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Immobilization of vaginal *Lactobacillus* in polymeric nanofibers for its incorporation in vaginal probiotic products

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ABSTRACT

Probiotic products require high number of viable and active microorganisms during storage. In this work, the survival of human vaginal *Lactobacillus gasseri* CRL1320 and *Lactobacillus rhamnosus* CRL1332 after nanofiber-immobilization by electrospinning with polyvinyl-alcohol, and during storage was evaluated. The optimization of bacterial immobilization and storage conditions using bioprotectors (skim milk-lactose and glycerol) and oxygen-excluding packaging was carried out, compared with lyophilization. After electrospinning, a higher survival rate of *L. rhamnosus* (93%) compared to *L. gasseri* (84%) was obtained in nanofibers, with high viable cells ($>10^7$ colony-forming unit/g) of the two probiotics in nanofibers stored at -20°C up to 14 days. The storage in oxygen-excluding packaging was an excellent strategy to extend the shelf-life of *L. rhamnosus* (up to 1.7×10^8 CFU/g) in nanofibers stored at 4°C during 360 days, with no addition of bioprotectives, resulting similar to freeze-dried-cells. *L. rhamnosus* was successfully incorporated into polymeric hydrophilic nanofibers with a mean diameter of 95 nm. The composite materials were characterized in terms of morphology, and their physico-chemical and thermal properties assessed. Nanofiber-immobilized *L. rhamnosus* cells maintained the inhibition to urogenital pathogens. Thus, polymeric nanofiber-immobilized *L. rhamnosus* CRL1332 can be included in vaginal probiotic products to prevent or treat urogenital infections.

1. Introduction

The normal and healthy woman vaginal microbiota in fertile age is characterized by a complex microbial ecosystem with lactobacilli as the predominant species, which participate in maintaining vaginal pH values lower than 4.5. The acidic environment in combination with defensins and other antimicrobial substances (e.g. hydrogen peroxide, bacteriocins, biosurfactants) secreted by lactobacilli protect the urogenital tract from the colonization of potential pathogens, such as *Escherichia coli, Candida albicans* and *Staphylococcus aureus* (Mendling, 2016; Witkin, 2015). The pathogens are in low numbers in the healthy vagina or enter the tract by sexual and hygiene practices, or ascendant colonization from the rectum (Gupta et al., 1998; Kontiokari et al., 2004; Reid et al., 2004). Moreover, vaginal lactobacilli can prevent the pathogen colonization in vaginal epithelium via competitive adhesion, exclusion and/or interactions with local immune system (Borges et al., 2014; De Gregorio et al., 2016; De Gregorio et al., 2020a; Kovachev, 2018). Then, the use of probiotic products containing beneficial vaginal lactobacilli is a promising alternative for the prevention and treatment of urogenital tract infections (Nader-Macías and Juárez Tomás, 2015; Reid, 2017).

For the design of probiotic products for the urogenital tract, different requirements must be fulfilled, recommended by different scientific/government organism, such as a) isolation of microorganism from the same host and mucosal environment where they will be applied, b) correct phenotypic and genotypic identification of the probiotic strains, c) demonstration of their mechanisms of action in both *in vitro* and *in vivo* experimental models, d) safety assays, e) technological studies, and f) evidence of the efficacy and effectiveness of the microorganisms in clinical assays (Nader-Macías and Juárez Tomás, 2015).

Referred to technological studies, an adequate delivery system of probiotic microorganisms for the vaginal tract requires a high number of viable beneficial bacteria, and the maintenance of their long-term viability and stability of the beneficial characteristics. Moreover, the delivery systems must be patient-friendly and offer an effective local delivery (Iqbal and Dilnawaz, 2019; Nader-Macías and Juárez

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Tomás, 2015; Vitali et al., 2016). Different drying process, such as lyophilization, spray-drying and vacuum drying are frequently employed in order to get the long-term stability of microorganisms, mainly probiotics (Broeckx et al., 2017; Lavari et al., 2014; Vera Pingitore et al., 2015). Also, bioprotective substances are usually used to promote the resistance of the beneficial bacteria to those processes (Juárez Tomás et al., 2009; Vera Pingitore et al., 2015).

The drying processes decrease the water content and thus the weight of the formulation and the storage place, in a way to facilitate the handling of probiotic cells and their controlled dosing (Coghetto et al., 2016). Even though lyophilization and spray-drying are the drying systems most frequently applied (Broeckx et al., 2016; Juárez Tomás et al., 2009; Vera Pingitore et al., 2015), the applicability of the powder obtained from these processes is limited to vaginal tablets, capsules and insert (De Gregorio et al., 2020b; Vitali et al., 2016; Wagner et al., 2015). In addition, the lyophilization includes several drawbacks as long time required for drying, low energy efficiency, high cost of purchase and maintenance of the equipment, and sensitivity of the product to the freezing and other processing-related stresses (Walters et al., 2014).

On the other side, several works have evaluated the encapsulation of lactobacilli for their potential inclusion in different probiotic applications (Juárez Tomás et al., 2015; Silva et al., 2016; Ningtyas et al., 2019); however, the microcapsules containing *Lactobacillus* must be dried to prevent spoilage, and decrease of the number and activity of viable cells during storage. A method applied also to dry microcapsules is lyophilization (Juárez Tomás et al., 2015). More recently, electrospinning has been widely proposed as a simple and fast alternative method for the immobilization of probiotic bacteria within polymeric solid materials (Ceylan et al., 2018; Fung et al., 2011; López-Rubio et al., 2009; Nagy et al., 2014; Škrlec et al., 2019; Zupančič et al., 2019). This technique allows a simple step process at room temperature, of main importance in the probiotic area, providing advantages on other technological processes, such as spray drying and lyophilization, that require different steps and critical temperatures (very high or low) to dry the microorganisms (Walters et al., 2014).

Electrospinning is a technique applied to obtain fibrous materials with small diameters, large surface area per mass unit, high-interconnected porosity and gas permeability, and small pore size in an easy and convenient way. The process applies a strong electric field to a polymeric solution leading to a series of instabilities that cause fast solvent evaporation, generating thus nanoscale fibers (Lagenaur et al., 2011; Liu et al., 2017; Wang et al., 2008). The electrospinning shows several advantages as great versatility, easy manipulation of the processing parameters to design and obtain complex architecture materials, spatial arrangements, and adjustable morphologies for a wide variety of applications. Nowadays, electrospinning is considered as a scalable technology for industrial applications by using multiple nozzles, needleless variations, and other strategies (Kostakova et al., 2009; Persano et al., 2013).

The properties of the nanofiber obtained by electrospinning, as uniform morphology and composition, nanometric diameter, very large surface areas, high porosity, added to an easy to perform procedure, offer many advantages to be widely exploited for the innovative delivery of probiotic bacteria. However, most of the scientific publications have applied the process to strains isolated from food, human intestine, infant feces or unidentified sources (Ceylan et al., 2018; Fung et al., 2011; Heunis et al., 2010; Nagy et al., 2014; Škrlec et al., 2019). Up today, the stability (viability and activity) of vaginal probiotic strains during immobilization by electrospinning in nanofibers, and their subsequent storage has scarcely been reported (Nagy et al., 2014). The incorporation of bioprotective substances during the process has barely been explored (Škrlec et al., 2019). Then, more studies in this field, in order to go further in the understanding of the effects of the electrospinning process, bioprotective substances, and environmental parameters on the vaginal probiotic viability and functionality after the application of the process and during storage, are required.

Lactobacillus gasseri CRL 1320 and *L. rhamnosus* CRL1332 [*Lacticaseibacillus rhamnosus* according to Zheng et al. (2020)] were isolated from healthy woman vagina in our research group, genetically identified and selected by their beneficial properties such as inhibition to urogenital pathogens (e.g. *C. albicans, St. agalactiae*, and *S. aureus, E. coli*), biofilm formation, self-aggregation and/or hydrophobicity (De Gregorio et al., 2019, 2014; Juárez Tomás et al., 2011; Leccese Terraf et al., 2012; Ocaña et al., 1999b). Also, technological properties (resistance to osmotic and thermal stress, simulated vaginal and gastrointestinal condition, and/or lyophilization process) (Juárez Tomás et al., 2009; Marchesi et al., 2020; Silva et al., 2019, 2020) and safety protocols in mice, and in Phase I clinical assay were previously carried out (De Gregorio et al., 2012; De Gregorio et al., 2020a). In order to advance with the design of vaginal probiotic products, the present work aimed to evaluate the electrospinning process as a method to immobilize probiotic vaginal strains within nanofibers by determining the viability and maintenance of beneficial properties of bacteria after the process and during storage under different conditions. Also, to optimize the bacterial immobilization and storage conditions by using bioprotectors (skim milk-lactose and glycerol) and oxygen-excluding packaging, compared with the freeze-dried process. Then, to characterize morphologically, physicochemically, and biologically the nanofibers obtained post-electrospinning and during storage.

2. Material and methods

2.1. Microorganisms and culture conditions

L. gasseri CRL1320 and *L. rhamnosus* CRL1332 from the Centro de Referencia para Lactobacilos Culture Collection (CRL, Tucumán, Argentina), originally isolated from healthy human vagina (Ocaña et al., 1999a), and selected for their beneficial, technological properties and safety (De Gregorio et al., 2014, 2019, De Gregorio et al., 2020a; Juárez Tomás et al., 2011; Leccese Terraf et al., 2012; Marchesi et al., 2020; Ocaña et al., 1999b; Silva et al., 2019) were employed in this work. *Lactobacillus* strains were grown in De Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, France) (De Man et al., 1960) at 37°C for 16 h and subcultured twice in the same medium at 37°C for 12 h before use.

