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RESEARCH ARTICLE

Cell-surface binding domains from *Clostridium cellulovorans* can be used for surface display of cellulosomal scaffoldins in *Lactococcus lactis*

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Abstract

Engineering microbial strains combining efficient lignocellulose metabolization and high-value chemical production is a cutting-edge strategy towards cost-sustainable 2nd generation biorefining. Here, protein components of the *Clostridium cellulovorans* cellulosome were introduced in *Lactococcus lactis* IL1403, one of the most efficient lactic acid producers but unable to directly ferment cellulose. Cellulosomes are protein complexes with high cellulose depolymerization activity whose synergistic action is supported by scaffolding protein(s) (i.e., scaffoldins). Scaffoldins are involved in bringing enzymes close to each other and often anchor the cellulosome to the cell surface. In this study, three synthetic scaffoldins were engineered by using domains derived from the main scaffoldin CbpA and the Endoglucanase E (EngE) of the *C. cellulovorans* cellulosome. Special focus was on CbpA X2 and EngE S-layer homology (SLH) domains possibly involved in cell-surface anchoring. The recombinant scaffoldins were successfully introduced in and secreted by *L. lactis*. Among them, only that carrying the three EngE SLH modules was able to bind to the *L. lactis* surface although these domains lack the conserved TRAE motif thought to mediate binding with secondary cell wall polysaccharides. The synthetic scaffoldins engineered in this study could serve for assembly of secreted or surface-displayed designer cellulosomes in *L. lactis*.

KEYWORDS
biorefinery, cellulosic biomass, cellulosome, metabolic engineering, S-layer homology domain

1 | INTRODUCTION

Lignocellulose is the most abundant raw material on the Earth. Its low price makes it an ideal feedstock for 2nd generation biorefining aimed at replacing fossil-derived production of fuels and chemicals.[1] However, lignocellulose has been selected to be recalcitrant to microbial and enzyme activity; therefore, its conversion through biological process is both technically and economically challenging.[1] Nowadays, industrial fermentation of lignocellulose is complex and expensive since multiple bioreactors in series are generally required.[2,3] Development of single-step fermentation (i.e., consolidated bioprocessing, CBP) of biomass is considered as one of the most promising strategies to reduce the costs of 2nd generation biorefinery and make them competitive with those of oil refinery.[3,4] The most straightforward path to achieve this goal is by using microbial strains that can directly ferment plant biomass and produce high-value compounds with high efficiency. Since such microbes have not been found in nature, so far, metabolic engineering can hopefully be used to develop them through gene modification techniques.[5,6]

Recombinant cellulolytic strategies (RCS) aim at endowing cellulolytic ability in microbial strains producing high-value compounds. RCS have been applied to several microbial models such as

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Saccharomyces cerevisiae, *Kluyveromyces marxianus* and *Zymomomans mobilis* (i.e., ethanol producers), *Clostridium acetobutylicum* (i.e., solvent producer), *Corynebacterium glutamicum* (i.e., glutamate producer) and lactic acid bacteria (LAB).[1,5,7] LAB have significant industrial application in production of lactic acid (LA) and as probiotics.[8] In addition, LAB have been considered as candidates for synthesizing other high-value compounds such as ethanol, polyhydroalkanoates, polyols, and exopolysaccharides.[9] However, LAB generally cannot directly ferment complex carbohydrates, namely few of them can ferment starch and no LAB isolated so far can depolymerize lignocellulosic feedstocks.[7] Attempts to engineer minimal (hemi)cellulase systems in LAB strains have therefore been reported (extensively reviewed in[7]). RCS are based on mimicking natural biochemical systems for plant biomass depolymerization, consisting of multiple enzymes with different substrate specificities and catalytic mechanisms.[5] These approaches generally refer to two main paradigms, that is, the non-complexed model of aerobic microorganisms, and the complexed (i.e., cellulosome) model of anaerobic strains.[10] In particular, cellulosomes are among the most efficient machineries for the degradation of lignocellulosic biomass, owing to close proximity between enzymes and microbial cells resulting in improved synergistic activity.[11] For biotechnological purposes, designer cellulosomes based on artificial scaffoldins have been used to control the composition and architecture of cellulosomes and reduce the size of these complexes so as to diminish burden related to their heterologous expression.[12,13]

Previously, we have expressed two non-complexed glycoside hydrolases, that is, a β-glucosidase (BglA) and an endoglucanase (EngD), from *Clostridium cellulovorans* in *Lactococcus lactis* enabling *L. lactis* to directly ferment cellodextrins up to 8 glucose units to LA with high efficiency.[14] Use of *C. cellulovorans* as a source of cellulolytic enzymes for expression in *L. lactis* was chosen owing to: i) similar GC content (31.2% in *C. cellulovorans* vs. 35.3% in *L. lactis*), which suggests similar codon usage;[15,16] similar growth temperatures (37°C for *C. cellulovorans* vs. 30°C for *L. lactis*). Both these characteristics may facilitate expression and activity of *C. cellulovorans* proteins in *L. lactis* with respect to proteins derived from other established cellulolytic models such as *Thermobifida fusca* or *Clostridium thermocellum* which show higher GC content and/or higher growth temperature.[12,17] Actually, both *C. cellulovorans* BglA and EngD were biosynthesized and efficiently secreted using their original signal peptides; thus, suggesting that genes from *C. cellulovorans* could be expressed in *L. lactis* without any prior modification/optimization.[14]

