PROBIOTICS APPLICATION FOR POTENTIAL FEMININE HYGIENE PRODUCTS

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The aim of the present research was to develop probiotic delivery systems intended for short-term application in feminine hygiene products. For this, freeze-dried and fresh probiotics (*Lactobacillus paragasseri* K7), encapsulated into hydroxy-β-cyclodextrins, with and without inulin used as a prebiotic, were at first electrospun onto inert polypropylene carrier fabrics, in order to establish the optimal spinning conditions and confirm the successful formation of fibers. The characteristics of the functionalized materials were analyzed by Fourier transform infrared spectroscopy (ATR-FTIR), X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM). In order to evaluate the functionality of the probiotic delivery systems, in the subsequent stage of the research, the optimum electrospinning formulation was deposited, under the defined optimal conditions, on a different carrier material, namely, a cellulose-based cotton stripe, to get a preliminary demonstration of the suitability of the developed material for its intended application as a feminine hygiene product. For this, the antioxidant properties of the materials and the probiotic release were observed. Experimental results confirmed that the material (cellulose-based cotton stripe/deposited nanofiber) possessed antioxidant properties and released probiotics within 4 hours, being in agreement with the recommended use of such products. This preliminary research underscores the potential usability and applicability of the developed material for tampon use, considering its anti-inflammatory property and beneficial effects in maintaining healthy vaginal microbiota.

Keywords: probiotics, electrospinning, cyclodextrins, inulin, SEM, XPS

INTRODUCTION

In recent decades, there has been significant progress in the development of novel drug delivery systems.[1] These advanced systems hold great promise for enhancing the efficacy of existing antimicrobial agents.[2] One notable example is the use of electrospun nanofibers, which can trigger the rapid release of specific drugs.[3] These nanofibers possess a porous structure with a large surface area and small intrafibrous pore size, offering a novel approach to delivering bioactive compounds.[4] This unique structure also provides a platform for influencing cell behavior.

Despite these advancements, the incorporation of bioprotective substances, such as probiotics, in the drug delivery process remains an underexplored area.[5] Further research is needed to better understand how the electrospinning process, bioprotective substances, and environmental factors impact the viability and functionality of vaginal probiotics.[6]

Probiotics are live microorganisms with potential health benefits, when administered in sufficient amounts, have garnered research attention.[7] However, their effectiveness depends on factors, such as route of administration, probiotic strain, and duration of use. Careful selection of components and production processes is crucial, as they can influence probiotic viability.[8] Probiotics have demonstrated positive effects in addressing vulvovaginal candidiasis, with specific strains like *Lactobacillus*

paragasseri K7, showing potential in maintaining gut homeostasis, regulating the immune system, and preventing various infections.[9,10]

The application of probiotics extends beyond internal health. Probiotics play a role in the development of sanitary materials, such as tampons and pads, contributing to the maintenance of physiological pH in the vaginal mucosa.[11] Additionally, probiotic-loaded nanomaterials, including those electrospun, show promise in the preventive treatment of conditions like diabetic foot, where accelerated wound healing and cell proliferation are crucial.[12] These approaches underscore the importance of advanced drug delivery systems and probiotics in improving health outcomes.

Polymeric nanofiber-immobilized *Lactobacillus rhamnosus* CRL1332 has been included in vaginal probiotic products for prevention or treatment of urogenital infections.[13] A high number of viable cells (log CFU/g nanofibers > 9.5) was obtained in the nanofibers after electrospinning. The viability of the lactobacilli after electrospinning on corn starch and sodium alginate was 94.1% of the original population.[14] The probiotic, antagonistic, antioxidant and immunomodulatory potential of isolated lactic acid bacteria (LAB) was also reported.[15,16] Moreover, prebiotics have been reported to offer some unique advantages, such as enhancing probiotics activity.[17] Thus, researchers found that the addition of prebiotic inulin improved the mucoadhesive properties of microcapsules.[17]

