Microbial Cell Factories

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Modified vaginal lactobacilli expressing fluorescent and luminescent proteins for more effective monitoring of their release from nanofibers, safety and cell adhesion

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Abstract
Electrospun nanofibers offer a highly promising platform for the delivery of vaginal lactobacilli, providing an innovative approach to preventing and treating vaginal infections. To advance the application of nanofibers for the delivery of lactobacilli, tools for studying their safety and efficacy in vitro need to be established. In this study, fluorescent (mCherry and GFP) and luminescent (NanoLuc luciferase) proteins were expressed in three vaginal lactobacilli (*Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenii*) and a control *Lactiplantibacillus plantarum* with the aim to use this technology for close tracking of lactobacilli release from nanofibers and their adhesion on epithelial cells. The recombinant proteins influenced the growth of the bacteria, but not their ability to produce hydrogen peroxide. Survival of lactobacilli in nanofibers immediately after electrospinning varied among species. Bacteria retained fluorescence upon incorporation into PEO nanofibers, which was vital for evaluation of their rapid release. In addition, fluorescent labelling facilitated efficient tracking of bacterial adhesion to Caco-2 epithelial cells, while luminescence provided important quantitative insights into bacterial attachment, which varied from 0.5 to 50% depending on the species. The four lactobacilli in dispersion or in nanofibers were not detrimental for the viability of Caco-2 cells, and did not demonstrate hemolytic activity highlighting the safety profiles of both bacteria and PEO nanofibers. To summarize, this study contributes to the development of a promising delivery system, tailored for local administration of safe vaginal lactobacilli.

Keywords Vaginal lactobacilli, Electrospinning, Nanofibers, NanoLuc luciferase, Bioluminescence, Fluorescent proteins, Caco-2 cells

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Introduction

Vaginal lactobacilli play an important role in protecting the host from urogenital infections. They are the predominant species in the human vagina and are crucial for maintaining normal microbial homeostasis [1]. The most important lactobacilli in the healthy vagina are *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii* and *Lactobacillus iners*. Each of the above-mentioned lactobacilli species, together with other vaginal microbial species, belongs to distinct community state type (CST) [2, 3]. Five CSTs have been described, of which CST I, II, III and V are dominated by lactobacilli, while the CST IV contains a diverse group dominated by strictly anaerobic bacteria from the genera *Atopobium*, *Gardnerella*, *Parvimonas*, *Prevotella*, *Sneathia*, and several other taxa associated with bacterial vaginosis [2]. CST varies with age, phase of the menstrual cycle, and is influenced by several lifestyle factors, including type of diet and alcohol consumption, relationships with one or more partners, hygiene habits and drug use [4].

Lactobacillus species are Gram-positive, facultatively anaerobic bacteria and are a hallmark of a healthy vagina. They inhibit the progression of vaginal pathogens by adhering to the epithelial mucus and thereby preventing pathogen colonization [5]. Lactobacilli also produce antimicrobial compounds such as lactic acid, hydrogen peroxide, bacteriocins and biosurfactants [6]. A reduction in the number of lactobacilli leads to vaginal dysbiosis, which causes an overgrowth of the CST IV microbiota, thus leading to vaginal infections. Re-establishing the normal vaginal microbiota with lactobacilli as probiotics can prevent the progression of vaginal infections. Many lactobacilli, especially the most dominant *L. crispatus*, have shown positive effects against vaginal pathogens [7–10].

The use of lactobacilli as probiotics is more commonly associated with gut health than vaginal health. Apart from the limited number of effective and well-studied strains, the lack of a suitable delivery systems for local administration hinders the use of lactobacilli for the treatment of vaginal infections. The vaginal delivery systems currently in use have their limitations as they can cause discomfort, leakage, inaccurate dosing and short residence time [11]. The ideal vaginal delivery system should be adherent, non-toxic and able to maintain the stability of the administered drug [12]. Nanofibers, produced by electrospinning [13], present such potential delivery systems for various drugs [14–16], including probiotics [17–19]. Some studies have already reported the incorporation of vaginal probiotics into various nanofiber formulations [20–23].

To evaluate the suitability of nanofibers for vaginal delivery of lactobacilli, their fate after release from the nanofibers has to be monitored. Fluorescent proteins have been used for decades to visualize living cells. Genes encoding fluorescent proteins can be inserted into lactobacilli using genetic engineering. Thus far, several studies have reported engineering of fluorescent lactobacilli and their use in imaging lactobacilli in vitro and in vivo [24–27]. Recently, we have reported the expression of fluorescent proteins with different spectral properties, such as green fluorescent protein (GFP), blue fluorescent protein (mTagBFP2), red fluorescent protein (mCherry) and infrared fluorescent protein (IRFP) in lactobacilli and their incorporation into nanofibers [28, 29]. Fluorescent proteins have also been used to visualize the adhesion of lactobacilli to the surface of epithelial cells [26].

In addition to fluorescence, chemiluminescence can also be used for cell imaging. This imaging technique is based on an enzyme catalyzed reaction, in which an enzyme oxidizes a substrate resulting in light emission. The most common enzyme for luminescence reactions in living cells is firefly luciferase with its substrate luciferin [30]. There are several examples in which this combination was applied for imaging lactobacilli [31–34]. Recently, NanoLuc (Nluc) luciferase with better properties has been introduced [35], but has not been expressed in lactobacilli yet. On the downside, overexpression of recombinant proteins can cause metabolic burden for bacteria, disrupting the strain's normal metabolic functions [36]. Therefore, the engineered and non-engineered lactobacilli should be compared to exclude interference with the probiotic properties of lactobacilli.

In our previous studies we genetically engineered four lactobacilli species (three vaginal lactobacilli *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenii* and control *Lactiplantibacillus plantarum*), evaluated the expression of four fluorescent proteins with different spectral properties, and incorporated them in polyethylene oxide (PEO) nanofibers [29]. We have also tested the stability of these lactobacilli in different nanofiber formulations [23]. The aim of the present study was to upgrade the existing knowledge by assessing the metabolic burden of the recombinant protein expression in the four lactobacilli, tracking the release kinetics of the lactobacilli from PEO nanofibers, testing their effect on epithelial cells viability, hemolytic activity, autoaggregation, hydrophobicity, hydrogen peroxide production and their adhesion to epithelial cells. For that purpose, we chose only two fluorescent proteins (mCherry and GFP) from previous work, and additionally introduced a luminescent (NanoLuc luciferase) protein as a novel tool for bacteria quantification.

Experimental procedures

Bacterial strains and culture conditions

L. crispatus ATCC 33820, *L. gasseri* ATCC 33323, *L. jensenii* ATCC 25258 and *L. plantarum* ATCC 8014 were

grown in De Man, Rogosa, and Sharpe (MRS) medium (Merck, Darmstadt, Germany) at 37 °C in anaerobic bags (GasPakTM EZ; Becton Dickinson, Franklin Lakes, NJ, USA) or jars (AnaeroGenTM 2.5 L; Thermo Scientific, Waltham, MA, USA). MRS was supplemented with 10 µg/mL chloramphenicol (MRSC10) if needed. The engineered lactobacilli were kept frozen at −80 °C in MRS medium supplemented with 20% glycerol for long-term storage. For culturing, they were transferred from frozen stocks to solid MRSC10 medium and grown anaerobically at 37 °C for 2–3 days. Then they were inoculated (single colony) into 5 mL of liquid MRSC10 medium and grown for one day at 37 °C. Bacterial culture was then prepared by inoculating the over-night culture (1:50 dilution) in fresh MRSC10 medium and incubating at 37 °C with aeration (shaking at 180–200 rpm). The four lactobacilli were grown to the late exponential or early stationary phase and centrifuged at 4400 × *g* for 10 min at 4 °C (Centrifuge 5702 R; Eppendorf, St. Louis, MO, USA) and resuspended in phosphate-buffered saline (PBS, pH 7.4), unless noted otherwise. *Lactococcus lactis* NZ9000 and *Escherichia coli* DH5α were utilized as intermediate hosts for cloning. *L. lactis* was grown at 30 °C in M17 medium (Merck) supplemented with 0.5% (v/v) glucose (GM-17), while *E. coli* was grown at 37 °C, with aeration in a lysogeny broth medium. All bacteria were stored at −80 °C for long-term storage.

Molecular cloning and plasmid construction

Plasmid pNZ-ldh-mCherry [29] containing *ldh-mCherry* was used to transform the four *Lactobacillus* species. Additionally, *L. crispatus* was also transformed with pMEC276 [37] containing *ldh-GFP* (for clarity, indicated as pNZ-ldh-GFP). Overlap-extension (OE) PCR [29, 38] with KOD Hot Start DNA polymerase (Merck Millipore, Burlington, MA, USA) was used to construct plasmid harboring the luciferase NanoLuc (Nluc). pMSP: Nluc [39] was used as a template for the *nluc* gene, whereas pNZ-ldh-mCherry was used as a template for the *ldh* promoter. The fused DNA fragment was inserted into pJET1.2/blunt vector and transformed into *E. coli* DH5α competent cells. Fused *ldh-nluc* was digested with XbaI/BglII restriction enzymes (Thermo Scientific, Waltham, MA, USA) and ligated into the pNZ8148 [40] using T4 DNA ligase (Thermo Scientific, Waltham, MA, USA). The new plasmid (designated as pNZ-ldh-Nluc) was electroporated into *L. lactis* using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA, USA). Plasmids were isolated from *E. coli* and *L. lactis* using NucleoSpin Plasmid (Macherey and Nagel, Düren, Germany) with an additional lysozyme treatment (50 mg/mL) when isolating from *L. lactis*. The engineered plasmids were transformed into the four lactobacilli using previously described electroporation protocols [29].

Growth characteristics of engineered and non-transformed lactobacilli

The potential effect of recombinant protein (mCherry, GFP and Nluc) expression on the growth of lactobacilli was evaluated by assessing their growth curves, growth rates and lag times. The culturing and optical density measurements were conducted in a microplate reader (Sunrise; Tecan, Salzburg, Austria) and analysed with the Baranyi and Roberts model using DMFit 3.5 software [41] as previously described [23, 42]. Briefly, fresh overnight cultures of engineered and NT lactobacilli were diluted (1:50) in 200 µL MRS media. Each strain was cultivated in triplicate in 96 well microplates covered with sealing film and incubated at 37 °C for 48 h with absorbance readings taken every 2 min.