For the inhibition assays, urogenital pathogens isolated from women were kindly provided by Dr. Virginia Ocaña [*Streptococcus* (*St.*) *agalactiae* BII, *S. aureus* NH1 and F1, and *E. coli* 16] from the Nuevo Hospital "El Milagro" (Salta, Argentina), and Dr. Cristina Gaudioso de Allori (*C. albicans* C2) from the Acción Social Universidad Nacional de Tucumán (ASUNT, Tucumán, Argentina). The pathogens were cultured in yeast extract, peptone, tryptone, Tween 80 and glucose (LAPTg) broth, at 37°C for 12 h and subcultured at 37°C in the same medium for different time periods up the late exponential phase.

All the microorganisms were stored in milk-yeast extract [% (w/v): 13 nonfat milk, 0.5 yeast extract and 1 glucose; Britania Laboratories, Argentina] with 20% glycerol (Cicarelli Laboratories, Argentina) at -20°C.

2.2. Immobilization of vaginal Lactobacillus cells

Vaginal *Lactobacillus* cells were concentrated to be used in the immobilization process. To set up the protocol, in the first stage, *L. gasseri* CRL1320 or *L. rhamnosus* CRL1332 three times subcultured in MRS broth were centrifuged (6000 g for 10 min at 4°C), washed twice and resuspended in saline-10% glycerol, in order to obtain concentrated bacterial cells (around 10^{10}–10^{11} CFU/ml). In a second stage, a similar procedure, but using only *L. rhamnosus* CRL1332 was applied, where cells were resuspended in distilled water or different bioprotective agents: a) 12% (w/v) reconstituted skim milk and 24% (w/v) lactose, b) skim-milk-lactose solution plus 10% glycerol, or c) water plus 10%

glycerol.

Electrospinning technique was applied for *Lactobacillus* immobilization following the procedure reported by De Gregorio et al. (2017) with modifications. Polyvinyl alcohol (PVA) (Vetec™ reagent grade, 87-90% hydrolyzed, Mw 3000-7000; Sigma-Aldrich, USA) polymeric solution (30% w/w) was prepared. PVA powder was dissolved in 10 ml of distilled water at 80°C until complete dissolution, autoclaved and stored at 4°C.

The vaginal *Lactobacillus* suspensions were mixed homogeneously with 30% PVA polymer solution [1:1 (v/v)] (final concentrations: 15% PVA, 5% glycerol and/or 6% skim milk-12% lactose). The bacterial suspensions mixed with polymers were electrospun during 1 h in homemade electrospinning equipment by using a 10 ml syringe connected to a stainless steel needle (22G). A grounded collector was lined with aluminum foil to collect the electrospun fibers. Electrospinning parameters were set as follows: flow rate, 0.4 ml/h; tip to collector distance, 12 cm; voltage, 12 kV. The environmental temperature was 24 ± 2°C, with the relative humidity controlled at 40%.

2.3. Lyophilization of L. rhamnosus CRL1332

L. rhamnosus CRL1332 cells (10^{10}–10^{11} CFU/ml) resuspended in distilled water or in the following bioprotectors: a) 6% (w/v) reconstituted skim milk and 12% (w/v) lactose, b) skim milk-lactose solution plus 5% glycerol, or c) water plus 5% glycerol, were frozen at -70°C for 24 h and later dried in a chamber type freeze-drier (Lyovac GT2; Leybold, Köln, Germany) for 16 h at 0.3 mbar (1 bar = 100 kPa). The yielded products contain < 1% residual moisture.

2.4. Storage conditions

In a first stage, nanofibers with immobilized *L. gasseri* CRL1320 or *L. rhamnosus* CRL1332 were distributed in sterile plastic microtubes, placed in plastic bags and stored at room temperature, 4°C and -20°C for 2 months (0, 7, 14, 28 and 56 days).

In the second stage, different systems (bacterial cell suspensions mixed with PVA and nanofibers-immobilized or freeze-dried cells of *L. rhamnosus* CRL1332) were also distributed aseptically into sterile plastic microtubes, but at this time placed in vacuum sealed aluminum bags, and stored at room and refrigeration (4°C) temperatures for 1 year.

2.5. Viability of vaginal lactobacilli in PVA-solutions and nanofibers and freeze-dried

The number of *Lactobacillus* viable cells before and after immobilization and freeze-drying processes, and along the storage was determined for all the conditions assayed. Survival rates during immobilization or lyophilization were expressed as NAI/NBI or NAL/NBL, where NAI and NBI are the log CFU/g after and before immobilization, respectively, and NAL and NBL are the log CFU/g after and before lyophilization, respectively (De Gregorio et al., 2017; Juárez Tomás et al., 2015).

The number of viable cells of lactobacilli was determined using the drop-plate method (Herigstad et al., 2001). Briefly, serial ten-fold dilutions were prepared in saline from *Lactobacillus*-polymer solutions, *Lactobacillus*-loaded nanofibers and *L. rhamnosus* CRL1332 lyophilized powder. 20 µL drops of each dilution were inoculated on agar MRS plate and incubated at 37°C for 48 h. The data were expressed as $\log_{10}$ colony-forming unit (CFU)/g.

2.6. Nanofiber characterization

The morphological, physicochemical and biological characterization of nanofibers (with skim milk-lactose and no-bioprotective agents) was carried out in the samples immediately after the electrospinning process and during storage.

2.6.1. Scanning electron microscopy

The morphology of nanofibers was evaluated by scanning electron microscopy (SEM). The samples were affixed to sample stubs and sputtered with gold. Micrographs were then obtained using a JEOL® (6460LV) microscope (Oberkochen, Germany). In a similar way, the morphology of *L. rhamnosus* CRL1332 cells in planktonic state, previously dehydrated as described by Silva et al. (2019), was evaluated in a Zeiss model SUPRA 55VP equipment (Oberkochen, Germany). Nanofiber mean diameters were calculated by measuring at least 80 different fibers across three SEM images with Image-Pro Plus 6.0 software. The diameters of *Lactobacillus*-loaded nanofibers were measured at places without bacterial cells. Also, the *L. rhamnosus* CRL1332 length and width into the nanofibers and in planktonic state were compared with Image-Pro Plus 6.0 software. Data were expressed as mean diameter ± standard error.

2.6.2. Contact angle measurements

The surface polarity of nanofibers was determined by water contact angle measures. For this protocol, nanofiber membranes were placed on a slide and 50 µl of milli-Q water was added for each determination. 60 measurements were taken every 0.5 seconds using a goniometer (ramé-hart Automated Tilting Base, Succasunna, NJ 07876 USA) and the water contact angle was calculated for each sample by triplicate. A contact angle with value $> 65^{\circ}$ was considered hydrophobic, while an angle $\leq 65^{\circ}$ as hydrophilic (Vogler, 1998).

2.6.3. Thermal analysis

The thermal degradation of the nanofiber membranes was characterized by thermogravimetric analysis (TGA) using Shimadzu TGA-DTGA 50 thermal analyzer (Kyoto, Japan). Samples of approximately 10 mg were heated from 25°C to 600°C with a heating rate of 10°C/min under nitrogen atmosphere.

The thermal events were also detected by differential scanning calorimetry (DSC) using a Perkin-Elmer Pyris 1 Differential Scanning Calorimeter (Waltham, Massachusetts, USA). Samples loaded and weighed (5-10 mg) into aluminum cells with a pin hole were scanned from 25°C to 250°C at a heating rate of 10°C/min, under nitrogen atmosphere. An empty aluminum cell was used as a reference.

2.6.4. Fourier transforms infrared spectroscopy (FTIR)

Surface functional groups of the nanofiber components and their interactions were evaluated by FTIR (Nicolet 6700 Thermo Fisher Scientific instrument, Waltham, Massachusetts, USA), using attenuated total reflectance mode (ATR-FTIR). Spectra were recorded at a resolution of 2 cm^{-1} with an average of 64 scans in the wavenumber range of 4000 to 400 cm^{-1}

2.6.5. Biological characterization of nanofibers

The antimicrobial activity of *L. rhamnosus* CRL1332 against different urogenital pathogens (*C. albicans* C2, *S. aureus* NH1, *S. aureus* F1 and *St. agalactiae* BII, and *E. coli* 16) was determined. For this assay, 2 mg nanofiber containing immobilized *L. rhamnosus* CRL1332 were inoculated in MRS broth at 37°C for 24 h and subcultured twice in the same medium at 37°C for 12 h. Pure cultures of *L. rhamnosus* CRL1332 were grown and evaluated under the same culture conditions as control. Three independent experiments were performed.

The ability of nanofiber-immobilized *L. rhamnosus* CRL1332 to inhibit different urogenital pathogens was determined applying the agar overlay technique (De Gregorio et al., 2019; do Carmo et al., 2016). Briefly, MRS agar plates were inoculated with 10 µl *L. rhamnosus* CRL1332 suspensions from nanofibers or pure cultures (control) and incubated at 37°C for 24 h under microaerophyllic conditions. After incubation, 10 ml of melted-agarized culture media (specific for each pathogen, containing 10^6 CFU *Candida* and 10^7 CFU of the other

Table 1
Viability of *L. gasseri* 1320 and *L. rhamnosus* CRL1332 to the immobilization process in nanofibers by electrospinning technique.

