Living biointerfaces based on non-pathogenic bacteria to direct cell differentiation

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Genetically modified Lactococcus lactis, non-pathogenic bacteria expressing the FNIII7-10 fibronectin fragment as a protein membrane have been used to create a living biointerface between synthetic materials and mammalian cells. This FNIII7-10 fragment comprises the RGD and PHSRN sequences of fibronectin to bind α5β1 integrins and triggers signalling for cell adhesion, spreading and differentiation. We used L. lactis strain to colonize material surfaces and produce stable biofilms presenting the FNIII7-10 fragment readily available to cells. Biofilm density is easily tunable and remains stable for several days. Murine C2C12 myoblasts seeded over mature biofilms undergo bipolar alignment and form differentiated myotubes, a process triggered by the FNIII7-10 fragment. This biointerface based on living bacteria can be further modified to express any desired biochemical signal, establishing a new paradigm in biomaterial surface functionalisation for biomedical applications.

Understanding the behaviour of cells on synthetic surfaces is of foremost interest to engineer microenvironments that direct cell adhesion, proliferation and differentiation. To favour the interaction with cells, surfaces of synthetic biomaterials have been functionalized with a broad range of proteins, fragments, peptides and growth factors including fibronectin (FN), laminin, vitronectin and others, by using physical and chemical strategies1–3. However, these passive coatings can by no means provide the dynamic stimuli required to orchestrate cell responses and organise the formation of a new tissue at the material interface. Significant efforts have focused on engineering materials that recapitulate characteristics of the ECM, such as the presentation of cell adhesive motifs or protease degradable cross-links, in order to direct cellular responses4,5. Photoactivable RGD adhesive peptides were used to investigate the effect of the density and time point of ligand presentation on cell adhesion, proliferation and differentiation6. Similarly, enzyme-responsive surfaces that present cell adhesive RGD sequences on-demand, that is, by enzymatic hydrolysis of inactive RGD containing precursors that carry cleavable steric blocking groups, have been used to spatiotemporal controlled attachment of cells7. However, the development of a cell/material interface able to provide biological stimuli upon demand, a functional dynamic interface between stem cells and synthetic materials, has not been established yet. We hypothesised that non-pathogenic bacteria can colonise the surface of a broad range of synthetic materials and can be genetically modified to constitutively express or secrete the desired adhesive proteins and factors to a living cell population upon external demand. This work shows that non-pathogenic bacteria that constitutively express a fibronectin fragment enhance cell differentiation. However, our long-term goal is to transform this living interface into a dynamic system able to secrete other proteins and growth factors upon demand.

We have recently shown that genetically modified, non-pathogenic bacteria, can play the role of a (living) biointerface between mammalian cells and synthetic biomaterials and we chose Lactococcus lactis subsp. cremoris as host for membrane expression of the fibronectin FNIII7-10 fragment8. This fragment contains the RGD adhesion motif (10th repeat of the type III fragment) and the PHSRN synergy site (9th repeat of type III fragment), both of them necessary to promote α5β1 integrin mediated adhesion9. Lactococcus lactis is a gram-positive, non-pathogenic bacterium, with GRAS (Generally Regarded as Safe) status and with low production of exopolysaccharides, a mandatory condition to ensure the accessibility of the displayed FNIII7-10 fragments on the bacteria membrane. Moreover, this low exopolysaccharide production is not an obstacle for this strain to develop stable...
biofilms: these bacteria firmly attach to synthetic surfaces and establish communities\textsuperscript{10,11}. This results in a stable layer of bacteria on the material surface which present FNIII\textsubscript{7-10} fragments\textsuperscript{8}.

Mammalian cells interact with FN via integrins, a family of transmembrane receptor proteins that anchor them to the ECM. Integrin-mediated adhesion is a complex process that involves the organization of focal adhesion clusters that link the ECM to the cytoskeleton. Focal complexes are mature adhesion sites that contain structural proteins (vinculin, paxillin, talin, tensin) and signalling molecules (FAK, focal adhesion kinase, Src)\textsuperscript{3,12,13}. We have previously shown that FN null fibroblasts (FN\textsuperscript{-/-}) adhered on this living biointerface based on \textit{L. lactis} expressing FNIII\textsubscript{7-10}, develop focal adhesions and promote FAK-based signalling\textsuperscript{8}.

