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Electrospun Fibers with Lactobacillus Acidophilus: A Potential In Vitro Solution Against Gardnerella Infections

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ABSTRACT

Bacterial vaginosis (BV) is a prevalent condition, impacting a third of women globally. It's marked by reduced healthy lactobacilli and increased growth of certain anaerobes like Gardnerella. Even though antibiotics can be taken orally or applied vaginally to treat BV, about 50% of the patients will have the condition return in less than 6 months. High concentrations of Lactobacillus acidophilus have been linked to health benefits. Given the recurrence rates after treating BV, there's growing interest in using beneficial bacteria either as an alternative or as a supplementary treatment. This research wanted to demonstrate the potential for a new long-lasting delivery method for L. acidophilus. Results indicated that polyethylene oxide (PEO) fibers containing L. acidophilus can be combined with poly(lactic-co-glycolic acid) (PLGA) fibers in a 1:1 ratio. This structure subsequently releases L. acidophilus, which remains metabolically active, produces lactic acid, and can counteract Gardnerella. When these probiotic-rich fibers were consistently cultured in MRS broth and refreshed daily, they continued to produce active L. acidophilus for up to a week. The amount of lactic acid and associated pH measurements reflected the concentration of L. acidophilus from the fibers, underscoring their metabolic activity. In lab tests with vaginal cell cultures, the L. acidophilus fibers hindered Gardnerella growth in proportion to their dilution, showcasing their bacteria-killing capability. Introducing VK2/E6E7 cells to these L. acidophilus fibers showed only a slight reduction in cell viability compared to cells that weren't treated. In summary, the findings highlight the potential of using electrospun fibers as a viable means to administer vaginal probiotics in a durable format.

Keywords: Bacterial vaginosis, Nanofibers, Lactobacillus acidophilus, Gardnerella, Poly(lactic-co-glycolic acid), Polyethylene oxide

1 INTRODUCTION

Vaginal microbial environment plays a crucial role in upholding ideal sexual and reproductive health by preventing the invasion of harmful bacteria and viruses. In the late 1800s, German gynecologist Albert Doderlein extensively researched the vaginal flora. He determined that healthy flora primarily consisted of gram-positive bacilli, which were later recognized as the Lactobacillus genus. Modern research has shown that for a large portion of premenopausal women, lactobacilli make up 70–90% of the vaginal microbiota. The rest is primarily made up of both facultative and obligate anaerobes. Bacterial vaginosis (BV) is a condition marked by a decrease in Lactobacillus levels and a surge in anaerobes like Gardnerella [1]. Around a third of women globally suffer from BV. In the U.S., African-American and Mexican-American women are more susceptible compared to white women. While the common symptoms like altered vaginal discharge and smell might appear minor, BV has been linked to more serious health concerns [2,3]. Issues associated with BV include sexually transmitted infections, complications during early pregnancy, and preterm labor. While the root cause of BV remains unclear, research has shown that as the number of lactobacilli decreases, harmful bacteria multiply. Lactic acid, an organic acid produced by lactobacilli, lowers the vaginal pH, creating a more acidic environment that's unwelcoming for less acid-tolerant anaerobes. Lactobacilli also compete with other microbes for nutrients in the vaginal milieu, thereby reducing the viability of harmful species. Additionally, the production of enzymes like arginine deaminase further starves anaerobic bacteria of vital amino acids [4]. Presently, the primary treatments for BV include antibiotics like metronidazole,

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clindamycin, and tinidazole, which can be taken orally or applied vaginally [1]. Even though the indications of BV generally decrease during treatment, research indicates that fewer than half of the women maintain a long-term absence of BV, leading to a relapse in as many as 50% of women within half a year of treatment. To tackle this challenge, probiotics are being explored either as a substitute or supplementary approach. Specifically, Lactobacillus acidophilus is viewed as a species that reinforces the stability of the regular vaginal flora and is associated with beneficial sexual and reproductive results [5–7]. Much like antibiotics and other substances applied vaginally, there's a need for a long-lasting, user-friendly, and efficient delivery method for probiotics. Existing probiotic offerings come in the form of vaginal suppositories, tablets, and rings [8–10].

Vaginal probiotic tablets have been created, showing potential as a BV remedy when inserted vaginally daily for a week. However, one notable drawback of both suppositories and tablets is the need for daily or twice-daily applications, which can compromise convenience and possibly reduce patient adherence. There's an ongoing effort to produce dosage forms that offer prolonged probiotic release, aiming for a single application to deliver treatment locally for several days to weeks. One such method that has shown consistent release is a pod-intravaginal ring (IVR). This is made of a silicone ring housing "pods" of sodium carboxymethyl cellulose filled with probiotics. In lab tests, this pod-IVR released up to 1.4×108 colony forming units (CFUs) daily for 21 days. However, like other long-term release methods, IVR systems might be susceptible to yeast attachment and the development of bacterial biofilms [11, 12].