Measurement of luminescence

To evaluate the luminescence as a function of the concentration, five serial, five-fold dilutions of the dispersion of bacteria with pNZ-ldh-Nluc were prepared in PBS. Number of colony forming units (CFU) was also determined using the drop-plate method [43] in which five 10 µL drops of serial ten-fold dilutions were pipetted onto MRSC10 agar plates and incubated anaerobically at 37 °C for 2–3 days. Luminescence was measured in duplicate in 96-well white plates (Corning Incorporated COSTAR −96 Well Assay Plate, White Plate, Clear Bottom with Lid Tissue Culture Treated Polystyrene) using a microplate reader (luminescence mode; integration time, 1 s; settle time, 150 ms) and Nano-Glo Luciferase assay kit (Promega, Madison, United States). Bacterial dispersions (25 µL) were mixed with 25 µL reagent and luminescence signal was measured after 3 min. Non-transformed (NT) bacteria were used as controls. Precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and linear concentration range were determined as previously described [39]. Briefly, dispersion of lactobacilli with different concentrations were prepared from standard culture in PBS (with drop-plated determined concentration). Dilutions (5-fold) were made in PBS. Different concentrations were used to determine linear concentration range, which was then used for the construction of the calibration curve and linear regression for the quantification of samples with unknown concentration. The limit of detection (LOD) and limit of quantification (LOQ) were determined as the means of six measurements of the blank increased by 3 standard deviations (LOD) and by ten standard deviations (LOQ), and calculated using the calibration curve. The accuracy was determined by calculating the relative error of determination of three known concentrations of lactobacilli, each as two biological and three technical repeats. The precision was determined by calculating the coefficient of variation of determination

of three known concentrations of lactobacilli, each as six technical repeats.

Production of hydrogen peroxide by lactobacilli

Production of hydrogen peroxide by lactobacilli was assessed using a chromogenic method [44] on MRSC10 agar plates supplemented with, 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma Aldrich, Darmstadt, Germany) and horseradish peroxidase (Thermo Scientific, Waltham, MA, USA). Fresh overnight cultures of engineered lactobacilli with pNZ-ldh-mCherry or pNZ-ldh-GFP in the case of *L. crispatus* were transferred onto the modified MRS agar medium using a sterile inoculation loop and incubated at 37 °C for 48 h under anaerobic conditions. After incubation, the plates were exposed to air for 30 min, resulting in the appearance of blue colonies, indicating hydrogen peroxide production.

Autoaggregation and hydrophobicity by lactobacilli

Autoaggregation and hydrophobicity were evaluated by centrifuging the over-night cultures of fluorescent lactobacilli, washing them twice and resuspending them to an OD_{600} of 0.5. Autoaggregation was determined by recording bacterial OD_{600} at hourly intervals over a period of 6 h. Hydrophobicity was determined by extracting the bacteria with n-hexadecan as previously described [18].

Electrospinning of nanofibers with lactobacilli

Because of the different growth kinetics and stationary cell concentrations [23], the four lactobacilli were grown as described above in different volumes, namely *L. gasseri* and *L. plantarum* in 500 mL, *L. crispatus* in 1000 mL, and *L. jensenii* in 300 mL. Preparation of bacteria and electrospinning parameters depended on the type of experiment.

Electrospinning of nanofibers with lactobacilli for evaluation of release kinetics, scanning electron microscopy and cell adhesion

Lactobacilli were washed twice with cold distilled water and resuspended in 10 mL of cold distilled water. Bacterial dispersion was added into vials with 400 mg of PEO powder (Mw 900 kDa; Sigma Aldrich, Darmstadt, Germany). The mixture was stirred at 400 rpm at 4 °C over-night in the dark to dissolve polymer and obtain a polymer concentration of 4% (w/v). Bacterial dispersions were loaded in 5 mL syringes and attached to a pump which is linked to an electrospinning machine (Spinbox, BioInicia SL, Valencia, Spain). Electrospinning was performed horizontally and the nanofibers were collected on a metal collector covered with aluminium foil. The electrospinning conditions were as follows: flow rate 350–500 µL/h, applied voltage 14–16 kV, distance between needle and collector 15 cm, humidity ~ 30% and room temperature.

Electrospinning of nanofibers with lactobacilli for evaluation of viability

Lactobacilli were washed once with different solutions: *L. plantarum* and *L. gasseri* with PBS, *L. jensenii* with 4% (w/v) sucrose and *L. crispatus* with 0.9% (w/v) NaCl. The bacteria were then resuspended in 5 mL water and mixed with 5 mL 8% (w/v) PEO solution for 15–30 min at 4 °C to obtain 10 mL 4% (w/v) homogeneous bacterial-polymer dispersion. Electrospinning was performed as previously described with some modifications: flow rate: 150–250 µL/h, applied voltage 10–13 kV, distance between needle and collector 15 cm, humidity ~ 30% and room temperature.

Scanning electron microscopy

The morphology of engineered (harbouring pNZ-ldh-mCherry, pNZ-ldh-GFP (for *L. crispatus*) and pNZ-ldh-Nluc) and NT lactobacilli in PEO nanofibers was examined using scanning electron microscopy (Supra 35 VP, Carl Zeiss, Oberkochen, Jena, Germany), as previously described [23, 29]. Briefly, nanofibers (nine distinct combinations) were observed at 1 kV using a secondary electron detector.

Release kinetics of fluorescent lactobacilli

The four engineered lactobacilli harboring pNZ-ldh-mCherry were incorporated separately into PEO nanofibers. Three nanofiber mats (50 ± 5 mg) were added in three separate vials that contained 5 mL PBS. The vials were incubated at 37 °C with stirring at 50 rpm and after 5, 10, 20, 30, 45, 60, 90 and 120 min, a 400 µL aliquot was withdrawn from each vial and replaced with fresh PBS buffer. The withdrawn aliquot was divided into two 200 µL samples and transferred to 96-well black, flat-bottomed plates. The fluorescence was measured using a microplate reader (Infinite M1000; Tecan, Männedorf, Switzerland) at an excitation/emission wavelengths of 587 nm/610 nm for mCherry. NT bacteria were used as controls.

Viability of engineered lactobacilli in dispersion and in nanofibers

Serial ten-fold dilutions of lactobacilli-polymer dispersion or dissolved nanofiber mats (5 ± 1 mg) were prepared. Lactobacilli were diluted in PBS (*L. plantarum* and *L. gasseri*), 0.9% (w/v) NaCl (*L. crispatus*) or 4% (w/v) sucrose (*L. jensenii*). To enable comparison with nanofibers, the viability from dispersion was normalized to CFU/g dry mass as previously described [23]. Nanofiber mats were dissolved in 300 µL solution and the viability

was determined by the drop plate method as described above.

Hemolytic activity of lactobacilli

Hemolytic activity was evaluated using defibrinated sheep blood agar plates (Oxoid, Blood agar Base, Thermo Fisher Scientific, Waltham, MA, USA). The four lactobacilli were streaked onto the plates with sterile inoculation loop. Separately, PEO nanofibers (5 mg) and PEO suspension without bacteria were added to individual plates. All plates were incubated at 37 °C for 48 h under anaerobic conditions.

Caco-2 monolayer preparation

Caco-2 cells were suspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and seeded at a density of 5×10^4 cells per well and volume of 0.2 mL on collagen-coated 96-well plates and at a density of 2×10^5 cells per well and volume of 0.5 mL on collagen-coated 24-well plates. Collagen coating was performed just prior to addition of Caco-2 cells, according to the following protocol. Each well was filled with 1 mL of collagen (20 µg/mL, type I solution from rat tail, Sigma-Aldrich), incubated for one hour, aspirated and carefully rinsed twice with Dulbecco's PBS (DPBS, Gibco). Cells were cultured for a minimum of 21 days before analysis, replacing the media every 2 days, at 37 °C with 5% CO_2.

Viability of Caco-2 monolayer after co-incubation with fluorescent lactobacilli in dispersion or in nanofibers

Caco-2 cell medium was removed from the 24-well plate and replaced with fresh pre-warmed RPMI 1640 medium with L-glutamine and HEPES (Lonza, Basel, Switzerland) containing engineered lactobacilli with pNZ-ldh-mCherry or pNZ-ldh-GFP in the case of *L. crispatus* (OD_{600} of 1.0). In the case of nanofibers, RPMI was also replaced and 5±1 mg of nanofiber mats with fluorescent lactobacilli were added. Empty PEO nanofibers and Caco-2 cells without bacteria or nanofibers were used as controls. All samples were assayed in duplicate in four different plates which were incubated at different time points (2, 4, 6 and 24 h) at 37 °C with 5% CO_2. After incubation, the cells were washed once with PBS and detached with 75 µL of Triple select composed of 0.25% (w/v) trypsin and 0.53 mM EDTA for 6 min. Triple select was then inhibited with pre-warmed 10% FBS prepared in RPMI medium. The cells were then pipetted into a sterile Eppendorf tube and diluted with trypan blue (1:1). Live-dead cells were then counted microscopically (CKX53, Olympus) using a hemocytometer and the percentage of live-dead cells was calculated.

Adhesion of fluorescent bacteria from nanofibers to Caco-2 monolayer

Caco-2 monolayer was grown at 37 °C with 5% CO_2 and seeded in 24-well tissue culture plates with sterile coverslips at the bottom of each well [45]. Nanofiber mats (5±1 mg) loaded with lactobacilli expressing mCherry (*L. plantarum*, *L. gasseri* and *L. jensenii*) and GFP (*L. crispatus*) were added in duplicate to each well. Controls included 5±1 mg PEO nanofibers without bacteria, and Caco-2 cells without bacteria or nanofibers. The cells with nanofibers were incubated for 2 h at 37 °C with 5% CO_2. Cells were then gently washed three times with PBS to remove unattached lactobacilli and were fixed with 4% (v/v) paraformaldehyde for 20 min at room temperature and washed again three times with PBS. The samples were mounted with IBIDI mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Waltham, MA, USA) and were visualized under confocal microscope (LSM-710, Zeiss). Multiple focal planes (z-stack) were collected to provide three-dimensional data of the monolayer and attached bacteria.