The vaginal microbiota plays an important role in the impairment of colonization by pathogens, which cause infections.[18] Considering this, probiotic products containing vaginal lactobacilli represent a novel strategy to restore the vaginal microbiota and thus treat infections.[19] It has been found that probiotics may have a positive effect in the treatment of bacterial vaginosis.[20]

In this work, the potential of nozzle-free electrospinning technology for the development of probiotic delivery systems tailored for short-term applications, such as feminine hygiene products, has been tested. A forward-looking approach involves enhancing these systems by introducing prebiotics into the nanocoating to encapsulate probiotics, thereby creating symbiotic agents, representing a promising avenue for future development.[21] To explore this direction, in this study, inulin, a prebiotic, was incorporated to support the growth and stability of *Lactobacillus paragasseri* K7. The bacterial release was monitored over a short timeframe, aligning with the recommended use of products, such as tampons, for a maximum of 4 hours, with practical changes occurring every 2 hours. Further, the electrospun samples deemed optimal were subjected to comprehensive characterization using advanced techniques, such as scanning electron microscopy (SEM), attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR), and X-ray photoelectron spectroscopy (XPS) measurements. This multifaceted analysis contributes to a thorough understanding of the structural and chemical characteristics of the developed probiotic delivery systems, paving the way for advancements in short-term applications, particularly in feminine hygiene materials.

EXPERIMENTAL

Materials

The standard solution used for electrospinning consisted in 10% hydroxy-β-cyclodextrin solution (Sigma-Aldrich, Darmstadt, Germany). Polyethylene oxide (PEO; Sigma-Aldrich, Darmstadt, Germany) 5% solution was prepared to adjust viscosity. PEO served as a medium for viscosity adjustment of the cyclodextrin solution.

The *Lactobacillus paragasseri* K7 (ZIM 105, CCM 7710) strain was prepared and applied in lyophilized (LP) and fresh (FP) forms. The probiotic originated from faces of one-week-old breastfed baby and was described previously.[10,22]

Inulin (100% inulin fibers, Fmed.eu, Maribor, Slovenia) was added into the probiotic to gain a prebiotic effect. In this form, it stimulates the growth of beneficial bacteria.[23]

Encapsulation of *L. paragasseri* was performed by adding lyophilized cells into 20 mL of PEO and 10 mL of cyclodextrin solution. In the next step, the same solution was prepared, and 3.3 g of inulin was added. In the third step, the same procedure was applied for solution preparation, but lyophilized probiotics were replaced by fresh probiotics.

ABTS reagent (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma-Aldrich, Darmstadt, Germany) was used to prepare the solution for determining the antioxidant activity.

Cellulose tampon stripes produced by Tosama, Domžale, Slovenia, were used as support for electrospinning under optimized conditions, for testing the probiotic release. The optimum electrospinning conditions were determined using a polypropylene support. The polypropylene was a standard Pegatex® S non-woven base material, kindly provided by Pegas Nonwovens (Znojmo, Czech Republic) in the form of 100% polypropylene (PP) fiber mesh.

Electrospinning

Electrospinning was performed with a NanoSpider NS LAB 500 (Elmarco, Liberec, Czech Republic) via the needle-free technique. The bathtub filled with the polymer solution (30 mL), containing the spinning electrode, was placed in the device. The polypropylene (PP) material (30 cm x 21 cm) was placed on the upper rounded collecting electrode and used as a support for collecting the formed fibers.

The solution samples for electrospinning were characterized in terms of conductivity, pH, viscosity, and surface tension. The following equipment was used for these tests: an HPC 227K conductivity meter (Mettler Toledo, Columbus, OH, United States), a SevenCompact pH meter (Metler Toledo, Columbus, OH, United States), a rotation and capillary rheometer (Fungilab, Barcelona, Spain), and a Tensiometer K12 (Krüss GmbH, Hamburg, Germany).

Besides the physical properties of the polymer solutions, environmental and technological parameters may also influence the formation of electrospun fibers. Hence, the optimization of the electrospinning procedure was performed, varying the processing parameters, such as voltage (U) and distance between electrodes (d), as well as the environmental conditions, such as temperature (T) and relative humidity (RH).