Electrospun fibers present a potentially viable alternative for dosage delivery. These fibers are produced by introducing a voltage to a droplet of a polymer solution, which establishes an electrical disparity between the polymer and a grounded receiver. This activity induces instability in the polymer blend, elongates the polymer, and facilitates the solvent to evaporate, leading to the gathering of fibers on the spindle. In terms of scaling up production, electrospinning presents a potentially economical method. It allows for the adjustment of fiber thickness, pore dimensions, and the type of polymer used. This flexibility facilitates the controlled release of active substances, possibly including live bacterial probiotics. Crucially, when it comes to delivering live cells, electrospun fibers have shown both chemical and mechanical resilience within a biocompatible framework [13]. Past research studies on female sexually transmitted diseases have utilized electrospun fibers infused with active substances to curb infections caused by human immunodeficiency 1 (HIV-1) and herpes simplex virus 2 (HSV-2) [14].

This research seeks to create new electrospun fiber formulations embedded with a high concentration of live vaginal bacteria (probiotics), aiming ultimately to rejuvenate vaginal lactobacillus populations and decrease the recurrence of BV. The hypothesis was that a fiber mesh structure, made up of an equal ratio of poly(lactic-co-glycolic acid) (PLGA) and polyethylene oxide (PEO), would facilitate the sustained presence of metabolically active bacteria for a minimum of 7 days when continuously cultured in a lab setting. Over this period, the production of D- and L-lactic acid and pH shifts were monitored to assess the bacteria's metabolic activity derived from the fibers. Additionally, the potential harmful effects on vaginal epithelial cells and the ability to counteract Gardnerella by the fibers loaded with L. acidophilus were investigated.

2 MATERIALS AND METHODS

2.1 Bacterial cultures

L. acidophilus was obtained from the American Type Culture Collection. L. acidophilus was cultivated using MRS broth and agar plates enhanced with Tween 80. On the other hand, Gardnerella was grown using New York City III (NYC III) broth and agar plates, which included 10% heat-inactivated horse serum. The NYC III agar plates were infused with 1 mg/mL streptomycin sulfate salt, enabling the selective cultivation of streptomycin-resistant Gardnerella.

Bacterial samples were prepared by spreading frozen stock onto a suitable agar plate with an inoculation loop. Both L. acidophilus and Gardnerella took about 24 hours of incubation before colony growth could be seen. When colonies became visible, one was picked from the plate and immersed in 1 mL of either MRS or NYC III broth inside a 1.5 mL microcentrifuge tube. Tubes housing L. acidophilus and Gardnerella were kept at 37 °C for another 24 hours. A correlation was established between OD600 and the respective CFU counts. One unit of OD600 for L. acidophilus and Gardnerella corresponded to 5.8×10^7 and 1.3×10^9 CFU/mL, respectively.

2.2 Vaginal epithelial cell cultures

The VK2/E6E7 cell line, which are immortalized vaginal epithelial cells, were looked after using base media enriched with various components including rh insulin, rh epidermal growth factor, L-glutamine, epinephrine, extract P™, hydrocortisone hemisuccinate, triiodothyronine, PS transferrin, phenol red, and gentamicin. After the cells were treated with trypsin, they were neutralized with a blend of Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 media (DMEM/F-12, mixed in equal parts) supplemented with 10% fetal bovine serum (FBS) and a dose of penicillin/streptomycin. For tests involving mammalian cells, the VK2/E6E7 cells were spun at 200 RCF for a duration of 5 minutes and then reintroduced into the aforementioned media, but without the addition of gentamicin. After quantifying the cells, they were allocated

into 48-well plates with a concentration of 25,000 cells in each well.

2.3 Electrospun nanofiber fabrication

To produce plain fibers, PLGA (at 15% weight by weight) was dissolved in 3 mL of hexafluoro-2-propanol (HFIP), and PEO (at 5% weight by weight) was dissolved in 3 mL of MRS broth, then left to incubate at 37 °C overnight. For fibers that incorporated probiotics, 150 mg of PEO by weight was mixed with 2.5 mL of MRS broth to create a PEO mixture. The bacterial density in the MRS solution was gauged using the Nanodrop 2000 by contrasting a diluted bacterial culture with a blank MRS. Upon determining the concentration, a specific volume containing the targeted bacterial quantity (5×10^7 CFU per mg for 150 mg PEO) was concentrated and re-dissolved in 0.5 mL of MRS after removing the remaining liquid. Right before the electrospinning process, this 0.5 mL of bacteria-rich MRS was blended with the PEO mixture. All equipment touching the fibers, such as needles, syringes, and the electrospinner spindle, were sterilized through autoclaving.

During the electrospinning process, two syringes filled with either PLGA or PEO were positioned in syringe pumps, set opposite to each other, with an 8 mm diameter mandrel situated in between them. The distances from the needle tips to the mandrel were 18 cm for the PLGA syringe and 15 cm for the PEO syringe. Positive electrical charges of 18 kV and 25 kV were applied to the PLGA and PEO syringe needles, respectively. Both syringes maintained a flow rate of 0.3 mL/hr, leading to a 1:1 fiber composition. As the polymers extruded and the voltages were applied, they both accumulated on the mandrel at the same time, forming the desired mesh structure.

2.4 Nanofiber morphology

Morphology of PLGA:PEO electrospun fibers (with and without probiotics incorporated) was evaluated using Field emission scanning electron microscopy (FE-SEM). Fiber samples were cut in approximately 3 mm square samples and placed on carbon tape. Samples were then sputter coated with a palladium gold alloy and imaged using FE-SEM (MIRA3 TESCAN, Czech Republic) using secondary electron detector with 2,500× magnification power and 25 kV.