Adhesion of luminescent bacteria from dispersion and nanofibers to Caco-2 monolayer

Bacterial attachment was also evaluated using the Nluc bioluminescence assay. Bacterial dispersions in RPMI medium (OD_{600} of 2.0 and 1.0 for *L. plantarum*, *L. gasseri* and *L. jensenii;* OD_{600} of 3.0 and 1.5 for *L. crispatus*) were incubated with Caco-2 cell monolayer. Caco-2 cells were grown in white sterile 96-well plates as described in Sect. Caco-2 monolayer preparationScanning electron microscopy. Culture media was replaced with 125 µL of fresh pre-warmed RPMI containing transformed or NT bacteria, or no bacteria. The plate was then incubated for 2 h at 37 °C with 5% CO_2. After the incubation, the cells were washed with PBS three times to remove the unattached bacteria. Then, 100 µL of 2 mM EDTA was added for 20 min to detach the cells from the plate. The cells were then transferred to a new 96-well plate, where the substrate from Nano-Glo Luciferase assay kit was added. The luminescence was measured as described above.

Similarly, attachment of bacteria from nanofibers was evaluated by incubating 5±1 mg or 2±1 mg PEO nanofiber mats loaded with luminescent lactobacilli with a Caco-2 cell monolayer. Empty PEO nanofibers and Caco-2 cells without nanofibers were used as controls.

Statistical analysis

Statistical analyses were performed using Prism 10.00 (GraphPad software, San Diego, CA, USA). Student's t test and one-way ANOVA with multiple pairwise comparison were used to define the significance of differences. The results were presented as means±standard deviation (SD).

Results

Growth characteristics of engineered and non-transformed vaginal lactobacilli

The growth of the lactobacilli differed when cultured under the same conditions (Fig. 1). *L. plantarum* demonstrated the highest average maximum OD of 1.12, followed by *L. crispatus* with an average maximum OD of 0.83, *L. gasseri* 0.27 and *L. jensenii* 0.14. The expression of recombinant proteins mCherry, Nluc and GFP influenced the growth of the lactobacilli. *L. plantarum* expressing mCherry reached lower maximum OD of 0.76. The metabolic burden imposed by these proteins was also evident in *L. gasseri*, where Nluc expression prolonged the lag phase and limited bacterial growth to a maximum OD of 0.19, while mCherry expression led to a slightly higher maximum OD of 0.22. On the other hand, the metabolic burden of the recombinant protein expression was minimal in *L. jensenii* and *L. crispatus*, with the engineered strains showing similar growth curves to those of the NT strains.

Differences in lag time and growth rate were also observed among the strains (Fig. S1), highlighting the impact of recombinant protein expression on lactobacilli. The engineered *L. plantarum* and *L. gasseri* exhibited longer lag times compared to the non-engineered strains. In contrast, in *L. jensenii* there was no significant difference between engineered and NT strain, while in *L. crispatus*, the engineered strains showed shorter lag time. The impact on growth parameters can be related to the amount of recombinant protein expression in the four species, as we previously demonstrated [29]. Higher expression of recombinant proteins in *L. plantarum* and *L. gasseri* likely imposed a greater metabolic burden, resulting in slower growth.

Hydrogen peroxide production in engineered vaginal lactobacilli

Vaginal lactobacilli produced hydrogen peroxide when plated on MRSC10 plates supplemented with TMB and horseradish peroxidase. The recombinant proteins did not affect the production of hydrogen peroxide (Fig S2.). *L. jensenii* was the fastest producer with blue colonies appearing after only 1 min of air exposure, followed by *L. gasseri* (5 min) and *L. crispatus* (8 min). *L. plantarum* showed no colour change, indicating an absence of hydrogen peroxide production. The most intense colour change was also observed in *L. jensenii*, confirming it as the best hydrogen peroxide producer among the four lactobacilli.

Nanofibers contain engineered and non-transformed vaginal lactobacilli

The expression of recombinant proteins did not influence the morphology of bacteria following incorporation in nanofibers (Fig. 2). NT, fluorescent and luminescent bacteria were observed as characteristic thickenings along the nanofibers, and no differences between engineered and NT lactobacilli could be established.

Fluorescent lactobacilli encoding fluorescent proteins were incorporated into PEO nanofibers and fluorescence was confirmed with a fluorescent confocal microscope. Discrete fluorescence signal corresponding to bacterial cells was colocalized with fibrous structures observed in bright-field mode. No fluorescence was observed in the empty PEO nanofibers (Fig. 3).

Release kinetics and viability of fluorescent vaginal lactobacilli in PEO nanofibers

To monitor the release kinetics, lactobacilli expressing mCherry were incorporated into PEO nanofibers. The fluorescence signal from the released bacteria allowed real-time study of their release from the nanofibers. Approximately 20–60% of the bacteria were released already within the first 10 min of incubation. After 30–45 min, the fluorescence intensity plateaued, indicating that almost all of the bacteria had been released and the nanofibers had dissolved (Fig. 4a).

The viability of fluorescent protein-labelled lactobacilli decreased following their incorporation into nanofibers. This was most pronounced with *L. crispatus*, for which viability was reduced by 4.73 log CFU/g after electrospinning. Less pronounced decrease in viability was observed with other three species (1.85 log CFU/g for

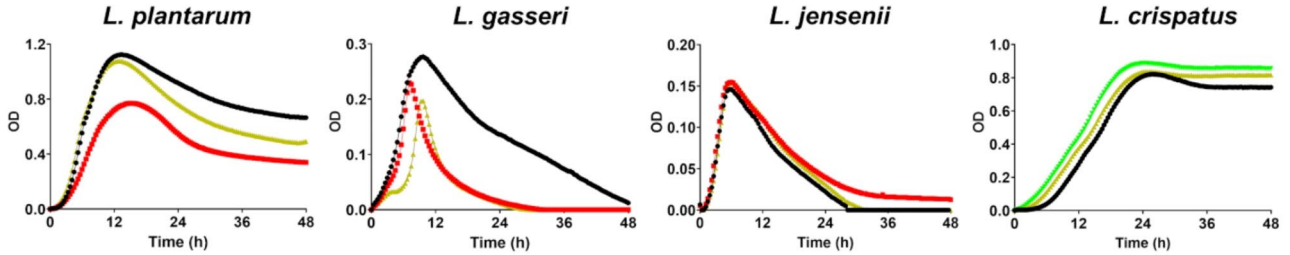

Fig. 1 Growth curves of engineered lactobacilli expressing mCherry (red lines), Nluc (dark yellow lines) and GFP (green lines). NT strains (black lines) were used as control

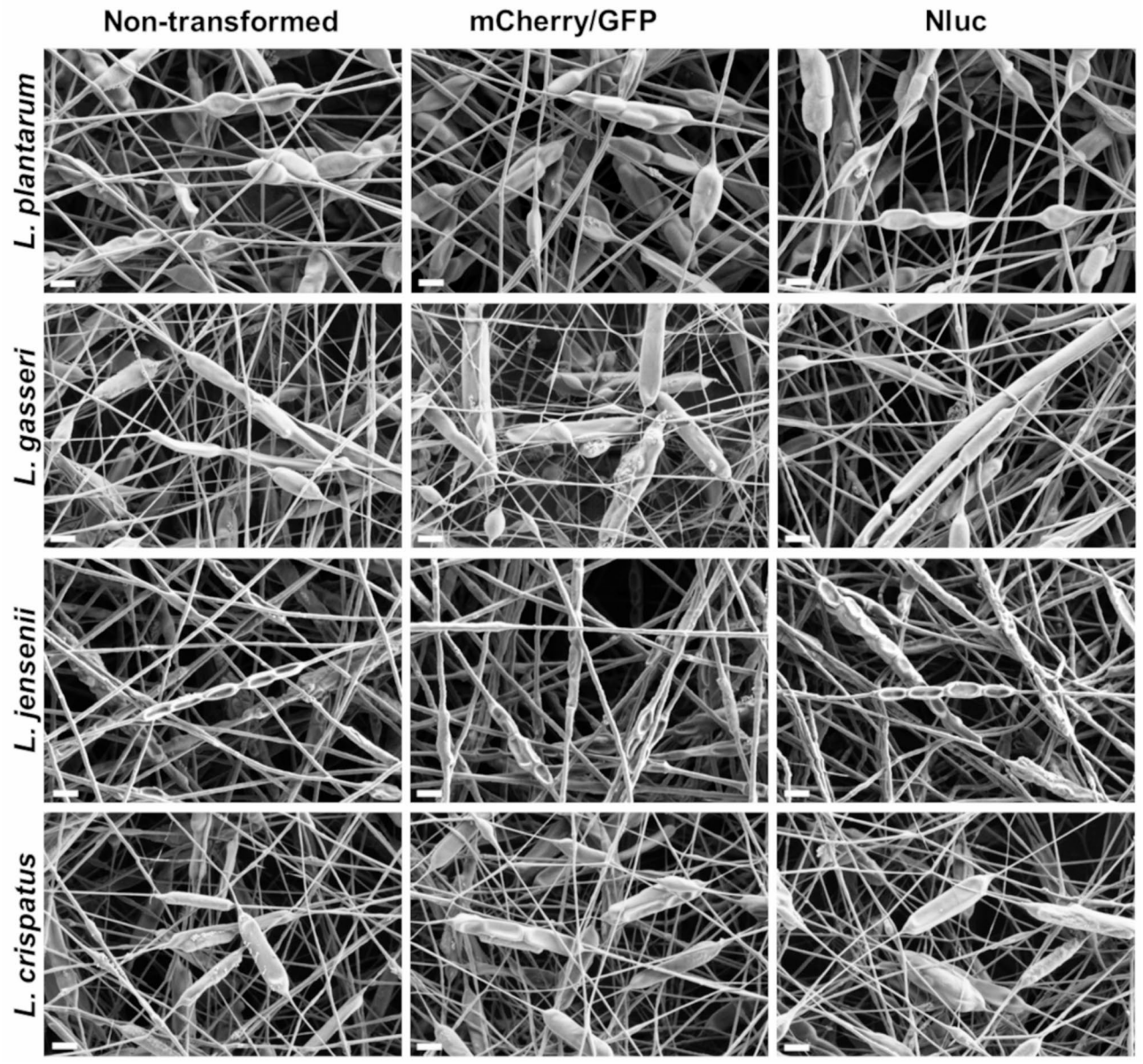

Fig. 2 Scanning electron microscopy of incorporated engineered and non-engineered vaginal lactobacilli into PEO nanofibers. The samples were imaged at a magnification of 20,000x. The scale bar in the bottom left represents 1 µm

L. plantarum, 1.30 log CFU/g for *L. gasseri* and 1.56 log CFU for *L. jensenii*; Fig. 4b).

Safety of fluorescent vaginal lactobacilli and PEO nanofibers assessed on defibrinated sheep blood agar plates and in Caco-2 monolayer model

The safety of lactobacilli and PEO were first evaluated on defibrinated sheep blood agar plates. After 48 h of incubation under anaerobic conditions, the bacteria and PEO (in suspension and nanofibers) did not show any hemolytic activity (Fig. 5a).