Microorganisms	log CFU/g pre-electrospinning[a]	log CFU/g post-electrospinning[b]	Survival rate (NAI/NBI)[c]
L. gasseri CRL1320	9.44 ± 0.07	7.90 ± 0.02*	0.84 ± 0.01[d]
L. rhamnosus CRL1332	9.44 ± 0.13	8.82 ± 0.23	0.93 ± 0.02[c]

[a] Data represent the average values of viable cell (log CFU/g ± standard error) of *L. gasseri* CRL1320 and *L. rhamnosus* CRL1332 from spinning mixtures used in three independent experiments prior to the immobilization process.
[b] Data represent the average values of viable cells (log CFU/g ± standard error) of *L. gasseri* CRL1320 and *L. rhamnosus* CRL1332 from nanofibers obtained in three independent experiments after the immobilization process. Statistically significant differences between viable cells before and after immobilization are indicated by * ($p < 0.05$).
[c] The survival rate during immobilization is expressed as NAI/NBI, where NAI and NBI are log CFU/g after and before immobilization, respectively. Different letters indicate statistically significant differences ($p < 0.05$) in the survival rate between the *Lactobacillus* strains.

pathogens) were added over the grown *Lactobacillus* agar. Agarized Sabouraud Dextrose (Britania Laboratories, Argentina), MacConkey (Britania Laboratories, Argentina), Todd Hewitt (Becton Dickinson, Le Pont de Claix, France) and Brain Heart Infusion (Difco, USA) were the media employed to *C. albicans, E. coli, St. agalactiae* and *S. aureus*, respectively. The plates were again incubated aerobically at 37°C for 24 h for pathogens growth. Inhibition zones over or around *L. rhamnosus* CRL1332 colonies indicated the antimicrobial activity. The diameters of the inhibition halos were measured.

2.7. Statistical analysis

The analysis of variance (ANOVA) using a general linear model was applied to determine the main and interaction effects of the factors evaluated in each one of the following processes: a) immobilization: point of the immobilization process (pre- and post-electrospinning), and immobilization conditions (nanofibers containing immobilized *L. gasseri* CRL1320 or *L. rhamnosus* CRL 1332, or nanofibers with *L. rhamnosus* CRL 1332 immobilized in presence or absence of bioprotective substances); b) lyophilization: point of the freeze-dried process (pre- and post-lyophilization), and freeze-dried conditions (freeze-dried *L. rhamnosus* CRL 1332 free cells in presence or absence of bioprotective substances); c) storage: immobilized *Lactobacillus* strains (*L. gasseri* CRL1320 and *L. rhamnosus* CRL 1332), and systems in which *L. rhamnosus* CRL1332 were stored (nanofibers in presence or absence of bioprotective substances, and freeze dried cells in presence or absence of lyoprotectors) at different temperatures and times. The number of *Lactobacillus* viable cells (log CFU/g) was the response of interest analyzed in the different protocols applied.

ANOVA using a general linear model was also applied to study: a) the effects of the condition in which nanofibers were obtained (pure PVA, *L. rhamnosus* CRL1332 cells re-suspended in water and mixed with PVA, skim milk-lactose mixed with PVA or *L. rhamnosus* CRL1332 cells re-suspended in skim milk-lactose) and storage (360 days at room or refrigeration temperatures) on the nanofiber diameters; and b) the effects of the condition in which *L. rhamnosus* CRL1332 was inoculated (cells included in nanofibers with or without protective substance, and recently obtained free bacterial cells) to perform the antimicrobial assay.

In each analysis, significant differences ($p < 0.05$) between mean values were determined by Tukey's test, using MINITAB statistical software (version 16 for Windows).

3. Results and discussion

3.1. Immobilization of vaginal Lactobacillus in nanofibers. Cell viability during the process and storage of the nanofibers

In the first stage of setting up the immobilization technique, the viability of *L. gasseri* CRL1320 and *L. rhamnosus* CRL1332 was assessed before and after the electrospinning process to determine their resistance. Concentrated suspensions of lactobacilli containing 15% PVA plus saline with 5% glycerol were employed as described by De Gregorio et al. (2017) and Salalha et al. (2006) who showed the protective effect of glycerol on the microbial survival during immobilization-induced stress. Also, PVA was selected, taking into account that it is a mucoadhesive, biocompatible, and hydrophilic polymer, which generates a high oxygen barrier when dry, protecting thus the bacterial bioactivity (López-Rubio et al., 2009). Furthermore, this polymer was extensively studied for multiple biomedical uses such as cartilage and orthopedic applications, and for delivery of vaginal and transdermal drugs (Baker et al., 2012; Gonzalez et al., 2012; Iqbal and Dilnawaz, 2019; Sharma et al., 2016). In addition, in the same way than lactobacilli, PVA is a generally recognized as safe (GRAS) compound (López-Rubio et al., 2009), which also supports its safe application as nanocarrier for vaginal probiotic delivery (Nagy et al., 2014). As expected, the exposition of *L. gasseri* CRL1320 and *L. rhamnosus* CRL1332 to PVA in the spinning mixture did not affect the bacterial cell viability, even after several days of contact with the polymer (data not shown).

The bacterial viability during the electrospinning process could be eventually affected by drastic changes in the osmotic environment due to the fast evaporation of solvents upon electrospinning (López-Rubio et al., 2009). The viable cell numbers of lactobacilli before and after the immobilization process are shown in **Table 1**. The results indicate that the two strains resisted the electrospinning process evidencing that the viability of *L. rhamnosus* CRL1332 was not affected during the immobilization, whereas *L. gasseri* CRL1320 viable cells were significantly reduced in approximately 1.5 log unit. Therefore, a higher survival rate was obtained for *L. rhamnosus* CRL1332 compared to *L. gasseri* CRL1320 (93% and 84%, respectively) (**Table 1**). The results obtained are similar to those of several authors who obtained between 68 to 90% survival rates following immobilization by electrospinning of *Bifidobacterium animalis* subsp. *lactis* Bb12 and *L. gasseri* into PVA-nanofibers (Amna et al., 2013; López-Rubio et al., 2009), *Lactobacillus acidophilus* into PVA and poly(vinyl pyrrolidone) nanofibers (Nagy et al., 2014), and into agrowaste-based nanofibers (Fung et al., 2011), *L. rhamnosus* in PVA and sodium alginate-based nanofibers (Ceylan et al., 2018), and *Lactobacillus plantarum* into poly(ethylene oxide) (PEO) nanofibers (Heunis et al., 2010; Škrlec et al., 2019). Most of the strains evaluated by different authors were isolated from a wide variety of sources different to human vagina, except one *L. acidophilus* (strain number: B1075) (Nagy et al., 2014), being a different specie to the strains evaluated in this work, supporting thus the originality of our research.

Although *L. gasseri* CRL1320 and *L. rhamnosus* CRL1332 strains were able to survive to the drastic osmotic change occurring during the electrospinning process, the higher post-process survival obtained for *L. rhamnosus* CRL1332 could be supported by its high resistance to the osmotic stress, recently reported by our research group (Silva et al., 2019). *L. rhamnosus* CRL1332 grew in high NaCl concentrations, showing optimal growth parameters and maintaining its properties after the stress applied (Silva et al., 2019). In a similar way, Zupančič et al. (2019) immobilized different *Lactobacillus* species (as *L. gasseri* and *L. rhamnosus*) in PEO-nanofibers and observed 0 to 3 log CFU/mg

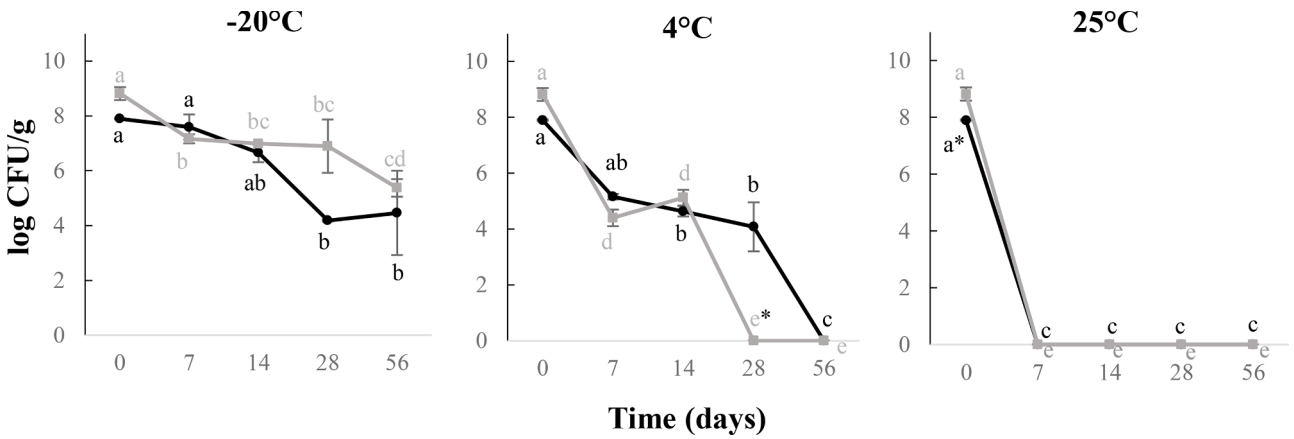

Fig. 1. Viability of *L. gasseri* CRL1320 (●) and *L rhamnosus* CRL1332 (■) in nanofibers during storage for 56 days at different temperatures (-20, 4 and 25°C). Different letters indicate statistically significant differences ($p < 0.05$) in the log CFU/g mean values of the same *Lactobacillus* strain at different time of storage at the same temperature, and between different temperatures at the same storage time. Grey/black letters represent the differences referred to the same color plot. (*) indicates statistically significant differences ($p < 0.05$) in the mean values of log CFU/g between strains of lactobacilli, at the same temperature and storage time.

loss in their viability after electrospinning, depending on the species. These authors correlated the higher post-process viability with a higher hydrophobicity of the lactic acid bacteria surface, and, in a different way than our results, they obtained a higher post-process viability in *L. gasseri* compared to *L. rhamnosus* (0.09 and 1.14 log CFU/mg loss, respectively). In the present work, the higher bacterial survival was not directly related with the surface hydrophobicity, because *L. gasseri* CRL1320 was more hydrophobic than *L. rhamnosus* CRL1332, as previously demonstrated (Ocaña et al., 1999a). Thus, these results highlight the importance of assaying each one of the electrospinning conditions for each one of the probiotic *Lactobacillus* strains under study, supporting again a strain-specific behavior, as widely demonstrated by different type of studies (De Gregorio et al., 2019; Marchesi et al., 2020; Silva, 2019, 2020).