This work investigates the potential of this living interface based on \textit{L. lactis} expressing a FN fragment as a membrane protein to direct cell differentiation by evaluating myogenic differentiation\textsuperscript{14}. As previously reported\textsuperscript{8}, a modified \textit{Lactis} (MG1363) was engineered to express FNIII\textsubscript{7-10} as a membrane protein. The used construct features the Usp45 secretion peptide, GFP and the FNIII\textsubscript{7-10} fragment containing the RGD adhesion (module III\textsubscript{10}) and PHSRN synergy site (module III\textsubscript{9}). The construct is anchored into the bacterial peptidoglycan layer by the protein A anchor (spaX). This anchor protein is covalently linked to the tetrapeptides of the peptidoglycan layer. \textit{L. lactis} hardly produces any exopolysaccharide, which granted exposure of FN fragments to mammalian cells. C2C12 differentiated into myoblasts triggered by the FNIII\textsubscript{7-10} fragment displayed on the bacterial membrane.

Results
We have first characterised the formation of the \textit{L. lactis} biofilm on glass, including the amount of FN that is available for cell interaction. Then we have addressed the stability of the biofilm after different days, that results in using a strategy to diminish bacterial metabolism and prevent uncontrolled proliferation. Afterwards, we have explored C2C12 interactions on this living interface, first in the short-term (cell adhesion, cell morphology) and then in the long-term (differentiation and related signalling).

Figure 1 | Schematic of living biointerfaces. C2C12 murine myoblasts were seeded on top of \textit{L. lactis} biofilms that express a FNIII\textsubscript{7-10} fragment as a membrane protein. The construct features the Usp45 secretion peptide, GFP and the fibronectin fragment FNIII\textsubscript{7-10} that contains both RGD (FNIII\textsubscript{10}) and PHSRN synergy sites (FNIII\textsubscript{9}). The construct is inserted into the bacterial peptidoglycan layer by the protein A anchor (spaX). This anchor protein is covalently linked to the tetrapeptides of the peptidoglycan layer. \textit{L. lactis} strain forms stable biofilms with very low exopolysaccharide production, which granted exposure of FN fragments to mammalian cells. C2C12 differentiated into myoblasts triggered by the FNIII\textsubscript{7-10} fragment displayed on the bacterial membrane.
efficiently promote the localization of FNIII 7-10 in the bacterial membrane. We have confirmed the presence of FNIII 7-10 by the tagged GFP, which shows fluorescent rings defining the perimeter of each bacterium (Supp Fig. S1). Fig 2a shows bacterial morphology at high magnification by SEM. Additional SEM and AFM images are included in Supp Fig. S1. We have quantified the amount of FNIII 7-10

Figure 2 | Visualisation and quantification of FNIII 7-10 in the bacterial membrane. (a) Low voltage SEM image of L. lactis-FN adhered to a glass surface showing the glycocalyx and the adhesion fimbriae. Scale bar size is 200 nm. (b) – (d) Western blot analysis of membrane fractions of L. lactis using protein extracts from 75, 50 and 25 µL of a stationary phase culture and 750, 500 and 250 ng of a purified FNIII 7-10 fragment as standards (quantification curve shown in d). Proteins isolated from bacteria have higher molecular weight due to GFP, the Usp45 and the spaX fragments (e) Quantification of FNIII 7-10 on the bacteria membrane using ELISA with HFN7.1 antibody. Biofilm activity is roughly equivalent to a surface coated with a solution of 20 µg/mL of plasma fibronectin (300 ng/cm²). L. lactis-empty shows negligible fibronectin activity (1000 µL of bacterial culture was used both for L. lactis-empty and L. lactis-FN, that correspond to a surface area coverage of 27.6 ± 8.5%). (f) ELISA of bacterial biofilms (L. lactis-FN) from fresh GM17-E medium and incubated in DMEM with tetracycline 10 µg/mL for 1 and 4 days respectively. ANOVA shows no statistically significant differences between samples. All data presented as mean ± SD.
available on *L. lactis* membranes using two techniques that provide
complementary information. First, we performed fractionation and
Western blot analysis for bacterial membrane proteins and
subsequent quantification using a recombinant FNIII 7-10 fragment
as the part of the bacteria lysate ascribed to membrane proteins
(Fig. 2b). Image analysis of the Western blot bands reveal that the
total amount of FNIII 7-10 increases as the volume of the sample
(number of colonies) does. Based on this, the approximate density
of FN fragment per unit volume of bacteria is 0.98 μg/mL, which
corresponds approximately to 9.8 μg/10^8 CFU (Fig. 2c,d). However,
considering the role of this living interface in interacting with
mammalian cells, is also important to calculate the availability of
FNIII7-10 after bacteria have been immobilised on a synthetic
material. To do so, we have performed an enzyme-linked
immunosorbent assay (ELISA) with a monoclonal antibody to probe the availability of the
flexible linker between the 9th and 10th type III repeats of FN 17.