Samples

Table 1 presents the nomenclature for the functionalized electrospun samples. A polypropylene mesh was used as support material to understand the formation of fibers by electrospinning and study their characteristics. Cellulose tampon stripes (TFCS) were used for the final application of these electrospun fibers, *i.e.*, to produce a cellulose support for a fiber network to achieve probiotic delivery.

Table 1
Denotation of samples

Denotation	Meaning
FFPS	Electrospun β-cyclodextrin solution containing fresh probiotic on PP mesh
FPS	Electrospun β-cyclodextrin solution containing lyophilized probiotic on PP mesh
FPIS	Electrospun β-cyclodextrin solution containing lyophilized probiotic and inulin on PP mesh
TFCS	Electrospun β-cyclodextrin solution containing lyophilized probiotic and inulin on cellulose tampon stripe

Characterisation

A Perkin Elmer Spectrum GX NIR-FT ATR-FTIR spectrophotometer was used to record the spectra of the samples from 4000 to 600 cm^{-1}. Resolution was set at 4 cm^{-1} and spectral acquisition was 1 cm^{-1}. A total of 16 scans were performed per sample.

The morphology of electrospun samples was observed by a FE-SEM Supra VP 35 (C. Zeiss AG, Germany). The dry sample was attached to an aluminium carrier, using a conductive carbon stripe. Palladium (Pd) was used for improving conductivity. Prior to placing the sample into the apparatus, it was blown with nitrogen for preventing contamination. The applied voltage was 1 keV, with variable working distance and 30 μm aperture.

Quantitative surface chemical composition of electrospun samples was determined by X-ray photoelectron spectroscopy (XPS), using an XPS spectrometer PHI-TFA 5600 XPS (Physical Electronics Inc., USA). The XPS spectrometer irradiated the sample with monochromatic X-ray light and the characteristic peaks for the elements present on the sample surface to a depth of about 6 nm were recorded. The base pressure in the XPS was 6 x 10^{-8} Pa.

The antioxidative potential was determined indirectly by using the ABTS radical cation.[24] The method of antiradical activities is based on the reduction of the ABTS•+ radical, which is analyzed spectrophotometrically at a wavelength of 734 nm (Shimadzu UV-1800 Spectrophotometer (UV-VIS)). To produce a solution of ABTS•+, 2 mL of potassium persulfate solution was added to an ABTS stock solution (98 mL) and this radical solution was kept in a stoppered flask in the dark at room temperature for 12–16 h before use in actual measurements. A sample solution (10 μL) was added into 200 μL of ABTS•+ reaction solution, and then the mixture was incubated for 60 min at room temperature in the dark. An equivalent volume of phosphate buffer (50 mM, pH 7.4) was used as the control sample. The antioxidant capacity can be determined by the decrease of absorption at the wavelength of 734 nm. The change in absorbance was measured. The inhibition (*Inh*, %) was calculated relative to the absorbance of the control sample (*A*c) at 734 nm, according to Equation (1):

$$Inh = \frac{Ac - As}{Ac} \cdot 100 \quad (1)$$

where *A*s is the absorbance of the remaining concentration of ABTS• in the presence of the material sample.

The release of lyophilized and fresh probiotics from the fibrous material, with and without inulin, was studied. To determine the number of embedded and alive bacteria in the electrospun product at the initial stage, a sample (64 cm^2) was placed in a sterile plastic vessel immediately after electrospinning and vigorously vortexed. After serial dilutions in PBS buffer (pH 7.4), the sample was spread onto MRS agar (Fluka, Germany) and incubated at 37 °C for 48 hours

in an anaerobic atmosphere, before counting CFU/mL. The spontaneous release of bacteria from the same size of the electrospun material was assessed after incubating the sample in the buffer PBS (pH 7.4) for 2, 4, and 24 h. The probiotic release (%) was expressed as the ratio between the CFU counts determined after each sampling time and the CFU counts at the initial stage (at 0 h) multiplied by 100. Three replicates of each sample were measured.