The cellulolytic system of *C. cellulovorans* mainly consists of a cellulosome which synergistically collaborates with non-complexed enzymes.[16,18] The present study was focused on the expression of components of the *C. cellulovorans* cellulosome in *L. lactis* and, in particular, on scaffolding proteins (also called scaffoldins) and proteins involved in anchoring the cellulosome to the cell surface. An additional advantage of using *C. cellulovorans* as a source of plant-biomass depolymerizing enzymes is that this bacterium can ferment all the main plant polysaccharides (namely cellulose, hemicelluloses and pectins)[19,20] while other cellulolytic microorganisms have more restrained substrate range (e.g., *C. thermocellum* can metabolize cellulose only[21] and *Clostridium cellulolyticum* cannot directly use pectin[19]). Since cellulosome assembly through interaction between scaffoldins and enzyme subunits is generally species-specific,[22] use of *C. cellulovorans* as cellulase source can provide a larger cellulosomal enzyme panel, without the need of extensive protein engineering. Scaffoldins are pivotal elements of the cellulosome architecture owing to the multiple functions they provide.[11] Primary scaffoldins are usually able to bind enzyme subunits through multiple cohesin (Coh) domains.[23] Additional domains may enable scaffoldins to bind to the substrate (through carbohydrate binding modules, i.e., CBM) and/or anchoring the microbial surface through covalent or non-covalent linkages.[11] Cellulosomes may contain from one to several scaffoldins contributing to these different functions. Although most cellulosomes described so far are tethered to the cell surface, cell-free scaffoldins and/or cellulosomes have been observed in *C. thermocellum*, *Clostridium clariflavum* and *Acetivibrio cellulolyticus*.[11] The main scaffoldin of *C. cellulovorans* cellulosome is CbpA which consists of a family 3 CBM (that can bind crystalline cellulose and chitin), nine Coh domains and four hydrophilic domains (Figure 1A).[24] The latter belong to the pfam PF03442 (pfam.xfam.org) of carbohydrate binding domains X2 (X2). Tandem associated CBM 3 and X2 domains have been found to promote the hydrolysis of insoluble polysaccharides.[25] X2 modules have been described to bind to different polysaccharides, such as cellulose and chitin,[18,19] and those found in bacterial cell walls,[25] but also to possibly stabilize the structure of the neighboring Coh domains.[26] More in detail, the binding affinity of CbpA X2 domains for *C. cellulovorans* cell wall fragments is lower than that for cellulose or chitin.[24] Surface-display of *C. cellulovorans* cellulosomes is probably mediated by multiple proteins. Another main player is the endoglucanase E (EngE) (Figure 1A).[27] N-terminal sequence of EngE comprises three tandem repeated S-layer homology (SLH) domains showing high homology with the S-layer protein RsaA from *Caulobacter crescentus*.[27,28] Moreover, the small scaffoldins CbpB, CbpC and HbpA and the endoglucanase G may further help anchoring the cellulosome to the *C. cellulovorans* surface.[29–31] However, the main role of CpbA and EngE in *C. cellulovorans* cellulosome structure and function is supported by the fact that they are among the most abundant components of this complex.[32] As for other SLH domain-containing proteins, experimental evidence has been brought that EngE does not bind peptidoglycan but secondary cell wall polysaccharides (SCWPs).[24,27,33] The mechanism of binding of CbpA X2 modules is different, but it is not known yet.[24]

In the present study, different scaffoldins have been engineered by using domains issued from CbpA and EngE of *C. cellulovorans*, with special attention to X2 and SLH domains because of their possible involvement in cell-surface anchoring. The genes encoding these recombinant proteins were expressed in *L. lactis* IL1403 so as to test the ability of their protein products to being secreted and displayed at the cell surface. A common general structure of the cell wall is found in Gram-positive bacteria such as Clostridia and LAB (recently, also renamed as parietal monoderm bacteria).[33,34] It consists of a thick layer of peptidoglycan decorated with other polysaccharides and glycopolymers (e.g., teichoic and lipoteichoic acids) and proteins. However, specific chemical modification and/or composition and/or decoration of

