PEARL: Protein Eluting Alginate with Recombinant Lactobacilli

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ABSTRACT

Engineered living materials (ELMs) made of bacteria in hydrogels have shown considerable promise for therapeutic applications since they offer the possibility to achieve controlled and prolonged release of complex biopharmaceuticals at low costs and with reduced wastage. While most therapeutic ELMs use *E. coli* as the living component due to its large genetic toolbox, most live biotherapeutic bacteria in clinical trials are lactic acid bacteria due to the native health benefits they offer. Among these, lactobacilli are the largest family of probiotic bacteria that are being investigated for their therapeutic potential in almost all sites of the body that host a microbiome. A major factor limiting the use of lactobacilli in ELMs is their limited genetic toolbox.

In this study, we build upon our recent work to expand the genetic programmability of a probiotic lactobacillus strain (*Lactiplantibacillus plantarum* WCFS1) for protein secretion and integrate it into a simple, cost-effective, and biocompatible alginate bead encapsulation format to develop an ELM. We demonstrate the controlled release of a recombinant protein for up to 14 days from this ELM, thereby terming it PEARL - Protein Eluting Alginate with Recombinant Lactobacilli. Notably, encapsulation of the lactobacilli offered multiple benefits such as preventing bacterial outgrowth, stabilizing protein release profiles over time, and preventing potential cytotoxicity caused by bacterial metabolites. These findings demonstrate the mutual benefits of combining recombinant lactobacilli with alginate for the controlled release of proteins for biomedical applications.

Keywords: Engineered living materials, lactobacillus, secretion, alginate, synthetic biology

INTRODUCTION

Engineered living materials (ELMs) are composites made of living entities integrated with materials. [1,2] The living entities in ELMs enable them to be designed with life-like abilities of adaptation, regeneration, biosynthesis, growth, etc. Additionally, the possibility to genetically reprogram these abilities to optimally suit a desired application has propelled the rapid development of ELMs in various domains ranging from architecture to medicine. ELMs have advanced in the medical field, especially due to the benefits they offer to deliver therapeutics. [3,4,5] Since the human body naturally supports microbial survival, ELM activities can potentially be sustained in the long term. The microbes can be engineered to produce high-value biopharmaceutical drugs (proteins, peptides, biomacromolecules) for delivery at the disease site. In most studies conducted to date, *E. coli* has been the predominant organism encapsulated within ELMs. For example, a living material ('PATCH') has been demonstrated to encapsulate lysostaphin-secreting *E. coli* to inhibit the growth of *S. aureus*, including the methicillin-resistant staphylococcus aureus (MRSA) strain. [6] In another study, an *E. coli* strain engineered to light-responsively produce an anti-bacterial compound, deoxyviolacein. After encapsulation, the strain demonstrated drug release over 40 days. [7] This approach avoids the need to externally produce, purify, encapsulate, and store these often-fragile biopharmaceuticals, thereby reducing the costs and waste associated with these processes.

To achieve these benefits, therapeutic ELMs primarily use bacteria as their living entity, since they grow fast, have simple nutrient requirements, can be encapsulated at high densities, and are often naturally beneficial to the host in the form of probiotics. In fact,

bacteria have been developed for over two decades as engineered live biotherapeutics that can be administered directly within the body to produce and deliver drugs on-site. [8] While clinical trials have shown that the engineered bacteria are safe for use in humans, proving their efficacy has been a major hurdle blocking their approval as therapeutic products. [9] A fundamental challenge in achieving therapeutic efficacy is ensuring that the right density of the engineered bacteria is viable and active when they reach the target site. Furthermore, this must be balanced with the need for biocontainment of the bacteria, preventing them from invading unwanted sites in the body or environmental niches where they can potentially disturb the local ecology. ELMs satisfy these needs since they can provide a conducive and protected environment for the bacteria to grow to large population sizes while physically confining them within the material.[10,11,12,13] Most therapeutic ELMs are based on probiotic *E. coli* and only a few studies cover other probiotic or GRAS (generally recognized as safe) strains like *Lactococcus lactis*,[14] *Bacillus subtilis* [15] and *Corynebacterium glutamicum* [16]. Interestingly, most engineered live biotherapeutic candidates currently in clinical trials are lactic acid bacteria. This is because lactic acid bacteria are the largest group of commensals and probiotics, beneficial not only for the gut but also other organs hosting a microbiome. In particular, the *Lactobacillus* family has the largest number of probiotic strains, many of which are being explored for therapeutic applications. [17] To date no ELMs containing lactobacilli have been reported yet despite their potential as therapeutics, possibly due to their limited genetic programmability.

Recently, a few research groups, including ours, have made major strides in expanding the genetic programmability of a prominent lactobacillus strain, *Lactiplantibacillus*

plantarum WCFS1 [18]. We developed a direct cloning methodology to speed up the plasmid-based engineering of this strain by circumventing the need for an intermediate host. [19] Using this strategy, we have identified the strongest constitutive promoter and repressor reported to date and characterized several toxin-antitoxin systems for antibiotic-free plasmid retention. [20,21] Other groups have reported the use of sakacin-inducible, xylose-inducible, and lactose-inducible systems to express heterologous proteins in *Lactobacillus plantarum* WCFS1. [22] To facilitate the secretion of these proteins, various endogenous and exogenous signal peptides have been explored, enabling the secretion of different proteins. [23] In addition to secretion, the anchoring of these secreted proteins has garnered significant interest in recent years, particularly in the development of bacterial vaccines. In this context, antigens have been secreted and anchored to various components of the bacterial cell surface using endogenous LysM and LPXTG moieties, enhancing their potential as vaccine candidates. [24] Nevertheless, the performance and containment of these engineered bacteria within biocompatible materials under physiologically relevant conditions is yet to be shown. Encapsulation of *L. plantarum* in hydrogels and food-based materials has been done for their delivery as probiotics. [25,26] These materials were not designed to contain the bacteria but rather to deliver them in the gut.

In this study, we have investigated how *L. plantarum* WCFS1 can be securely contained within a hydrogel. To do this, simple core-shell alginate beads were fabricated to trap the bacteria, and we showed that they sustained their viability for at least 14 days. We further expanded the genetic programmability of this bacterium by optimizing genetic parts needed to drive high-level secretion of a nuclease (NucA) protein. Encapsulation

of the engineered lactobacilli in alginate beads helped to stabilize the release profiles of the secreted protein and sustained this activity over at least 14 days. Due to this prolonged protein elution capability, we hereafter term this ELM as PEARL - Protein Eluting Alginate with Recombinant Lactobacilli. Finally, encapsulation additionally aids in preventing cytotoxicity that can potentially be caused by metabolites of *L. plantarum* as confirmed by biocompatibility tests performed using mammalian cells and highly sensitive Zebrafish embryos. These results set the stage for the development of PEARLs towards therapeutic applications.

EXPERIMENTAL SECTION

Strain, media and plasmids

L. *plantarum* WCFS1 strain was used in this study. De Man, Rogosa and Sharpe (MRS) medium (Carl Roth GmbH, Germany, Art. No. X924.1) was used to grow the strain. Engineered *L*. *plantarum* WCFS1 strains were grown in MRS media with 10 µg/mL of erythromycin (Carl Roth GmbH, Art. No. 4166.2) at 37°C and 250 revolutions per minute (rpm) for 16 to 20 h. *E. coli* Nissle 1917 was maintained in Luria-Bertani (LB) media (Carl Roth GmbH, Art. No. X968.1). Engineered *E*. *coli* Nissle 1917 was grown in LB media supplemented with 200 µg/mL of erythromycin at 37°C, 250 rpm for 16 to 20h. The pLp_3050sNuc plasmid was the backbone vector used in this study (Addgene plasmid # 122030).

Molecular biology

The following enzymes and kits were purchased from NEB: Q5 High Fidelity 2X Master Mix for PCRs, NEBuilder® HiFi DNA Assembly Cloning Kit for Gibson Assembly, Quick Blunting Kit for DNA phosphorylation and the T4 DNA Ligase enzyme for DNA ligation. DNA oligos were purchased from Eurofins Genomics GmbH (Köln, Germany) (**Table S1**). Synthetic genes were synthesized as eBlocks from Integrated DNA Technologies (IDT) (Coralville, USA) and were codon optimized using the IDT Codon Optimization Tool. Plasmids were extracted using the plasmid extraction kit from Qiagen GmbH (Hilden, Germany). The DNA purification kit was purchased from Promega GmbH (Walldorf, Germany). The ladder used for agarose gels was the Generuler 1 Kb DNA Ladder from Thermo Fisher Scientific.

L. plantarum WCFS1 direct cloning transformation

L. plantarum WCFS1 electrocompetent cells were prepared as described in [19]. All the clonings were done following the direct cloning transformation method adapted for *L. plantarum* WCFS1. [19]

Nuclease Assay (in DNase medium with Methyl green)

Qualitative Assay

To analyze the secretion of nuclease by genetically modified bacteria, the bacteria were first grown overnight in MRS media supplemented with 10 µg/mL erythromycin at 37°C, 250 rpm and the OD_{600} was measured the following day. The samples were

subcultured in 4 mL of antibiotic-supplemented fresh MRS media at an initial OD600 of 0.1 and incubated at 37°C until an OD of 1. Then, 10 µL of the undiluted bacterial cultures were spotted on DNAse agar (Altmann Analytik GmbH, Germany) supplemented with 10 µg/mL of erythromycin and incubated at 37°C for 24 h. The discoloration zone surrounding the spotted area, indicative of nuclease activity was captured and documented using the BioRad Gel Documentation System.

