentage ductility increased with increasing molecular weight. Comparing blank fibers to bacteria-loaded fibers, the ultimate tensile strength increased at low HA concentrations but decreased at high concentrations. Incorporating *Lactobacillus* spp. also increased the ductility (%) of the fibers. This suggests that HA contributes ductile properties to the fibers, thereby reducing stiffness.

The thermal stability of the electrospun nanofibers was analyzed using TGA and DTA. The curves, respectively, shown in Fig. 2A and B indicate the percentage weight loss as a function of temperature.

The result of the thermogravimetric analysis (TGA) indicates that the presence of lactobacilli cells contribute significantly to initial weight loss, as evidenced by 12.61% in D1 and 13.60% in D2 around 100–200 $^{\circ}\text{C}$, while also resulting in higher final residual masses, specifically 21.53% in D1, 23.03% in D2, and 12.22% in D6 at 700 $^{\circ}\text{C}$. Formulations with higher molecular weight HA (1,200,000 Da) in D3, D4, D5, and D6 show greater major weight loss rates (84.83–93.18%) between 300 and 500 $^{\circ}\text{C}$, compared to those with lower molecular weight

HA (100,000 Da) in D1 and D2, which show major weight loss rates of 63.37–65.86% in the same temperature range. Higher molecular weight HA enhances thermal stability, shifting primary decomposition to higher temperatures (400–450 °C), this is similar to a review by Snetkov et al. [27], where it was found that high molecular weight HA improves the thermal properties of the biopolymer. *Lactobacillus* spp. cells contribute to initial weight loss at lower temperatures and result in higher residual masses. Overall, the impact of HA concentration on major weight loss percentages varies significantly across formulations, with both high and low concentrations demonstrating varied effects on decomposition outcomes.

The differential thermogravimetric analysis (DTA) of PVA/HA nanofibers (D1–D6) showed clear thermal characteristics that were affected by the molecular weight and concentration of HA, as well as the presence of lactobacilli. Samples containing low molecular weight HA (D1 and D2) demonstrated primary decomposition occurring at temperatures between 300 and 320 °C, whereas samples containing high molecular weight HA (D3–D6) revealed enhanced thermal stability, with the primary decomposition occurring at higher temperatures between 400 and 450 °C. The introduction of *Lactobacillus* spp. cells (D4 and D6) resulted in intricate multi-stage breakdown profiles, with D6 exhibiting prominent peaks at temperatures ranging from 300 to 350 °C and 400 to 450 °C. D5 and D6, despite having a lower percentage of HA (0.004%), exhibited consistently high decomposition temperatures. The notable characteristics observed were distinct peaks in D4 and D5 at around 450 °C, with D5 exhibiting a different and sharp spike after decomposition. The results show the effect of the molecular weight and biological components of HA on the thermal characteristics of PVA-based composites.

Research has shown that incorporating freeze-dried *Lactobacillus coryniformis* Si3 into sucrose-based formulations with polymers such as poly(vinyl)pyrrolidone K90 and Ficoll 400 can increase the amorphous matrix stability, thus improving the overall stability of the product including thermal stability [36].

The DSC thermograms for the formulations are presented in Fig. 2C. The second peak observed around 200 °C for samples D1, D3, D4, and D6 corresponds to the melting transition temperature of PVA [37]. The DSC thermogram for sample D5 indicates an endothermic reaction, suggesting that the composite material exhibits a glass transition temperature at 110.99 °C and melting transitions at 268.487 °C and 281.023 °C. In comparison, sample D4 displays two endothermic peaks at 113.488 °C and 190.8 °C, indicating that the material undergoes at least two distinct thermal events when heated. These findings are consistent with

the previous studies. For instance, Zulkifli et al. [38] reported glass transition and melting transition temperatures of 104.72 °C and 222.26 °C, respectively, for a PVA-based composite scaffold used in tissue engineering. Guirguis and Moselhey [39] observed a glass transition temperature of 88.1 °C and a melting temperature of 209.6 °C. Furthermore, Sgorla et al. [40] noted glass transition and melting transition temperatures of 95.55 °C and 222.98 °C, respectively, for polymeric films produced from cross-linked hyaluronic acid.

FTIR spectra were used to identify peak shifts indicative of interactions within the polymeric composites of the nanofibers. The FTIR spectra of the formulations are shown in Fig. 3A. The major peaks were detected around 3400, 2900, 1750, 1250, 1100, and 800 cm^{-1} . These peaks were found to be present in all formulations.

The weak band around 3400 cm^{-1} can be explained by the O–H and N–H stretching vibrations of the N-acetyl side chain of hyaluronic acid (HA) and polyvinyl alcohol (PVA) [41, 42]. The bands detected at approximately 2900 cm^{-1} could be attributed to the C–H stretching vibrations of the CH_2 – group in HA and PVA. A similar peak was also reported by Kharazmi et al. [43]. The bands at 1750 cm^{-1} could be explained by the asymmetric C=O and symmetric C–O stretching of the carboxyl groups in HA.