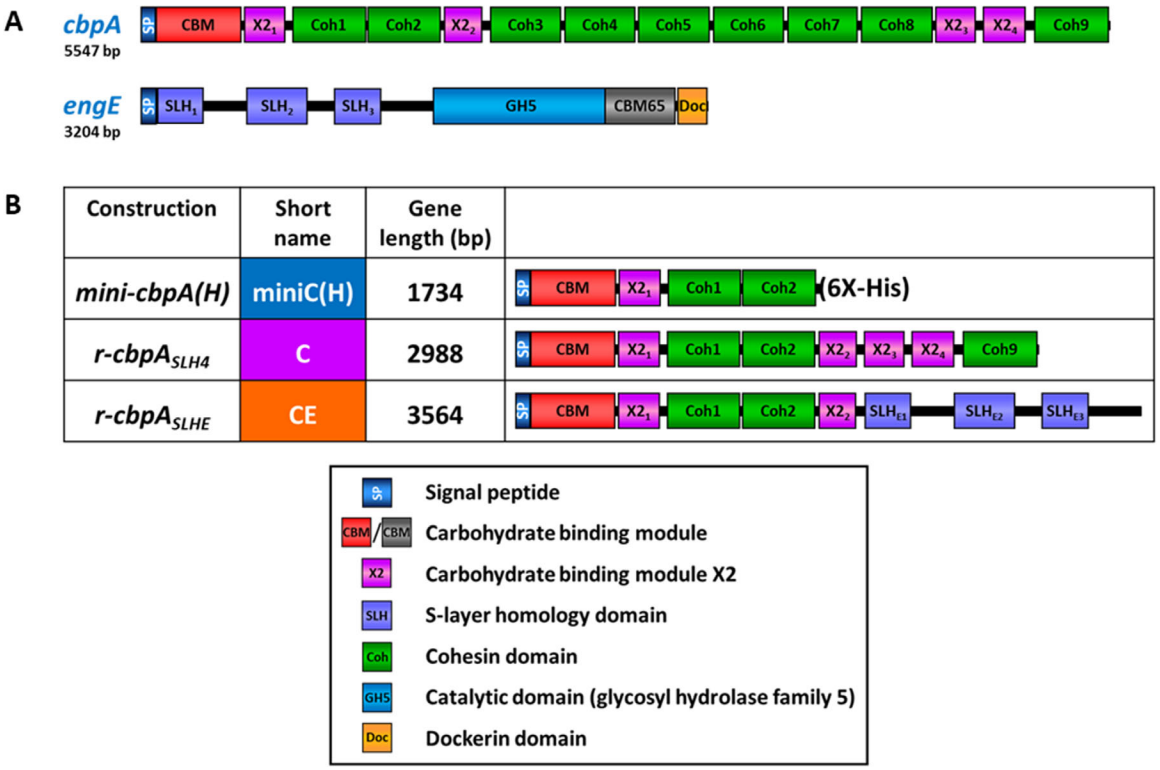

FIGURE 1 Schematic representation of the tertiary structure of: A) the cellulosomal main scaffoldin CbpA and endoglucanase E (EngE) from *C. cellulovorans*; B) the recombinant scaffoldins obtained in this study by assembling protein domains derived from *C. cellulovorans* CbpA and EngE. Carbohydrate binding modules (CBM) mediate linkage with cellulose or other polysaccharides. The function of X2 domains is still not clear and may include binding with different polysaccharides, stabilizing the structure of neighboring cohesin (Coh) domains and promoting hydrolysis of crystalline cellulose. S-layer homology (SLH) domains enable binding with bacterial cell wall polysaccharides. Coh domains mediate binding with enzymes or other protein components carrying a dockerin (Doc) domain. EngE is equipped with a catalytic activity supported by its family 5 glycosyl hydrolase (GH5) domain and a Doc domain

cell-wall components characterizes each bacterial strain.[34] *L. lactis* IL1403 seems to have a simpler cell wall structure than other *L. lactis* strains since it includes only one type of SCWP that consists of a linear backbone of rhamnose irregularly substituted with a trisaccharide carrying glycerophosphate groups.[35] Additionally, the structure of a poly (glycerolphosphate) teichoic acid of *L. lactis* IL1403 was recently determined.[35] These characteristics significant affect the physiology of *L. lactis*, such as the electric charge distribution on cell surface which influences surface protein binding.[34]

In this study, we were able to introduce three recombinant scaffoldins with different combinations of X2/SLH domains in *L. lactis*. Two of them were secreted in the extracellular medium, while the third one was displayed at *L. lactis* surface showing that cell-surface binding domains of *C. cellulovorans* are also able to recognize structural motifs on *L. lactis* cell wall.

2 | EXPERIMENTAL SECTION

2.1 | Bacterial strains and media

The bacterial strains used in this study are listed in Table S1. Growth media and culture conditions were previously described.[14] If not otherwise stated, recombinant *Escherichia coli* harboring pMG36eaΔ-based vectors were grown in LB medium supplemented with 100 μg mL^{-1} ampicillin. Recombinant *L. lactis* strains harboring pMG36eaΔ-based vectors were grown at 30°C in GM17 medium plus 5 μg mL^{-1} erythromycin without shaking.[14]

2.2 | Recombinant scaffoldin gene construction, cloning and transformation of *L. lactis* IL1403

Enzymes and protocols for DNA amplification, digestion, ligation, purification and transformation were previously described.[14] Plasmids and primers used in this study are listed in Tables S1 and S2, respectively.

DNA fragments of interest were amplified from genomic DNA (gDNA) isolated from overnight cultures of *C. cellulovorans* as previously described.[14] *mini-cbpA* (*miniC*), encoding the N-terminal fraction of CbpA (consisting of its CBM, X2$_1$, and Coh1, 2 domains, Figure 1B) was amplified by using the cbpA-D/mini-cbpA-R primer pair (Table S2). A slightly modified version of *mini-cbpA* encoding a miniC with a 6 X His tag at its C-terminus (miniCH) was amplified by means of the cbpA-D/mini-cbpAHis-R primer pair (Table S2). *r-cbpA*$_{X24}$ (*C*) and *r-cbpA*$_{SLHE}$ (*CE*) fusion genes were constructed in two steps. In parallel, i) the 5′

fragment of *cbpA* (encoding CBM, $X2_1$, Coh1, Coh2 and $X2_2$ domains) was amplified with the cbpA-D/linker$X2_2$-R primer pair (Table S2); thus, generating the portion encoding the N-terminal part of C/CE; ii) the fragment encoding the C-terminal fragment of C (consisting of $X2_3$, $X2_4$ and Coh9 domains of CbpA) was amplified by using the linker$X2_3$-D/cbpA-R primer pair (Table S2); iii) the fragment encoding the C-terminal fraction of CE (comprising SLH_1, SLH_2 and SLH_3 domains of EngE) was amplified by using the linker EngE-D/EngE-R primer pair (Table S2). Fragments encoding N- and C-terminal portions of C and CE, respectively, were assembled through fusion PCR. For assembling C, cbpA-D/cbpA-R primer pair was used while cbpA-D/EngE-R primer pair was used for CE (Table S2). *miniC(H)*, *C* and *CE* were cloned in the pMG36eaΔ *E. coli- L. lactis* shuttle vector between the SacI and XbaI sites[14]; thus, obtaining pMiniC(H), pC and pCE plasmids, respectively. These vectors were transformed into *E. coli* TOP10 (Invitrogen, Thermo Fischer Scientific) and transformant selection was performed on LB agar plates supplemented with 100 μg mL^{-1} ampicillin.

pMiniC(H), pC and pCE were extracted from the corresponding *E. coli* strains and transformed in electrocompetent *L. lactis* IL1403 cells as previously described[14]; thus, obtaining *L. lactis* miniC(H), *L. lactis* C, and *L. lactis* CE, respectively.

2.3 | Production of anti-scaffoldin specific antibodies

MiniCH was purified from culture supernatants of *L. lactis* miniCH. Bacterial cells were grown overnight in 2 L of GM17 medium. Biomass and culture broth were separated by centrifugation (3005 × *g*, 25 min, 4°C). Proteins in acellular supernatant were precipitated by adding $(NH_4)_2SO_4$ until 80% saturation and stirring overnight at 4°C. Precipitated proteins were recovered through ultracentrifugation (53,792 × *g*, 30 min, 4°C), re-suspended in 50 mM potassium phosphate buffer pH 6 and concentrated by Vivaspin 20 ultrafiltration devices (Sartotius Stedim Biotech, Goettingen, Germany) with 30 kDa cut off polyethersulfone (PES) membrane. MiniCH scaffoldin was purified through Immobilized Metal Affinity Chromatography by using Chelating Sepharose Fast Flow (GE Healthcare Life Science) with immobilized Ni, according to manufacturer's instructions. Elution of the mini-scaffoldin was performed by using 300 mM imidazole in 50 mM sodium phosphate buffer pH 7. The purified protein (1.5 mg) was sent to Eurogentec (Seraing, Belgium) for production of polyclonal anti-miniCH specific antibodies in rabbit.