Quantitative Assay

Standard curve: Overnight grown culture of WCFS1 - Empty vector (EV, bacteria carrying a plasmid based on the p256 origin of replication and the erythromycin resistance). was diluted to the OD 0.1 in DNase agar + methyl green medium. Using the 0.1 OD DNase media prepared in the previous step as the solvent, serial dilutions of Nuclease protein (Sigma Aldrich) were made. In our experiment, the standard curve was plotted in the range of 200 nm - 1.56 nM (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 nM). The final volume of each concentration was 1 mL and was prepared in 1.5 mL Eppendorf tube. Once the protein dilutions were transferred to the Eppendorf tube, they were covered with aluminum foil to protect the bleaching of methyl green from exposure to light. The tubes were stacked in a 37°C incubator with 250 RPM shaking for 24 hours.

After 24 hours, 200 mL from each eppendorf/concentration were pipetted in duplicates into a black 96-well plate with transparent bottom. The fluorescence of samples was measured at Ex/Em of 633/668 nm at Z position of 19000 um and Gain of 100.

Quantification from culture: To measure the concentration of the secreted nuclease, overnight bacterial cultures (in MRS broth) were subcultured to an OD600 of 0.1 in MRS broth. Once the OD reached 1, bacteria equivalent to OD 0.1 were transferred to an autoclaved DNase medium supplemented with erythromycin of 1 mL volume and incubated at 37ºC and 250 rpm shaking conditions for 24 h. The DNase Broth was obtained by agar precipitation of the commercial DNase Agar and the final formulation comprised Tryptose (20 g/L), Sodium Chloride (5 g/L), Calf Thymus DNA (2 g/L), Methyl Green (0.05 g/L). After 24 h, the bacterial cultures were centrifuged at 13000 rpm (15700 × g) and 200 µL of the respective supernatants were added to the clear bottom 96-well microtiter plate (Corning® 96 well clear bottom black plate, USA). The samples were analyzed in the Microplate Reader Infinite 200 Pro (Tecan Deutschland GmbH, Germany) for methyl green-DNA complex-based fluorescent intensity (Exλ / Emλ = 633 /668 nm). The z-position and gain settings for recording the fluorescence were set to 19000 µm and 100 respectively. Serial dilutions of the purified nuclease protein in DNase broth incubated under the same conditions were used to plot the standard curve for estimating the protein concentration.

PEARL fabrication - Encapsulation in alginate core-shell hydrogels

Fabrication of alginate cores:

3 wt% alginate solution was prepared by dissolving sodium alginate (Sigma-Aldrich 71238) in sterile MilliQ water followed by sterilization by heating it to 120°C for 30 minutes. A bacterial culture (approximately 10^8 cells in LB medium) was mixed with 3 wt% alginate solution in a 1:2 volume ratio to obtain a bacterial-alginate core solution of 2 wt%. The bacterial culture was supplemented with 1% v/v Alcian blue to enhance the visualization

of alginate-bacteria core beads during manual fabrication. The bacteria-alginate core mixture was vortexed thoroughly to ensure uniformity. Droplets, each with a volume of 10 µL, were pipetted and dropped into a 5 wt% $CaCl_2$ solution (Ionic crosslinker). The droplets were left to polymerize and solidify in $CaCl_2$ solution for 15 minutes. Solidified core beads (roughly 2 mm in diameter) were collected and washed thrice with sterile Milli-Q water to remove the remnant $CaCl_2$ solution and bacteria on the surface of the beads.

Shell coating:

1.5 wt% alginate solution for shell coating was prepared by mixing 3 wt% alginate stock solution and Milli-Q water in a 1:1 volume ratio followed by vortexing. Alginate cores from the last step of the previous section were dipped into the shell solution. Alginate bead surrounded by shell solution (roughly 25 µL) was collected with a pipette tip, whose tip was cut with a sterile blade to increase the size of the opening, followed by dropping into the cross-linker solution. The core-shell alginate beads were left to solidify for 15 minutes. The beads were collected, washed with sterile Milli-Q water, and transferred to a well plate with a nutrient medium.

Tracking changes in PEARLs using microplate reader

PEARLs (alginate beads) after fabrication were immediately transferred to a 96-well plate containing wells filled with 200 µL of respective media (LB medium for OD_{600nm} measurements and DNase medium for nuclease quantification). Growth kinetics along with tracking of fluorescence expression was done for 72 hours using Tecan microplate reader with settings for Excitation/Emission wavelengths (Ex/Em for mCherry (587/625 nm) / mScarlet3 (569/600 nm) /Nuclease (633.668 nm), Manual gain 80-100, Z-position

19000 μm and integration time of 20 μs). Data was normalized and analyzed using GraphPad prism.

Detection of bacterial escape

Tracking of escape or leakage of bacteria from alginate beads was done in three ways: 1) Transfer of alginate bead onto nutrient agar plate immediately after fabrication and observe whether colony outgrowth is observed or not; **(Figure S4a)** 2) Alginate beads incubated in LB medium overnight at 37 °C incubator followed by spotting of 10 μL volume supernatants from triplicates of beads on Nutrient agar plates at regular intervals (Day 1,2,3..). 3) Setting up the OD_{600} measurement of alginate beads immersed in 200 μL of nutrient medium in a well of a 96-well plate to detect even a slight increase in turbidity of surrounding media due to bacterial escape and subsequent growth (**Figure 1g**).

Bacterial recovery from PEARLs

To determine the viable population of bacteria within the alginate core after 14 days, alginate macro beads were treated with 200 μL 25 mM of EDTA (pH 8) in a 1.5 mL Eppendorf for 30 minutes with shaking in a thermomixer (30 °C, 1000 rpm). The mixture containing EDTA, dissolved alginate, and bacteria was centrifuged at 8000 rpm for 5 minutes. EDTA supernatant was discarded, bacterial pellet was resuspended in 100 μL of nutrient broth and plated on an agar plate supplemented with erythromycin (10 μg/mL). The agar plate was incubated was incubated in a 37 °C static incubator for 48 hours followed by imaging and automated counting of colonies to determine CFU/mL.[27]

Evaluation of the cytocompatibility of PEARLs

Cell culture:

Normal human dermal fibroblast (NHDF) cells were purchased from Lonza, Germany. The cells (passages between 10-12) were cultured in growth medium (FBM™ Fibroblast Growth Basal Medium (Lonza, Germany) supplemented with 1% penicillin−streptomycin (P/S) and FGM-2 SingleQuots supplements (containing insulin, hFGF-B, GA-1000, 2% FBS)). All cells were incubated at 37°C and 5% CO_2. The medium was exchanged every other day. The cells were sub-cultured after growing to 60−80% confluency.

PEARL supernatants for cytotoxicity assays:

To evaluate the cytocompatibility of PEARLs, supernatants (200 µL in FGM) following their incubation in FGM were collected after 24 hours and used to treat the NHDFs. Cells (34,000 cells/well) were seeded in sterile µ-Plate Angiogenesis 96 well-plates (Ibidi, Germany) in growth medium (70 µL) and allowed to attach overnight (16 h, 37°C, 5% CO_2). Then, the medium was removed, cells were washed twice with DPBS, and treated with supernatants from the PEARLs and incubated overnight at 37°C, 5% CO_2. The conditions investigated were: “Alginate” (supernatants from empty gels without bacteria), “WT” (supernatants from wild type bacterial gels), “P_{tec}_3050” (supernatants from high secreting NucA bacterial gels), “P_{tlpA}_3050” (supernatants from low secreting NucA bacterial gels), “Alginate+Nuc” (supernatants from alginate gels loaded with 50 nM of nuclease), “Nuc control” (medium containing 0.5 nM, 5 nM or 50 nM nuclease).

Cytotoxicity assays:

Lactate dehydrogenase (LDH) assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) was performed to quantify the percentage of cell death after treatment following the manufacturer's instructions. Briefly, 30 µL of supernatant from each well was transferred to a new 96-well plate, and 30 µL of LDH substrate was added. Samples were incubated in the dark for 30 min at room temperature. Then, 30 µL of the stop solution was added and the absorbance at 490 nm was read using a plate reader (TECAN Spark). A negative control of cells cultured on cell growth medium, and a positive control of cells treated with 37.5% Triton X-100 (lysis control) were also analyzed. The blanks used were medium background absorbance readouts at 490 nm. The percentage of cell death was calculated as:

$$\mathbf{100x\ [Sample_{LDH\ release} - blank)/\ (lysis\ control_{LDH\ release} - blank)]}.$$

Cell viability assays:

AlamarBlue assay (Invitrogen) was used to measure cell viability after treatment, following the manufacturer's guidelines. Briefly, alamarBlue reagent (10% v/v in growth medium) was added to treated cells (cells incubated for 24 h with bacterial alginate beads supernatants) and incubated for 2 h. The medium was then transferred to black 96-well plates. Fluorescence (Ex/Em 570/600 nm) was analyzed using a plate reader (TECAN Spark). Controls of cells cultured on cell growth medium and blanks with only medium were also analyzed. To calculate cell viability, fluorescence values were normalized with the values of the control as follows:

$$\mathbf{[(Sample_{fluorescence} - blank)/\ (Control_{fluorescence} - blank)]}$$

Cell staining:

Cells were fixed with 4% paraformaldehyde (PFA, in DPBS) for 20 minutes at room temperature and washed twice with DPBS. Then, cells were permeabilized with 0.1% Triton X-100 (in DPBS) for 5 minutes. After permeabilization, samples were washed twice with DPBS. Then cells were blocked using blocking buffer (1% bovine serum albumin (BSA) in DPBS). For actin staining, cells were incubated with alexaFluor 488-Phalloidin (dilution 1:400 in blocking buffer), and for nuclei staining, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (dilution 1:1000 in blocking buffer) for 1h at room temperature and protected from light. After staining, samples were washed three times with DPBS. Images were taken using a Leica DMI6000 B epifluorescence microscope at 20X magnification. Image analysis was carried out using ImageJ software (version). Nuclei were counted using ImageJ to quantify cell density from the images. Images were converted to 8-bit and the Gaussian blur filter was selected with a ball radius of 2. After that, the find maxima tool was applied with a noise tolerance of 20. Maxima points found were counted (one per nucleus detected in the image). Finally, the number of nuclei per area was calculated.