Additionally, the safety assessment of lactobacilli in dispersion (Fig. 5b) and incorporated in nanofibers (Fig. 5c) was evaluated in Caco-2 cell model by determining Caco-2 cell viability 2, 4, 6 and 24 h after addition. After 2, 4 and 6 h, the viability of Caco-2 cells co-incubated with dispersion of lactobacilli did not drop significantly; the exception was *L. plantarum* which caused slight, but significant decrease in viability after 4 and 6 h of incubation in comparison to control Caco-2 cells. After 24 h of incubation, there were no differences in viability between control cells and cells incubated with lactobacilli. When incorporated in nanofibers, *L. gasseri*, *L. crispatus* and *L. jensenii* again caused no significant decrease in the viability of Caco-2 cells. However, significant decrease in viability was observed with nanofibers containing *L.*

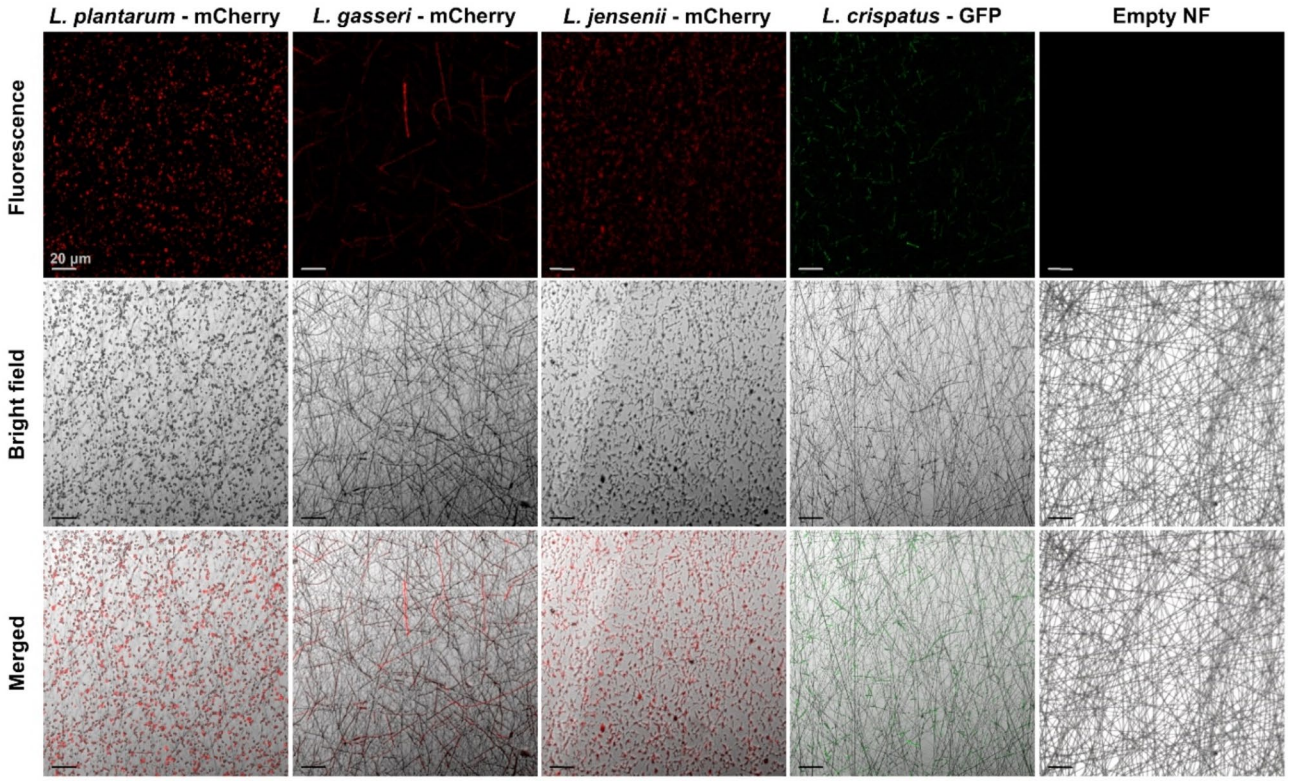

Fig. 3 Representative confocal microscopy images of mCherry-labelled lactobacilli *L. plantarum*, *L. gasseri* and *L. jensenii* and GFP-labelled *L. crispatus* incorporated into PEO nanofibers. Empty PEO nanofibers are shown for comparison

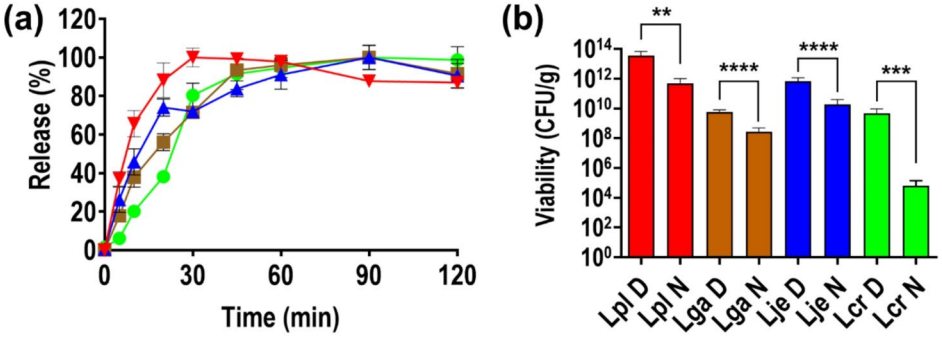

Fig. 4 Kinetics of release of vaginal lactobacilli from PEO nanofibers determined by measuring fluorescence (**a**), and the influence of electrospinning on the viability of fluorescent lactobacilli (**b**). Percentage of the released bacteria was determined based on the highest overall fluorescent signal. Viability in dispersion was normalized to dry mass to enable comparison with viability in nanofibers. Lpl, *Lactiplantibacillus plantarum* (red); Lga, *Lactobacillus gasseri* (brown); Lje, *Lactobacillus jensenii* (blue); Lcr, *Lactobacillus crispatus* (green); D, dispersion; N, nanofibers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Student's t test) relative to bacterial dispersion

plantarum. In comparison to control Caco-2 cells, nanofibers containing *L. plantarum* decreased Caco-2 cell viability for 13.4%, 10.4% and 25.6% after 4, 6 and 24 h, respectively. *L. plantarum* therefore seems to be more detrimental for Caco-2 cells.

Autoaggregation and hydrophobicity of lactobacilli

The autoaggregation and surface hydrophobicity of engineered lactobacilli expressing mCherry (or GFP in the case of *L. crispatus*) (Fig. 6) were analyzed as an indicator of intestinal cell adhesion. While autoaggregation did not differ among the species significantly (Fig. 6a), their surface hydrophobicity did, with *L. gasseri* exhibiting the highest surface hydrophobicity at 95.8%, followed by *L. jensenii* at 87.3%, *L. crispatus* at 21.4%. *L. plantarum* had the lowest hydrophobicity at 2.8% (Fig. 6b).

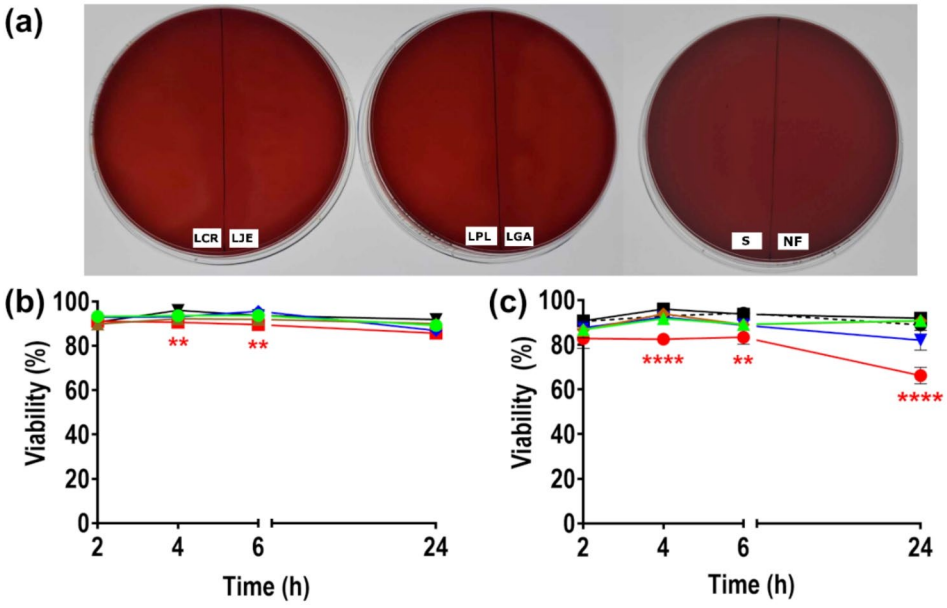

Fig. 5 (**a**) Hemolytic test of bacteria (*Lactiplantibacillus plantarum* (LPL); *Lactobacillus gasseri* (LGA); *Lactobacillus jensenii* (LJE); *Lactobacillus crispatus* (LCR)), PEO suspension (S) and PEO nanofibers (NF). Percentage of live Caco-2 cells after 2, 4, 6 and 24 h incubation with bacterial dispersion (**b**) or nanofibers (**c**). Caco-2 cells were incubated with *Lactiplantibacillus plantarum* (red); *Lactobacillus gasseri* (brown); *Lactobacillus jensenii* (blue); *Lactobacillus crispatus* (green). Controls are shown in black (full line – no addition, dotted line – nanofibers without bacteria). ANOVA with multiple pairwise comparison was performed for each time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; relative to control (Caco-2 cells without bacteria or nanofibers)

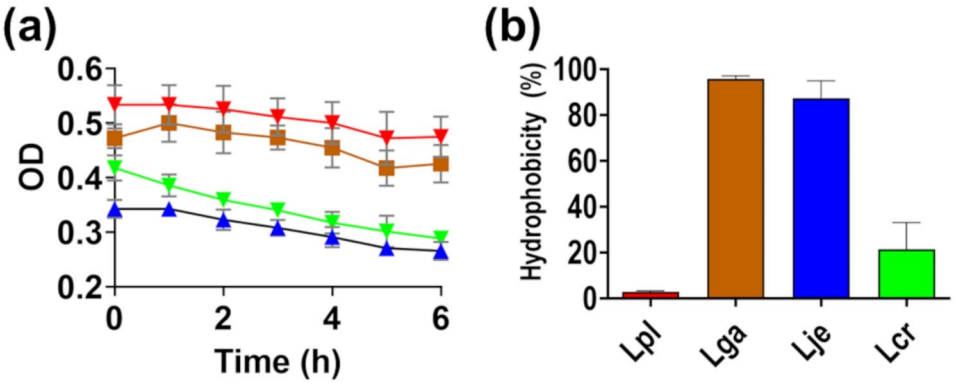

Fig. 6 (**a**) Autoaggregation of *Lactiplantibacillus plantarum* (red); *Lactobacillus gasseri* (brown); *Lactobacillus jensenii* (blue); *Lactobacillus crispatus* (green) monitored over a period of 6 h. Surface hydrophobicity (**b**) of *Lactiplantibacillus plantarum* (Lpl); *Lactobacillus gasseri* (Lga); *Lactobacillus jensenii* (Lje); *Lactobacillus crispatus* (Lcr) shown as a percentage and determined through extraction with n-hexadecane

Adhesion of fluorescent lactobacilli from nanofibers to Caco-2 cells

The fluorescent lactobacilli adhered to the Caco-2 cells following their release from nanofibers, as confirmed by confocal microscopy. Caco-2 cells were visualized using fluorescence staining of nuclei (DAPI). The adhesion of bacteria to the top of the cell monolayer was confirmed using Z-stack imaging. Reconstructed 3D images were rotated and tilted to better visualize the position of bacteria relative to Caco-2 cells (Fig. 7).