2.4 | Protein quantification

Protein concentration was determined by the 2-D Quant Kit (GE Healthcare Life Science, Chicago, IL) and/or the DC Protein Assay Bradford protein assay (Biorad), using Bovin Serum Albumin (BSA) as the standard.

2.5 | Cellulose binding assay

Cellulose Binding Assay was performed on extracellular fraction of *L. lactis* cultures as previously described[14] with slight modifications. Recombinant *L. lactis* cells were grown in 50 mL of GM17 until mid ($OD_{600\,nm} = 2$) exponential phase. Culture broths were separated from biomass by centrifugation (3005 × *g*, 20 min, 4°C), syringe filtered (0.45 μm cut off) and incubated with 100 mg of crystalline cellulose (Sigmacell, Sigma-Aldrich) for 1 h at 25°C. After centrifugation (3005 × *g*, 10 min, 4°C), pellets were washed twice with ice-cold 50 mM potassium phosphate buffer pH 6 and re-suspended in 100 μL of SDS-PAGE loading buffer.[14] Samples were then boiled and centrifuged to remove cellulose and supernatants were analyzed by SDS-PAGE and gels were stained with Coomassie Brilliant Blue as previously described.[14]

2.6 | Detection of scaffoldins displayed on the *L. lactis* cell surface by immunofluorescence

Scaffoldin adhesion to cell surface was analyzed through immunofluorescence microscopy. Recombinant *L. lactis* cells were grown until the middle exponential phase ($OD_{600nm} = 2$) while *C. cellulovorans* was grown in CCM medium[14] plus 0.5% cellobiose until $OD_{600nm} = 0.7$. Cells were harvested, washed twice with phosphate-buffer saline (PBS) (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4, pH 7.4) (3005 × *g*, 10 min, 4°C) and re-suspended in 800 μL PBS + 200 μL Fixing Buffer (12% formaldehyde, 150 mM Na_2HPO_4). Samples were incubated 15 min at room temperature with mild agitation and then 1 h in ice. After recovery by centrifugation (3005 × *g*, 5′ min, 4°C), cells were washed three times with cold PBS and re-suspended in a volume of GTE buffer (25 mM Tris-HCl pH8, 10 mM EDTA, 50 mM glucose) so that the OD_{600nm} of this suspension was around 1. 50 μL of this suspension was placed on polylysine-coated microscope slides and dried. Slides were washed with cold PBS and blocked by incubation with 2% BSA dissolved in PBS at 37°C for 15 min in pre-warmed moist chamber. After further washing with PBS, anti-miniCH antibody (1:3000 dilution) was added onto the slides to detect scaffoldins on the cell surface. Samples were incubated 1 h at 37°C in moist chamber. After washing with PBS, goat anti-rabbit IgG antibody conjugated to Atto 488 Dye (5 μg mL^{-1}, Sigma-Aldrich) was added and incubated 1 h at 37°C in moist chamber. Slides were then washed again with PBS and DNA was stained with 3 μg mL^{-1} Propidium Iodide (15 min, 37°C, in moist chamber). After one last washing with PBS, samples were dried and covered with DABCO mix mounting medium (19.5 mL glycerol, 24 mL H_2O, 9.6 g polyvinyl alcohol, 2.5 g 1,4-diazabicyclo[2.2.2]octane (DABCO), 48 mL Tris-HCl 0.2 M pH 8.5) and a coverslip. Fluorescence images were taken using an Olympus Fluoview 200 laser scanning confocal system (Olympus America Inc., Melville, NY, USA) mounted on an inverted IX70 Olympus microscope, equipped with 60× Uplan Fl (NA 1.25) oil-immersion objective. The antibody conjugated to Atto 488 Dye and Propidium Iodide dyes were excited with a

Ar/Kr laser at 488 and at 568 nm, respectively. Images acquired at 60× magnification were processed and analyzed with ImageJ software (Rasband, W.S., U.S. National Institutes of Health, Bethesda, MA).

2.7 | Detection of bacterial cell adhesion to cellulose membrane

In order to evaluate the binding of recombinant *L. lactis* strain to cellulose mediated by heterologous scaffoldins, cells were incubated with a cellulose membrane and detected with fluorescence microscope or counted in a Burker chamber. Additionally, the same analysis was performed on *E. coli* cells since they should not be able to bind to cellulose.

Recombinant *L. lactis* and *E. coli* TOP10 cultures were grown until the middle exponential phase $OD_{600nm} = 2$. Cells were separated from culture broth through centrifugation (3005 × *g*, 10 min, 4°C) and washed twice with cold PBS. Pellet was re-suspended in a volume of 2% BSA in PBS so that the $OD_{600nm} = 20$. In the meanwhile, a square slice (around 1.44 cm^2) of cellulose dialysis membrane (cut off 30 KDa, Sigma-Aldrich) was leaned on glass slides, coated with 2% BSA in PBS and incubated 15 min at 30°C in pre-warmed moist chamber. After incubation, membrane was washed with cold PBS. Thirty microliter of the cell suspension was dropped onto BSA-treated membrane and incubated 1 h at 30°C in moist chamber. In order to remove not attached bacteria, membrane was washed four times with PBS. Membrane-attached cells were then detected by fluorescence microscopy or cell counting.

For fluorescence microscopy observation, 0.2 $\mu g\ mL^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI) was added onto the membrane and incubated 15 min at 30°C in moist chamber to stain DNA. After washing with PBS, membrane was covered with DABCO mix mounting medium and a coverslide. Cells were detected with an Olympus IX50 fluorescence microscope.

To quantify the amount of cells attached to cellulose membrane, membrane was stained for 1 min with Gram's crystal violet solution (Sigma-Aldrich), further washed with PBS and placed into a Burker chamber. Cells in the 0.0025 mm^2 squares were counted through a Wild Leitz GMBH microscope (40× magnification). Three independent determinations (i.e., biological replicates) were performed for each *L. lactis* strain. For each biological replicate, seven squares were analyzed.