Zebrafish embryo-based Assays

Zebrafish Strain and Maintenance:

Husbandry of adult zebrafish (ZF) was carried out in accordance with internal guidelines based on the German Animal Welfare Act (§11 Abs. 1 TierSchG). Embryos of wild-type ZF from the AB strain used for all investigations originated from the HIPS in-house zebrafish facility. The AB strain was originally purchased from the European Zebrafish

Resource Center at Karlsruhe Institute of Technology (EZRC, KIT Karlsruhe, Germany). All experiments were carried out before 5 days post-fertilization (dpf) as these early devolvement stages are not considered as animal experiments according to the EU Directive 2010/63/EU. [28] ZF embryos were maintained at 28 °C in fresh 0.3× Danieau's medium (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$, 1.5 mM HEPES, 1.2 µM methylene blue, pH 7.1–7.3) until they were used for the tests. At a maximum of 5 dpf, all embryos were euthanized by hypothermal shock and consecutive freezing.

Biotests:

Three distinct types of alginate beads were produced based on the encapsulated bacterial strains: 1) eight alginate beads containing *L. plantarum* wild type, 2) eight PEARLs with *L. plantarum* P_{tec}_3050 Nuclease (which secretes the highest level of nuclease), 3) eight empty alginate beads without bacteria in the core and 4) eight PEARLs with *L. plantarum* P_{tec}_3050 Nuclease (which secretes the highest level of nuclease) preincubated for 4 days in the ZF medium prior to test start in modified 0.3x Danieau's medium (pH 8 with 1% v/v sucrose) to assess the effect of maximum secretion of bacterial secretome on ZF embryos. These beads were fabricated in the morning on the day of experiment and placed in a flat-bottom 48-well plate and incubated in modified 0.3× Danieau's medium to maintain pH stability and promote bacterial growth. As a positive control, culture supernatant from *L. plantarum* (OD_{600nm} 1 equivalent) was incubated in modified 0.3× Danieau's medium for 96 hours at 37°C with shaking, to evaluate the effect of the highest concentration of non-encapsulated bacterial secretions on ZF embryos. The 48-well plate, containing alginate beads immersed in 350 µL of modified 0.3× Danieau's medium per

well, was further incubated till the addition of three zebrafish embryos per well for subsequent testing.

At 1 dpf ZF embryos were de-chorionated by treating the eggshell with pronase (1 mg/mL; Sigma, P5147-1G, lot: SLCF6250) for approx. 6 minutes. When the chorion started to disintegrate, the digestion process was stopped by washing the embryos with fresh Danieau's medium 3-times, which did not contain methylene blue in order to prevent inhibition of *L. plantarum* in the following test. Three de-chorionated embryos each were transferred to a well pooled in a volume of 50 µL pure medium. The survival as well as sub-lethal effects such as morphological, physiological and behavioral alterations were investigated with a microscope and recorded at test start and after 24, 48, 72, and 96 hours (test end) corresponding to 1, 2, 3, 4 and 5 dpf, respectively. The following conditions were tested: a negative control containing only pure enriched Danieau's medium, empty alginate beads with no bacteria in the core, alginate beads containing wild-type *L. plantarum*, PEARLs containing *L. plantarum* harboring P_{tec}_3050 nuclease and the last ones being alginate beads with *L. plantarum* P_{tec}_3050 nuclease pre-incubated for 4 days prior to test start on the plate. Each condition was tested in 8 replicates, each containing 3 embryos resulting in 24 embryos for each condition.

In a separate test, three different concentrations of cell-free supernatant serving as positive control of bacterial secretome were tested, the pure supernatant as well as two dilutions containing 50 and 25 %, and a negative control containing only pure modified Danieau's medium. The supernatant was diluted using a modified Danieau's medium.

The test was performed in 96-well plates with each well containing 300 µL of test solution and one embryo each. Each condition was tested in 20 replicates.

All tests were performed in parallel using ZF embryos from the same batch.

Bioinformatics analysis

InterProScan was used to identify the signal peptides within proteins encoded in the genome of *L. plantarum* WCSF1. Signal P6.0 was used to predict the signal-peptide-driven secretion of NucA.

Statistical analysis

Statistical analysis was done using GraphPad Prism 7.0 software. Student's T-tests were used to determine significant differences between the means of the groups. For the cell experiments, the goodness of fit of the data was tested via Normality Shapiro-Wilk test. Normal distributed populations were analyzed using analysis of variance (ANOVA) test followed by a Tukey's *post hoc* test to correct for multiple comparisons. Non-Gaussian populations were analyzed via the non-parametric Kruskal-Wallis test followed by a Dunn's *post hoc* test to correct for multiple comparisons.

Differences among groups are indicated as: p-values <0.05 (*), p-values <0.01 (**), p-values <0.005 (***), p-values <0.001 (****), and differences among groups not statistically significant (**ns**).

RESULTS AND DISCUSSION

1) PEARL fabrication and characterization of encapsulated *L. plantarum*

To study the behavior, secretory performance, and biocompatibility of encapsulated probiotic lactobacilli, we developed the PEARL format, which is an easy-to-fabricate alginate core-shell macro-bead ELM as shown in **Figure 1a**. *L. plantarum* WCFS1 was encapsulated in an alginate bead by manually dropping a viscous alginate-bacteria mix into a calcium chloride bath to form the core gel as previously described (**Figure 1b**). [29] A polysaccharide-staining dye, Alcian Blue, was added to this mix to improve the visibility of the core gel for the next step. These beads were then submerged in a viscous alginate solution. The core bead surrounded by the alginate solution was then dropped into the calcium chloride bath to form a thick protective shell layer.

To study growth and containment of probiotic Lactobacilli within PEARLs by light microscopy, *Lactobacillus plantarum* WCFS1 was engineered to constitutively express mScarlet3, a stable fluorescent protein (**Figure 1c**). After the fabrication of PEARLs with roughly 10^6 cells (equivalent to OD_{600nm} of 0.01) per bead, they were incubated in LB medium at 37 °C, in which bacterial growth and mScarlet3 protein expression were monitored using timelapse microscopy. We observed that bacterial growth occurs within the core of the PEARL (**Figure 1d**). In terms of protein expression, mScarlet3 fluorescence was detected only in the core of the PEARL (**Figure 1e**).

To quantify bacterial growth and mScarlet3 expression, an increase in optical density at 600 nm (OD_{600nm}) and fluorescence intensity (Ex/Em - 569/600 nm) were measured

over 72 h in a microplate reader **(Figure 1g** & **1f)**. The results showed that encapsulated *L. plantarum* grows during this time but stays contained within the core as outgrowth into the medium was not observed. In contrast, this PEARL format was unable to contain *E. coli* Nissle 1917 (**Figure 1h**) beyond 8 h, as observed by the rapid increase in OD_{600nm} values. This indicates that alginate is particularly suited to contain *L. plantarum* when fabricated in a core-shell format.

Similarly, the fluorescence intensity associated with mScarlet3 expression was observed to increase over 72 h only within the beads and not outside. In comparison, when the bacteria are grown freely in the medium, the fluorescence signal is seen throughout the well with some accumulation in the middle, possibly due to sedimentation of the bacteria in that region. Plotting mean fluorescence intensity of the well over time shows that produced mScarlet3 steadily increases in the PEARLs over the first 20 h after which it plateaus. In comparison, this occurs for about 30 h in the non-encapsulated bacteria and at a higher magnitude since fluorescence was detected throughout the well. The shorter duration of fluorescence increase and lower magnitude in the PEARLs is possibly also caused due to limited oxygen availability within the hydrogel preventing mScarlet3 fluorophore formation. [30] Apart from ensuring containment, supporting biomass growth, and enabling protein expression, the PEARLs also help in preventing pH of the medium dropping below 6, which occurs when the bacteria are cultured without encapsulation **(Figure S4b)**.