2.5 Retrieving living L. acidophilus from fibers introduced into MRS medium

We aimed to examine the extraction of live bacteria from the electrospun fibers and their capacity to grow in MRS. Evaluating the potential for an extended release of metabolically active bacteria was crucial. To achieve this, we monitored the active bacterial recovery in consecutive daily cultures over a week. Here's how the process was carried out: Initially, 8–10 mg of fiber was positioned in 5 mL centrifuge tubes and then submerged in 5 mL of MRS broth. This mixture was incubated at 37 °C under anaerobic conditions. On each subsequent day for a week, 5 mL of the used MRS broth, which now housed the Lactobacillus, was carefully pipetted out and replenished with an equal volume of fresh MRS broth. This Lactobacillus-containing suspension was then systematically diluted and dabbed onto an MRS agar plate. After a 48-hour incubation at 37 °C, the number of CFUs was counted. This procedure was conducted using cultures from three distinct fibers. Each of these fibers was produced separately on different days. From each fiber, three samples were tested, and each of these samples had three technical replicates.

2.6 Quantification of pH changes in cultures of L. acidophilus-loaded naofibers

Daily evaluations of pH and lactic acid production were conducted in the consecutive fiber cultures in MRS at the aforementioned intervals. The pH was assessed in the clear supernatants extracted from the cultures at each designated time, using a pH meter with a precision up to 0.01 pH units. The levels of L- and D-lactic acid were ascertained using a lactic acid detection kit, which has a sensitivity threshold of 1.5 μg/mL.

2.7 Inhibition of Gardnerella growth by L. acidophilus-loaded nanofibers

Tests were conducted to determine how various dilutions of supernatant from cultured fibers loaded with L. acidophilus impacted the growth of Gardnerella, especially in the presence of VK2/E6E7 vaginal epithelial cells. Fibers cultured in MRS media contain both L. acidophilus and potential antibacterial substances. At the 24-hour and 7-day marks, these cultures were spun at 3000g, and the 5 mL of used MRS media was extracted. The sedimented bacteria were then reconstituted in 5 mL of antibiotic-free NYC III media and then systematically diluted, yielding 12 distinct dilutions. In a 48-well plate, where each well had roughly 90% grown VK2/E6E7 cells and 250 μL of antibiotic-free cell solution, 250 μL from each diluted fiber culture was added to three individual wells. This setup was incubated in a 5% CO2 environment at 37 °C for an hour, priming the VK2 cells to resist harmful bacteria. After this, 250 μL of free Gardnerella at a concentration of 1×10^6 CFU/mL was introduced to every well, followed by incubation in a 5% CO2 setting at 37 °C. Post 24 and 48-hour incubation, the supernatants were discarded and the cells rinsed twice with sterile 1X PBS. Each well was then treated with 1 mL of sterile water and kept at 37 °C in a 5% CO2 environment for 30 minutes to break down the cells. The resulting cell mixture from every well was then methodically diluted,

and 5 μL of each dilution was spread onto respective NYC III (containing 1 mg/mL streptomycin sulfate salt) and MRS agar plates. After being incubated in an anaerobic setting at 37 °C for 48 hours, the colony forming units were counted for every dilution, leading to the calculation of CFUs/mL.

All control samples were grown alongside fully grown VK2/E6E7 cells and in 250 μL of antibiotic-free NYC III media. The control groups consisted of free L. acidophilus at a concentration of 5×10^7 CFU/mL combined with Gardnerella at 1×10^6 CFU/mL, and Gardnerella at 1×10^6 CFU/mL by itself. A blank mesh fiber, which didn't contain any bacteria, was produced and its supernatant served as a negative control (with the expectation that it wouldn't affect Gardnerella's growth). Additionally, free Gardnerella at a concentration of 1×10^6 CFU/mL was used as a control measure. For each experiment, fibers from three separate batches were examined, and every batch consisted of three technical duplicates. To illustrate the capacity of the L. acidophilus -infused fibers to surpass the growth of Gardnerella, the methods used in the inhibition test were employed. The difference, however, was that varied dilutions of the supernatant, which contains L. acidophilus from the cultured fibers embedded with L. acidophilus, were combined with Gardnerella and VK2/E6E7 cells without an initial defensive priming step. The controls remained consistent with those used in the inhibition test.

2.8 *VK2/E6E7 viability*

The MTT assay, which employs the chemical 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, was utilized to evaluate the cytotoxic effects of fibers infused with L. acidophilus. VK2 vaginal epithelial cells were arranged at a concentration of 300,000 cells per well in a 12-well plate with an antibiotic-free medium. They were then incubated for 24 hours at 37 °C with 5% CO2, resulting in roughly 50% cell coverage. Both blank and fibers loaded with L. acidophilus (weighing 2.5 mg) were introduced to cells set in triplicate and left to incubate under the same conditions for durations of 24 and 72 hours. Cells only treated with media served as a negative control, while those treated with 10% DMSO acted as the positive control. Post the 24 and 72-hour incubation periods, 100 μL of the MTT agent was added to every well and incubated under the same conditions for 4 hours. After this, 550 μL of a lysis solution was added and the plates were left to incubate overnight. The absorbance levels were measured at 570 nm using the SYNERGY Microplate Reader by Biotek Instruments Inc and were then adjusted to match the absorbance of cells that hadn't been treated.