2.8 | Statistical analyses

Data were analyzed by means of the Student's t-test and considered as significantly different when $p < 0.05$.

2.9 | Protein sequence analysis

Protein sequence alignments were performed with Geneious version 8.1 (Biomatters) (http://www.geneious.com). Search for SLH conserved motif was performed through the dedicated PROSITE tool (https://prosite.expasy.org). Blast search was performed at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Protein sequence logo was generated through WebLogo3 software (http://weblogo.threeplusone.com/).

3 | RESULTS

3.1 | Construction of recombinant scaffolding proteins

Three recombinant scaffoldins were constructed in this study by using protein domains of CbpA and EngE from *C. cellulovorans* as building blocks (Figure 1B). Each of these recombinant scaffoldins contains the 578 aa N-terminal portion of CbpA comprising its: original signal peptide, carbohydrate binding module (CBM), first X2 domain ($X2_1$) and first two cohesin domains (Coh1, 2). This corresponds to the whole structure of miniC scaffoldin (Figure 1B). A second version of miniC was also constructed which contains a 6 histidine-tag at its C-terminus (miniCH). C and CE scaffoldins were constructed by fusing miniC, respectively, with: $X2_{2,3,4}$ and Coh9 of CbpA (C); the $X2_2$ of CbpA and the three SLH domains of EngE ($SLH_{1,2,3}$) (CE) (Figure 1B). Scaffoldin C, therefore, contains all the X2 domains of CbpA. Additionally, Coh9 was included in the design of this recombinant scaffoldin since previous studies had established its high binding affinity for dockerin domains of key cellulosomal enzymes of *C. cellulovorans*.[36] Both X2 domains of CbpA and SLH domains of EngE were reported to bind *C. cellulovorans* cell wall fragments.[24,27] MiniC, C and CE recombinant scaffolding proteins contain 1, 4 and 5 domains potentially anchoring bacterial cell wall through non-covalent binding, respectively (Figure 1B).

The genes encoding miniC(H), C and CE were constructed, cloned in the pMG36eaΔ *E. coli-L. lactis* vector [14] and finally transformed in *L. lactis* IL1403; thus, obtaining *L. lactis* miniC(H), C and CE, respectively.

3.2 | Growth parameters of recombinant *L. lactis* strains

Final biomass (OD_{600nm}) and specific growth rate (μ) of *L. lactis* miniC(H), C and CE were determined (Figure 2). Growth parameters of recombinant strains were the same as the parent *L. lactis* strain (pMG36eaΔ), except for *L. lactis* C which showed significant ($p < 0.05$) reduction of growth rate (32%) and final biomass (6%).

3.3 | Recombinant scaffoldin secretion by *L. lactis*

L. lactis miniC, C and CE were grown in GM17 medium and harvested in the middle exponential phase. The presence of recombinant scaffoldins in the extracellular medium was analyzed by using the cellulose binding assay.[14] Actually, each scaffoldin engineered in this study contains a CBM at its N-terminus with high affinity for cellulose; thus, enabling their selective precipitation by incubation with crystalline cellulose. The extracellular fraction of *L. lactis* harboring the empty pMG36eaΔ

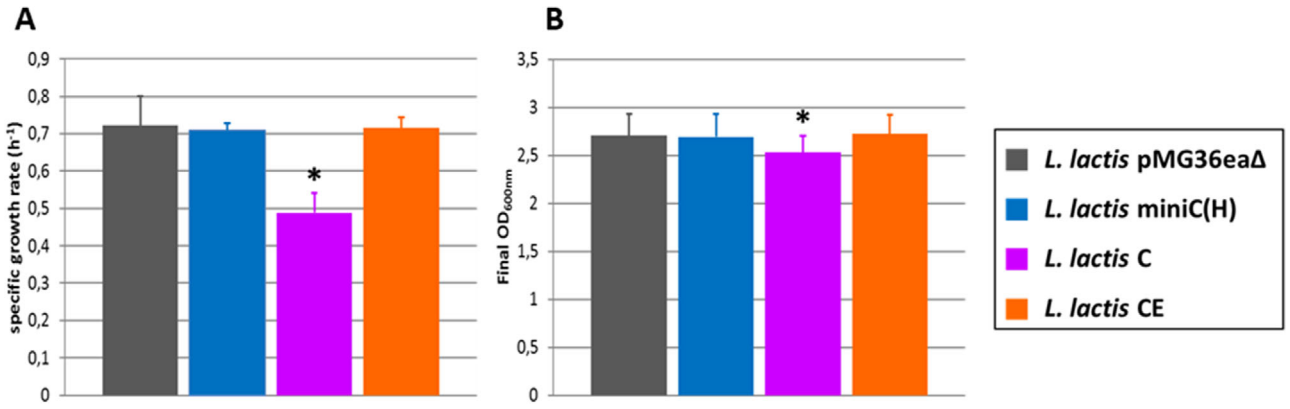

FIGURE 2 Specific growth rate (A) and final biomass (OD_{600nm}) (B) and of *L. lactis* strains expressing engineered scaffoldins obtained in this study. Three independent replicates were performed for each *L. lactis* strain. Symbol * indicates data that significantly ($p < 0.05$) differ from those measured in the parent (*L. lactis* pMG36eaΔ) strain

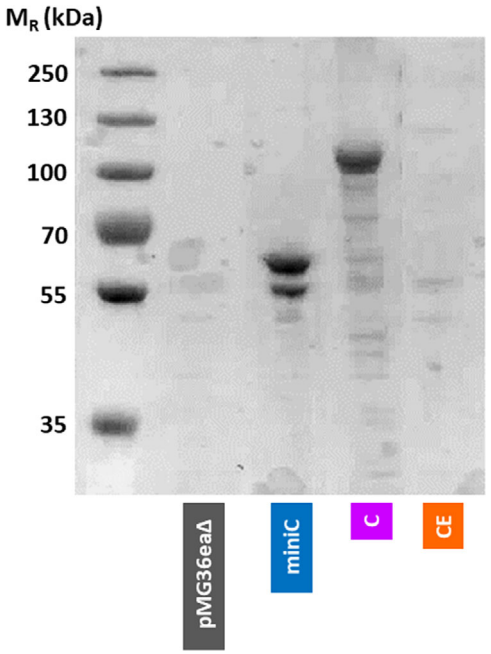

FIGURE 3 Secretion of recombinant scaffoldins by *L. lactis*. The extracellular fraction of *L. lactis* harboring the empty pMG36eaΔ vector (pMG36eaΔ) and *L. lactis* miniC, C, and CE, harboring the plasmids encoding the recombinant scaffoldins, was analyzed by cellulose binding assay, followed by SDS-PAGE. A band corresponding to the molecular mass of scaffoldins miniC (61 kDa) and C (104 kDa) was present in the extracellular medium of *L. lactis* miniC and C cultures, respectively. A faint band corresponding to the M_R of scaffoldin CE is likely present in the extracellular extract of *L. lactis* CE. Additional bands with lower M_R are also present, which likely correspond to products of partial hydrolysis of the engineered scaffoldins