RESULTS AND DISCUSSION

Optimization of electrospinning

The optimal parameters were determined as 21 ±1 °C, 45 ±2% humidity, 50 kV, and 0.040 ±0.005 mA, at a constant distance between the electrodes of 210 mm. The solutions prepared for electrospinning at the above-mentioned optimal parameters were subjected to conductivity, viscosity and surface tension measurements and the results are listed in Table 2. Figure 1 presents cellulose tampon stripes used as support for electrospun lyophilized probiotics. The electrospinning of the TFCS sample – on the cellulose support – (Fig. 1) took longer, around 90 min, because of the thicker tampon tape, but the fibers were thinner compared with the other samples deposited on the PP reference material.

Basic physical properties of the liquid formulations for electrospinning are presented in Table 2. The values represent an average of three measurements, with standard deviations. Comparing FPS with FPIS solutions shows that viscosity increased with the addition of inulin, which is in accordance with the results reported in another study.[23] Inulins are a group of naturally occurring polysaccharides, with quite long macromolecules, which increase solution viscosity. Consequently, the firmness of the material also increased.

Table 2
Conductivity (σ), viscosity (η) and surface tension (γ) of liquid formulations used for electrospinning

Polymer solution used in electrospinning	σ (uS/cm)	η (mPas)	γ (mN/m)
FPS	1935 ± 5	516.7 ± 6.2	53.9 ± 1.2
FPIS	1451 ± 5	539.1 ± 5.8	54.6 ± 2.1
FFPS	994 ± 5	441.5 ± 6.2	53.2 ± 2.1

Figure 1: Cellulose tampon stripe with electrospun lyophilized probiotic and inulin (TFCS)

Results of characterisation

The SEM micrographs of the formed nanofibrous structure are presented in Figure 2 (a-d). Figure 2 (a) presents the polypropylene (PP) mesh used as support for electrospinning of the nanofibers. The fiber diameter was measured to be about 31 μm and the fiber surface was rough. The presence of microscopic particles was observed, which can be formed during the production of PP fibers. When comparing the electrospun samples, it may be noted that sample FPS (Fig. 2 (b)) produced better nanofibers, compared with FPIS (Fig. 2 (c)). In the case of FPIS, a surface film was formed on the PP support, while FPS formed thin nanofibers, possessing a diameter of 0.5 μm. In contrast to FPS, some larger spots were observed in the micrograph of FPIS (Fig. 2 (c)), which probably represent probiotic deposition. Due to the generation of a surface film structure after electrospinning FPIS, it was not possible to determine the fiber diameter. Since a probiotic is

much smaller in size, it was not observed, however, it could be covered by the fibers or entrapped into the porous structure of the thin film.

FFPS (Fig. 2 (d)) formed nanofibers with clearly defined individual beads, unlike FPIS (Fig. 2 (c)). The diameter of FFPS nanofibers was around 0.4 μm and a smooth fiber surface is observed. The fiber diameter is affected by electrical voltage.[24] As, in our study, it was maintained constant, the diameters of the nanofibers were comparable. However, the probiotic addition can also change the diameter significantly.[6,13] When inulin was added to the PEO solution containing the probiotic, the coating effect occurred during the spinning process. Also, a decrease of electrical conductivity was noticed for FPIS, compared to FPS. The conductivity decreased from 1935 μS/cm to 1451 μS/cm, which consequently, led to the formation of the macromolecular film. Higher values of conductivity caused the jet to be stretched, having a positive effect on the nanofiber formation, while lower conductivity obstructed nanofiber formation. A similar situation was reported in another study, where a film was obtained by electrospinning PA solution with inulin.[26]