Figure 2e shows the amount of this integrin-binding domain on
the surface of the living interface is similar to adsorbing FN from a
solution of concentration 20 μg/mL on glass, a surface density of FN
ca. 300 ng/cm^2^18,19. As we will use tetracycline containing medium
(TC) to maintain a stable biofilm on the material surface during
experiments with mammalian cells, and since TC might inhibit
bacterial protein expression, we have assessed that there is no
influence of TC on FNIII 7-10 expression as a membrane protein
(Fig 2f).

In summary, the engineered living interface presents a high den-
sity of FNIII7-10 available on the cell surface, seeking to promote focal
adhesion organisation and trigger cell fate.

**Biofilm formation and stability.** We next examined the stability of the living interface on the material surface. This strain, once the
biofilm has been established, is able to continue proliferating as
long as there is a sufficient supply of nutrients.

*L. lactis* is a homofermentative strain that metabolises any carbon
source found in the medium, glucose in our experiments, to L-(+)-
lactic acid, in an anaerobic fashion20. This production of lactic acid
leads to a decrease in the pH of the culture medium up to ~4,
seriously compromising the viability of the aboveground cells.
Here arises the need to control the pH of the medium, by inhibiting
the bacterial metabolism. We have found that tetracycline used at low
concentrations, 10 μg/mL, is enough to inhibit the bacterial replica-
tion without impacting mammalian cell metabolism21,22 (Fig 3 and

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**Figure 3 | Biofilm metabolism and viability.** To assess the metabolism of the bacterial community inside the biofilm in conditions compatible with the
culture of C2C12 cells, biofilms of *L. lactis* FN and *L. lactis*-empty were produced. Then, GM17-E medium was changed to either DMEM, DMEM
with 1% penicillin/streptomycin (P/S) or DMEM with 10 μg/mL tetracycline (TC). After 1, 2 and 4 days of culture in DMEM, DMEM + P/S and
DMEM + TC, viability was tested using the LIVE/DEAD BacLight bacterial viability kit, where viable bacteria are stained in green and non-viable in red.
(a) Representative images after 4 days. Scale bar size is 25 μm. (b) Image quantification shows that viability is not affected by either the use of antibiotics or
the culture time. No statistically significant differences among conditions were found. Data is presented as mean ± SD. (c) Simultaneously, bacterial
metabolism was inferred by measuring the pH of the medium as a function of time, to assess the production of lactic acid. Addition of tetracycline
maintained the pH stable and above 7.5. The use of P/S was enough to maintain the pH above 6.5. In antibiotic-free DMEM, the pH dropped to
a minimum of 4 due to the transformation of glucose in the medium into lactic acid.
results on cell differentiation later in this work). Tetracycline is a broad-spectrum antibiotic that binds to the 30S subunit of the microbially ribosomes, inhibiting the protein synthesis by blocking the attachment of the charged aminoacyl-tRNA to the ribosomal A site\(^6\). Conversely, penicillin acts inhibiting the peptidoglycan cell-wall remodelling, ultimately leading to cell breakdown. As we only intend to maintain the cell population under control, it is counterproductive to promote cell lysis, and, as side effect, the modification of the biofilm morphology. Moreover, tetracycline is more effective controlling the pH at low concentrations and is nontoxic to mammalian cells\(^22,23\) (see also our results on cell behaviour afterwards).