When evaluating the *Lactobacillus* survival into nanofibers during the storage for 56 days at different temperatures (-20, 4 and 25°C), the viability of the strains was affected by the two conditions (time and temperature) under assay. When refrigerated at -20°C, a higher bacterial viability and a longer survival for the two strains were observed (**Fig. 1**). However, in this condition, a decrease of cell viability to values below 1×10^7 CFU/g [minimum number of viable cells required in probiotic products (Hill et al., 2014; ISAPP (International Scientific Association for Probiotics and Prebiotics), 2013)] was evidenced in *L. gasseri* CRL1320 and *L. rhamnosus* CRL1332 at 28 and 56 days, respectively (**Fig. 1**). In a similar way, several authors have evidenced that the viability of nanofiber-immobilized lactobacilli decreases through the time, while lower temperatures showed a long-term bacterial preservation (Amna et al., 2013; Fung et al., 2011; Nagy et al., 2014; Škrlec et al., 2019). These results can be explained by the slow bacterial metabolisms of the cells at low temperatures (Broeckx et al., 2017).

3.2. Optimization of the immobilization process of L. rhamnosus CRL1332 in nanofibers and subsequent storage. Comparison with the lyophilization process

The effect of the storage on the viability and functionality of the nanofiber-immobilized lactobacilli is an essential evaluation to be performed when planning to apply a scale-up process, and also to determine if the probiotic strains maintain their beneficial characteristics at the time of use (Amna et al., 2013). The application of stabilizer or bioprotective substances and packaging systems excluding oxygen can improve the stability of living lactic acid bacteria (Morgan et al., 2006). Different bioprotectors, such as lactose, skim milk, sucrose, and trehalose are frequently introduced to enhance the bacterial viability during the storage (Juárez Tomás et al., 2009; López-Rubio et al., 2009; Škrlec et al., 2019). Recently, Škrlec et al. (2019) optimized the incorporation of *L. plantarum* (isolated from an undefined source) into PEO-nanofibers, and showed that the viability was improved by the addition of trehalose as protectant. Also, Lopez Rubio et al. (2009) successfully achieved the encapsulation of *B. animalis* in PVA nanofibers with skim milk by electrospinning, indicating that the bacterial cells remained viable within the fibers for 40 days at room temperature, and for 130 days at refrigeration conditions. Then, given the low numbers of viable *Lactobacillus* cells obtained in nanofibers stored at different temperatures in the assays described previously, in this second stage, bioprotective substances were added to the spinning mixtures in order to increase the bacterial viability in the nanofibers during the storage at refrigeration and room temperatures. From this point, only *L. rhamnosus* CRL1332 was selected to apply these new protocols, supported by the higher resistance of this strain to the electrospinning process than *L. gasseri* CRL1320.

The protective substances evaluated in the spinning polymeric mixtures were skim milk-lactose, skim milk-lactose-glycerol or glycerol in distilled water. Skim milk-lactose was used since it is a cryoprotectant successfully used in our laboratory to preserve the stability (viability and functionality) of lyophilized lactobacilli over the time (Juárez Tomás et al., 2009). Glycerol was again evaluated because it is able to enter in the bacteria by facilitated diffusion without chemical modification, and in this way, it could protect the cells from the fast evaporation of solvents that occurs during electrospinning (De Gregorio et al., 2017). Saline was replaced by distilled water to reduce the number of ions interacting with the polymer (Juárez Tomás et al., 2015). Also, nanofibers only with PVA dissolved in distilled water were evaluated to immobilize *L. rhamnosus* CRL1332, supported by the results obtained by Nagy et al. (2014) who encapsulated huge numbers of *L. acidophilus* into PVA-nanofibers and showed a long shelf life at 7 and -20°C during 90 days of storage.

On the other hand, the lyophilization technique, considered as the gold standard to preserve bacterial viability, was applied by using the same bioprotective substances to compare the efficiency of the two processes. The nanofibers and powders obtained from electrospinning and lyophilization, respectively, were later stored in vacuum-sealed aluminum bags in order to reduce the ambient wet and oxygen levels that could affect the bacterial stability (Kurtmann et al., 2009; Nagy et al., 2014).

Before the immobilization process, the combination of *L. rhamnosus* CRL1332 with the spinning mixtures with or without bioprotective agents and stored at refrigeration and room temperatures, was evaluated over the time. The number of *L. rhamnosus* CRL1332 viable cells

Table 2
Viability of *L. rhamnosus* CRL1332 during electrospinning and lyophilization processes.

Condition[a]	Processes					
	Electrospinning			Lyophilization		
	log CFU/g pre-electrospinning[b]	log CFU/g post-electrospinning[c]	Survival rate (NAI/NBI)[d]	log CFU/g pre-lyophilization[b]	log CFU/g post-lyophilization[c]	Survival rate (NAL/NBL)[d]
skim milk-lactose	9.99 ± 0.06	9.96 ± 0.53	0.99 ± 0.04[g]	10.11 ± 0.01	10.77 ± 0.01	1.07 ± 0.01[g]
skim milk-lactose-glycerol	9.87 ± 0.08	9.89 ± 0.03	1.00 ± 0.01[g]	9.56 ± 0.08	10.65 ± 0.04	1.11 ± 0.01[g]
glycerol	10.00 ± 0.12	10.10 ± 0.05	1.01 ± 0.01[g]	10.07 ± 0.05	8.04 ± 0.21*	0.79 ± 0.02[h†]
water	9.98 ± 0.04	9.53 ± 0.04	0.95 ± 0.01[g]	10.14 ± 0.01	7.80 ± 0.01*	0.77 ± 0.01[h†]

[a] Condition evaluated in mixtures employed for electrospinning and lyophilization processes.
[b] Data represent the average values of viable cells (log CFU/g ± standard error) of *L. rhamnosus* CRL1332 from employed mixtures in three independent experiments prior to the electrospinning and lyophilization processes.
[c] Data represent the average values of viable cells (log CFU/g ± standard error) of *L. rhamnosus* CRL1332 from nanofibers and powders obtained in three independent experiments by electrospinning and lyophilization, respectively. Statistically significant differences between viable cells before and after processes (electrospinning or lyophilization) are indicated by * ($p < 0.05$).
[d] Survival rate during electrospinning or lyophilization was expressed as NAI/NBI or NAL/NBL, where NAI and NBI are the log CFU/g after and before immobilization, respectively, and NAL and NBL are the log CFU/g after and before lyophilization, respectively. Different letters indicate statistically significant differences ($p < 0.05$) between survival rates of the different conditions evaluated in each process. Statistically significant differences ($p < 0.05$) in survival rates between the different processes for the same condition are indicated by †.

was stable in all the mixtures stored at refrigerated temperature up to 30 days with no significant differences to the initial time. However, it significantly decreases at 7 days when all the mixtures were stored at room temperature (**Fig. S1**). These results demonstrate that is possible to keep the spinning mixtures for a certain time period before applying the immobilization process.

When applying the electrospinning technique to immobilize *L. rhamnosus* CRL1332 cells in PVA with and without bioprotective substances, no significant differences in the viable cell number before and after immobilization process was observed in each one of the assayed conditions (**Table 2**). A high number of viable cells (log CFU/g nanofibers > 9.5) was obtained in the nanofibers post-electrospining. On the other side, no significant differences in *L. rhamnosus* CRL1332 survival rates (≥ 0.95) between the different conditions assayed were observed ($p > 0.05$) (**Table 2**). These results are different from those published by Škrlec et al. (2019) who achieved a lower decrease of *L. plantarum* cell viability with sucrose or trehalose added to PEO nanofibers when compared to PEO nanofibers with no bioprotectants after the electrospinning. On the other side, the results obtained in this stage demonstrate that bioprotective substances, such as for example glycerol, were not required to preserve the viability of *L. rhamnosus* CRL1332 during the immobilization process, in an opposite way to the data reported by De Gregorio et al. (2017) and Salalha et al. (2006). These authors have shown the glycerol protective effect only on gram-negative bacteria (De Gregorio et al., 2017; Salalha et al., 2006), which suggests that the differences in the chemical composition of the gram-positive cell wall could provide a higher electrospinning resistance to *Lactobacillus* strains.