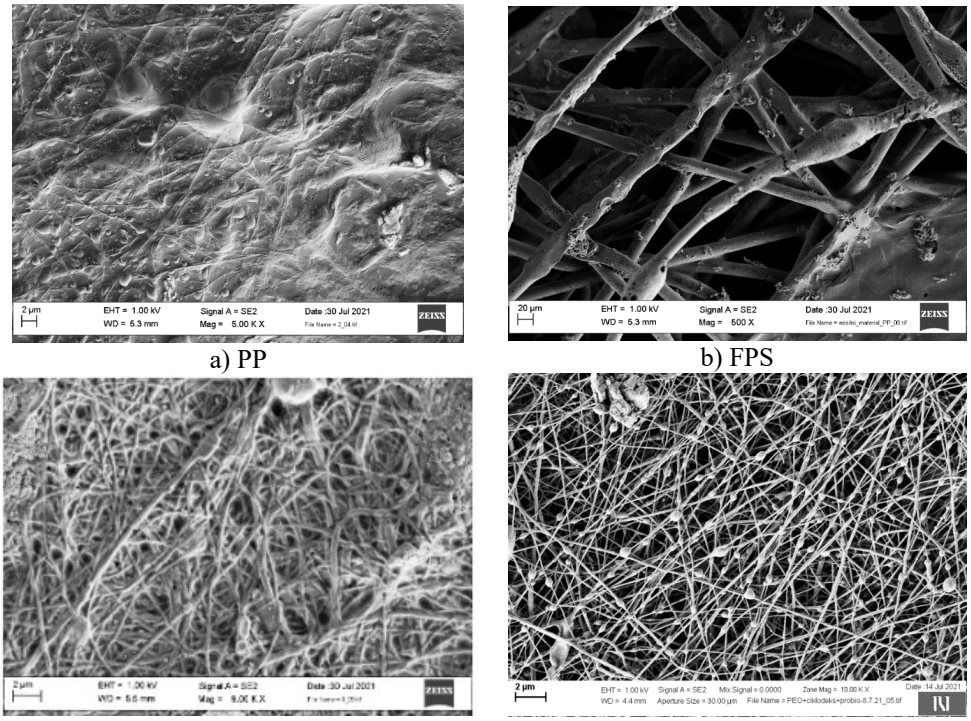

Figure 2: SEM images of electrospun samples

Table 3
Surface elemental analysis (%) of the samples

Sample	C	O	Na	P	K	Si
PEO	70.8	22.0	0.6	2.4	3.1	-
FPS	79.6	13.7	1.9	1.9	2.6	0.3
FPIS	72.5	19.5	2.0	2.4	2.6	0.9
FFPS	64.2	34.4	0.7	0.7	-	-

Table 3 presents the surface elemental analysis of the samples. The results show the presence of carbon (C) and oxygen (O) in the samples, with smaller amounts of natrium (Na), phosphorus (P), potassium (K) and silica (Si). An increased percentage of C can be noted in all the samples, but the % was lower in sample FFPS. The content of O increased in FFPS, while it was lower in other samples. An increased content of minerals, such as Na, has been also reported before in inulin.[25] The Na content is the highest in sample FPIS containing inulin. Thus, effective

encapsulation of probiotics and inulin is confirmed by the higher concentration of carbon and oxygen, as well as the higher Na content in the samples.

Figure 3 presents the FTIR spectra of PP, FFPS and FP (fresh probiotic). The spectrum of PP was taken as reference, with known peaks, for comparing with those of FP and sample FFPS. Thus, it can be noted that in the spectra of both PP and FFPS, the peaks at 2877 cm^{-1}, corresponding to –OH, and at 1466 cm^{-1}, indicating the presence of the CH_3 group, were significant. The peaks at 3308 and 1637 cm^{-1} were not found in the mentioned samples, only in the spectrum of FP. Therefore, it could be concluded that the fresh probiotic did not survive on the sample FFPS.