vector was analyzed as the negative control. Cellulose-bound proteins were analyzed by SDS-PAGE (Figure 3). Protein bands corresponding to the molecular mass of miniC (61 KDa) and C (104 KDa) were detected in the extracellular medium of *L. lactis* miniC and C, respectively. This indicates that both these proteins are biosynthesized and secreted. Secreted amounts of miniC are around 4.5 μg mg^{-1} biomass dry weight (as determined by 2 D quant kit), this value should be similar for scaffoldin C also (Figure 3). These amounts are consistent with secreted levels of *C. cellulovorans* EngD in *L. lactis* (*engD* had been cloned under the same transcriptional promoter).[14] Also a faint band corresponding to the expected molecular mass of scaffoldin CE (125 KDa) was likely present in the extracellular medium of *L. lactis* CE cultures (Figure 3). Additional bands with lower M_R are also present in the extracts of all the scaffoldin-expressing strains, which most probably correspond to partially hydrolyzed scaffoldins.

3.4 | Analysis of surface-displayed scaffoldins in *L. lactis*

The presence of miniscaffoldins on the surface of recombinant *L. lactis* was analyzed by two different approaches: i) immunofluorescence on whole recombinat *L. lactis* cells; ii) ability of recombinant *L. lactis* to adhere to cellulose membranes. The first methodology took advantage from the fact that specific anti-scaffoldin antibodies were obtained in this study. The second approach exploited the presence of a CBM in each recombinant scaffoldin engineered in this study. In parallel, the same analyses were performed on *L. lactis* harboring the empty pMG36eaΔ vector, as the negative control.

3.4.1 | Immunofluorescence assays

Bacterial cells were fixed onto glass slides and incubated with primary (anti-scaffoldin) antibodies and fluorophore-labeled secondary antibodies. Propidium iodide was used to localize cells. This analysis was performed on *L. lactis* miniC(H), C and CE but also on *L. lactis* pM36eaΔ and on *C. cellulovorans* cells which served as negative and positive control, respectively (Figure 4). These analyses showed that scaffoldins are displayed on the surface of *C. cellulovorans* and *L. lactis* CE, while no