After the freeze-dried process, a significant post-processing loss of *L. rhamnosus* CRL1320 viability was obtained when water and water-glycerol were used. Then, the comparison of the survival rates of the two processes (lyophilization and electrospinning) showed better results in the immobilization in nanofibers for all the conditions assayed (**Table 2**).

During the drying processes, the most common site of damage is the bacterial membrane, because of the water loss produced from phospholipid membranes and proteins (Liu et al., 2018; López-Rubio et al., 2009). Then, the excellent *L. rhamnosus* CRL1332 survival rate obtained during electrospinning (in all the assayed conditions) and lyophilization (with skim milk-lactose), could be explained by the fact that PVA (in nanofibers) and skim milk-lactose (both in powders and nanofibers) substitute the water molecules by forming hydrogen bonds around the polar and charged groups in the lipid bilayer and the membrane proteins, and thereby stabilize the native structure of *L. rhamnosus* CRL1332 in absence of water (Vitali et al., 2016). Taking into account these results, the electrospinning represents a promising method for the incorporation of vaginal probiotic into nanofibers, allowing in a single step obtained dry bacteria and a solid delivery system, offering thus significant advantages over lyophilization.

3.2.1. Viability of immobilized and freeze-dried L. rhamnosus CRL1332 during storage

The viability of immobilized and freeze-dried *L. rhamnosus* CRL1332 was significantly affected by the different factors under evaluation: storage system (nanofibers and powders at the different conditions assayed), temperature (refrigeration and room) and time (0, 7, 30, 60, 120, 240, 360 days) (**Fig. 2A**).

The highest survival of *L. rhamnosus* CRL1332 during the storage was observed in systems prepared with skim milk-lactose (either powder or nanofibers) and with no bioprotector (in nanofibers) (**Fig. 2A**). On the other hand, the bacterial viability was significantly higher in all the systems after storage at 4°C than at room temperature, observing a significant decrease of the cell viability at different storage times (**Fig. 2A**). These results were expected considering that marketed products containing living organism must be stored frequently at 2–8°C.

Taking into account the differences evidenced in *L. rhamnosus* CRL1332 viability at the two storage temperatures, the bacterial viable numbers were determined for each temperature and storage times in the different systems (**Fig. 2B**). At refrigeration temperature, significant differences of *L. rhamnosus* CRL1332 survival kinetics were observed between the systems studied. The bacteria in powders from skim milk-lactose (P-SM-L) evidenced the best survival kinetic ($p < 0.05$), followed by nanofibers without protectives (N-W) and with skim milk-lactose (N-SM-L). After 360 days storage, the highest number of viable cells was obtained in P-SM-L (2×10^{10} CFU/g). In this system, the viable cell numbers did not decrease significantly during the storage time. Also, N-W, N-SM-L and powder from water (P-W) have shown a high number of viable cells (1.82×10^{9}, 6.11×10^{8} CFU/g and 5×10^{8}, respectively) at 240 days, with significant reduction of 0.98, 1.86 and 1.60 log unit at 360 days. On the other hand, significant reduction of the cell viability (≥ 1 log unit) was evidenced in powder and nanofibers from skim milk-lactose-glycerol (P-SM-L-G and N-SM-L-G) at 60 storage days, and in nanofibers and powder with glycerol (N-G and P-G) at 7 days (**Fig. 2B**). Thus, in cells stored at 4°C, the differences between *L. rhamnosus* CRL1332 cell viability submitted to electrospinning or lyophilization were minimal. These data indicate that the storage in a type of packaging that excludes oxygen is an excellent strategy to extend the shelf-life of *L. rhamnosus* CRL1332, without adding

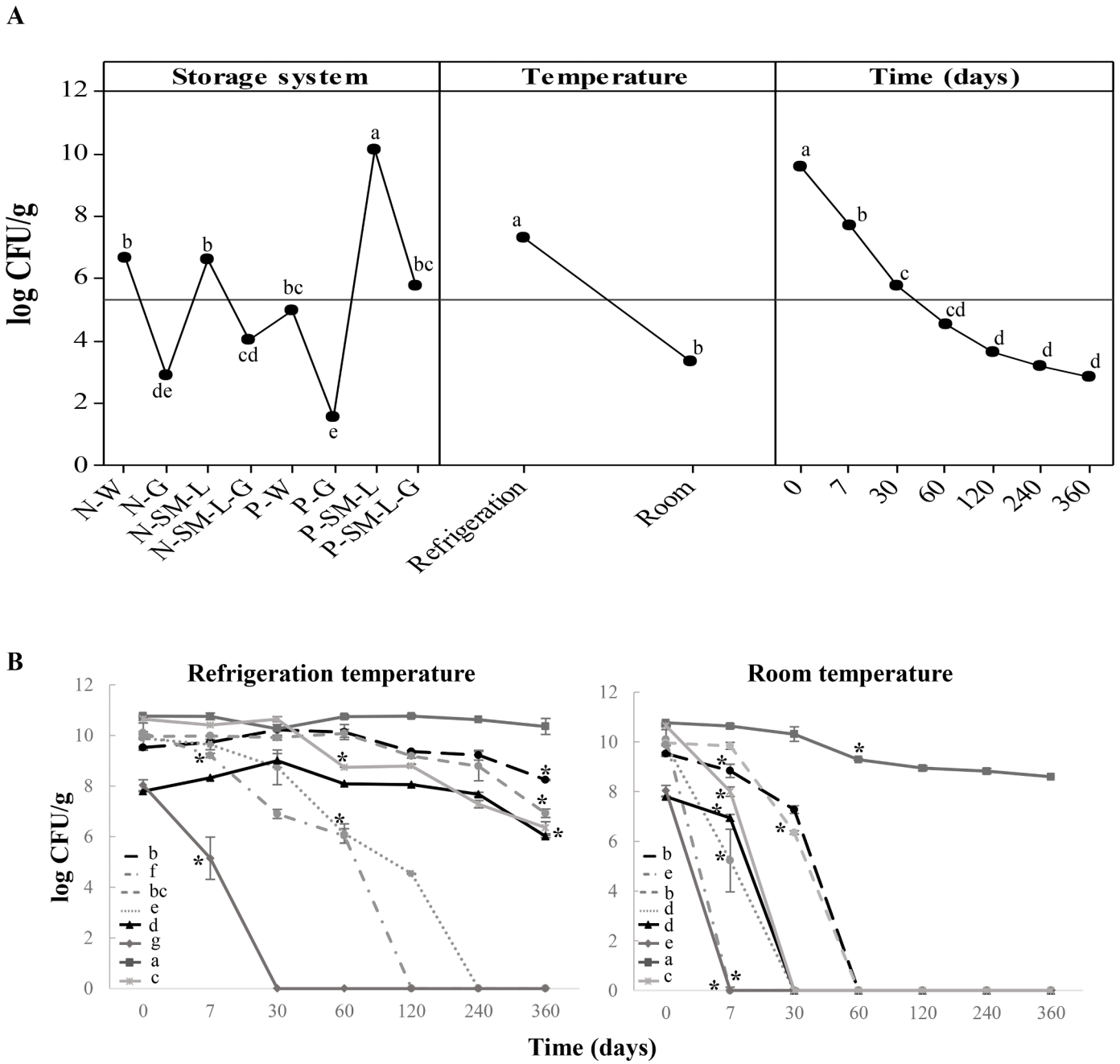

Fig. 2. Viability of immobilized and lyophilized *L. rhamnosus* CRL1332 in nanofibers and powders, respectively, during storage for 360 days at room and refrigeration (4°C) temperatures. (**A**) Main effects of storage system, temperature and time on *L. rhamnosus* CRL1332 survival during storage. In the "Storage system" panel, each point shows the log CFU/g mean value for each system at all the storage times and temperatures. In the "Temperature" panel, each point shows log CFU/g mean value for each temperature, at all the storage times and systems. In the "Time" panel, each point shows log CFU/g mean value for each time at all the conditions and temperatures. In each panel, different letters represent statistically significant differences ($p < 0.05$) in the log CFU/g mean values between levels of each factor assayed (system, temperature, or time), according to Tukey's test. (**B**) The data indicate the mean values of the viable cell number (log CFU/g) in nanofibers or powders. Different letters on the left of each plot indicate statistically significant differences ($p < 0.05$) in the kinetics of storage of the studied systems for each one of the temperatures assayed (refrigeration o room temperature). Symbols or lines referred to each curve were included on the left of each plot in order to add the statistical analysis in a more understandable way. (*) indicates a statistically significant decrease ($p < 0.05$) in the number of viable cells in the same system and storage temperature, according to Tukey's test. Storage conditions (systems) included: bacterial cells immobilized in nanofibers obtained with PVA dissolved only in water (N-W, ●—●) or with addition of protectors, as glycerol (N-G, ●···●), skim milk-lactose (N-SM-L, ●—●) or skim milk-lactose-glycerol (N-SM-L-G, ●···●); and powders of lyophilized free cells in water (P-W, ▲—▲) or with protectors as glycerol (P-G, ◆—◆), skim milk-lactose (P-SM-L, ■—■) or skim milk-lactose-glycerol (P-SM-L-G, ✳—✳).

bioprotective substance, mainly for nanofibers.