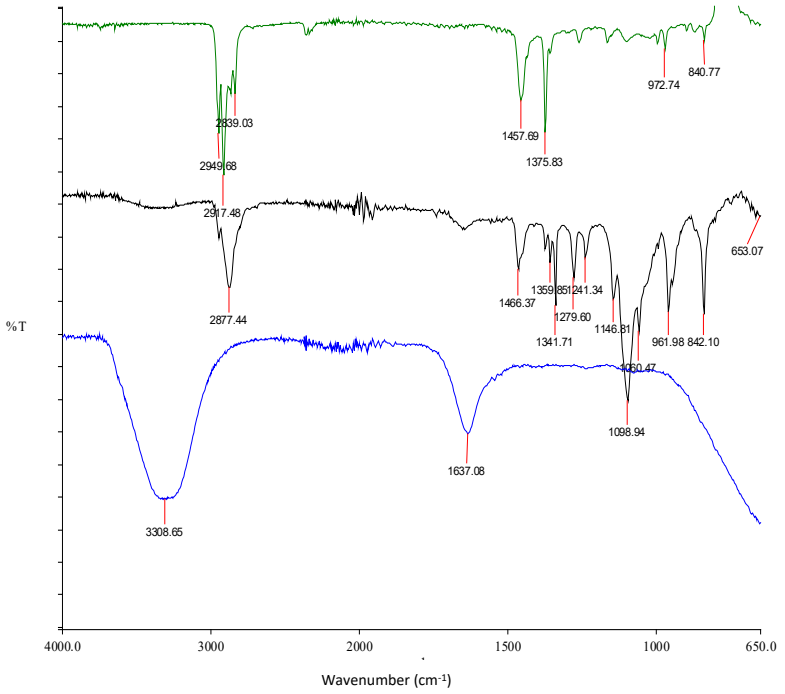

Figure 3: FTIR spectra of PP (upper), FFPS (middle) and FP (lower)

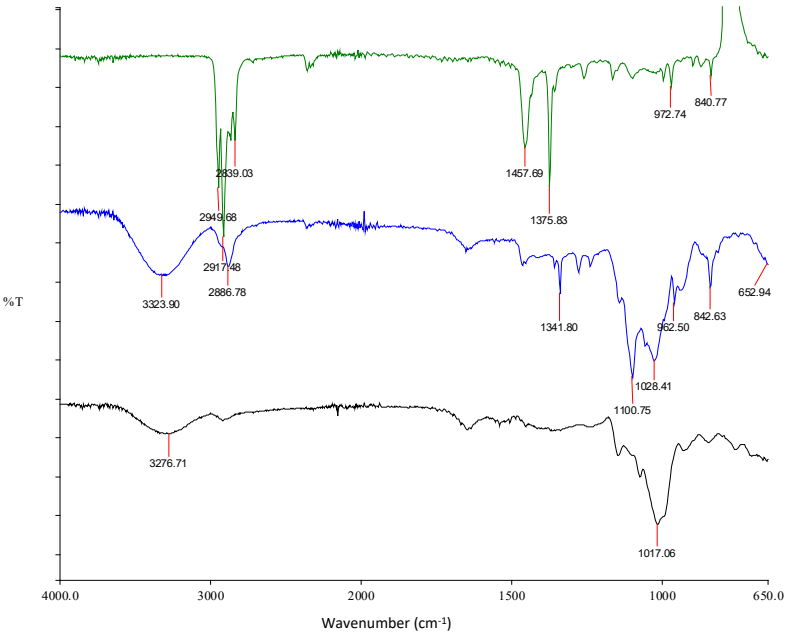

Figure 4: FTIR spectra of PP (upper), FPS (middle) and LP (lower)