Peaks around 1750 cm^{-1} can also be due to carboxylic acid groups present in the cell wall of *Lactobacillus* spp., and in particular, Amiri et al. [44] reported similar observations for *Lactobacillus acidophilus*-loaded lactose/whey protein nanocomposite. The peak around 1100 cm^{-1} could be due to the C–O–C bond in PVA [45] while the peak at 1250 cm^{-1} could be explained by the C–H wagging which was similarly reported by Asran et al. [46]. The peak at 800 cm^{-1} was due to the C–C stretching vibration of PVA [47]. Jipa et al. [48] reported a similar peak for PVA bacteria cellulose films.

The peak at 1250 cm^{-1} could also have been attributed to the amide bonds from the peptides in the *Lactobacillus* spp. cells as reported by Oust et al. [49], who noted that *Lactobacillus* spp. are typically identified in the region between 1400 and 720 cm^{-1} . Feng et al. [50] reported peaks in the range of 900–1300 cm^{-1} which was attributed to nucleic acids and the proteins in *Lactobacillus* spp.

Similar peaks were also reported by Ceylan et al. [12], for lactobacillus-loaded electrospun fibers consisting of sodium alginate and poly(vinyl alcohol). Other studies conducted by Škrlec et al. [25], and Xu et al. [51] observed similar peaks in lactobacillus-loaded electrospun fibers, and this was taken as evidence of the successful encapsulation of *Lactobacillus* spp.

The XRD patterns were studied to evaluate the crystallinity of the nanofibers. Figure 3B shows the diffractograms of the nanofibers as obtained from XRD. Evaluation of the diffractograms for all formulations showed that HA was completely amorphous with no clear crystalline peaks. The amorphous nature of HA has been cited by earlier research [40, 52, 53].

Figure 3B shows that there was a single major peak that occurred at a 2θ value of around 20° and was attributed to PVA. This pattern was common to formulations D1, D5, and D6 which are the formulations with the least amount of HA (0.004% HA), and introduced some level of crystallinity to these formulations.

These findings mirror those that were cited by Dumitriu et al. [54] and Hamad et al. [55]. Uma Maheshwari et al. [56] reported characteristic peaks at 2θ values of 19.5° and 20.1° for PVA/HAp/PCL bilayer composites, and they attributed the peaks to the presence of PVA. Nasar et al. [57] also reported a diffraction peak at 2θ value of 19.8° which was due to the semicrystalline nature of PVA. In comparing the diffraction patterns of all formulations, it can be seen that the presence of HA was possibly responsible for the non-crystalline nature of the electrospun fibers.

This study has been focused on improving the viability of *Lactobacillus* spp. by incorporating PVA/HA polymers to enhance their beneficial effects.

After the development of nanofibers containing probiotics, it is important to ensure the long-term viability of the probiotic strains for optimal therapeutic efficacy. Probiotic bacteria underwent an electrospinning process, which could have potentially affected their survivability. Therefore, a viability analysis was conducted using the pour plate counting method.

To ascertain their resistance to the electrospinning conditions, the viability of *L. fermentum* was evaluated in the polymer solution (before immobilization) and electrospun nanofibers (after immobilization) by the plate method.

Two formulations, namely, D2 and D4, were utilized for assessing the viability of *Lactobacillus* spp. in the electrospun fibers. The primary factor for selecting D2 and D4 formulations for studying the viability of *Lactobacillus* spp. was the significant difference in the molecular weight of hyaluronic acid (HA) used. Specifically, D2 contained HA with a molecular weight of 100 kDa, whereas D4 utilized HA with a much higher molecular weight of 1200 kDa. This comparison aimed to investigate the impact of hyaluronic acid (HA) molecular weight on the survival of lactobacillus within electrospun fibers.

The viability of lactic acid bacteria decreased by more than 2-log units, potentially due to the use of only 0.035 g and 0.042 g of total nanofibers from

formulations D2 and D3, respectively, in the experiment. For instance, 0.042 g of nanofiber (D4) contained 0.2% of the expected cell count, whereas 0.035 g of nanofiber (D2) contained only 0.1% of the total cells added to the polymer solution. This discrepancy could account for the lower-than-anticipated colony-forming units, as a significant number of cells may have remained on the unused nanofibers during the viability assessment. Therefore, it is recommended that a larger portion of nanofibers be utilized to potentially enhance the presence of lactobacilli cells.

Observations revealed that formulation D4, with a higher molecular weight of HA, exhibited twice the percent increase in *Lactobacillus* spp. viability. Di Cerbo et al. [58] discovered that HA concentrations between 0.5 and 0.125 mg/mL enhance bacterial growth, and high molecular weight HA appears to improve bacteria's survival on various lactic acid bacteria (LAB) strains. This underscores the potential influence of the molecular weight of HA on bacterial viability and growth.