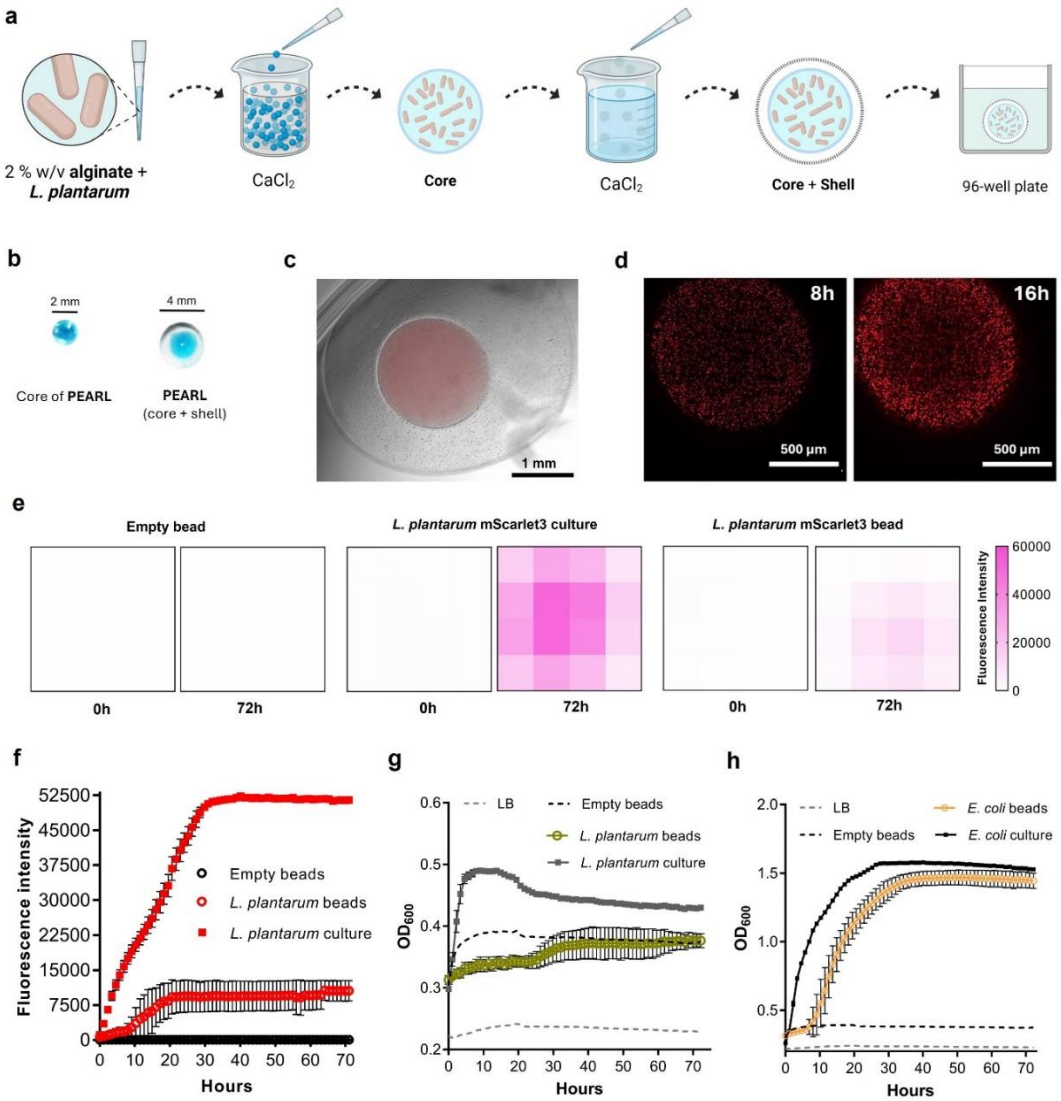

Figure 1: Lactobacilli-based alginate macro beads design and fabrication: **a)** Schematic representation of manual fabrication of alginate-based PEARL. It depicts the step-by-step making of PEARL. **b)** Macroscopic image of the core comprising alginate and bacteria; shell made with alginate. **c)** 4x Brightfield microscopic image of an alginate bead in a 96-well plate **d)** 10x Fluorescence microscopy image of alginate macro beads at 8h and 16h, depicting the growth and fluorescence of bacterial colonies expressing mScarlet3 reporter protein within the core. **e)** 2D heatmap-based representation of fluorescence signal recorded from wells with PEARLs and bacterial cultures at 0-hour and 72-hour. **f)** 72-hour kinetic measurements of fluorescence expression from *L. plantarum* expressing mScarlet3 in PEARLs and non-encapsulated culture control (n=4, mean ± SD). **g)** 72-hour growth kinetic measurements depicting OD_{600} values of encapsulated *L. plantarum* in PEARLs and non-encapsulated culture control (n=4, mean ± SD). **h)** 72-hour growth kinetic measurements depicting OD_{600} values of encapsulated *Escherichia coli Nissle* in PEARLs and non-encapsulated culture control (n=4, mean ± SD).

These promising results showed that *L. plantarum* could grow and produce a heterologous protein in a contained manner within the PEARLs. For application as a therapeutic ELM, it is desirable that the bacteria secrete proteins, and these proteins diffuse out of the material. In the next sections, we engineer robust protein secretion from *L. plantarum* using strong promoters recently discovered by our group [20] and demonstrate their functionality in PEARLs.

2) Enhancing protein secretion in probiotic lactobacilli

Identification and selection of endogenous signal peptides (SPs)

Protein secretion in bacteria is enabled by SPs at the N-terminal of peptides that are recognized by the cellular secretion machinery, which transports the proteins across the cell membrane. While several SPs have been identified in *L. plantarum*, it was necessary to identify optimal candidates compatible with our novel promoters and drive higher secretion of protein of interest compared to the reported signal peptides. For this, we screened the protein sequences encoded by more than 200 endogenous genes via InterProScan until we identified 40 SPs. As a reporter protein, we chose NucA, a nuclease from *Staphylococcus aureus*, which can be easily detected in the medium using a simple DNA degradation assay and has been extensively used to characterize secretion in gram-positive bacteria. [23, 31, 32] SignalP6.0 was used to predict NucA secretion mediated by all SPs [33]. The predictions of 5 SPs with varying secretion levels, henceforth termed SP1-5, were experimentally verified. We additionally included one of the most efficient and used SPs from the literature, Lp_3050 in the testing for comparison [24]. NucA secretion was assessed using DNase agar and medium assays (**Figure 2a**).

Characterization of novel endogenous SPs using nuclease assay.

The novel strong promoters for *L. plantarum* recently discovered in our lab are P_{tlpA} and P_{tec} (5x and 40x stronger than previously reported) [21, 20]. To avoid the risk of overwhelming the secretion machinery in *L. plantarum*, we initially tested the ability of the weaker promoter out of the two, P_{tlpA}, to drive the production and secretion of NucA fused to each of the SP (SP1-5 and Lp_3050) (**Figure 2b**). Secretion was semi-quantitatively characterized using a DNase agar plate assay in which active extracellular NucA creates a dark halo around secreting colonies by degrading fluorescently labeled DNA in the agar. Only three SPs: Lp_3050, SP3 and SP5 were found to secrete detectable amounts of active NucA after 24 hours of growth on DNase agar plates. Two parameters were measured to compare the performance of each SP in the DNase agar plates - the area of the halo and the decrease in fluorescence intensity within this halo. Based on this analysis, SP3 was found to generate the biggest halo (~4 cm^2) and the largest drop in fluorescence (**Figure S5a**), followed by LP_3050. Next, we tested the possibility of increasing SP3 and Lp_3050-driven secretion using the P_{tec} promoter (**Figure 2c**). We observed that both SP3 and Lp_3050 NucA-driven secretion could be increased when P_{tec} was the promoter driving the gene expression since bigger and darker halos (**Figure S5b**) were detected for P_{tec} compared to P_{tlpA}. These results proved that NucA secretion can be increased by replacing the Lp_3050 signal peptide for SP3 under P_{tlpA} or swapping P_{tlpA} by P_{tec} for both Lp_3050 and SP3. Yet, the best promoter-SP combination was found to be P_{tec}_Lp_3050.

To quantify the amount of NucA secreted by these promoter-SP combinations, we used liquid DNase media instead of the agar, in which the bacteria were allowed to grow for 24

hours at 37°C with shaking (**Figure 3d**). This enabled us to accurately measure the drop in fluorescence intensity in the medium and interpolate the values in a NucA standard curve (**Figure S6a**). Once again, NucA secretion was observed only with SP3, SP5 and Lp_3050, validating the results obtained with the agar plates (**Figure 2b**). SP3 mediated better secretion than Lp_3050 when driven by P_{tlpA} (~20 nM and ~15 nM, respectively). However, both SP3 and lp_3050-driven secretion levels could be significantly increased when P_{tlpA} was swapped by P_{tec} (~24 nM and ~55 nM, respectively) (**Figure 3e**). Interestingly, with P_{tec}, lp_3050 performed better than SP3, indicating that SPs may perform differently when expression is driven by different promoters.

Based on the growth curve of *L. plantarum* WCFS1 in LB medium (**Figure 1e)**, it is evident that the bacteria grow exponentially for about 6 h beyond which they reach their saturation phase due to limitation of nutrients. This change in growth phase can influence protein production and secretion, so we quantified NucA secretion at mid (3 h) and late log phase (6 h) in addition to the late saturation phase (24 h). We observed that for all the promoter/SP combinations, most of the protein (>60% approximately) is secreted within first 6 h, suggesting that the promoters are most active in the log phase of growth (**Figure 2a**). Furthermore, most of the secretion from the P_{tlpA} constructs occurs over the first 6 h, whereas from the P_{tec} constructs, most secretion occurs in the first 3 h (**Figure S6b**). We examined both the promoter's activity using another reporter, mCherry, in the different growth phases, and we observed that P_{tec} is much more active in the early and mid-log phase, which supports our results obtained with the NucA where most of the protein is produced (**Figure S6c**). Such high levels of production and secretion in the hours timescale is well-suited for short-term protein delivery within the body.