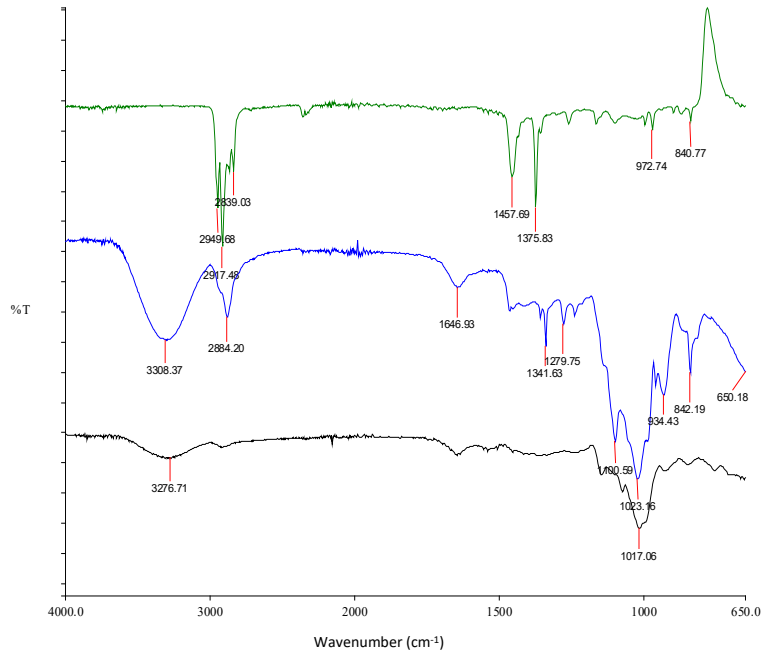

Figure 5: FTIR spectra of PP (upper), FPIS (middle) and LP (lower)

Figure 4 presents the FTIR spectra of PP, FPS and LP (lyophilized probiotic). Again, the spectrum of PP was taken as reference, for comparison with those of LP and the formulation FPS. Considering the addition of PEO in the composition of FPS, several peaks appear indicating its presence, as presented in our previous study,[26] *i.e.* the peaks at 2879 cm^{-1}, and between 1300 and 1000 cm^{-1}, are characteristic of the C–OH group, and the peak around 1241 cm^{-1} is typical of C–O–C. The peak appearing at 1466 cm^{-1} is assigned to CH_3 and at the one at 842 cm^{-1} – to the monoester.

The non-woven PP sample (reference substrate for collecting electrospun nanofibers) shows multiple characteristic signals for C-H bond vibrations, *e.g.* at 2949 cm^{-1} and 2889 cm^{-1}, at 1457 cm^{-1} vibration for $–CH_2$ bond is noted and at 1375 cm^{-1} – the vibration characteristic of $-CH_3$.

The spectrum of LP shows typical peaks at 3276 cm^{-1} and 1017 cm^{-1}, characteristic of –OH and C-OH groups, respectively. The spectra of FPS and LP were very similar, and it could be concluded that lyophilized probiotic is attached to the PP surface in the FPS sample.

Figure 5 presents the comparative FTIR spectra of PP, FPIS and LP. The spectrum of LP shows characteristic peaks at 3308 cm^{-1} and 1023 cm^{-1}. In the spectrum of FPIS, the most intensive peaks could be seen around 1190 cm^{-1} and 1023 cm^{-1}, due to C-O-C and C-O groups, indicating the presence of lyophilized probiotic attached to FPIS.[27] The FTIR spectrum of inulin was discussed in our previous study.[26] Briefly, the peak at 3290 cm^{-1} corresponds to the –OH group. In the region between 3000 cm^{-1} and 2700 cm^{-1}, the peak at 2933 cm^{-1} appears, attributed to CH_2 stretching, and the peak at 1017 cm^{-1} indicating the presence of C-O-C bending.

To conclude, the FTIR analysis of the electrospun samples demonstrated the presence of inulin and probiotics. The characteristic peak for carbohydrates around 1600 cm^{-1}, signifying the C=O group, was consistently observed in all electrospun samples. Additionally, the presence of the C-H group around 2884 cm^{-1} further supported the successful integration of inulin and probiotics. This is also in agreement with the XPS results for elemental analysis.

Further, the study aimed to assess the antioxidant properties of the developed nanofibers encapsulating probiotics. The antioxidant properties of the samples were determined using the ABTS method. Table 4 presents the inhibitory effect of the samples after 15 and 60 min. The absorbance of PP after did not show any antioxidant properties. However, with the incorporation of the probiotic into the electrospinning formulation (sample FPS), the antioxidant activity reached 100% ABTS radical inhibition after 60 min. The incorporation of inulin together with the probiotic into the formulation (sample FPIS) did not affect the antioxidant activity – again, 100% radical

inhibition was recorded. In sample TFCS, the absorbance decreased only slightly, as the sample provided around 11% radical inhibition.

The data in Table 4 indicate that the probiotic has a strong antioxidant effect. This is also in agreement with previous reports stating that more than 80% antioxidant activity was recorded for *Lactobacillus* strains.[28] The antioxidant effect is very important for further applications of the developed materials.[11] The inhibition is much lower on the TFCS cellulosic stripes, probably because the support material is porous, which might have led to the entrapment of *Lactobacillus* within the pores, which hindered their release.