Quantification of lactobacilli via expression of Nluc and establishment of Nluc assay

The expression of Nluc luciferase in all tested vaginal lactobacilli was confirmed by comparing the luminescence signals of engineered bacteria and NT bacteria at different concentrations (Fig. 8). Increase in luminescence intensity as a function of bacterial concentration was observed in engineered bacteria only, indicating that Nluc assay can be used to quantify vaginal lactobacilli concentration.

To enable the luminescence quantification of lactobacilli for each recombinant species, Nluc assay was established and validated. The accuracy and precision of the

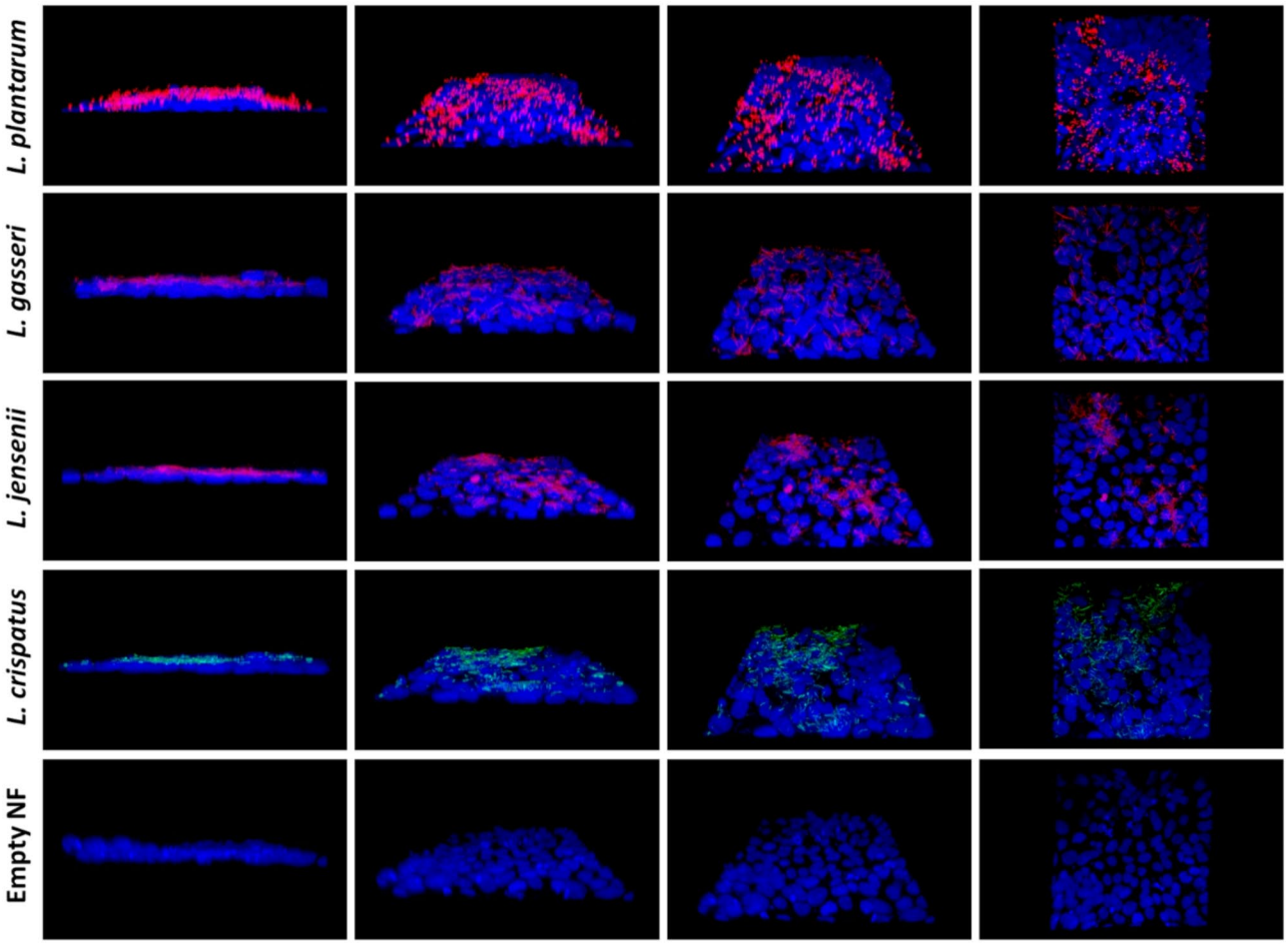

Fig. 7 Z-stack of confocal images of Caco-2 cells with fluorescent lactobacilli adhered to the top of the layer

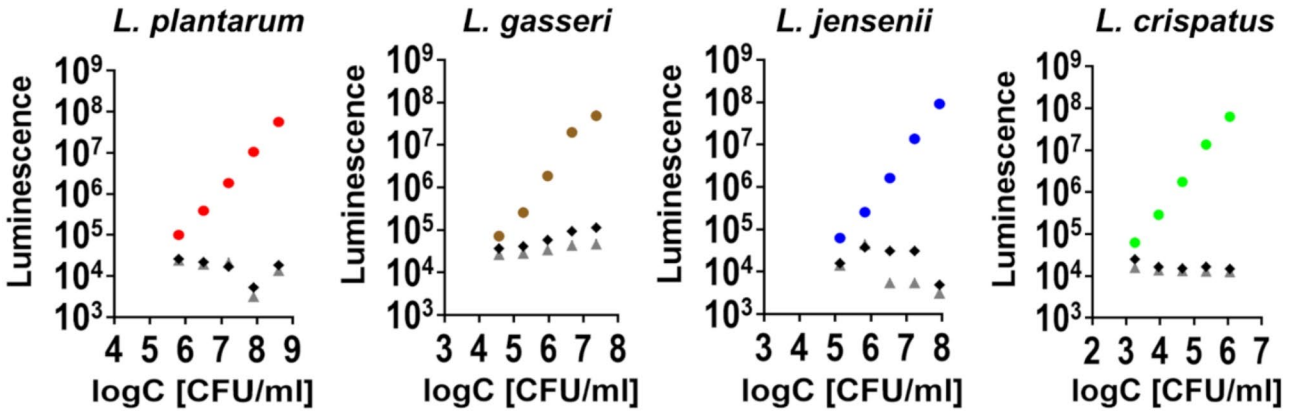

Fig. 8 Luminescence intensity of engineered vaginal lactobacilli as a function of bacterial concentration. *Lactiplantibacillus plantarum* (red circles), *Lactobacillus gasseri* (brown circles), *Lactobacillus jensenii* (blue circles), *Lactobacillus crispatus* (green circles). Non-transformed bacteria (black diamonds) and phosphate-buffered saline (grey triangles) were used as controls

luminescent lactobacilli were determined for three different concentrations. Accuracy was determined, and four samples had a relative error above the desired 30%, with only one being unacceptably high (70.8%). Precision was analyzed in six technical repeats and only one sample had coefficient of variation above the desired 10%. This indicates that precision and accuracy of the assay are appropriate for vaginal lactobacilli determination. All

parameters, including LOD, LOQ and linear concentration range are shown in Table 1. Linear concentration ranges that were used for the preparation of calibration curve extend over 3 log units of bacterial concentration for all species.

Use of Nluc assay to quantify attachment of bacteria from dispersion or nanofibers to Caco-2 cells

Adhesion of vaginal lactobacilli to Caco-2 monolayer, observed with fluorescent bacteria (Fig. 7), was confirmed with Nluc-expressing bacteria using Nluc assay (Fig. 9). The assay was used to quantify the adhesion of bacteria both from dispersion and from nanofiber mats. The luminescence signal depended on the bacterial concentration or mass of nanofibers that were added to the cells, with higher concentration and mass yielding significantly higher luminescence signal. NT species, empty PEO NF, Caco-2 cells without bacteria or nanofibers and PBS were used as controls and showed significantly lower luminescence compared to the engineered species in dispersion or nanofibers.

Bacterial concentration was calculated using calibration curve. Bacterial adhesion to Caco-2 cells differed between species and their concentrations. Higher adhesion was observed for *L. gasseri* (10–50% range) and *L. crispatus* (5–10% range), while the adhesion of *L. plantarum* (1–5% range) and *L. jensenii* (0.5-1% range) were lower (Table 2). Nluc was also used to determine bacterial adhesion after their release from nanofibers. The concentration of bacteria adhered to Caco-2 cells again differed between species and the quantity of nanofibers added. Only concentration of adhered bacteria was determined, and therefore the percentage of adhered bacteria was not calculated. The highest concentration of adhered bacteria was observed for *L. plantarum* $(5.73 \pm 2.67) \times 10^8$, and the lowest for *L. crispatus* $(2.20 \pm 0.99) \times 10^4$ (Table 2).