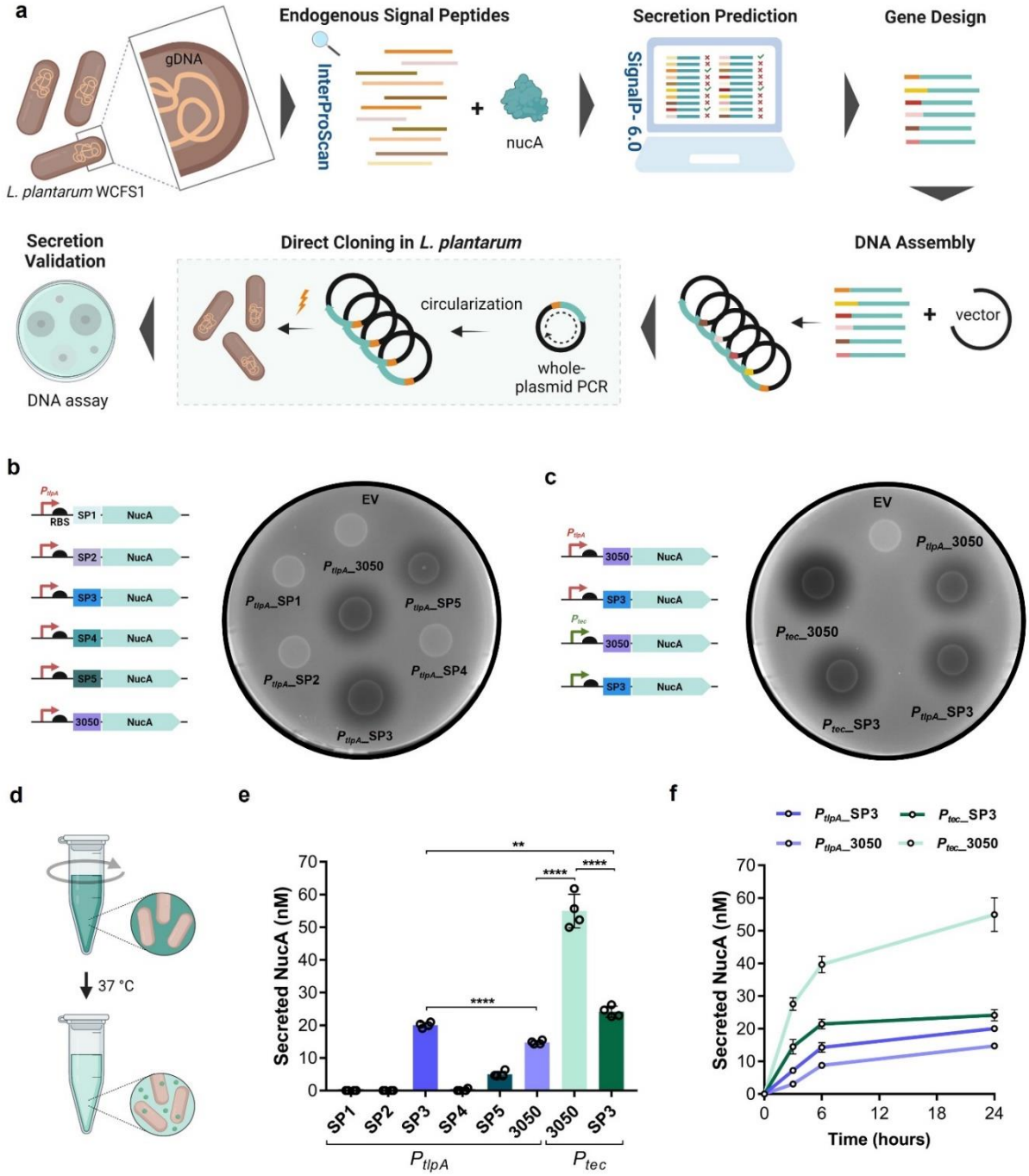

Figure 2: Workflow depicting quantification of recombinant protein secretion in liquid culture: **a)** Flow scheme representing the screening of endogenous signal peptides followed by gene design and molecular cloning in *L. plantarum* WCFS1 to validate the secretion of staphylococcal nuclease (17 kDa), a reporter protein commonly used for secretion studies. **b)** Schematic of genetic constructs of P_{tlpA} promoter driving the expression of nuclease, each

tagged with one of six different signal peptides and EV bacteria (as negative control); to the right is the image of DNase agar plate illustrating the secretion of nuclease from 10 μL of bacterial spots. **c)** Schematic of genetic constructs of P_{tlpA} and P_{tec} promoters driving the expression of nuclease, each tagged with two best signal peptides (3050 and SP3) from this study; to the right is the image of DNase agar plate illustrating the secretion of nuclease from 10 μL bacterial spots of these genetic constructs. **d)** Graphical representation of liquid culture-based assay to quantify the nuclease secretion from engineered bacterial strains: Nuclease secreted from bacteria depolymerizes the DNA, thereby breaking down the methyl green-DNA complex in media resulting in loss of green coloration. **e)** Quantification of secreted nuclease in DNase medium after 24 hours of incubation for all the secretion-based genetic constructs screened in this study (n = 3, mean ± SD). **f)** Timepoint-based quantification (n =3, mean ± SD) (3h, 6h, 24h) of secreted nuclease (in nanomolar, nM) in DNase medium for two best constructs of each promoter (P_{tec}_3050, P_{tec}_SP3, P_{tlpA}_3050, P_{tlpA}_SP3).

3) Assessing protein release from PEARLs

Nuclease release from PEARLs was assessed by two different methods: 1) by placing the PEARLs in the center of a DNase agar plate and imaging the agar plate at regular time intervals, and 2) by placing the PEARLs in a well containing DNase media followed by fluorescence measurements (**Figure 3a**). In DNase agar plates, we observed halo formation at 24 h after placing the bead in the center of the plate for all the NucA-secreting clones based on the P_{tlpA}/P_{tec} promoters and SP3/lp_3050 SPs (**Figure 3b**). The halos kept increasing over the next 14 days, suggesting continuous nuclease secretion from the bead. We observed the same secretion trend for the different promoter/SP combinations as observed in culture (**Figure 3b**), with the P_{tec}_3050 strain producing the biggest and darkest halos. We also noticed that the bacteria remained contained in the bead and secretion was sustained for at least 14 days (**Figure 3c**). However, it is to be noted that since the PEARLs in this study are manually fabricated, if the core is misaligned in the shell leading to a thin shell on one edge, outgrowth of the bacteria, indicated by colonies outside the bead, is observed within 1-2 days. This occurred in approximately

10% of the PEARLs that were fabricated, and these constructs were not included in any of the analyses.

To quantify nuclease secretion from the PEARLs, DNA degradation in liquid DNAse media was measured over 3 days under static incubation conditions. Non-encapsulated bacterial cultures with the same number of cells as in the alginate core of the PEARLs (10^6) were included in the experiment for comparison. Activity of the secreted nuclease was detectable from the three-hour mark **(Figure S7a)** and it increased over 72 h. Interestingly, nuclease secretion from non-encapsulated bacteria was distinctly non-monotonic, with a rapid increase within the first 8 h and then a much slower increase over the next 72 h (**Figure 3d**). In the case of P_{tec}_3050, another increase in secretion rate was observed around 24 h, and the final plateauing around 45 h is attributed to the complete degradation of the probe DNA in the DNase medium. It is suspected that these different secretion profiles are related to the different growth phases that these bacteria go through during this period associated with metabolic changes and possible biofilm formation (**Figure S6c**) [34]. In contrast, nuclease release profiles from the PEARLs were found to be much more stable with a largely monotonic rate observed till at least 24 h followed by a gradual plateauing most likely caused by the decrease in DNA substrate availability (**Figure 3F, S7b-e**). Even the differences in protein secretion rates observed between the different promoter-signal peptide combinations seem to have considerably reduced during the first 24 h. Furthermore, even though the grown biomass within the PEARLs is not expected to exceed that of the non-encapsulated bacteria, it was observed that nuclease release from the PEARLs was at least two-fold higher at 24 hours **(Figure 3f)**. This suggests that bacterial encapsulation promotes higher secretion rates on average

during the first 24 hours compared to non-encapsulated bacteria. This could be because encapsulation forces the bacteria to grow slower and thereby dedicate more of its metabolic resources towards the production and secretion of the recombinant protein [35]. Considering our previous results indicating that the P_{tec} and P_{tlpA} are most active in the early- to mid-log phases (Figure 2f), the results with the PEARLs suggest that the bacteria remain in a state equivalent to these growth phases within the alginate. This is possibly since bacterial growth is restricted by mechanical forces imposed by the hydrogel matrix rather than nutrient unavailability. Thus, while the population size is not necessarily increasing, the bacteria probably have access to nutrient levels similar to what they would have in liquid culture at early- or mid-log phase. Thus, in the PEARL format, the most productive phase of the engineered bacteria is prolonged.