2.9 *LDH release assay*

The integrity of the cell membrane was assessed by detecting the release of the internal enzyme called lactate dehydrogenase (LDH). The amount of LDH in VK2 epithelial cell supernatants was determined using the CytoTox96® non-radioactive cytotoxicity test, following the guidelines provided by the manufacturer. VK2/E6E7 cells were spread at a concentration of 300,000 cells per milliliter in each well of a 12-well flat-bottomed plate. This setup was then incubated at 37 °C and 5% CO2 for 24 hours. Both empty and L. acidophilus-loaded fibers were introduced to the cell samples arranged in triplicate, and left to incubate for both 24 and 72 hours at 37 °C in a 5% CO2 environment. From the treated cell samples, 50 microliters of the supernatant were mixed with the LDH substrate provided in the kit and left to sit at room temperature for 30 minutes. The reactions were halted by adding 50 μL of a stop solution. LDH activity was then assessed by checking the optical density of the mixture at 490 nm. As reference points, cells treated with 1 ng of staurosporine served as the positive control, while those treated only with medium were the negative control. The release of LDH was quantified by comparing the absorbance of each test sample to the negative control, determining it as a fold change.

2.10 *Statistical analyses*

All experimental sample comparisons were statistically analyzed using a one-way ANOVA, paired with the Tukey's multiple comparison test (with a significance level of $p \leq 0.05$). This analysis was conducted using GraphPad Prism software, version 9.3.1.

3 RESULTS

3.1 *Fiber morphology*

Figure 1 displays the FE-SEM photographs of both empty and probiotic-infused PLGA:PEO mesh fibers. Both types of fibers, whether they contained bacteria or not, consisted of 15% w/v PLGA in HFIP and 5% w/v PEO in MRS broth. Beading was absent in both fibers due to the previously fine-tuned electrospinning conditions. The average thickness of the empty PLGA and PEO fibers were 1.84 $\pm$ ondingly. When L. acidophilus was added to the fibers, the average thicknesses were 1.81 $\pm$ 0.32 μm for PLGA and 339 $\pm$ 114 nm for PEO.

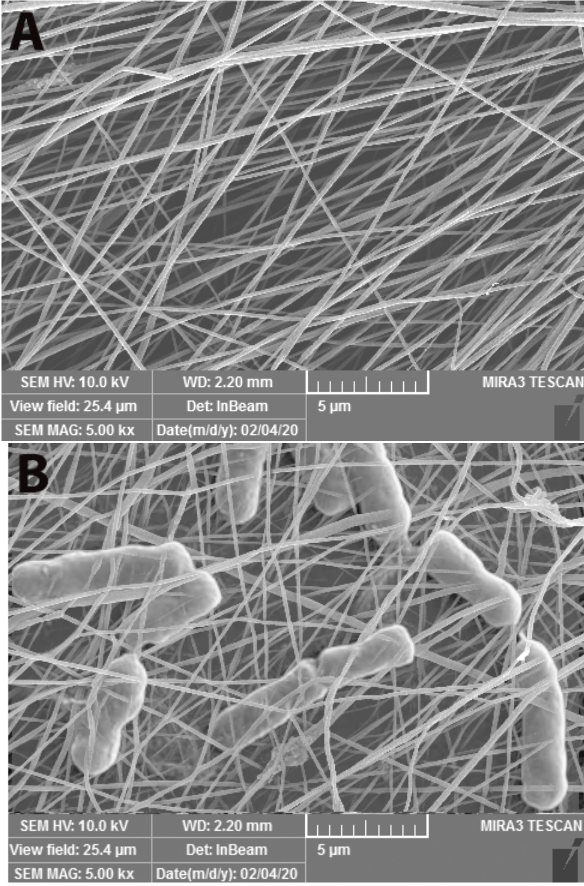
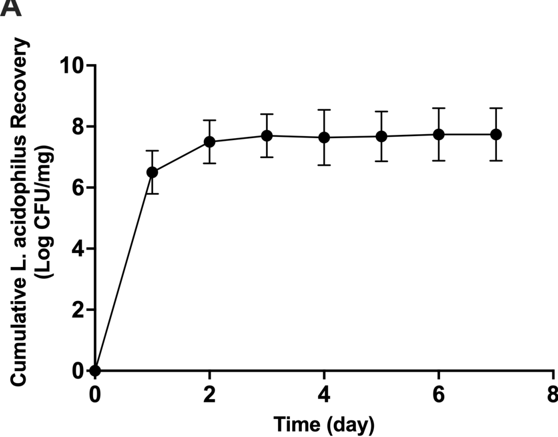


Fig. 1. FE-SEM micrographs of the electrospun nanofibers. On the left, we see empty fibers, while on the right, fibers loaded with L. acidophilus made of a 1:1 ratio of PEO:PLGA are presented. The presence of rod-shaped bacteria, marked in yellow, indicates bacterial inclusion. A scale bar indicates 5 µm. Images for both types of fibers were taken from the side facing away from the mandrel, making it possible to compare their consistent structures.