Discussion

Genetic modification of lactobacilli to express fluorescent or luminescent proteins is an effective tool to track the spatial localization and abundance of these bacteria. This is particularly useful when studying lactobacilli in solid dosage forms, such as nanofibers. In this study, we used three vaginal lactobacilli *L. crispatus* ATCC 33,820, *L. gasseri* ATCC 33,323, *L. jensenii* ATCC 25,258 and a control *L. plantarum* ATCC 8014 to express the fluorescent proteins mCherry and GFP, as previously described [29], and also engineered these bacteria to express the luminescent protein Nluc. *L. crispatus* showed relatively low expression of mCherry and was replaced by a GFP producing strain. Poor transformation and protein expression in *L. crispatus* has already been observed by other authors [46, 47]. In contrast, Nluc was expressed comparably by all lactobacilli species. The expression of

Table 1 Luminescence assay of vaginal lactobacilli at different concentrations expressing Nluc. The results are shown as accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) and concentration range

		Concentration 1	Concentration 2	Concentration 3
L. plantarum	Concentration of bacteria (CFU/mL)	1.07×10^8	2.14×10^7	4.28×10^6
	Accuracy (%)	8.4	24.4	33.0
	Precision (%)	3.2	14.8	7.1
	LOD (CFU/mL)	2.22×10^5		
	LOQ (CFU/mL)	5.51×10^5		
	Linear concentration range (CFU/mL)	$6.42 \times 10^5 - 4.01 \times 10^8$		
L. gasseri	Concentration of bacteria (CFU/mL)	6.24×10^6	1.25×10^6	2.50×10^5
	Accuracy (%)	70.8	21.9	41.3
	Precision (%)	1.6	3.6	2.3
	LOD (CFU/mL)	3.45×10^4		
	LOQ (CFU/mL)	6.25×10^4		
	Linear concentration range (CFU/mL)	$3.74 \times 10^4 - 2.34 \times 10^7$		
L. jensenii	Concentration of bacteria (CFU/mL)	2.27×10^7	4.53×10^6	9.07×10^5
	Accuracy (%)	3.3	20.0	0.1
	Precision (%)	7.3	1.8	1.9
	LOD (CFU/mL)	1.89×10^5		
	LOQ (CFU/mL)	4.34×10^5		
	Linear concentration range (CFU/mL)	$1.36 \times 10^5 - 8.50 \times 10^7$		
L. crispatus	Concentration of bacteria (CFU/mL)	4.28×10^5	8.55×10^4	1.71×10^4
	Accuracy (%)	32.7	16.8	14.2
	Precision (%)	5.0	9.9	6.9
	LOD (CFU/mL)	3.24×10^2		
	LOQ (CFU/mL)	4.73×10^2		
	Linear concentration range (CFU/mL)	$1.82 \times 10^3 - 1.14 \times 10^6$		

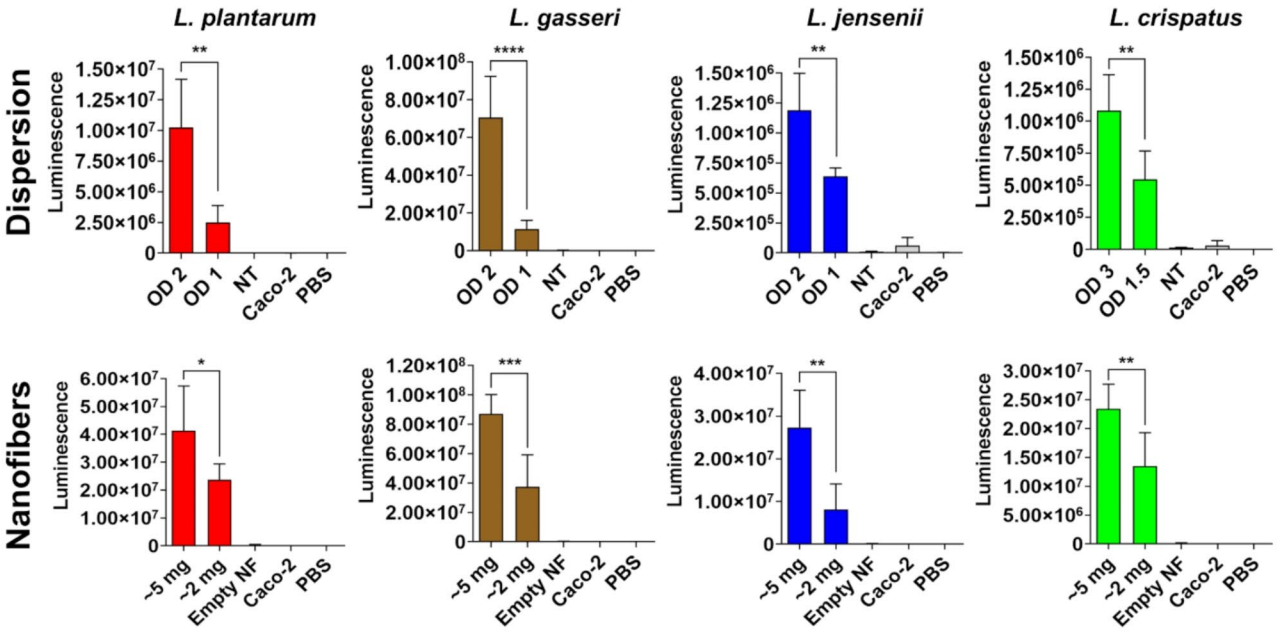

Fig. 9 Luminescence intensity of vaginal lactobacilli adhered to Caco-2 cells following their addition in dispersion or in nanofibers. NT (non-transformed species), Caco-2 cells without bacteria and PBS (phosphate-buffered saline) were used as controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Student's t tests)

Table 2 Luminescence assay of vaginal lactobacilli expressing Nluc adhered to Caco-2 cells in dispersion or in nanofiber mats. The data are presented as means ± standard deviation (SD)

		Dispersion		Nanofibers	
		Concentration 1	**Concentration 2**	**Mass 1**	**Mass 2**
L. plantarum	Added (CFU/ml)	1.03×10^{9}	5.13×10^{8}	~5 mg	~2 mg
	Adhered (CFU/ml)	$(4.57\pm1.73)\times10^{7}$	$(1.50\pm0.72)\times10^{7}$	$(5.73\pm2.67)\times10^{8}$	$(3.60\pm0.91)\times10^{8}$
	Adhered (%)	4.4	2.9	/	/
L. gasseri	Added (CFU/ml)	1.07×10^{8}	5.33×10^{7}	~5 mg	~2 mg
	Adhered (CFU/ml)	$(4.54\pm1.29)\times10^{7}$	$(7.25\pm2.83)\times10^{6}$	$(7.87\pm1.07)\times10^{6}$	$(3.97\pm1.92)\times10^{6}$
	Adhered cells (%)	42.6	13.6	/	/
L. jensenii	Added (CFU/ml)	7.60×10^{7}	3.80×10^{7}	~5 mg	~2 mg
	Adhered (CFU/ml)	$(6.13\pm1.34)\times10^{5}$	$(3.43\pm0.33)\times10^{5}$	$(2.34\pm0.63)\times10^{7}$	$(7.67\pm5.04)\times10^{6}$
	Adhered (%)	0.8	0.9	/	/
L. crispatus	Added (CFU/ml)	3.75×10^{4}	1.88×10^{4}	~5 mg	~2 mg
	Adhered (CFU/ml)	$(2.93\pm0.70)\times10^{3}$	$(1.37\pm0.55)\times10^{3}$	$(3.64\pm0.74)\times10^{4}$	$(2.20\pm0.99)\times10^{4}$
	Adhered (%)	7.8	7.3	/	/

recombinant proteins affected their growth characteristics, particularly in *L. plantarum* and *L. gasseri*. This effect is likely due to the higher levels of fluorescent protein expression observed in these strains [29], which led to increased metabolic burden. Vaginal lactobacilli are known for producing hydrogen peroxide [48]. Unlike growth, hydrogen peroxide production was not affected by the expression of recombinant proteins. The incorporation of vaginal lactobacilli into nanofibers was achieved with electrospinning, a method in which a flexible solid dosage form with potential for vaginal administration is produced. In this article, different electrospinning conditions were used to produce PEO nanofibers for the evaluation of release kinetics and viability. For the release kinetics, the process was optimized to increase the fluorescence of the bacteria in the nanofibers and the amount of nanofibers produced. The bacteria were washed with distilled water to remove ionic compounds that interfere with the electrospinning process and cause instabilities in the electrospinning jet [49]. To increase yield, the nanofibers were produced with higher flow rate and voltage. The bacteria and PEO powder were mixed overnight to allow oxygen to increase the fluorescence intensity. Oxygen plays a crucial role in the post-translational modification of fluorescent proteins by promoting autocatalytic cyclization in the primary structure

and increasing fluorescence [29, 50]. For viability assessment, the deleterious hypotonic washing conditions were replaced with PBS (*L. plantarum* and *L. gasseri*), 4% sucrose (*L. jensenii*) or 0.9% NaCl (*L. crispatus*); conditions that were previously shown to be particularly beneficial for viability [23]. Stirring time was minimized as it increased oxygen level [51] which is detrimental to anaerobic or facultatively anaerobic lactobacilli [52, 53]. The electrospinning conditions were also modified by decreasing the flow rate and voltage which positively impacts bacterial viability [28].

The engineered lactobacilli did not differ from the NT strains when incorporated into nanofibers and observed under the electron microscope. When incorporated, lactobacilli interact with the excipients of the nanofibers, which affect their viability [23]. Lactobacilli retained their fluorescence after incorporation into PEO nanofibers and after their release. As already observed with PEO nanofibers, the release of the lactobacilli was rapid. It was also species-independent, with complete dissolution of 50 mg of nanofibers occurring within 30 to 45 min for all strains. Vaginal infections are characterized with excessive discharge and fluid secretion [54], making rapid dissolution in water desirable. The rapid release of a large dose of probiotics over a short period of time can enhance their colonisation of target surfaces [17]. For optimal therapeutic effect, viable bacteria are essential. The four species survived electrospinning and were viable after their release from nanofibers. The viability of the lactobacilli differed between species, with *L. crispatus* exhibiting the lowest viability. These results are consistent with our previous findings, obtained with NT lactobacilli [23]. The engineered lactobacilli in this study had a lower viability than the NT lactobacilli in the previous study. This can be attributed to plasmid loss under stress conditions [55], which prevents growth on selective MRSC10 agar plates. This was particularly evident in engineered *L. crispatus* (3.37 log CFU/g less than NT) and *L. gasseri* (1.78 log CFU/g less than NT), while no significant difference in viability between engineered and NT bacteria was observed in *L. jensenii*.