Bifilms of different densities were formed on glass and remained stable after several days on the material surface. We have effectively shown that we can control the surface density of bacteria on the material surface by controlling the seeding volume (Supp Fig. S2). We selected high-density bacterial cultures to have the highest possible \(L.\) lactis density on the material surface readily available for cell differentiation experiments. We have assessed the stability of the layer of bacteria at different time points up to 4 days. This is the time needed for myogenic differentiation experiment afterwards. Fig. 3 shows live/dead staining for \(L.\) lactis grown on glass using different media (DMEM, DMEM + tetracycline (TC), DMEM + penicillin and streptomycin, stated as P/S). After 4 days of culture bacteria viability (measured by the ratio of live to total cells) is unaffected by the presence of small amounts of antibiotics in the culture medium and it is maintained at ca. 60% (Fig. 3b).

The temporal evolution of the pH in the different media used is shown in Fig. 3c. Only when the culture medium is supplemented with TC, acidification does not occur and the pH remains stable for four days (Fig 3c). We suggest that this acidification of the culture medium is a consequence of the \(L.\) lactis metabolism. As said before, \(L.\) lactis is a homofermentative strain that metabolises any suitable carbon source found in the medium -glucose in our experiments- to L-(+)-lactic acid. Then, the production of L-(+)-acid lactic, with a pK\(_a\) of 3.86\(^{24}\) must be responsible for the acidification of the culture medium. Addition of TC maintains a stable pH, which suggests that no L-lactic acid is produced and that, consequently, bacterial metabolism is inhibited.

The use of antibiotics in the culture medium diminished the density of bacteria on the material surfaces, as it can be inferred from Fig 3a and we have quantified in Supp Fig. S3. However, even if this is the case, the layer of bacteria that remains on the surface is viable and sufficient to support cell adhesion and cell differentiation, as it is shown afterwards. That is to say, even if the layer of bacteria attained on the material surface is not homogeneous (see e.g. Supp Fig S2), the attained bacterial surface involves the adequate expression of FN for mammalian cell interactions.

**Cell morphology, adhesion and differentiation on \(L.\) lactis.** The density of the biofilm directs area and roundness of mammalian cells. We first studied cell morphology on established biofilms of different densities on glass (Supp Fig. S2). Both cell area and roundness are directly related to the biofilm density, as shown in fig. 4. C2C12 cells seeded on biofilms of different densities showed increased area (Fig 4b), with minimal changes in cell roundness (Fig 4c). In addition, focal adhesions were formed on cells seeded on \(L.\) lactis-FN biofilms. The number of focal adhesion plaques increased with the density of bacteria on the surface (Supp Fig. S4). These results suggest that \(L.\) lactis-FN directs cell adhesion and cell morphology in the short-term, which is necessary before considering other long-term processes such as cell differentiation.

We previously showed that \(L.\) lactis modified to express FNIII\(_{7-10}\) as a membrane protein promotes focal adhesion formation and FAK signalling at similar levels as native FN in fibroblasts\(^5\). Now, we assessed the ability of this living interface to support cell differentiation by examining myogenic differentiation\(^26\). Sarcomeric myosin expression and cell bipolar alignment and fusion into myotubes, markers of myogenesis, were significantly higher on \(L.\) lactis modified with FNIII\(_{7-10}\) (Fig. 5a) as compared to \(L.\) lactis-empty (control bacteria that do not contain FNIII\(_{7-10}\)). Surprisingly, myogenic differentiation was higher on the modified \(L.\) lactis than on native fibronectin and collagen type I, which represents the gold-standard substrate for myogenic differentiation (Fig 5b).\(^{22,27}\) It is important to emphasise that these differences in myogenic differentiation are not due to differences in the number of cells on the substrates. It is known that a direct correlation between the number of cells on the surface and the level of myogenic differentiation has been found, however the number of cells on the modified bacteria is slightly lower than on the FN and Col I controls (Fig 5c).