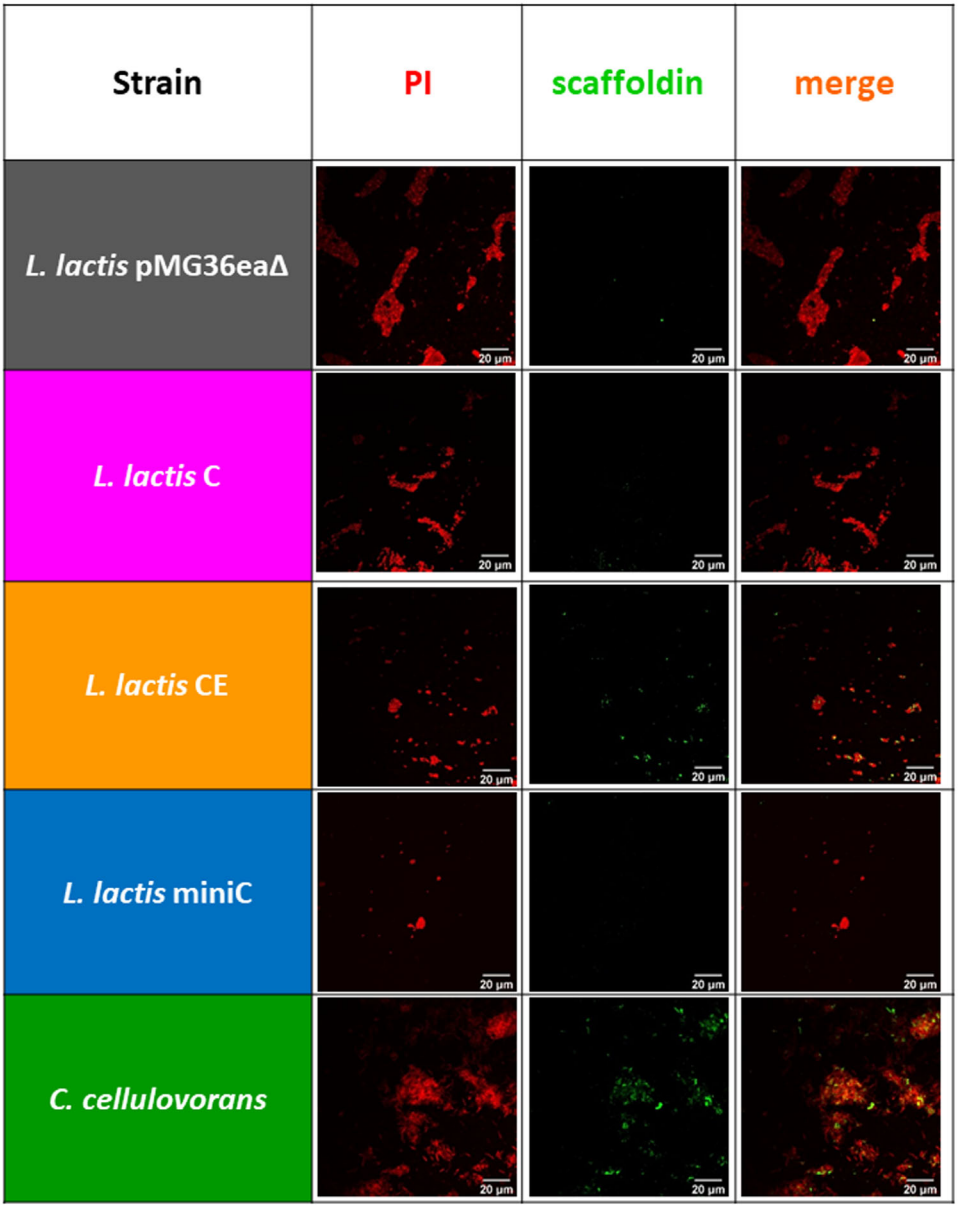

FIGURE 4 Detection of scaffoldins displayed at the surface of *L. lactis* and *C. cellulovorans* cells by immunofluorescence. Cells were stained with propidium iodide (PI) to detect DNA and localize cells and anti-scaffoldin (scaffoldin) antibodies. The merged images highlight the presence of scaffoldins on the surface of *C. cellulovorans* and *L. lactis* CE. Scale bars correspond to a length of 20 μm

fluorescence was detected on the surface of *L. lactis* pM36eaΔ, miniC(H) or C (Figure 4).

3.4.2 | Ability of recombinant *L. lactis* to adhere to cellulose

The CBM of CbpA from *C. cellulovorans* is present in each mini-scaffoldin engineered in this study. *L. lactis* cells displaying these scaffoldins at their surface should therefore be able to adhere to cellulose. In order to test this ability, *L. lactis* pM36eaΔ, miniC(H), C and CE were incubated with a transparent cellulose dialysis membrane and cells adhering to the membrane were visualized by DAPI. An additional control, that is, *E. coli* TOP10, was tested as further negative control since this strain should not be able to bind to cellulose. No *E. coli* (data not shown) and very few *L. lactis* pM36eaΔ, and C cells remained attached to the cellulose membrane, while this number was higher for *L. lactis* CE (Figure 5A–C). Cellulose membranes incubated with recombinant *L. lactis* cells were laid in a Burker chamber and the attached cells were counted. A higher number of *L. lactis* CE cells (8537 ± 2074 cells mm^{-2}) was able to adhere to the cellulose membrane with respect to *L. lactis* pM36eaΔ (1807 ± 1103 cells mm^{-2}) (p value $= 4.61 \times 10^{-14}$). According to these results, scaffoldin CE is displayed on the surface of *L. lactis* CE.

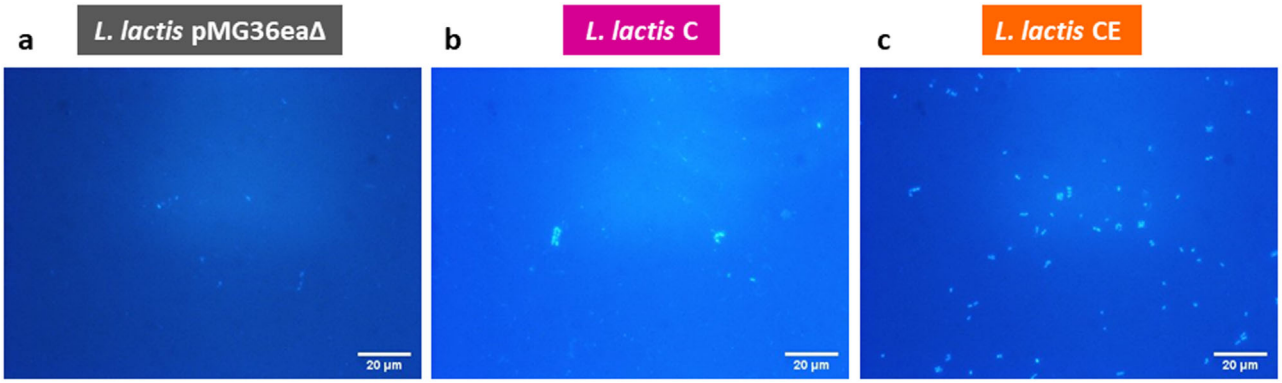

FIGURE 5 Adherence of engineered *L. lactis* strains to cellulose membranes. (A) *L. lactis* pMG36eaΔ; (B) *L. lactis* C; (C) *L. lactis* CE. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and images were acquired with an Olympus IX50 fluorescence microscope. Scale bars correspond to a length of 20 μm

4 | DISCUSSION

Cloning and expression of synthetic scaffoldins is an essential pre-requisite for introducing designer cellulosomes in heterologous microorganisms and has been performed in a number of microbial models[37,38] including different LAB.[12,17] Artificial scaffoldins allow precise control of designer cellulosome composition and architecture by mediating assembly of a minimal number of essential enzyme subunits; thus, reducing protein burden during heterologous expression.[12,13] In the present study, three engineered scaffoldins, named miniC, C and CE, were constructed by using protein domains derived from two of the main components of the *C. cellulovorans* cellulosome (i.e., the main scaffoldin CbpA and the endoglucanase EngE) and expressed in *L. lactis*. More in detail, these scaffoldins consist of: one CBM; 2 (miniC and CE) or 3 (C) Coh domains for anchoring enzyme subunits; 1 (miniC), 4 (C) or 5 (CE) domains possibly involved in anchoring the microbial cell surface. Our analyses clearly showed that all these proteins are biosynthesized and secreted by *L. lactis* (Figure 3). In addition, CE scaffoldin was able to bind to the *L. lactis* surface (Figures 4 and 5). Successful heterologous expression of miniC has previously been reported in *Bacillus subtilis*.[39] In the latter study, the original signal peptide was replaced with *sacB* signal sequence for efficient protein secretion in *B. subtilis* while in the present study the original signal peptide of CbpA from *C. cellulovorans* was kept. This indicates that signal peptides from *C. cellulovorans* can be recognized by *L. lactis* secretion system; thus, confirming our previous results on the expression of *C. cellulovorans* BglA and EngD in *L. lactis*.[14] More in general, the present results validate the initial assumption of this study, namely that *C. cellulovorans* genes can be expressed in *L. lactis* with few modifications, based on similar GC content and growth temperature of these bacteria. In the present study, scaffoldins including up to three Coh domains (i.e., that could potentially anchor up to three enzyme subunits) were transformed in *L. lactis*, which are among the largest scaffoldins introduced in LAB, so far. Scaffoldins supporting assembly of more sophisticated cellulosomes were engineered in *Lactobacillus plantarum* only.[12] In particular, the latter study took advantage of sharing cell-surface binding and enzyme binding functions among different scaffoldin molecules; thus, reducing protein burden in host cells. It is worth noting that functionality of Coh domains included in the synthetic scaffoldins engineered in the present study was not tested here. However, the main focus of the present study was on protein domains related to cell-surface binding.