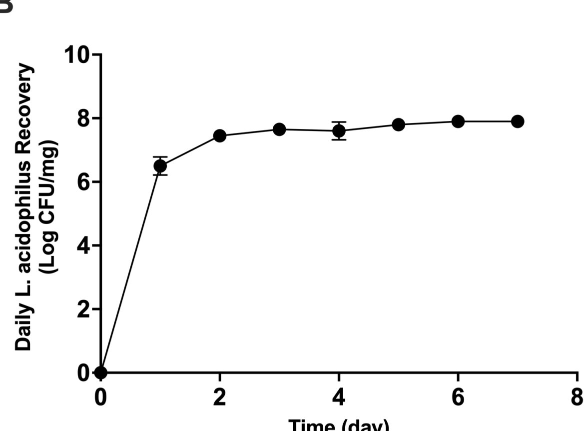


Fig. 2. Illustrates the CFU counts of L. acidophilus obtained from the mesh electrospun fibers each day for a week. (A) Within 24 hours, the fiber mesh containing the probiotic showcased a cumulative viable count of L. acidophilus reaching 10.11 × 106 CFU/mg. (B) For six successive days, L. acidophilus was cultured from probiotic-infused fibers at comparable or even greater levels. The average CFU counts plus or minus the standard deviation, obtained from the cultured fibers, are displayed. These counts are based on three separate fiber batch tests, and each of these batches was replicated technically three times.

3.2 *CFU of L. acidophilus cultured from mesh electrospun fibers*

Fibers, both empty and filled with probiotics, were created using equal parts of PLGA and PEO in a mesh fiber design. They were then cultivated in MRS media to retrieve living L. acidophilus. Every day for a week, the liquid above the settled fiber (supernatant) was gathered, diluted in series, and then spread on plates for analysis. At every interval, the leftover fiber material was rinsed and immersed in fresh media. L. acidophilus remained viable and was retrievable at each of these intervals for a span of 7 days, as shown in Fig.2. The daily evaluations of the amount of probiotics showed a total recovery of 1.45×10^9 CFU/mg after a week (Fig.2a), and an average daily retrieval rate of 2.1×10^8 CFU/mg throughout the 7-day period (Fig.2b).

3.3 *Electrospun fibers allow for the recovery of metabolically active L. acidophilus*

Both empty and fibers loaded with probiotics were introduced to MRS media to extract living bacteria. The cultures were then assessed daily for pH changes over a span of one week (as shown in Fig.3). The pH readings of cultures post-fiber incubation in MRS medium were notably reduced on days 1 (pH 5.95; $P < 0.05$) and 7 (pH 4.14; $P < 0.001$) of the serial culture in comparison to the initial pH of the medium (t = 0, pH 6.5). The decline in pH is in sync with the noted viable extraction of L. acidophilus, with the most significant drop in pH observed

between the first and second day.

Figure 3B presents the total production of D- and L-lactic acid from fibers infused with L. acidophilus. This outcome aligns with the significant retrieval of live L. acidophilus and the associated pH measurements. Over a period of 7 days, the serial cultures of these L. acidophilus -laden mesh fibers generated roughly 165 mM and 268 mM of D- and L-lactic acid per mg of fibers, respectively. In the cultures derived from the empty fibers, lactic acid was not detected.

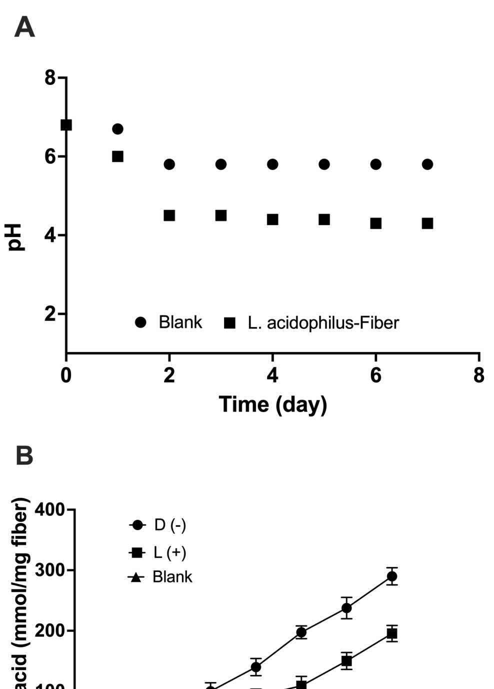
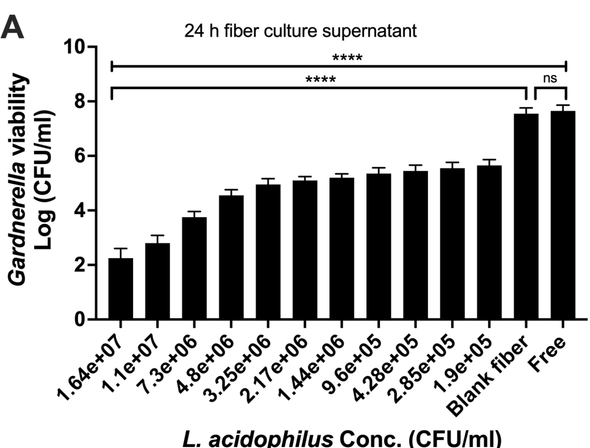


Fig. 3. Illustrates the pH and lactic acid content in the supernatant from fibers grown in MRS. The data is represented as the average ± standard deviation, based on three distinct fiber batches. Each of these batches was technically replicated thrice. (A) The pH of fiber cultures dipped to its minimum by the 4th day, and the residual fiber material kept producing acid-rich Lactobacillus cultures up to, at the very least, day 7. (B) The quantity of lactic acid correlated with the visible revival of L. acidophilus when the fibers were cultured in MRS solution.