Integrin-mediated cell adhesion triggers a cascade of intracellular signals such as the p38 mitogen-activated protein kinase (p38 MAPK) pathway, which is involved in the myoblast differentiation process, by promoting the activity of several transcription factors and regulating cell cycle withdrawal\(^{25,28}\). An intermediary step between adhesion and downstream targets, including MAPK pathways, is the phosphorylation of FAK\(^{28}\); as a result, FAK phosphorylation at Tyr397 plays a central role during myoblast differentiation in 2D cultures\(^{27,29}\). Initially, FAK phosphorylation at Tyr397 is transiently reduced—contributing to trigger the myogenic genetic program—but it is later activated, as it is central to terminal differentiation into myotubes\(^{30}\). In order to study the activation of FAK on the \(L.\) lactis expressing FNIII\(_{7-10}\) we examined the phosphorylation of Tyr397 by Western blot. FAK phosphorylation was similar on modified \(L.\) lactis and FN-coated glass (Fig 5d e), in good correlation with the high levels of cell differentiation found in this work (Fig 5b).

These results suggest that enhanced cell differentiation can be triggered by FNIII\(_{7-10}\) expressed on the membrane of \(L.\) lactis. The fact that the same experiments have been done with \(L.\) lactis-empty strain without significant levels of cell differentiation absolutely confirm FNIII\(_{7-10}\) expressed on the bacteria membrane as the trigger of differentiation.

**Discussion**

Engineering microenvironments to provide cells with dynamic cues, as occurs in the natural extracellular matrix, is a field of intensive research seeking to direct the differentiation of stem cells and advancement in strategies for tissue repair and regeneration\(^11\). Several approaches have been explored spanning from the simple functionalisation of material surfaces with cell adhesive motifs to the incorporation of protease-degradable fragments in polymer hydrogels that allow changes in the material structure as cells secrete proteases to remodel the ECM\(^7,14,32\). Dynamic surfaces have been engineered to present cell adhesive RGD sequences on-demand\(^7,33\).

From a different perspective, dynamic cell microenvironments have been engineered to present and release growth factors in a sustainable and controlled way to stimulate and support cell differentiation\(^34-40\). We have recently shown that non-pathogenic bacteria can be genetically modified to establish a functional interface between cells and synthetic materials, and we anticipate that this living interface could be further modified to instruct cells to perform different functions in a highly regulated manner\(^6\). As an initial step and proof of concept towards this long-term goal, we engineered food-grade \textit{Lactococcus lactis}, a gram-positive bacterium with very low production of lipopolysaccharides (LPS) - previously used for recombinant therapeutic proteins production, therapeutic drug delivery and vaccine production\(^{41}\) - to present a fibronectin fragment (FNIII\(_{7-10}\)) as a membrane protein. FNIII\(_{7-10}\) contains RGD and the PHSRN synergy sequences and promote cell adhesion and differentiation\(^42,43\). We have previously shown that fibronectin null fibroblasts (FN -/-) adhere, spread and develop focal adhesions on this engineered \(L.\) lactis expressing FNIII\(_{7-10}\) in a similar way as when seeded directly on a FN coating\(^4\).
Here, we show that this living interface that constitutively express a FN fragment is able to produce stable biofilms that support cell growth, cell adhesion and cell differentiation. The formation of the biofilm starts when some individual cells adhere to the surface, a process governed by weak non-specific forces - Van der Waals and polar Lewis acid-base interactions. This initial, reversible attachment becomes irreversible after bacteria make use of several cell-wall anchored proteins (CWAP) known as adhesins. In L. lactis, the most prominent ones are the CluA sex factor and the PrtP and NisP proteinases. These adhesins act modifying the bacterial surface properties and ultimately lead to an irreversible attachment of the bacteria to the surface. After bacteria attachment becomes irreversible, the force required to detach additional bacteria increases and more bacteria are recruited and co-aggregated to the existing ones. Afterwards, these bacteria proliferate and develop the biofilm itself. In this phase, a few hours after the initial irreversible attachment, a stable layer is formed. As L. lactis MG1363 is a strain with a low production of exopolysaccharides, biofilms are usually monolayered, lacking the horizontal stratified structure found in other species. Hence, the formation of L. lactis biofilms seems limited to a thin layer, usually a monolayer, of strongly attached bacteria to almost any abiotic surface, such as polymers, glass or metallic surfaces. Usually, the adhesion of the bacterium to the substrate is mediated by the bacterial fimbriae, also called adhesion pili. These fimbriae are also involved in the formation of the bacterial aggregates, as shown in Supp Fig. S1.