In our study, a preliminary essay on the probiotic release from the different electrospun samples was performed and the results are presented in Table 5. It may be noticed that 13.0, 11.2 and 3.7% of probiotics were released from FPS, FPIS, and TFCS materials, as detected in the buffer after 2 hours. This suggests that bacteria are very quickly spontaneously released from the material and are not firmly embedded within the fibers. Besides, these results also confirm that the bacteria survived the electrospinning process and may be immediately released into the surrounding medium.

In our experiment, the number of CFU released into the buffer did not increase over time. On the contrary, it decreased, which was unexpected, and may because of non-optimal conditions for a long-term probiotic release experiment in phosphate buffer. The FFPS material had the lowest number of viable bacteria attached to the material in the initial stage, and no alive bacteria could be detected after 2 hours of probiotic release. In this sample, the probiotics were fresh, non-protected, which may be a reason for a lower survival rate during electrospinning and, consequently, during the release assay.

Table 4
Antioxidant effect of samples

Sample	Inhibition (%) 15 min	Inhibition (%) 60 min
PP	0	0
FPS	100.0	100.0
FPIS	98.9	100.0
TFCS	0	10.8

Table 5
Concentration of probiotics in the solution used for electrospinning of different formulations and in PBS buffer solution by spontaneous release after 2, 4 and 24 h from electrospun materials

Sample	CFU/mL solution	CFU/mL 0 h	CFU/mL 2 h	CFU/mL 4 h	CFU/mL 24 h
FFPS	1.6×10^9	4.4×10^5	0	0	0
FPS	0.8×10^{11}	2.0×10^8	2.6×10^7	1.8×10^7	0.3×10^5
FPIS	0.4×10^{11}	1.6×10^8	1.8×10^7	0.9×10^7	0
TFCS	1.8×10^8	4.0×10^7	1.5×10^6	0.6×10^5	0

These preliminary results showed that the probiotics applied in this work were released in a culturable form within 4 hours of the material's application, which would make it suitable for practical use as feminine hygiene products. The quick release of almost 100% of probiotics from the electrospun material has been recently demonstrated for *Lactobacilus plantarum* strain.[5] Adding inulin to encapsulated probiotics has previously been shown to increase bacterial viability. However, inulin may also increase the size of particles formed between probiotic bacteria, resulting in a lower content of bioactive substances in the final material.[29] This also explains the lower number of bacteria in sample FPIS. Many viable bacteria in samples FPS, FPIS, and TFCS are consistent with previously reported values of log CFU/mL nanofiber above 9.[13]

CONCLUSION

Electrospun nanofibers incorporating probiotics were prepared through the nozzle-free technique and characterized. The incorporation of *Lactobacillus paragasseri* K7, in both lyophilized and fresh forms, with or without inulin, into nanofibers aimed to create a material with

controlled release properties on a PP support. The identical procedure, featuring the addition of probiotics, was replicated on cellulose tampon stripes. The initial phase involved determining the physical properties of the polymer solutions, followed by the optimization of technological and environmental parameters to ensure seamless production.

The preparation of the nanostructures was validated by various techniques, including SEM, FTIR and XPS. SEM micrographs revealed the formation of a film, rather than nanofibers, when the polymer solutions containing probiotics and inulin were subjected to a high electric field. This deviation was attributed to the reduced conductivity of the polymer solution due to the addition of inulin. While some beads were observed more prominently with inulin addition, the potential formation of nanofibers persisted. ATR-FTIR analysis and XPS results for the electrospun samples demonstrated the presence of inulin and probiotics within the elecrospun formulations. Preliminary assay results showed that the developed nanofibers encapsulating probiotics had antioxidant activity and that the samples containing lyophilized probiotics released the bacteria within 4 hours of the material's application. These findings collectively affirm the potential of probiotic delivery systems for short-term applications, particularly within a few hours, for practical use as feminine hygiene products.

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