At room temperature, the highest *L. rhamnosus* CRL1332 survival rate was obtained for P-SM-L. In this system, a significant decrease of viability (1 log unit) was observed at 60 days, however, a high number of viable cells (4×10^8 CFU/g) was kept up to 360 days. All the other systems under evaluation evidenced a significant reduction of viability from 7 storage days, except N-SM-L that decreased at 30 days. However, at this storage time, a higher number of viable cells (1×10^7 CFU/g) was observed in N-W compared to N-SM-L (2×10^6 CFU/g). After 60 days storage, no viable cells were recovered from all the systems under

study. In a different way, Nagy et al. (2014) reported that *L. acidophilus*-loaded PVA nanofibers stored at room temperature lost total biological activity after a week. These authors did not use packaging to exclude the oxygen, as applied in the present work.

The results obtained demonstrated that the storage at room temperature of lyophilized powder with skim milk-lactose was more efficient than electrospun nanofibers in all the condition under study. However, different bioprotective substances, such as trehalose, could be further assayed in order to increase the viability of *L. rhamnosus* CRL1332 in nanofibers stored at room temperature (Škrlec et al., 2019).

Even though the P-SM-L storage system have shown a higher bacterial viability than the systems with nanofiber-immobilized *Lactobacillus* in the different conditions evaluated, it must be pointed out that by using the electrospinning technique is possible to cover different devices for vaginal application (e.g. tampons, hygienic towels) with viable *Lactobacillus* (immobilized in adequate amounts in nanofibers) in a simple and unique step. This coating was successfully applied in several products (e.g. stent, soybeans, cellulose paper) with a wide variety of bioactive/substances immobilized in nanofibers through electrospinning (Cherpinski et al., 2019; De Gregorio et al., 2017; Kersani et al., 2019). Then, the *Lactobacillus* immobilization system proposed offers some administration advantages when compared with lyophilized powders, which require subsequent processing for their incorporation in the design of novel products.

The storage at -20°C was not included in this protocol since this temperature is not a frequent condition applied for the storage of probiotic vaginal product.

3.3. Characterization of nanofibers containing L. rhamnosus CRL1332

On the basis of the viability results obtained from the different immobilization conditions assayed, nanofibers from *L. rhamnosus* CRL1332 cells re-suspended in water or skim milk-lactose, mixed with PVA (CRL1332 + PVA and CRL1332 + SM-L + PVA, respectively) and subjected to electrospinning, were selected for their further characterization. Also, nanofibers prepared only with PVA, and with skim milk-lactose plus PVA (PVA and SM-L + PVA, respectively) without bacteria were included for comparison purposes.

3.3.1. Morphology of nanofibers

Nanofiber morphology can be affected by numerous compositional, processing and environmental parameters (Pelipenko et al., 2015; Škrlec et al., 2019). In this work, the incorporation of *L. rhamnosus* CRL1332 cells and the addition of skim milk-lactose into the polymer solution, as well as the effect of the storage at room and refrigeration temperatures were evaluated to determine if these factors affect the nanofiber morphology.

Single or dividing cells of *L. rhamnosus* CRL1332 were oriented along the nanofibers and distributed over the mats indicating a successful immobilization within the nanofiber with and without bioprotective substances (**Fig. 3**). A local widening was also observed in the nanofibers corresponding to the length and width of the bacterial shape in planktonic state (**Fig. 3A**). When analyzing the nanofiber morphology, the bead formation in some regions was observed. Moreover, fibers with significantly higher diameters were detected when skim milk-lactose was added (126.05 ± 1.83 nm) compared to nanofibers with no-protector (94.47 ± 1.77 nm) (**Fig. 3B**). In a similar way, Škrlec et al. (2019) reported diameters of 150 nm for PEO-nanofibers obtained with protectants (trehalose or sucrose), and diameters of 135 nm for PEO-nanofibers with no-protectants. Moreover, nanofibers with immobilized *L. rhamnosus* CRL1332 showed significantly lower diameters (112.20 ± 2.06 and 85.38 ± 1.56 nm for CRL1332 + SM-L + PVA-nanofibers and CRL1332 + PVA, respectively) compared to nanofibers without bacterial cells (**Fig. 3B**). In the same sense, Škrlec et al. (2019) and Zupančič et al. (2019) indicated that the addition of bacteria to PEO solutions did not only modify the viscosity, but also the dispersion conductivity, suggesting that a prominent influence of conductivity over viscosity might lead to the decreased nanofiber diameters in the bacteria-loaded nanofibers, supporting thus our results.

The storage times and temperatures did not significantly affect the diameters of all the nanofibers at 180 days (data not shown). Similar results were observed for PVA-nanofibers up to 360 storage days (**Fig. 3C,D**). However, an increase in the diameters of CRL1332 + PVA-nanofibers was evidenced at this point. In an opposite way, a reduction of the diameters was observed in nanofibers obtained with the protectors (SM-L + PVA and CRL1332 + SM-L + PVA) with longer storage time (**Fig. 3**). The fiber diameter variation could be clearly ascribed to the different chemical composition of the protector, which could undergo chemical changes during storage conditions.

On the other hand, slightly flatter fibers were observed in the SM-L + PVA-nanofibers at the 360 days storage, both at room and refrigeration temperatures. In the CRL1332 + SM-L + PVA-system, misshapen immobilized-bacteria were only evidenced at room temperature which is related with the viability loss at this storage temperature. Slighter wrinkles in the immobilized-bacteria were also observed in CRL1332 + PVA nanofibers stored to refrigeration temperature. In the same way, Amna et al. (2013) evidenced morphological changes in the microstructure of PVA-nanofibers-immobilized *L. gasseri* due to long-term storage.

3.3.2. Physico-chemical characterization

As expected, nanofibers obtained from pure PVA and CRL1332 + PVA showed to be highly hydrophilic since they were dissolved instantly when contacted with water. A decrease of the hydrophilic nature of nanofibers was evidenced with the incorporation of skim milk-lactose with and without *L. rhamnosus* CRL1332 (SM-L + PVA and CRL1332 + SM-L + PVA) since higher contact angles (57.70 ± 5.72 and 18.78 ± 0.10°, respectively) were obtained. This fact could be supported by the lipidic content of milk. Moreover, the functional groups on the *L. rhamnosus* CRL1332 surface and the enlargement of the exposed area of the fibers when surrounding the bacterial cells could lead to intermediate values for the loaded nanofibers. However, taking into account the values of their contact angles (less than 65°), these nanofibers still indicated an hydrophilic behavior. The highly hydrophilic nature of nanofibers should be pointed out to allow the quick and easy release of *L. rhamnosus* CRL1332 in products for vaginal delivery (Nagy et al., 2014; Rodrigues et al., 2015).

Thermogravimetric analysis (TGA) was used to characterize the thermal degradation of the nanofibers. The first derivative of the material weight loss as a function of the temperature is shown in **Fig. 4**. The representation of the rate of material weight change upon heating against temperature was used to simplify the thermograms readings in order to identify in a better way the different thermal processes in composite materials. After the dehydration processes of the adsorbed water produced around 100°C, the compositional differences between the samples led to variations in the degradation of the materials.

The nanofibers containing skim milk-lactose displayed an additional peak between 150°C and 250°C, encompassing several processes, namely the loss of crystallization water, anomerization and lactose melting and degradation (reported at 155°C, 177°C and 222°C, respectively for lactose monohydrate) (Listiohadi et al., 2009). In particular, CRL1332 + SM-L + PVA-nanofibers exhibited the earliest degradation.

The thermal decomposition of PVA polymer shows a two-stage process. Starting around its melting temperature, the first step showed a maximum at 315°C, related to the release of acetyl groups that were transformed into acetic acid molecules with subsequent in situ chain stripping (Amna et al., 2013). The incorporation of *L. rhamnosus* CRL1332 in CRL1332 + PVA-nanofibers and CRL1332 + SM-L + PVA-nanofibers slightly modified the shape of this peak in the first stage, probably because of the intermolecular interactions, though there was no modification of the maximum peak temperature. SM-L + PVA-