Once the biofilm has been successfully established, a need to control its proliferation arises, and we found that the monolayer can be controlled to remain in a stationary phase (using low amount of tetracycline) on the material surface during the time needed to complete the myogenic differentiation process (up to 4 days). We have quantified the amount of FNIII that is available on the surface of the bacterial and verified that it promotes cell adhesion, signalling and cell differentiation. Overall, this living interface based on non-

Figure 4 | Morphological features of C2C12 myoblasts related to biofilm density. A 3 h culture of C2C12 cells seeded on top of biofilms of different surface density. Briefly, 10,000 cells/cm² were seeded on top of L. lactis-FN biofilms of increasing density (covered surface area 4.6%, 11.4%, 19.1% and 27.6%), and L. lactis-empty biofilm produced with an surface density of 27.6%. Afterwards, cells were stained with a monoclonal anti-vinculin antibody (shown in red), whereas the actin cytoskeleton was stained with phallacidin (in green). Both nuclei of C2C12 cells and bacterial cells are shown in blue. (a) C2C12 cells seeded over L. lactis-empty biofilm showed no adhesion and remained in their original rounded shape. Cells seeded on L. lactis-FN biofilms showed good adhesion and well-developed focal adhesion plaques and actin cytoskeleton. Scale bar is 100 µm. (b) Quantification of cell area on the different biointerfaces suggests a correlation between cell area and the surface density of the biofilm. Area and roundness of at least 450 cells were quantified using the ImageJ software. (c) Quantification of cell roundness, defined as roundness = (4 • Area)/p • (major axis)². ANOVA test (N = 400) shows statistically significant differences between cells seeded on L. lactis-empty, FN-coated surfaces and glass.
pathogenic bacteria supports myogenic differentiation. Further genetic modification of this living interface is being currently done to engineer a dynamic system able to provide cells different temporal stimuli beyond cell adhesion, such as the delivery of growth factors and other molecules upon external demand. By engineering non-pathogenic bacteria to synthesise proteins and growth factors upon external stimuli, we aim at control the interface between synthetic biomaterials and stem cells to control self-renewal and differentiation, which can be then applied to several strategies to promote tissue repair and regeneration.

Methods
Genetic modification of *L. lactis*. The GFP-containing plasmid pGFP-C2 (Clontech) was used to construct a vector containing FNIII-10 downstream to GFP. FNIII-10 fragment was amplified from the plasmid pET15b-FNIII-10. The forward and reverse primers used for the FNIII-10 amplification were F1, with HindIII restriction site: aagcttaCCATTGTCTCCACCAACAAAC, and R1, with SalI restriction site: gtcgacttaTTCTGTTCGGTAATTAATGGAAA. The *lactococcal* plasmid PT1NX was used to clone the GFP-FNIII-10 fragment using the following primers: F2, with NgomIV restriction site gccggcATGGGTAAAGGAGAAGAACTTT; and R2: TTCTGTTCGGTAATTAATGGAAA. *Lactococcus lactis* MG136353 containing the PT1NX plasmid was grown as standing culture, in anaerobic conditions, at 30°C in M17 medium (Oxoid, Basingstoke, UK) supplemented with glucose (0.5% v/v, Sigma) and erythromycin (10 μg/mL, Sigma-Aldrich) (hereinafter GM17-E). The plasmid extraction was carried using a protocol described elsewhere. Competent *L. lactis* MG1363 cells were prepared growing the cells in rich medium supplemented with glycine (1% v/v). Cells were harvested when the culture reached an optical density of 0.6–0.8 measured at 600 nm, resuspended in glucose (0.5 M) with glycerol (10% v/v) and frozen at −80°C. Electroporation was performed at 2.5 kV using a BTX electroporator (Harvard Apparatus). In these conditions, with desalted DNA, the typical time constant was 4.5. Electroporated cells were transferred directly to 5 mL medium composed of M17, glucose (0.5 M), MgCl₂ (20 mM) and CaCl₂.
that, samples were washed twice with PBS and incubated for 1 h at 37°C at 110 min, with occasional vigorous vortexing. The extract was centrifuged at 7000 g for 5 min. A single colony from the GM17-E agar plate was inoculated into sterile GM17-E broth (Oxoid). The culture was grown until OD_{600} ~ 0.3, and then transferred to a 24 well multwell plate containing sterile glass coverslips in each well, using 300 μL and 1000 μL respectively in order to produce low and high areal density biofilms. The multwell plate was sealed with Parafilm and left at 30°C in anaerobic conditions for 48 h. After that, the planktonic (non-adhered) bacteria were removed by shaking the multwell plate at 150 rpm for 3 min followed with three washing steps with sterile ultrapure water. In the last wash, the ultrapure water was substituted with PBS supplemented with CaCl$_2$ and MgCl$_2$ (PBS + ). The same protocol was used for empty and FN-expressing strains.