3.4 Study of mesh fibers infused with L. acidophilus in a culture with Gardnerella and VK2/E6E7 vaginal Cells

We then investigated the influence of L. acidophilus sourced from fibers on the growth of Gardnerella in a lab-based setting alongside the VK2/E6E7 vaginal cell line. In summary, suspensions of L. acidophilus obtained from these fibers were gathered, diluted, and then introduced to VK2 cells as outlined in the experimental methods. Concurrently, or an hour later, 106 Gardnerella were incorporated into each well. The continual cultivation of the fibers infused with L. acidophilus underwent 12 consecutive dilutions, leading to free L. acidophilus concentrations ranging between roughly 105 and 107, as verified through parallel CFU counts. The viability of Gardnerella was assessed after a 24-hour incubation period alongside L. acidophilus-infused fiber cultures in the company of VK2 cells (Fig.4).



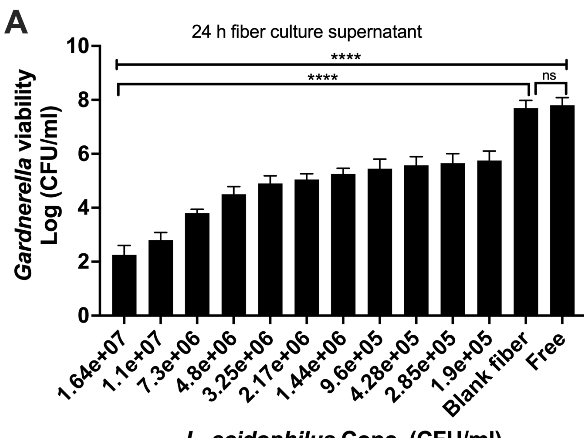
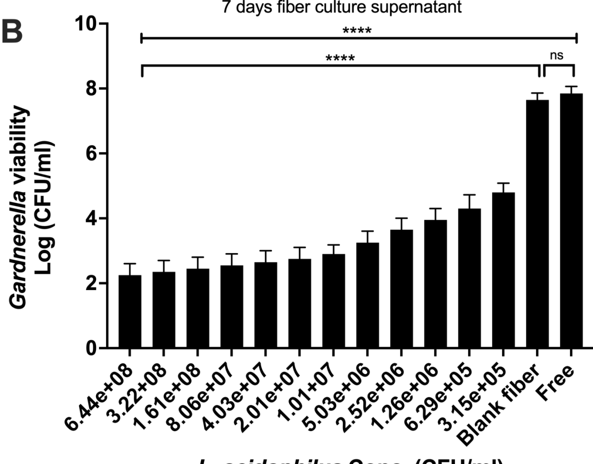
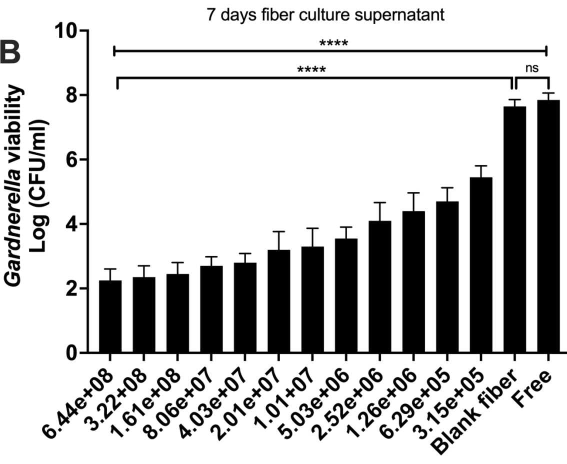


Fig. 4. Viability of Gardnerella in the presence of L. acidophilus from electrospun fibers. The figure depicts the effects of 24-hour and 7-day culture supernatants (with varied dilutions obtained from fiber incubation in MRS media) post 24-hour incubation with VK2 cells that were either (A) pre-conditioned for defensive protection or (B) not pre-conditioned against infections (as detailed in Methods). The logarithmic (CFU/mL) values were calculated based on the average and standard deviation from three separate batches of L. acidophilus-infused fibers. (****p $\leq$ 0.0001).