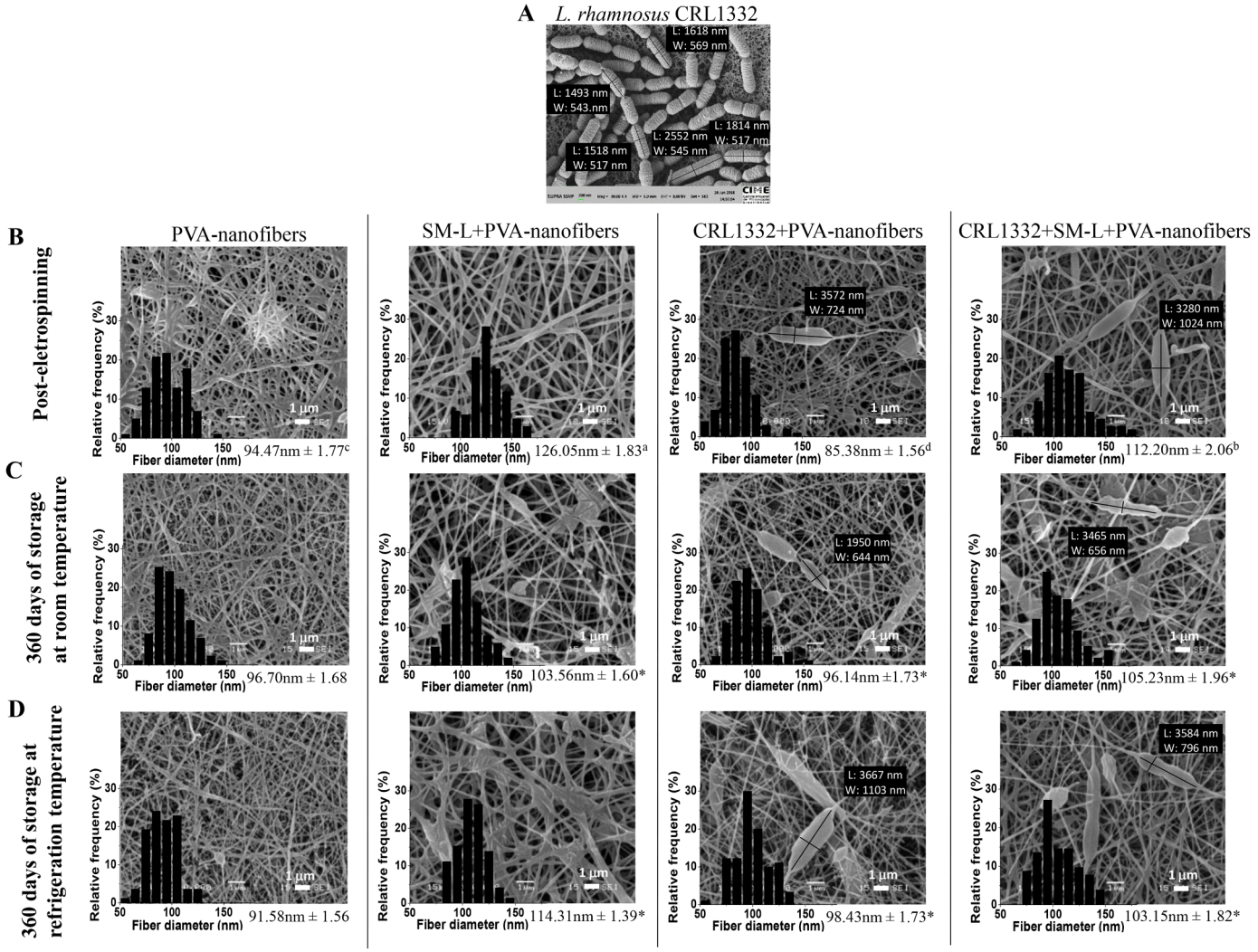

Fig. 3. Scanning electron microscopy of nanofibers obtained by electrospinning and stored at different temperature. (**A**) *L. rhamnosus* CRL1332 (CRL1332) in planktonic state. (**B**) Nanofibers obtained post-process and after 360 days of storage at (**C**) room and (**D**) refrigerate temperatures. Also, the size distribution histograms of the nanofibers in different conditions are included. Results represent two independent experiments. The average nanofiber diameters ± standard error are included in the histograms. Different letters indicate statistically significant differences ($p < 0.05$) in the diameters. (*) Indicates statistically significant differences between initial nanofiber diameters and those of each storage temperature ($p < 0.05$).

nanofibers exhibited the PVA main degradation peak with a maximum at 340°C, but this thermostability conferred by the skim milk-lactose phase to the nanofibers seems to be reversed when *L. rhamnosus* CRL1332 was incorporated. The second step in PVA degradation relates to the C-C backbone degradation, with a maximum at 465°C for PVA-nanofibers and at 438°C for CRL1332 + PVA-nanofibers. This slight advance of the peak maximum temperature for the backbone cleavage stage was similar for all the composite materials assayed in this work. Results show that the degradation features of each component inside the nanofibers are affected by the chemical interactions at the molecular level, and then processes are either accelerated or delayed.

The thermal characterization of the nanofibers was also carried out by DSC. **Table 3** shows the melting point (T_m), melting enthalpies (ΔH_m), and degree of crystallinity (χ_c) for PVA in the water-based nanofibers, and the glass transition temperatures (T_g) of all the electrospun nanofibers. The peak assigned to the polymer melting was detected around 194-195°C in the unloaded nanofibers. DSC thermograms performed to *L. rhamnosus* CRL1332 solid samples showed an asymmetric endothermic peak around 95-125°C (curve not shown), that could be assigned to the *Lactobacillus* decomposition according to the results published by Škrlec et al. (2019) by using freeze-dried *Lactobacillus*. However, no own bacterial thermal events were distinguished in the curves of the CRL1332 + PVA-nanofibers, probably due to the lower relative mass content and the overlap with the dehydration processes of the adsorbed water. Indeed, nanofibers without lactobacilli exhibited a T_g value at 100-107°C, probably associated with certain water contain. When incorporating *L. rhamnosus* CRL1332, a decrease in T_g was observed with respect to the unloaded nanofibers, probably because of the distortion of the polymer arrangement. This effect was particularly more marked for the water-based nanofibers, where CRL1332 + PVA-nanofibers exhibited also lower melting enthalpy (ΔH_m) and crystallinity than the PVA-nanofiber counterpart. Skrlec et al. (2019) reported a similar effect for *L. plantarum* incorporated to PEO electrospun nanofibers, leading to lower temperatures, melting enthalpies and crystallinity values for the polymer peak in the loaded systems, as a consequence of the interactions between components.

On the other hand, the addition of skim milk-lactose as bioprotector led to a change in the shape of the polymer melting peak, given that degradation processes of the additive occur in this range as well, as shown by TGA. Skim milk-lactose-containing nanofibers presented T_g values slightly higher to their water-based counterparts. These results suggest that particles like sugar and proteins from skim milk-lactose could enhance the chain rigidity in the amorphous phase of the

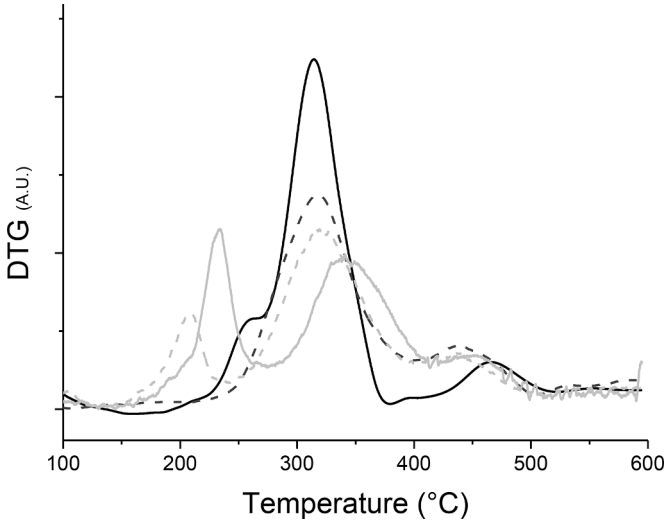

Fig. 4. First derivative of the material weight loss as a function of temperature (DTG) curve of nanofibers neat and loaded with *L. rhamnosus* CRL1332 (CRL1332) obtained by electrospinning. Curves of nanofibers from pure PVA (—), CRL1332 + PVA (---), SM-L + PVA (—) and CRL1332 + SM-L + PVA (---).

nanofibers (López-Rubio et al., 2009).

When analyzing the thermal events of nanofibers during the storage at different temperatures, no differences were found in the melting peak temperature for CRL1332 + PVA-nanofibers stored for 12 months. When stored, the T_g values did not follow a defined trend, but the transition seemed more marked and broader in all the refrigerated systems. A more marked glass transition temperature is associated with a more defined amorphous polymer phase. In other words, the variation in heat capacity is increased in refrigerated systems (not shown).

The characteristic functional groups in the nanofibers were studied by ATR-FTIR. The spectra of pure PVA, CRL1332 + PVA, SM-L + PVA, and CRL1332 + SM-L + PVA-nanofibers obtained post-electrospinning are shown in Fig. 5. A small band around ~1040 cm^{-1} was detected in the spectrum of CRL1332 + PVA-nanofibers which is absent in PVA-nanofibers. It could correspond to the δ(COH) vibrations of the cell wall peptidoglycan of immobilized bacteria (Kiwi and Nadtochenko, 2005; López-Rubio et al., 2009). However, the *L. rhamnosus* CRL1332 incorporation was more difficult to identify in CRL1332 + SM-L + PVA-nanofibers, where the lactose with an absorption range of 930 to 1190 cm^{-1} presented intensity large enough to mask the peptidoglycan region (Solís-Oba et al., 2011). On the other hand, the amide I (1600-1680 cm^{-1}) bands were only detected in CRL1332 + PVA, CRL1332 + SM-L + PVA and SM-L + PVA-nanofibers, probably related to the protein and peptide contents of the bacterial surface and milk (Fig. 5). Additionally, one broad band was detected for amide II groups in the SM-L + PVA-nanofibers in the range of 1555-1565 cm^{-1}; while two weak shoulders (~1545 cm^{-1}) were visible for the two CRL1332-loaded nanofibers. Interactions of *L. plantarum* with the excipients within PEO electrospun nanofibers were proposed to explain the modifications in the shape and vibrational frequencies of the amide I peaks (Škrlec et al., 2019). When evaluating the spectra of all the nanofibers, no significant changes were detected after the storage at room temperature. However, a decrease in the intensity of the band at 1333 cm^{-1} was observed for the refrigerated samples, that could be assigned to the in-plane O-H bend (Smith, 1999) (**Fig. S2**). Refrigeration could have restricted the molecular mobility during storage, thus modifying the hydrogen bonding interactions between components within the nanofibers.