**Biofilm viability and metabolism.** For bacterial viability studies, after 1, 2 or 4 days, biofilms were gently washed twice with sterile NaCl 0.85% w/v solution and incubated for 30 min using the BacLight LIVE/DEAD kit (Life Technologies), containing SYTO9 (5 μM) and propidium iodide (30 μM) in NaCl 0.85% w/v. Afterwards, samples were washed and mounted using BacLight Oil mounting medium (Life Technologies). Bacteria were counted using Fiji – ImageJ software, using at least four microscope fields at 40× magnification for every sample. The viability was determined as the ratio between the viable and total number of bacteria.

For metabolic analysis, biofilms were prepared following the biofilm formation protocol. Afterwards, the medium was changed to either DMEM or DMEM containing tetracycline (TC, 10 μg/mL) or DMEM containing 1% penicillin-streptomycin (PS). Biofilms were kept at 37°C in a humidified atmosphere under 5% CO$_2$, and the pH was monitored as a function of time (0, 0.5, 1, 1.5, 2, 3, 4, 5, 24 and 48 h) using a pH-meter (Eutech Instruments).

**AFM and SEM imaging of the biofilms.** For SEM imaging, bacterial biofilms were produced as described in the *biofilm formation* section. Freshly washed biofilms were fixed in 2.5% glutaraldehyde in phosphate buffer (PB, 0.1 M, pH 7.3) for 14 hours at 4°C. The samples were washed twice with PB, then dehydrated in an ethanol/water mixture of 50%, 70%, 80%, 90%, 95% and 100% for 10 minutes each. The 100% ethanol step was repeated three times. Afterwards, samples were immersed twice in hexamethyldisilazane (Sigma) and air-dried for 10 minutes. Samples were stored in vacuum until imaging in the microscope. Prior to imaging, samples were sputter-coated with 1 nm of gold and observed using a Zeiss Ultra 55 Field Emission SEM (Zeiss Microscopy, Germany) with 1 kV of gun acceleration voltage.

For AFM, a culture in stationary phase was washed twice with sodium acetate (100 mM, pH 4.8) and three millilitres of the resulting bacterial suspension with the ultrapure water was substituted with PBS supplemented with CaCl$_2$ and MgCl$_2$ (PBS + Ca$^{2+}$, Mg$^{2+}$). For AFM imaging, biofilms were prepared following the biofilm formation protocol. Afterwards, the medium was changed to either DMEM or DMEM containing tetracycline (TC, 10 μg/mL) or DMEM containing 1% penicillin-streptomycin (PS). Biofilms were kept at 37°C in a humidified atmosphere under 5% CO$_2$, and the pH was monitored as a function of time (0, 0.5, 1, 1.5, 2, 3, 4, 5, 24 and 48 h) using a pH-meter (Eutech Instruments).

**Surface density of FN - ELISA.** 3 ml from a 24 h standing culture of *L. lactis*-FN and *L. lactis*-empty grown in sterile GM17-E, with a bacterial density of 10$^{10}$ cfu/mL, were filtered through a porous polycarbonate membrane with a mean pore size of 0.8 μm (Millipore). The membrane was gently washed with the same sodium acetate solution to remove the non-trapped bacteria, while avoiding dewetting. The wet sample was imaged in a Multimode 8 (Nikon) with 1000× magnification, transformed to an 8-bit grayscale bitmap (Fiji - ImageJ software) and segmented using the Trainable Weka Segmentation plugin to create a binary mask, for both DAPI and Cy3 channels. Total nuclei per image were counted using the particle analysis command. Then, the segmented DAPI channel image was subtracted from the Cy3 channel segmented image, and the remaining nuclei were counted and assigned to non-differentiated cells. The fraction of differentiated cells was calculated subtracting the non-differentiated nuclei from the total nuclei count.