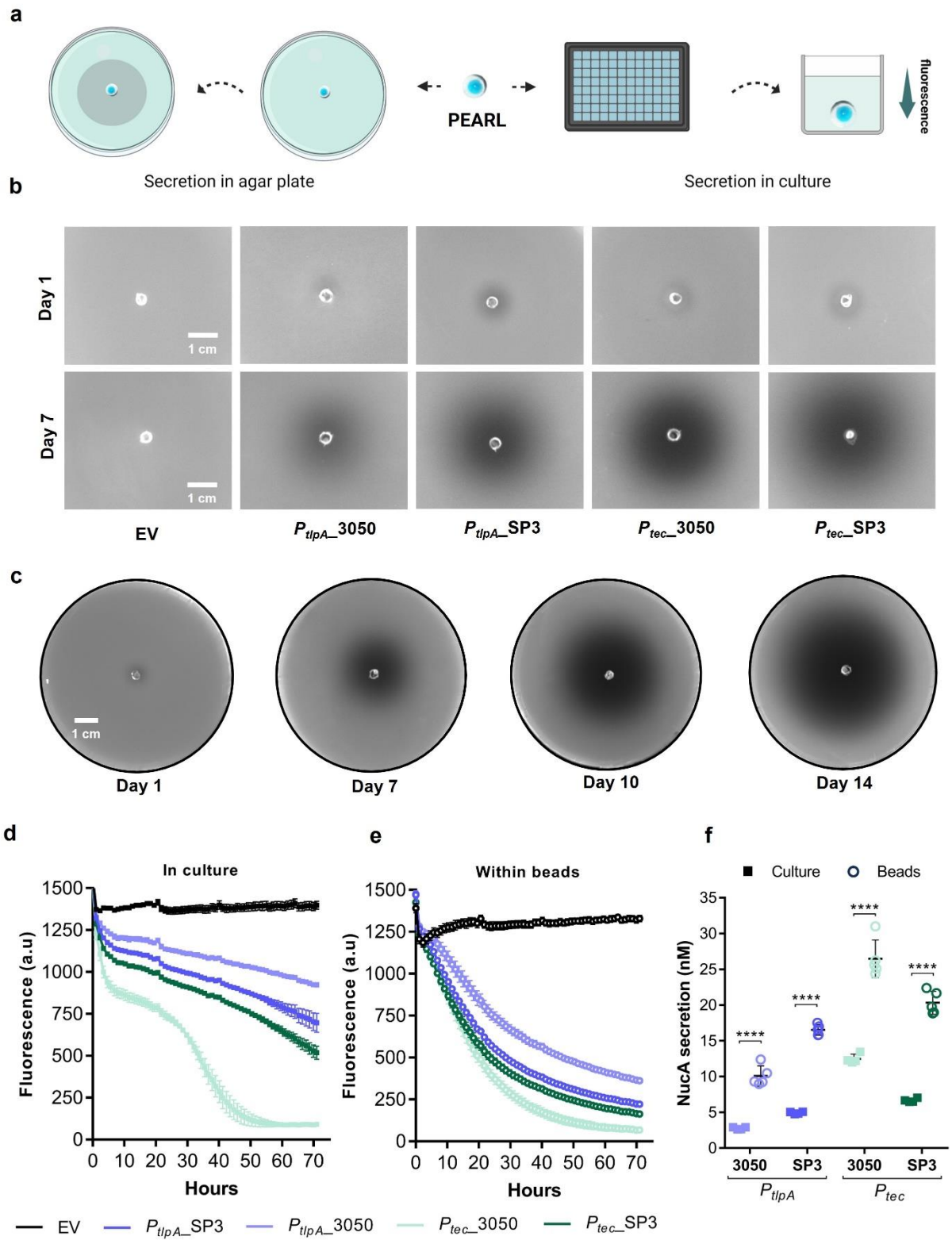
a
PEARL
fluorescence
Secretion in agar plate
Secretion in culture
b
Day 1
Day 7
1 cm
EV
P_{tlpA}_3050
P_{tlpA}_SP3
P_{tec}_3050
P_{tec}_SP3
c
1 cm
Day 1
Day 7
Day 10
Day 14
d
In culture
Fluorescence (a.u)
Hours
e
Within beads
Fluorescence (a.u)
Hours
f
Culture
Beads
NucA secretion (nM)
****
3050
SP3
3050
SP3
P_{tlpA}
P_{tec}
EV
P_{tlpA}_SP3
P_{tlpA}_3050
P_{tec}_3050
P_{tec}_SP3

Figure 3: Characterization of Engineered living material's release profile: **a)** Graphical representation of assays done to validate and quantify the secreted nuclease from alginate beads using DNase agar plate and by fluorescence kinetics in culture. **b)** DNase agar plate-based qualitative assay to track the nuclease secretion and bacterial containment within PEARLs on Day 1 and Day 7. **c)** Nuclease secretion from PEARL embedded in DNase agar plate captured on Day 1, 7, 10, 14 indicating the bacterial viability up to 14 days **d)** Quantification of nuclease secretion with reference to the drop in fluorescence in wells with culture for 72 hours (3 days) (n=5, mean ± SD). **e)** Quantification of nuclease secretion with reference to the drop in fluorescence in wells with alginate beads for 72 hours (3 days) (n=5, mean ± SD). **f)** Plot showing the comparison of secreted nuclease (n=5, mean ± SD) after 24 hours in culture and from alginate beads for the two best genetic constructs of each promoter (P_{tec}_3050, P_{tec}_SP3, P_{tlpA}_3050, P_{tlpA}_SP3).

4) Polycistronic gene expression from PEARLs

Since our strongest promoter, P_{tec}, was capable of driving high levels of NucA secretion and fluorescent protein expression, we tested its ability to do both simultaneously. Being able to encode strong production of two proteins at once would improve the versatility of programming *L. plantarum*. For this, we inserted the mScarlet3 gene downstream of the NucA gene, separated by a ribosome binding site (**Figure 4a**). With non-encapsulated bacteria, we observed that the expression of mScalet3 dropped to roughly half when compared to the strain encoding only mScarlet3 production. In contrast, NucA secretion did not significantly decrease compared to the strain only encoding NucA secretion (**Figure 4b**). Both reporters could also be detected well in a DNAse agar plate when grown in static (**Figure 4c**). In the PEARL format, mScarlet3 could be detected inside the bead and continuous secretion over 7 days was also detected using DNAse agar plates (**Figure 4d**). These experiments confirmed that the P_{tec} promoter can be used to drive strong expression of two independent genes in parallel, secretion is not compromised by doing this, and this functionality is maintained in the PEARLs.

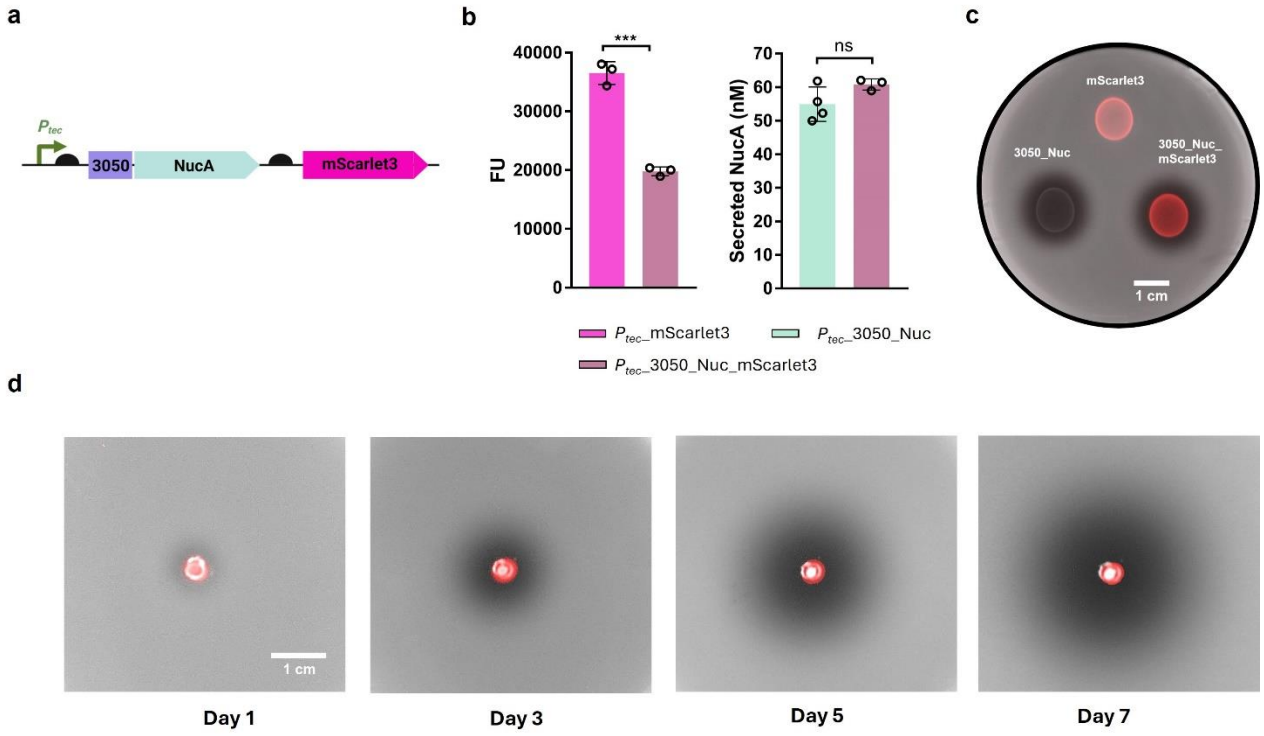

Figure 4: Polycistronic gene expression from PEARLs. **a)** Scheme of the polycistronic gene based on NucA and mScarlet3 genes. **b)** The left plot shows the decrease in fluorescence expression (mean ± SD) by placing the mScarlet3 gene downstream NucA gene (in comparison to mScarlet3-producing *L. plantarum* WCFS1). The right plot shows the levels of secreted NucA (n= 3/4, mean ± SD) for the polycistronic-engineered bacteria and single NucA secreting bacteria (P_{tec}_3050). **c)** DNA agar plate showing bacteria expressing only mScarlet3, secreting only NucA, and expressing and secreting mScarlet3 and NucA, respectively, after 24 hours of growth. **d)** PEARL containing the polycistronic engineer strain. Expression of both genes could be detected from day 1 and kept increasing over the days.