Lactobacilli are frequently used as probiotics and usually considered safe [56]. To confirm safety of the vaginal species and PEO nanofibers, we investigated their potential for hemolysis. The spread of bacteria on defibrinated sheep blood agar plate is a standard method for assessing hemolytic activity of potential probiotic strains. The absence of hemolytic activity of lactobacilli suggests that they do not produce hemolytic enzymes, which could otherwise damage the mucosal membrane and facilitate the infiltration of pathogens and toxins [57]. To further investigate the safety of vaginal lactobacilli and nanofibers, we co-incubated them with Caco-2 cells. Caco-2 cells are not vaginal in origin and do not recapitulate the properties of vaginal tissue; however, they are a well-established epithelial cell model, commonly used in the studies of probiotic safety and adhesion [58]. Recently, a cell model for studying immunogenicity of nanofibers has been developed based on peripheral blood mononuclear cells, which can provide relevant results on the safety of nanofibers [59]. The epithelial cell model can provide information not only on the safety, but also on the fate of administered agent. Here, we were able to show that both dispersed and nanofiber-incorporated vaginal lactobacilli did not decrease cell viability. The only exception was the control *L. plantarum*, in which a significant decrease in Caco-2 viability was observed after 24 h of incubation. This decrease may be attributed to the production of D-lactic acid, which is toxic to mammalian cells [60], and has previously been shown to be particularly high in *L. plantarum* [61]. The safety of *Lactobacillus acidophilus* from PEO nanofibers has also recently been demonstrated in in vitro and in vivo models [21]. In addition to safety, adhesion was also tested. Autoaggregation and surface hydrophobicity indicate adhesion capabilities and were evaluated prior to adhesion of lactobacilli to epithelial cells. Vaginal lactobacilli *L. gasseri* and *L. jensenii* showed high surface hydrophobicity which is crucial for adhesion to epithelial cells [62]. Adhesion to Caco-2 monolayer was demonstrated here for all lactobacilli tested, regardless of whether they were added in dispersion or in nanofibers. The adhesion of vaginal lactobacilli has been observed previously and has been shown to be influenced by surface layer proteins and carbohydrates [63]. Using confocal microscopy and z-stack imaging of washed Caco-2 monolayers, we demonstrated that the lactobacilli tested in this study adhered to the upper surface of the monolayer. However, quantification of adhesion using fluorescence proved to be more difficult, and chemiluminescence was introduced instead.

Chemiluminescence is the emission of light as a result of a chemical reaction. In this study, we engineered the four lactobacilli to express the luciferase enzyme Nluc. Compared to fluorescence, luminescence has a higher sensitivity and selectivity, because it is not excited by external light, resulting in low background signal and a lower signal-to-noise ratio [64]. The expression of Nluc was confirmed in all lactobacilli species, and the bioluminescence signal was found to be concentration-dependant over a wide range of concentrations (3 to 4 log units), similar to what was previously observed in *L. innoucua* [39]. This allowed the introduction of a Nluc assay with high sensitivity (LOD varied from 3.24×10^2 CFU/mL for *L. crispatus* to 2.22×10^5 CFU/mL for *L. plantarum*), acceptable accuracy and good precision. The limitation of the Nluc assay is that it can also detect non-viable cells, which can lead to discrepancies with the CFU count, which can vary depending on the species. In

the case of the oxygen-sensitive *L. crispatus*, a low LOD could be the result of cells that contain active Nluc, but are not viable.

The Nluc assay was used to quantify the adherence of lactobacilli from dispersions or nanofibers to Caco-2 monolayer. To minimize the background signal of non-adhered lactobacilli, we carefully detached the Caco-2 cells with EDTA [65] and transferred them to a fresh plate, so that only the specifically adhered lactobacilli could be detected. The total number of adhered bacteria correlated with the added concentration, while the percentage of adhered bacteria relative to the added bacteria remained relatively constant within species, but differed between species (from app. 0.5 to 50%), suggesting species-specific adhesion mechanisms. *L. gasseri* showed the highest adhesion to Caco-2 monolayer, correlating with its high hydrophobicity. The adhesion of lactobacilli from a dispersion was reproduced with the addition of lactobacilli in nanofibers. The Nluc assay thus proved to be suitable for testing the lactobacilli-loaded nanofibers in cell models.

Preliminary results shown in this article thus highlight the potential of both, fluorescent and luminescent vaginal lactobacilli, to be assessed in nanofibers and in cell models. However, it should be mentioned that the cell assays of safety and adhesion were limited to a single cell model (Caco-2), and release kinetics was determined in solution only. Future studies of release, adhesion and safety should thus involve more advanced and physiologically relevant cell culture models, as well as animal models to further improve and validate these findings.

Conclusion

To advance the application of nanofibers for the delivery of lactobacilli, tools for studying their safety and efficacy in vitro need to be established. We have shown that the expression of fluorescent and luminescent proteins in lactobacilli can provide complementary data in the Caco-2 cell model. Fluorescence was used to track the release of lactobacilli from nanofibers and confirmed their adhesion to the Caco-2 monolayer. However, quantification of adhesion was achieved by introducing a Nluc-based luminescence assay for all four lactobacilli species tested. The present study not only introduced a new methodology, but also preliminarily demonstrated the safety of nanofibers and their ability to deliver functional lactobacilli in an in vitro experiment. The tools developed will allow further, more detailed evaluation of the safety and efficacy of lactobacilli-loaded nanofibers in cell and animal models.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02612-w.

Supplementary Material 1

Author contributions

SS: performed the experimental work and wrote the draft of the manuscript. TVP: performed the experimental work. ŠZ: revised the manuscript, supervised the work. AB: revised the manuscript, planned and supervised the work.

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Data availability

The data produced and/or analysed in this study can be obtained from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The manuscript is the original work of the authors who mutually agreed on submitting the manuscript.

Competing interests

The authors declare no competing interests.

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References

1. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. *Lactobacillus* species as biomarkers and agents that can promote various aspects of vaginal health. Front Physiol. 2015;6:81.
2. Ma ZS, Li L. Quantifying the human vaginal community state types (CSTs) with the species specificity index. PeerJ. 2017;5:e3366.
3. Chee WJY, Chew SY, Than LTL. Vaginal microbiota and the potential of *Lactobacillus* derivatives in maintaining vaginal health. Microb Cell Fact. 2020;19:203.
4. Lebeer S, Ahannach S, Gehrmann T, Wittouck S, Eilers T, Oerlemans E, Condori S, Dillen J, Spacova I, Vander Donck L, et al. A citizen-science-enabled catalogue of the vaginal microbiome and associated factors. Nat Microbiol. 2023;8:2183–95.
5. Barrientos-Duran A, Fuentes-Lopez A, de Salazar A, Plaza-Diaz J, Garcia F. Reviewing the composition of vaginal microbiota: inclusion of nutrition and probiotic factors in the maintenance of eubiosis. Nutrients. 2020;12:419.
6. Boris S, Barbes C. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes Infect. 2000;2:543–6.
7. Stapleton AE, Au-Yeung M, Hooton TM, Fredricks DN, Roberts PL, Czaja CA, Yarova-Yarovaya Y, Fiedler T, Cox M, Stamm WE. Randomized, placebo-controlled phase 2 trial of a *Lactobacillus crispatus* probiotic given intravaginally for prevention of recurrent urinary tract infection. Clin Infect Diseases: Official Publication Infect Dis Soc Am. 2011;52:1212–7.
8. Ghartey JP, Smith BC, Chen Z, Buckley N, Lo Y, Ratner AJ, Herold BC, Burk RD. *Lactobacillus crispatus* dominant vaginal microbiome is associated with inhibitory activity of female genital tract secretions against Escherichia coli. PLoS ONE. 2014;9:e96659.
9. Atassi F, Pho Viet Ahn DL, Lievin-Le Moal V. Diverse expression of antimicrobial activities against bacterial vaginosis and urinary tract infection pathogens by cervicovaginal microbiota strains of *Lactobacillus gasseri* and *Lactobacillus crispatus*. Front Microbiol. 2019;10:2900.
10. Spurbeck RR, Arvidson CG. *Lactobacillus jensenii* surface-associated proteins inhibit *Neisseria gonorrhoeae* adherence to epithelial cells. Infect Immun. 2010;78:3103–11.
11. Borges S, Barbosa J, Teixeira P. Drug delivery systems for vaginal infections. In: Rahman AU, editor. Frontiers in Clinical Drug Research: anti-infectives. Sharjah, United Arab Emirates: Bentham Science; 2015. pp. 233–58.