**Immunofluorescence.** Cultures were fixed at 4°C with 70% ethanol, 37% formaldehyde and glacial acetic acid (20: 1: 1) for 15 min and then blocked with 5% goat serum in PBS + for 1 h at RT. The samples were incubated with MF-20 antibody against sarcosomic myosin (Developmental Studies Hybridoma Bank, University of Iowa, USA) at 1: 250 dilution in PBS + and 5% goat serum, followed by two washes with PBS + /Tween 20 (0.5% v/v) and another blocking step with 5% goat serum in PBS + for 10 min at RT. Then were washed twice with PBS + /Tween 20 (0.5% v/v) and incubated with rabbit anti-mouse Cy3-conjugated secondary antibody (Jackson Immunoresearch) at 1: 200 dilution in PBS + with 5% goat serum. Samples were then washed three times with PBS + /Tween 20 (0.5% v/v) and washed with Vectashield-DAPI, and observed under an epifluorescence microscope (Nikon Eclipse 80i).

Images from the fluorescence microscope (DAPI channel - nuclei, and Cy3 channel - sarcosomic myosin) of the C2C12 culture were acquired at 10× magnification, transformed to an 8-bit grayscale bitmap (Fiji - ImageJ software) and segmented using the Trainable Weka Segmentation plugin to create a binary mask, for both DAPI and Cy3 channels. Total nuclei per image were counted using the particle analysis command. Then, the segmented DAPI channel image was subtracted from the Cy3 channel segmented image, and the remaining nuclei were counted and assigned to non-differentiated cells. The fraction of differentiated cells was calculated subtracting the non-differentiated nuclei from the total nuclei count.

**FAK phosphorylation.** After 3 hours of culture, cells were lysed with RIPA buffer (Tris-HCl 50 mM, 1% Nonidet P-40, 0.25% Na deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor cocktail tablets (Complete, Roche). Proteins were concentrated using Microcon YM-30 Centrifugal Filter devices (Millipore) as manufacturer described. To determine FAK protein expression and its Tyr 397 phosphorylation, concentrated samples were subjected to 7% SDS-PAGE gel electrophoresis. Proteins were transferred to a positively charged PVDF membrane (GE Healthcare) using a semidyfer transfer cell system (Bio-Rad) and blocked by immersion in skimmed milk (5% w/v) in PBS for 1 h at room temperature. The blot was incubated with anti-FAK antibody (Upstate) and anti-pFAK antibody (Abcam) containing a rabbit anti-mouse Cy3 conjugated (Jackson Immunoresearch). 1: 100 dilution, with FITC-conjugated phallacidin (Life Technologies) diluted 1: 100 in 1% BSA/PBS + /Tween 20 (0.5% v/v) phosphorylated secondary antibody (Jackson Immunoresearch) incubated 1: 200 dilution, with FITC-conjugated phallacidin (Life Technologies) diluted 1: 100 in 1% BSA/PBS + /Tween 20 (0.5% v/v). The secondary antibody was incubated 1 hour at RT in absence of light. Cells were washed three times with PBS/Tween 20 (0.5% v/v) and mounted with Vectashield-DAPI (Vector Laboratories).

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distribution. Then, a non-parametric ANOVA followed by a Kruskal-Wallis test was performed, with p = 0.05.

Figure 5b and 5e, corresponding to the C2C12 differentiation assay and cell density respectively: A D’Agostino & Pearson omnibus normality test was done, finding that the data follows a Gaussian distribution. Then, a one-way ANOVA with p = 0.05 followed by a Tukey multiple column comparison test was performed.

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Author contributions
A.R.N. and M.S.S. designed the research. A.R.N. performed it and prepared all figures. A.R.N., P.R., A.S., A.J.G. and M.S.S. analysed the data. A.R.N. and M.S.S. wrote the paper.

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