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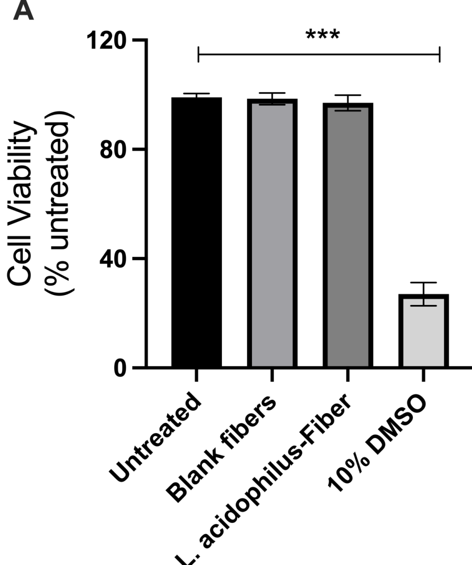
When L. acidophilus derived from 24-hour fiber supernatants was introduced to VK2 cells pre-conditioned for defense against infections, there was a noted decrease in Gardnerella viability compared to the standalone Gardnerella control after a 24-hour incubation (Fig.4a).

Specifically, a 1.9-log drop in Gardnerella's viability was observed when paired with the lowest L. acidophilus concentration from the fiber cultures, which was 1.95 × 105 CFU/mL. With the highest concentration, 1.64 × 107 CFU/mL, a 6.4-log decrease in Gardnerella viability was seen. In the same vein, continuous cultures of fibers infused with L. acidophilus that underwent 12 consecutive dilutions with free L. acidophilus concentrations between 105-108 CFU/mL from a 7-day fiber culture supernatant halted G. vaginalis growth after a day's incubation. The weakest concentration after the 12th dilution, 3.15 × 105 CFU/mL, led to a significant ($p \leq 0.0001$) 3.0-log decline in G. vaginalis viability compared to the control G.vaginalis, while the most potent concentration, 6.65 × 108 CFU/mL, caused a 6.7-log reduction.

However, when L. acidophilus was introduced to VK2 cells that were not pre-conditioned against infections (Fig. 4b), various dilutions of the 24-hour supernatant from cultured fibers significantly suppressed Gardnerella growth within 24 hours, recording 1.7- and 5.7-log reductions against the standalone Gardnerella control at the lowest and most potent supernatant concentrations from cultured fibers, respectively. Similarly, the 7-day continuous cultures of fibers had a pronounced effect ($p \leq 0.0001$) on Gardnerella growth. The observed reductions in Gardnerella viability when paired with the weakest and strongest concentrations of the 7-day supernatant from cultured fibers were 2.9-log and 7.6-log, respectively.

3.5 Testing fiber cytotoxicity on vaginal epithelial cells

The health of VK2 cells was examined post-application of both blank and L. acidophilus-infused fibers for 24 and 72 hours (the longest duration VK2 cells sustain without a media change). As depicted in Fig. 5a, both blank and L. acidophilus-infused fibers caused little decrease in cell health compared to untreated cells, indicating the fibers' safety for up to 3 days. Conversely, cells exposed to 10% DMSO experienced a notable decline in viability compared to controls (****$p \leq 0.0001$).



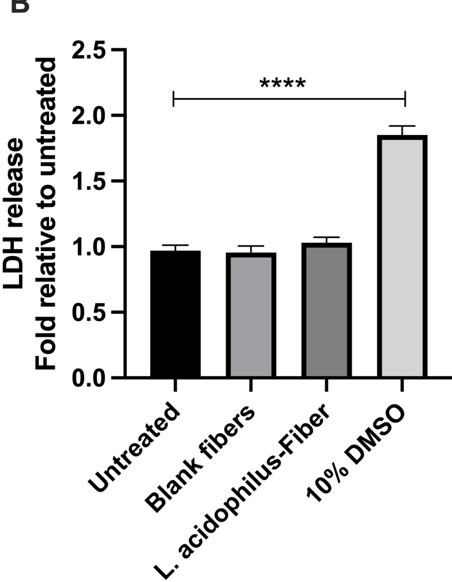


Fig. 5. Assessment of L. acidophilus-infused fibers' impact on vaginal keratinocytes (VK2/E6E7 cells). (A) Minimal cytotoxicity (measured as cell viability percentage against untreated cells) was detected in VK2/E6E7 cells through the MTT assay. (B) LDH release remained insignificant in VK2/E6E7 cells exposed to either blank or L. acidophilus-infused fibers over 24 hours, when compared to untreated cells. Conversely, staurosporine-treated cells displayed a substantial rise in LDH compared to those not treated. Data is displayed as average ± standard deviation (n = 5). Differences between experimental sets, evaluated using one-way ANOVA (Tukey test), are marked by ****$p \leq 0.0001$.

3.6 *Lactate dehydrogenase emission test*

To gauge cell membrane stability, the discharge of the intracellular enzyme LDH was examined from VK2 cells exposed to both blank and L. acidophilus-infused fibers for a day. After 24 and 72 hr incubation, VK2 cells that encountered either blank or L. acidophilus-containing fibers exhibited minimal LDH emission in comparison to the untouched control cells (Fig. 5b). Predictably, staurosporine, a strong cytotoxic substance, triggered a substantial LDH discharge, noticeably higher (****$p \leq 0.0001$) than the control cells.

4 DISCUSSION

The vaginal microbiome's composition, influenced by BV, is associated with outcomes like sexually transmitted diseases and complications during pregnancy. Although antibiotics effectively target harmful bacteria in approximately 80% of cases, over half of the women suffer from BV's recurrence within six months after treatment. In response to these challenges, the use of probiotics like L. acidophilus has emerged as a potential solution. They aim to reestablish and sustain a balanced vaginal setting, primarily by producing lactic acid and subsequently lowering the vaginal pH (3). Various intravaginal (topical) probiotic formats, including vaginal tablets, gels, suppositories, and rings, have been designed as potential substitutes for existing antibiotic treatments. The creation of new delivery methods for L. acidophilus aims to counter negative health effects linked to diminished vaginal Lactobacillus levels.