12. Valamla B, Thakor P, Phuse R, Dalvi M, Kharat P, Kumar A, Panwar D, Singh SB, Giorgia P, Mehra NK. Engineering drug delivery systems to overcome the vaginal mucosal barrier: current understanding and research agenda of mucoadhesive formulations of vaginal delivery. J Drug Deliv Sci Tec. 2022;70:103162.
13. Xue JJ, Wu T, Dai YQ, Xia YN. Electrospinning and Electrospun nanofibers: methods, materials, and applications. Chem Rev. 2019;119:5298–415.
14. Stojanov S, Berlec A. Electrospun nanofibers as carriers of microorganisms, stem cells, proteins, and nucleic acids in therapeutic and other applications. Front Bioeng Biotech. 2020;8:130.
15. Torres-Martínez EJ, Bravo JMC, Medina AS, González GLP, Gómez LJV. A summary of electrospun nanofibers as drug delivery system: drugs loaded and biopolymers used as matrices. Curr Drug Deliv. 2018;15:1360–74.
16. Stojanov S, Berlec A. Smart bionanomaterials for treatment and diagnosis of inflammatory bowel disease. Nanotechnol Rev. 2024;13:20240057.
17. Grilc NK, Zidar A, Kocbek P, Rijavec T, Colja T, Lapanje A, Jeras M, Gobec M, Mlinarič-Rasčan I, Gasperlin M, et al. Nanofibers with genotyped *Bacillus* strains exhibiting antibacterial and immunomodulatory activity. J Control Release. 2023;355:371–84.
18. Zupančič Š, Škrlec K, Kocbek P, Kristl J, Berlec A. Effects of electrospinning on the viability of ten species of lactic acid bacteria in poly(ethylene oxide) nanofibers. Pharmaceutics. 2019;11:483.
19. Hirscha E, Panteaa E, Vassa P, Domján J, Molnár M, Suhajda A, Andersen SK, Vigh T, Verreck G, Marosi GJ, et al. Probiotic bacteria stabilized in orally dissolving nanofibers prepared by high-speed electrospinning. Food Bioprod Process. 2021;128:84–94.
20. Silva JA, De Gregorio PR, Rivero G, Abraham GA, Nader-Macías MEF. Immobilization of vaginal *Lactobacillus* in polymeric nanofibers for its incorporation in vaginal probiotic products. Eur J Pharm Sci. 2021;156:105563.
21. Minooei F, Gilbert NM, Zhang L, Sarah NeCamp M, Mahmoud MY, Kyser AJ, Tyo KM, Watson WH, Patwardhan R, Lewis WG, et al. Rapid-dissolving electrospun nanofibers for intra-vaginal antibiotic or probiotic delivery. Eur J Pharm Biopharm. 2023;190:81–93.
22. Mahmoud MY, Wesley M, Kyser A, Lewis WG, Lewis AL, Steinbach-Rankins JM, Frieboes HB. *Lactobacillus crispatus*-loaded electrospun fibers yield viable and metabolically active bacteria that kill Gardnerella in vitro. Eur J Pharm Biopharm. 2023;187:68–75.
23. Stojanov S, Kristl J, Zupancic S, Berlec A. Influence of excipient composition on survival of vaginal lactobacilli in electrospun nanofibers. Pharmaceutics. 2022;14:1155.
24. Han XF, Wang L, Li W, Li BB, Yang YX, Yan HL, Qu L, Chen YL. Use of green fluorescent protein to monitor *Lactobacillus plantarum* in the gastrointestinal tract of goats. Braz J Microbiol. 2015;46:849–54.
25. Mohedano ML, Hernández-Recio S, Yépez A, Requena T, Martínez-Cuesta MC, Peláez C, Russo P, LeBlanc JG, Spano G, Aznar R, et al. Real-time detection of riboflavin production by *Lactobacillus plantarum* strains and tracking of their gastrointestinal survival and functionality in vitro and in vivo using mCherry labeling. Front Microbiol. 2019;10:1748.
26. Spacova I, Lievens E, Verhoeven T, Steenackers H, Vanderleyden J, Lebeer S, Petrova MI. Expression of fluorescent proteins in *Lactobacillus rhamnosus* to study host-microbe and microbe-microbe interactions. Microb Biotechnol. 2018;11:317–31.
27. Berlec A, Završnik J, Butinar M, Turk B, Štrukelj B. In vivo imaging of *Lactococcus lactis*, *Lactobacillus plantarum* and *Escherichia coli* expressing infrared fluorescent protein in mice. Microb Cell Fact. 2015;14:181.
28. Škrlec K, Zupančič S, Mihevc SP, Kocbek P, Kristl J, Berlec A. Development of electrospun nanofibers that enable high loading and long-term viability of probiotics. Eur J Pharm Biopharm. 2019;136:108–19.
29. Stojanov S, Plavec TV, Kristl J, Zupančič S, Berlec A. Engineering of vaginal lactobacilli to express fluorescent proteins enables the analysis of their mixture in nanofibers. Int J Mol Sci. 2021;22:13631.
30. Riggs P. Fusion protein. In: Maloy KH, editor. Brenner's Encyclopedia of Genetics. Elsevier; 2013. pp. 134–5.
31. Moon GS, Narbad A. Monitoring of bioluminescent *Lactobacillus plantarum* in a complex food matrix. Korean J Food Sci An. 2017;37:147–52.
32. Van Zyl WF, Deane SM, Dicks LMT. In vivo bioluminescence imaging of the spatial and temporal colonization of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the intestinal tract of mice. BMC Microbiol. 2018;18:171.
33. Oozeer R, Goupil-Feuillerat N, Alpert CA, van de Guchte M, Anba J, Mengaud J, Corthier G. *Lactobacillus casei* is able to survive and initiate protein synthesis during its transit in the digestive tract of human flora-associated mice. Appl Environ Microb. 2002;68:3570–4.
34. Daniel C, Poiret S, Dennin V, Boutillier D, Lacorre DA, Foligne B, Pot B. Dual-color bioluminescence imaging for simultaneous monitoring of the intestinal persistence of *Lactobacillus plantarum* and *Lactococcus lactis* in living mice. Appl Environ Microbiol. 2015;81:5344–9.
35. England CG, Ehlerding EB, Cai WB. NanoLuc: a small luciferase is brightening up the field of bioluminescence. Bioconjug Chem. 2016;27:1175–87.
36. Matsuyama C, Seike T, Okahashi N, Niide T, Hara KY, Hirono-Hara Y, Ishii J, Shimizu H, Toya Y, Matsuda F. Metabolome analysis of metabolic burden in *Escherichia coli* caused by overexpression of green fluorescent protein and delta-rhodopsin. J Biosci Bioeng. 2024;137:187–94.
37. Salome-Desnoulez S, Poiret S, Foligne B, Muharram G, Peucelle V, Lafont F, Daniel C. Persistence and dynamics of fluorescent *Lactobacillus plantarum* in the healthy versus inflamed gut. Gut Microbes. 2021;13:1–16.
38. Kadkhodaei S, Memari HR, Abbasiliasi S, Rezaei MA, Movahedi A, Shun TJ, Bin Ariff A. Multiple overlap extension PCR (MOE-PCR): an effective technical shortcut to high throughput synthetic biology. RSC Adv. 2016;6:66682–94.
39. Berlec A, Janez N, Sternisa M, Klančnik A, Sabotić J. Expression of NanoLuc luciferase in *Listeria innocua* for development of biofilm assay. Front Microbiol. 2021;12:636421.
40. Mierau I, Kleerebezem M. 10 years of the nisin-controlled gene expression system (NICE) in. Appl Microbiol Biot. 2005;68:705–17.
41. Baranyi J, Roberts TA. A dynamic approach to predicting bacterial growth in food. Int J Food Microbiol. 1994;23:277–94.
42. Stojanov S, Ravnikar M, Berlec A, Kreft S. Interaction between silver fir (Abies alba) wood water extract and lactobacilli. Pharmazie. 2021;76:614–7.
43. Herigstad B, Hamilton M, Heersink J. How to optimize the drop plate method for enumerating bacteria. J Microbiol Meth. 2001;44:121–9.
44. De Gregorio PR, Silva JA, Marchesi A, Nader-Macias MEF. Anti-*Candida* activity of beneficial vaginal lactobacilli in in vitro assays and in a murine experimental model. FEMS Yeast Res. 2019;19:foz008.
45. Plavec TV, Mitrović A, Nanut MP, Štrukelj B, Kos J, Berlec A. Targeting of fluorescent *Lactococcus lactis* to colorectal cancer cells through surface display of tumour-antigen binding proteins. Microb Biotechnol. 2021;14:2227–40.
46. Stephenson DP, Moore RJ, Allison GE. Transformation of, and heterologous protein expression in, *Lactobacillus agilis* and *Lactobacillus vaginalis* isolates from the chicken gastrointestinal tract. Appl Environ Microbiol. 2011;77:220–8.
47. Beasley SS, Takala TM, Reunanen J, Apajalahti J, Saris PE. Characterization and electrotransformation of *Lactobacillus crispatus* isolated from chicken crop and intestine. Poult Sci. 2004;83:45–8.
48. Miko E, Barakonyi A. The role of hydrogen-peroxide (H_2O_2) produced by vaginal microbiota in female reproductive health. Antioxidants. 2023;12:1055.
49. Josef E, Guterman R. Designing solutions for electrospinning of poly(ionic liquid)s. Macromolecules. 2019;52:5223–30.
50. Tsuji FI. Oxidation of a cyclic tripeptide by molecular oxygen and the development of fluorescence in the Aequorea green fluorescent protein. Int Congr Ser. 2002;1233:37–44.
51. Gao YX, Li HY, Liu J. Direct writing of flexible electronics through room temperature liquid metal ink. PLoS ONE. 2012;7(9).
52. Talwalkar A, Kailasapathy K. The role of oxygen in the viability of probiotic bacteria with reference to *L. Acidophilus* and *Bifidobacterium* spp. Curr Issues Intest Microbiol. 2004;5:1–8.
53. Gupta PK, McGrath C, Microbiology. Inflammation, and viral infections. In: Bibbo M, Wilbur D, editors. Comprehensive Cytopathology. Amstedram: Elsevier; 2008. pp. 91–129.
54. Kairys N, Carlson K, Garg M. Bacterial vaginosis. StatPearls. 2024.
55. Silva F, Queiroz JA, Domingues FC. Evaluating metabolic stress and plasmid stability in plasmid DNA production by *Escherichia coli*. Biotechnol Adv. 2012;30:691–708.
56. Plavec TV, Berlec A. Safety aspects of genetically modified lactic acid bacteria. Microorganisms. 2020;8:297.
57. Yasmin I, Saeed M, Khan WA, Khaliq A, Chughtai MFJ, Iqbal R, Tehseen S, Naz S, Liaqat A, Mehmood T, et al. In vitro probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of *Bifidobacterium* strains isolated from raw camel milk. Microorganisms. 2020;8:354.
58. Lea T. Caco-2 cell line. In: Verhoeckx K, Cotter P, Lopez-Exposito I, Kleiveland C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H, editors. The impact of food bioactives on health: in vitro and ex vivo models. 2015. pp. 103–111.

59. Zidar A, Zupančič S, Kristl J, Jeras M. Development of a novel in vitro cell model for evaluation of nanofiber mats immunogenicity. Int J Pharm. 2024;650:123696.
60. Pohanka M. D-lactic acid as a metabolite: toxicology, diagnosis, and detection. Biomed Res Int. 2020;2020:3419034.
61. Cappello C, Acin-Albiac M, Pinto D, Polo A, Filannino P, Rinaldi F, Gobbetti M, Di Cagno R. Do nomadic lactobacilli fit as potential vaginal probiotics? The answer lies in a successful selective multi-step and scoring approach. Microb Cell Fact. 2023;22:27.
62. Chantanawilas P, Pahumunto N, Teanpaisan R. Aggregation and adhesion ability of various probiotic strains and *Candida* species: an in vitro study. J Dent Sci. 2024;19:2163–71.
63. Boris S, Suárez JE, Vázquez F, Barbés C. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun. 1998;66:1985–9.
64. Baljinnyam B, Ronzetti M, Simeonov A. Advances in luminescence-based technologies for drug discovery. Expert Opin Drug Discov. 2023;18:25–35.
65. Lai TY, Cao J, Ou-Yang P, Tsai CY, Lin CW, Chen CC, Tsai MK, Lee CY. Different methods of detaching adherent cells and their effects on the cell surface expression of Fas receptor and Fas ligand. Sci Rep. 2022;12:5713.

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