5) Cytocompatibility investigation of bacterial alginate gels secreting nuclease

L. plantarum is known to produce lactic acid and other metabolites that can be cytotoxic to eukaryotic cells. [36] Several reports suggest that such cytotoxicity can be selective for cancer cells but we found it important to test if encapsulation of these bacteria in the PEARLs would alter this cytotoxicity. To evaluate this, we incubated the PEARLs in a medium optimized to support the growth of *L. plantarum* and human cells. Thus, during the 24 h period of this experiment, the bacteria are proliferating within the alginate beads

and secreting NucA (**Figure 3**). The supernatants from these cultures were used to treat human fibroblasts overnight (**Figure 5**). We compared the supernatants from PEARLs containing the P_{tec}_3050 (a high NucA producer, >25 nM, **Figure 3f**) and P_{tlpA}_3050 (a low NucA producer, <5 nM, **Figure 3f**) strains. We used supernatants from empty alginate macro beads, wild-type lactobacilli entrapped in the alginate macro beads, nuclease-laden alginate macro beads (50 nM), and medium containing 0.5, 5 and 50 nM of NucA as controls. First, we quantified the percentage of cell death by measuring the number of cells with damaged cell membranes using the lactate dehydrogenase assay (**Figure 5b**). A lysis condition where all cells were damaged with Triton X-100 was used as a positive control. All conditions investigated presented similar values of cell death around 20%, which were comparable to the negative control, untreated cells. Cell viability was also quantified as the number of metabolically active cells using alamarBlue assay (**Figure 5c**). Results show similar values of viability to the negative control. We also stained the actin cytoskeleton of the fibroblasts to assess cell morphology (**Figure 5d**). From the staining, we observed similar morphologies across conditions, with spread cells and mature actin fibers developed. Nuclei staining was quantified to obtain values of cell densities for the conditions evaluated. Cell densities were similar across conditions (~1600 cells/mm^2) indicating comparable cell confluency after treatment to the negative control **(Figure S8**).

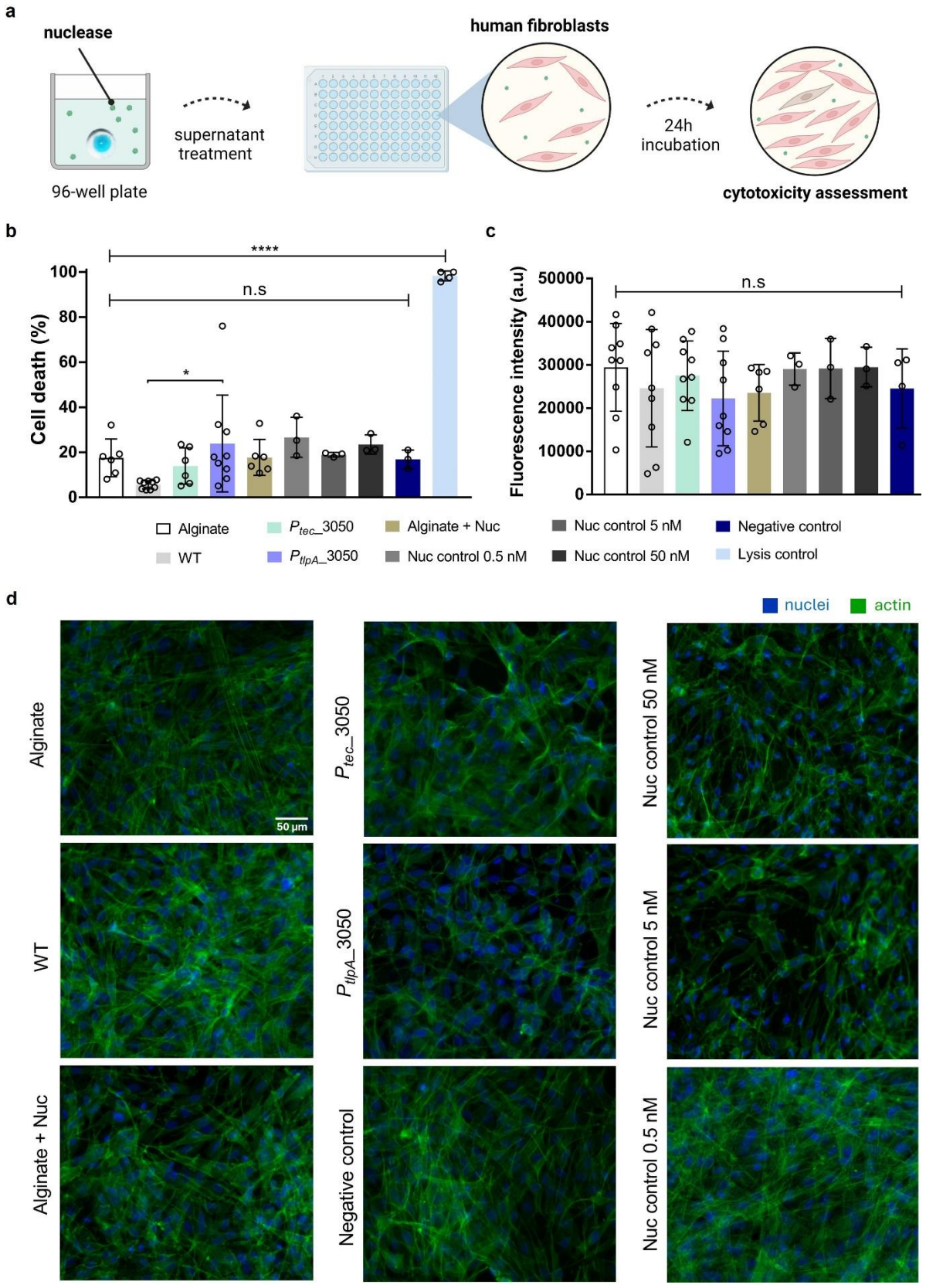
a
nuclease
96-well plate
supernatant treatment
human fibroblasts
24h incubation
cytotoxicity assessment
b
Cell death (%)
****
n.s
*
c
Fluorescence intensity (a.u)
n.s
Alginate
WT
P_{tec}_3050
P_{tlpA}_3050
Alginate + Nuc
Nuc control 0.5 nM
Nuc control 5 nM
Nuc control 50 nM
Negative control
Lysis control
d
nuclei
actin
Alginate
WT
Alginate + Nuc
P_{tec}_3050
P_{tlpA}_3050
Negative control
Nuc control 50 nM
Nuc control 5 nM
Nuc control 0.5 nM
50 µm

Figure 5: Cytocompatibility evaluation of nuclease-secreting bacterial alginate beads on human fibroblasts. **a**) Bacterial beads (and controls) were incubated in growth medium overnight to evaluate the compatibility potential on human fibroblasts. The collected supernatants were used to treat fibroblasts in culture for 24 h, after which viability assessment was performed. **b**) Percentage of cell death quantified after treatment with the supernatants via LDH assay (mean ± SD). **c**) Fluorescence intensity measured during alamarBlue assay after treatment with supernatants (mean ± SD, arbitrary units (a.u)). **d**) Representative fluorescence images of human fibroblasts after treatment (scale bar: 50 µm, blue: nuclei, green: actin).

6) Zebrafish Embryo toxicity test

Zebrafish (ZF) embryos <5 day post fertilization (dpf) were used as a highly sensitive *in vivo* acute toxicity invertebrate test model. Following OECD guideline 236 [37], such tests are usually performed in 24-well plates with one ZF embryo per well in a volume of 2 mL medium. In order to achieve a more robust exposure scenario, the test protocols were adapted in the following ways to be better suited for PEARLs:

Usually, the zebrafish embryos are exposed to test compounds while they remain inside the egg from which they hatch later during the test. However, the chorion can prevent substances from accessing the embryo. [38] To ensure that the embryos got into direct contact with any bacterial products released in the medium, they were released from the chorion before the start of the test. The de-chorionation took place at 1 dpf since this is the earliest time at which this treatment can be carried out reliably without harming the embryos, and which is also a common practice in drug screening. [39]

The ZF medium was adapted to support *L. plantarum* growth and metabolism by adding saccharose. Different sugars (Glucose, Galactose, Maltose, Arabinose, Fructose, Sorbitol, Xylose, Mannose and Saccharose) were added to the *L. plantarum* culture at 1% v/v concentration and after 24 hours, OD_{600nm} and pH of the cultures were measured. Saccharose supplementation was the only condition in which significant bacterial growth

(up to OD_{600} of 2) was seen along with the least drop in pH to 6. In all the remaining conditions with other sugars, either bacterial growth was compromised, or the pH decreased below 6, even down to 3.