Electrospun fibers made of polymers are gaining attention as a novel means for delivering active substances intravaginally. These fibers' flexibility in terms of polymer composition, diameter, and porosity makes them ideal for boosting the lifespan and growth of probiotics. They also appear to be a promising method for prolonged-release, similar to films but with the potential for weeks to months of delivery. Such a long-lasting release could encourage consistent use and reduce relapse chances. This research proposed that combining L. acidophilus with mesh fibers, which use both water-attracting and water-repelling polymers, could offer a platform for probiotic growth, stability, and extended availability. The fiber design aimed for prolonged delivery of L. acidophilus for BV treatment. The structural integration and form of L. acidophilus in these fibers were inspected using SEM compared to control fibers (Fig.1). After a week, the probiotic's total recovery rate was 109 CFU/mg, with a consistent daily recovery rate of over 108 CFU/mg (Fig.2). Such findings indicate the possibility of achieving the levels found in healthy vaginal samples, potentially addressing BV at a rate of 108 CFU/day.

Prior studies demonstrated that when the probiotic L. gasseri was integrated into a silicon device (IVR), it maintained a consistent recovery of 1.1 to 14 $\times$ 107 CFU daily over 21 days (10). In this case, L. acidophilus was extracted and cultivated in a culture medium, leading to increased bacterial counts in laboratory tests. When trying to retrieve and measure the bacteria in media without

nutrients, live L. acidophilus was not found (details not provided). Looking ahead, improving bacterial lifespan during fiber creation could enhance the delivery of the probiotic. For instance, designing a mesh fiber structure might allow probiotics located in water-attracting sections of the fibers to be more accessible on the exterior, rather than being trapped within the PLGA fibers. Thus, a more extensive examination of fiber makeup is anticipated to offer additional understanding.

The lactic acid levels and the pH of the cultured fiber's supernatant were measured because of their recognized antibacterial properties. Data indicates a decline from an initial pH level of 6.5 at the beginning to a pH of 4.1 by day 7 (Fig.3a), which aligns with the pH range for a healthy vagina. Furthermore, by the 7th day, the cultured fibers consistently generated significant quantities of both D- and L- lactic acid. The lactic acid produced (Fig.3B) is similar to the concentrations known to counteract BV [15, 16]. Fascinatingly, fibers loaded with L. acidophilus generated a greater quantity of D-lactic acid than L-lactic acid. It's well-documented that L. acidophilus typically produces more of the D-lactic acid isomer compared to other lactobacilli species. Earlier research has revealed that mammalian cells primarily produce L-lactic acid, suggesting that the dominant source of D-lactic acid comes from probiotics [17–19].

The ability of L. acidophilus-infused fibers to prevent and compete against the viability of Gardnerella was tested in the lab using a VK2 co-culture procedure. Supernatants from cultures of L. acidophilus-infused fibers, taken at 24 hours and 7 days, reduced the viability of Gardnerella by a minimum of 3-log after 24 hours, showing their potential to kill bacteria. To determine if there was a concentration-related effect on prevention, twelve different dilutions of the cultured fiber supernatant were examined. Results based on concentration revealed that after a 24-hour incubation with VK2 cells that were already set up to defend against infection, the supernatants from L. acidophilus-infused fibers reduced Gardnerella growth more than in situations where cells weren't pre-prepared for defense (Fig.4). This suggests that the initial presence of L. acidophilus in the VK2 culture may lead to lactic acid production which reduces the pH, creating an environment where Gardnerella cannot thrive. In general, these findings align with past research indicating that probiotics can restrict the growth of Gardnerella [20–22]. Finally, the safety of the L. acidophilus-infused PLGA:PEO mesh fibers was assessed after exposing VK2 cells to them for 24 and 72 hours. The results indicated no harmful effects when compared to cells that weren't treated (Fig.5).

5 CONCLUSION

This research assessed electrospun fibers loaded with L. acidophilus for the delivery of live lactobacilli that produce lactic acid and compete against Gardnerella. The findings underscore the promise of these L. acidophilus-infused fibers as a safe method for BV therapy. Electrospun fibers crafted from FDA-sanctioned polymers, namely PLGA and PEO, are capable of consistent probiotic recovery and production of lactic acid and hydrogen peroxide. One limitation of this preliminary study is its focus on a single probiotic strain in its tests against one type of anaerobic bacteria. Further studies could probe the potential harmful effects on vaginal epithelial cells by introducing other relevant cells, like immune cells and cultured epithelia. Additionally, understanding cellular reactions, such as the release of cytokines post probiotic introduction, remains crucial. In essence, the current methodology establishes foundational knowledge for the examination of the in vitro impact of mesh fibers for vaginal use. Subsequent research will delve into enhancing the lifespan of L. acidophilus, minimizing material usage, and ensuring its safety for human application. Achieving these goals will necessitate detailed probiotic delivery examinations in animal models for BV.

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