Only a fraction of *L. fermentum* cells survived encapsulation within nanofibers, as indicated by the drop in cell counts in both formulations D2 and D4 (Table 2). While a larger proportion of cells may have been encapsulated, the rigorous conditions of electrospinning such as high voltage and intense whipping or shearing action likely contributed to cell damage. It is also probable that after being enclosed, the cells lost their viability [59].

Due to the rapid evaporation of water during the electrospinning process, a decline in cell viability may also be linked to a fundamental change in the osmotic environment [60]. The same results were cited by earlier studies [12, 60], that developed electrospun alginate-based and starch-based core-sheath composite nanofibers [61, 62] to encapsulate viable probiotic cells. However, the probiotics' continued survival showed that it was able to withstand the high voltage and shear stress of the electrospinning process.

Table 2 Design of the electrospun formulations, showing different molecular weights and concentrations of hyaluronic acid (HA) used, along with 25% w/w polyvinyl alcohol (PVA)

Formulation	HA (100 kDa) (%w/v)	HA (1200 kDa) (%w/v)	<i>Lactobacillus</i> spp. (CFU/mL)
D1	0.004	–	150×10^6
D2	0.04	–	150×10^6
D3	–	0.04	–
D4	–	0.04	150×10^6
D5	–	0.004	–
D6	–	0.004	150×10^6

Three formulations, namely, D1, D2, and D3, were utilized for safety testing on animals. D1, D3, and D4 were selected for safety assessment based on their varying molecular weights of hyaluronic acid (HA), as well as the presence or absence of lactobacilli. This choice aimed to comprehensively evaluate how these factors influence the safety and potential effects of nanofibers on vaginal tissue, providing crucial insights for biomedical applications.

No electrospun fibers caused irritation or inflammation in the rats' vaginal epithelium during safety evaluation when compared with the vaginal epithelium of the control group (no treatment). A normal lamina propria with blood vessels and lymphocytes was seen in the histological section, except for animals that had received D1, who displayed a hypertrophied epithelium caused by estrogen-induced stratification and cornification. These findings highlight the normal structural and physiological state of vaginal tissue, showing no inflammation or apoptosis, the presence of vascular supply, and the functional role of glands in maintaining tissue health. The formulations were found to be safe for short-term use.

Conclusion

In this study, *Lactobacillus fermentum* was successfully encapsulated within hybrid electrospun nanofibers using the electrospinning technique. Our investigation was focused on evaluating the influence of different molecular weights and concentrations of hyaluronic acid (HA) on both the viability of lactobacilli and the characteristics of the electrospun fibers.

Surface morphology analysis confirmed the efficient encapsulation of *Lactobacillus* spp. within the fibers, revealing good porosity that supports sustained interaction between the probiotics and the vaginal environment. Furthermore, the nanofibers exhibited favorable thermal properties, ensuring stability during processing and storage.

The electrospinning of the polymer composite, combining polyvinyl alcohol (PVA) with varying molecular weights of hyaluronic acid (HA), resulted in a notable 2-log reduction in *L. fermentum* cell counts within the nanofibers.

Our findings suggest that the developed nanofibers are stable and generally well-tolerated by the vaginal epithelium in animal models. This innovative approach of employing PVA/HA electrospun fibers for *L. fermentum* encapsulation introduces a promising nanofiber-based delivery system. Such a system holds potential for addressing recurrent vaginal infections, including bacterial vaginosis, by delivering viable lactic acid bacteria effectively to the vaginal environment.

Abbreviations

PVA	Polyvinyl alcohol
HA	Hyaluronic acid
CFU	Colony-forming units
PEO	Polyethylene oxide
PVP	Polyvinylpyrrolidone
SEM	Scanning electron microscopy
TGA	Thermogravimetric analysis
DTA	Differential thermal analysis
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared spectroscopy
UTS	Ultimate tensile strength
MRS	De Man–Rogosa–Sharpe

Acknowledgements

We acknowledge the technical expertise of Edidiong Akang in the Department of Anatomy, College of Medicine, University of Lagos.

Author contributions

MOI, DAO, and POB carried out the experiment. MOI conceptualized and designed the project. DAO drafted the manuscript. DAO and POB carried out the analysis. TA, DT, and AET performed the SEM measurements. DAO, BO, and ATK performed the manuscript editing and final improvement. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the 2022 JEIVEN/MED AFRICA Foundation Grant USA, award number 01/2021/22/23.

Availability of data and materials

All data are available within the manuscript.

Declarations

Ethics approval and consent to participate

All experiments were carried out according to the institutional guidelines and were approved in writing by the Health Research Ethical Committee "CMUL/HREC/12/21/992" for studies involving animals.

Consent for publication

All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no conflict of interest. The funder had no role in the design of the study; in the collection, analysis, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

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Received: 17 May 2024 Accepted: 29 July 2024

Published online: 21 August 2024

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