The potential toxicity of non-encapsulated *L. plantarum* on ZF embryos was investigated using a cell-free supernatant containing the complete bacterial secretome. To decrease the needed production volumes, the test was performed in a 96-well plate, thereby decreasing the test volume from 2 to 0.3 mL per replicate. We observed that the pure supernatant instantly killed all ZF embryos within the first 24 hours (**Figure 6b**). On dilution to 50 % of the culture supernatant, 45 % of the ZF embryos died within the first 24h. After 48 hours all embryos were dead. On dilution to 25 % of the culture supernatant, 75 % survived after 24 h, 55 % after 48 h, and 50 % after 72 and 96 h. The results indicate that metabolic byproducts of *L. plantarum* are toxic to ZF embryos, similar to their reported effect on cancer cells. [35] Notably, the supernatant decreased the pH from 8 to values around 6, possibly due to lactic acid production by the bacteria. This low pH was measured in the 50% diluted samples but in 25% dilution, the pH was closer to neutral (**Table S2**). However, ZF embryos have a relatively high pH tolerance and should tolerate the observed pH changes [40]. Therefore, the observed mortality induced by the supernatant is considered to be induced rather by substances produced by *L. plantarum* than by decreased pH.

The effect of the PEARLs on ZF embryos were tested in 48-well plates to reduce the volume and increase the concentration of potentially released bacterial metabolic byproducts. The size of a well was small enough to establish direct contact between embryos and beads, but still big enough to prevent mechanical interferences such as

squeezing of the embryos. 8 PEARLs were tested for each condition with 3 embryos in the same well as one PEARL. The scheme of the experiment is shown in **Figure 6a**. Survival of the ZF embryos in the presence of the PEARLs was tested over 96 hours and compared to a negative control group that did not contain any PEARLs or alginate. In the first test group empty alginate beads without bacteria were tested. In the second group, core-shell alginate beads contained wild-type *L. plantarum*. The next group included PEARLs containing the P_{tec}_3050 strain, which secretes the highest level of nuclease. In all these groups, all embryos survived the 96 hours incubation period (**Figure 6b**). In an additional treatment group, the PEARLs containing the P_{tec}_3050 strain were incubated for 96 hours without ZF before the start of the test to increase the concentration of secretion products in the surrounding medium. In this treatment, one embryo died between test day 3 and 4 resulting in a survival rate of 95.8 % (**Figure 6b**). Such effects with a value lower than 10 % are considered natural alterations and cannot be related to toxicity. Thus, no significant reduction of survival due to the presence of PEARLs was observed during the 96h test duration. This was quite a surprising result considering the toxicity of the non-encapsulated bacteria. It is to be noted that the PEARLs did not considerably reduce the pH of the medium as it remained around 7.5 in all cases (Table S2). This indicated that encapsulation of the bacteria lowered its lactic acid production rate. While it is possible that alginate encapsulation slowed down metabolite diffusion into the medium, the results from **Figure 3d** indicate that diffusion of a secreted protein occurs within a matter of hours, so the 4-8 day periods of the last two test groups should have been sufficient for metabolite accumulation in the medium. Furthermore, we were able to clearly detect NucA secretion into the ZF medium after 24 hours (**Figure S9a**).

In parallel, the PEARLs incubated in ZF medium for 14 days were disintegrated using EDTA to recover the viable bacteria and plated on an MRS-agar plate. The number of colony forming units (CFU) was found to be around 10^5 CFU/mL (**Figure S9b**). Though this is lower than the initial number of cells that were encapsulated in the PEARLs (~10^6), it is to be noted that EDTA treatment could have killed some of the bacteria. [27] Additionally, six random colonies from the MRS agar plate were spotted on the DNase-agar plate, and secretion was observed from all colonies after 24 hours (**Figure S9c**). These results indicated that PEARLs could maintain the viability and activity of a bacterial population over 14 days and that encapsulation of *L. plantarum* in alginate possibly reduces or prevents the production of metabolites that can be cytotoxic to the zebrafish. Extensive analysis of the secretome from non-encapsulated bacteria and the PEARLs using metabolomic and proteomic methods could shed light on this mechanism.

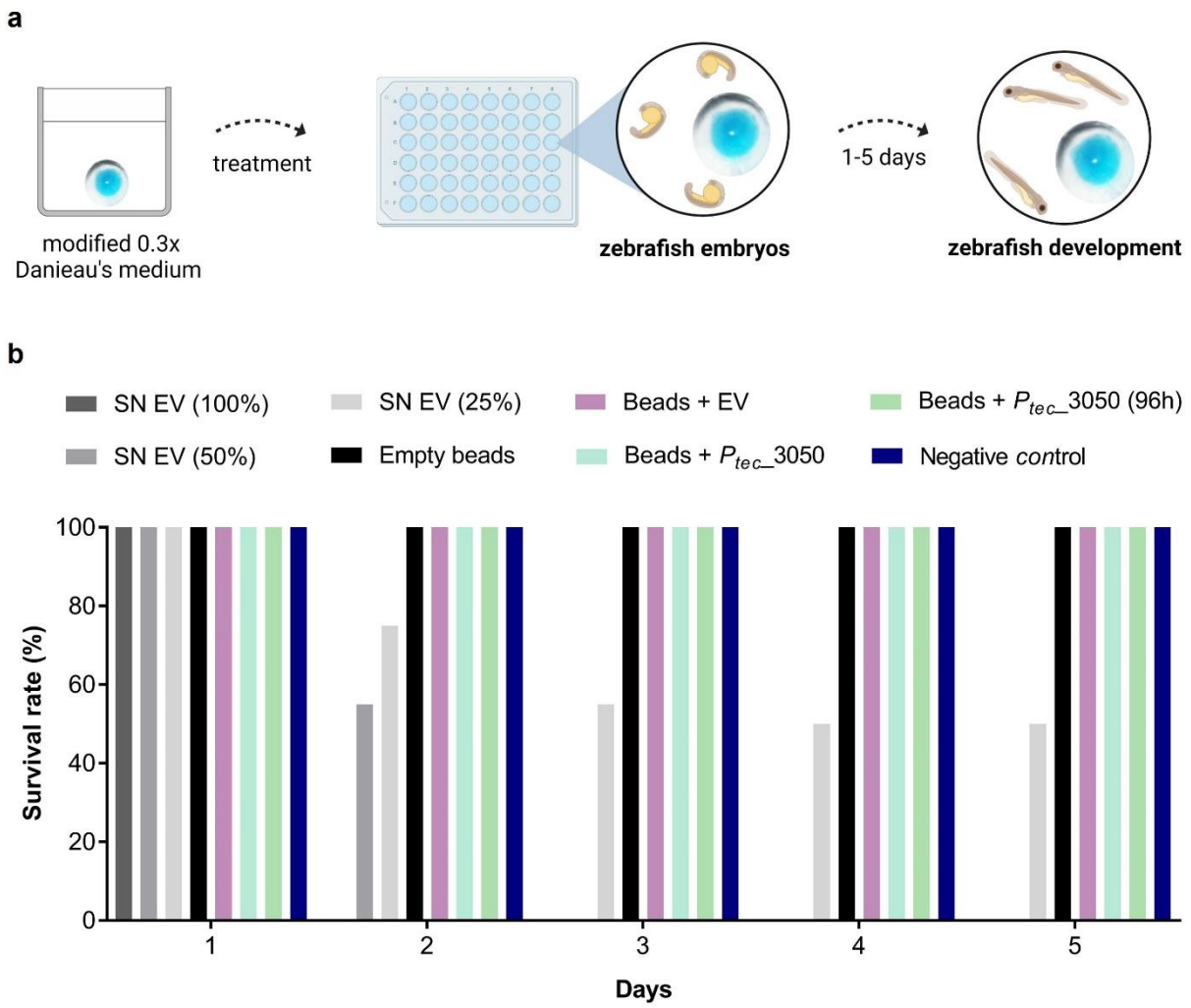

Figure 6: Cytotoxicity evaluation of nuclease-secreting alginate beads on Zebrafish embryos. **a)** Graphical representation of alginate beads incubated in 0.3x Danieu's medium followed by incubation of zebrafish embryos with beads in a 48-well flat bottom plate up to 5 days. **b)** Survival rates of zebrafish embryos exposed to three different concentrations of the cell-free supernatant (SN) containing the complete bacterial secretome (SN EV (100%), SN EV (50%), SN EV (25%) as a positive control, four different kinds of alginate beads: empty beads, beads + EV (containing wild-type *L. plantarum*), beads + P_{tec}_3050 (secreting the highest level of nuclease), beads + P_{tec}_3050 (96h) (pre-incubated for 96h before adding ZF embryos) and the negative control.

CONCLUSIONS

To our knowledge, this is the first study that comprises an ELM based on probiotic lactobacilli. We have developed a rapid, cost-effective, and safe encapsulation method that keeps probiotic bacteria viable and active while containing them. Characterization of these PEARLs has revealed interesting insights concerning protein secretion and cytotoxicity. For example, the exact amount of secreted protein from both non-encapsulated and encapsulated bacteria was measured in the range of 5 – 50 nM and, a majority of this was secreted within the first 6 h. Encapsulation as PEARLs offered multiple benefits like supporting long-term protein release (at least 14 days), stabilizing the release profiles, and improving release rates. Additionally, we discovered and characterized new genetic parts that improve the programmability of *L. plantarum* for protein secretion. The super strong constitute promoter, $P_{tec,}$ was able to drive high levels of protein secretion and additionally, support strong expression of two genes simultaneously. Finally, the cytotoxicity results using human cell lines and ZF suggest considerable suppression of toxic metabolite release when the bacteria are encapsulated in alginate. Altogether, these results suggest that PEARLs are a promising platform to be developed for therapeutic applications.

SUPPORTING INFORMATION

A file containing all the supporting information is available from the Wiley Online Library.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The information provided in the main text and supporting information are sufficient to reproduce the experiments described in this study. The raw and processed datasets along with relevant metadata are available from the corresponding author upon reasonable request.

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