3.3.3. Biological characterization of nanofibers

To know if the nanofiber-immobilized *L. rhamnosus* CRL1332 retained the biological functionality even after subjected to the high voltage of electrospinning and stored under refrigeration conditions, its inhibition to urogenital pathogens was evaluated. The *L. rhamnosus* CRL1332 antimicrobial effect obtained from nanofiber-immobilized or free cells is shown in Fig. 6. *L. rhamnosus* CRL1332 maintained the antimicrobial properties when immobilized in nanofibers both with and without bioprotective substances. No significant differences ($p > 0.05$) were evidenced in *C. albicans* C2 and *S. aureus* F1 inhibition patterns in nanofibers-immobilized or free lactobacilli. However, the inhibition of *E. coli* 16 and *S. aureus* NH1 was higher with nanofiber-immobilized *L. rhamnosus* CRL1332 compared to free cells. Only in the case of *St. agalactiae* BII, the immobilization of *L. rhamnosus* CRL1332 in nanofibers (without protective substances) slightly affected its inhibitory capability (significantly lower inhibition halos, $p < 0.05$ referred to control). Similar results of *L. rhamnosus* CRL1332 antimicrobial effect were observed from nanofibers stored during 180 days at refrigeration temperatures, demonstrating that these conditions did not affect the antimicrobial capacity of the strain (data not shown).

4. Conclusions

In the present work, *L. gasseri* CRL1320 and *L. rhamnosus* CRL1332, two probiotic strains isolated from healthy human vagina were successfully immobilized in polyvinyl-alcohol nanofibers by electrospinning. *L. rhamnosus* CRL1332 showed higher resistance to the immobilization process compared to *L. gasseri* CRL1320, and the two strains evidenced a high number of viable cells (~10^7 CFU/g) in nanofibers stored at -20°C up to 14 days. The shelf-life of *L. rhamnosus* CRL1332 was extended during 360 days at 4°C (up to 1.7 × 10^8 CFU/g,

Table 3
Thermal characterization of nanofibers by differential scanning calorimetry (DSC).

Nanofibers	Initial[a]				Storage time (360 days)[b]					
					Room temperature			Refrigeration temperature		
	T_m[c] (°C)	ΔH_m[d] (J/g)	T_g[e] (°C)	x_c[f]	T_m (°C)	ΔH_m (J/g)	T_g (°C)	T_m (°C)	ΔH_m(J/g)	T_g (°C)
PVA	194.92	33.10	103.14	39.68	194.55	30.35	65.76	195.06	25.28	98.62
CRL1332 + PVA	189.08	20.03	76.94	24.02	189.41	18.22	91.49	188.64	21.86	97.48
SM-L + PVA	*	*	107.41	*	*	*	66.01	*	*	90.52
CRL1332 + SM-L + PVA	*	*	105.09	*	*	*	84.28	*	*	89.86

[a] Thermal characterization of nanofibers obtained post-electrospinning.
[b] Thermal characterization of nanofibers storage during 360 days at room and refrigeration temperatures.
[c,d] Maximum melting temperature (T_m) and melting enthalpy (ΔH_m), respectively.
[e] Glass transition temperature (T_g) determined in the half of the transition.
[f] Degree of crystallinity (χ_c) = $\Delta H_m.100/[(w.\Delta H_m^0)]$; ΔH_m^0 is the melting heat for the 100% crystalline polymer, estimated to be ΔH_m^0 = 139 J/ g; [65]w is the weight fraction of polymeric material in the respective composite.
* T_m and ΔH_m of PVA were not calculated for nanofibers containing skim milk-lactose, as degradation of the additive takes place within the same temperature range.

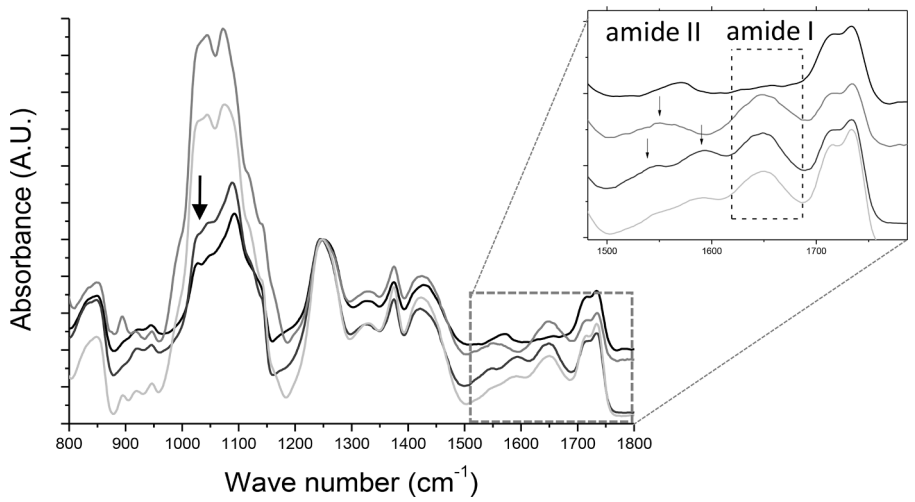

Fig. 5. ATR-FTIR spectra of unloaded and loaded nanofibers with *L. rhamnosus* CRL1332 (CRL1332) obtained by electrospinning. Spectra of nanofibers from pure PVA (black line), CRL1332 + PVA (dark grey line), SM-L + PVA (grey line) and CRL1332 + SM-L + PVA (light grey line).

data similar to freeze-dried) in nanofibers stored in oxygen-excluding packaging, and without the requirement of a bioprotective added. Hydrophilic nanofibers were obtained, highly suitable to an easy and quick release of *L. rhamnosus* CRL1332 in products for vaginal delivery. Moreover, nanofiber-immobilized *L. rhamnosus* cells maintained their ability to inhibit urogenital pathogens, they could be included in vaginal probiotic products to prevent or treat female urogenital infections. In summary, this study proposes a promising vaginal nanodelivery system based on the use of nanofibers loaded with probiotic vaginal *L. rhamnosus* CRL1332, which presents long shelf life and maintains the beneficial properties when stored at low temperature. Further studies should be performed to improve the survival of nanofiber-immobilized *L. rhamnosus* CRL1332 during storage at room temperature and to evaluate its interaction with mucosa vaginal components.

CRediT authorship contribution statement

Jessica Alejandra Silva: Data curation, Formal analysis, Methodology, Software, Validation, Writing - original draft. **Priscilla Romina De Gregorio:** Conceptualization, Methodology, Formal analysis, Funding acquisition, Investigation, Project administration, Software, Supervision, Writing - review & editing. **Guadalupe Rivero:** Data curation, Formal analysis, Methodology, Software, Writing - review & editing. **Gustavo A. Abraham:** Funding acquisition, Project administration, Supervision, Writing - review & editing. **María Elena Fátima Nader-Macías:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare there is no conflict of interest. The results obtained were included in a patent presentation (INPI, 2018, N° 20180103893).

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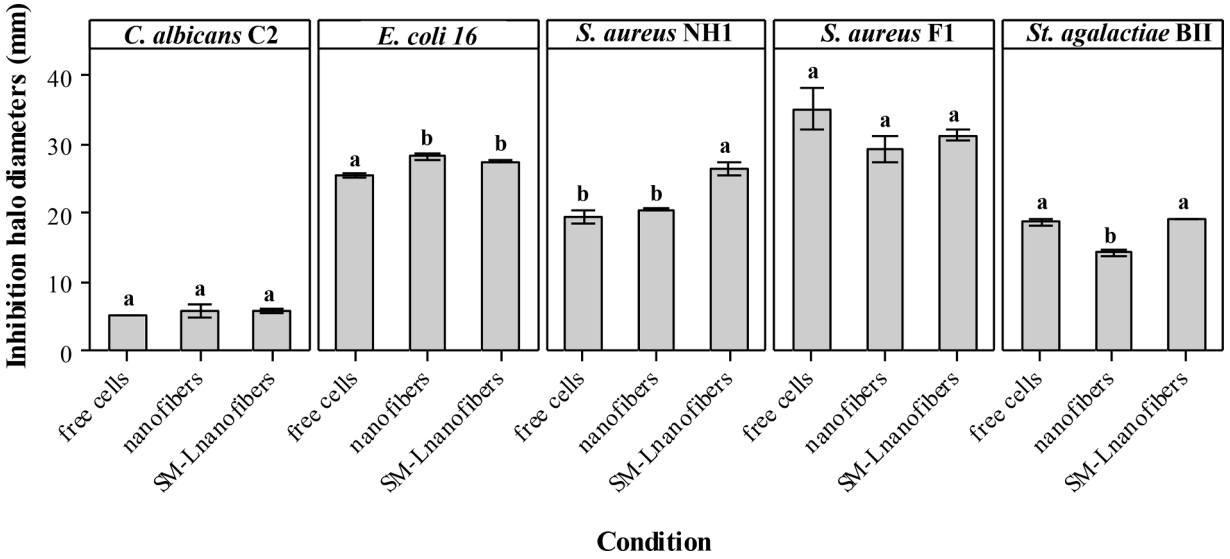

Fig. 6. Inhibition of urogenital pathogens by *L. rhamnosus* CRL1332 from pure cultures (free cells) and nanofibers without and with protective substance (nanofibers and SM-L-nanofibers, respectively). The data express the mean values ± standard error of the inhibition halo diameters. Different letters indicate statistically significant differences ($p < 0.05$) in the diameter of halos of the different conditions assayed for each pathogen, according to the Tukey test.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2020.105563.

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