A number of surface-anchoring domains has previously been used to display heterologous proteins on the cell surface of LAB through covalent (i.e., sortase-mediated) or non-covalent (e.g., through LysM modules) binding.[40–42] Examples of covalent binding of scaffoldins or cellulase system components to the LAB cell surface have also been reported.[12,17] In these studies, heterologous proteins have generally been engineered with established surface-display motifs suitable for specific LAB host. In the present study, an original approach was used to develop anchoring scaffoldins for *L. lactis* that used protein domains which are thought to mediate cell-surface anchoring of cellulosomes in *C. cellulovorans*. More in detail, the four X2 domains of CbpA and the three SLH domains of EngE were included in the structure of the synthetic scaffoldins engineered in this study. The three scaffoldins engineered in this study differ for both the type and number of putative cell-surface anchoring domains. All the scaffoldins containing X2 domains derived from CpbA only, that is, miniC(H) and C, were not able to bind to the *L. lactis* surface (Figures 4 and 5). The only scaffoldin able to anchor the *L. lactis* surface was CE, that included the three SLH domains of EngE (Figures 4 and 5). According to the structure of CE scaffoldin, it could not be excluded that its surface-binding ability derives from the combination of CbpA X2 and EngE SLH domains. Nevertheless, these results indicate at least that EngE-derived SLH domains can bind to *L. lactis* surface stronger than CbpA X2 domains, which is coherent with previous observations made on *C. cellulovorans*.[24] More in detail, recombinant scaffoldins including only CbpA $X2_{3\text{-}4}$ domains (Figure 1) showed 5.5-fold higher K_d and 2-fold lower binding capacity than EngE for *C. cellulovorans* cell wall.[24] Consistently, scaffoldin CE (including CbpA $X2_{1\text{-}2}$ and EngE SLH domains) was displayed on the *L. lactis* cells while scaffoldin C (including CbpA $X2_{1\text{-}4}$ domains) was not. These observations suggest that cell wall composition

of *L. lactis* and *C. cellulovorans* may have some similarity. Previous studies on *C. cellulovorans* also indicated that the higher the number of X2 domains in a protein the greater its binding affinity for the cell wall.[24] However, this did not have any major effect on *L. lactis* cell wall binding, since the degree of display of scaffoldins miniC(H) (one X2 domain) and C (four X2 domains) on *L. lactis* surface did not show any significant difference (Figures 4 and 5). Apart from binding affinity, it is known that the binding target(s) of CbpA X2 and EngE SLH domains on the cell wall of *C. cellulovorans* are different, since removal of SCWPs prevents EngE from binding to cell wall fragments while this does not influence CbpA X2 binding affinity.[24,27] Binding targets of CbpA X2 domains seem therefore absent or masked on the *L. lactis* surface.

A survey of the literature concerning S-layer proteins and SLH domains, indicates that there is still unclear and confusing understanding of their structure, their binding to target molecules in the bacterial cell wall and classification.[43] It has been speculated that SLH domains may have low sequence conservation between them because SLH binding sites in different bacterial species can significantly differ owing to the different composition of surface layer structure.[24,27,44] It has been shown that the C-terminal region of the S-layer protein SlpB (LcsB) of *Lactobacillus crispatus* K2-4-3 can also bind (although with different efficiency) to the cell wall of several other LAB (including different lactobacilli, *L. lactis* and *Streptococcus thermophilus*) but not that of *Lactobacillus casei*.[45] High amino acid sequence conservation characterizes the SLH domains of EngE from *C. cellulovorans* (more than 63% identity)[28] but their structure is not currently available. Recently, the crystal structure of the SLH domains of S-layer proteins Sap from *Bacillus anthracis*[46] and SpaA from *Paenibacillus alvei* CCM 2015T[47] has been determined; thus, allowing to identify the amino acid residues which are essential for binding the cell surface. In particular, a conserved TRAE motif (SLH domain residues 42–45) is thought to mediate SLH domain binding with the negatively charged pyruvate ketal commonly found in SCWPs. An analysis of the EngE-derived domain sequence through the dedicated PROSITE tool (https://prosite.expasy.org) could not detect the presence of the SLH domain pattern (PDOC00823). The mechanisms enabling EngE SLH domains to bind to the bacterial cell wall remains therefore elusive. SLH domains from EngE were aligned and the consensus sequence was submitted to BLAST search for homologous sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). EngE SLH domains show high sequence identity with domains belonging to 11 clostridial glycosyl hydrolases and one from *Herbinix luporum* (Figure S1). These domains could possibly constitute a subgroup or another group of cell-associated protein domains, with a mechanism of binding that significantly differs from the most established SLH domains and which deserves further investigations.

In conclusion, this study led to construction of three synthetic scaffoldins with biotechnological relevance since they could potentially be used for future assembly of designer cellulosomes in *L. lactis*. MiniC and C could mediate assembly of soluble secreted mini-cellulosomes while CE could support display of designer cellulosomes on the surface of *L. lactis*. This a remarkable progress in the field of RCS applied to LAB, which is relatively underdeveloped as compared to the number of studies on other microbial models.[7] As far as we know, recombinant scaffoldins able to bind the surface of LAB have previously been developed only by the research group coordinated by Professor Martin in Canada [17] and that directed by Professors Mizrahi and Bayer in Israel.[41] With respect to these studies, the present investigation represents a further step towards industrial application of recombinant cellulolytic LAB, since constitutive expression of scaffoldins (i.e., without the need of expensive inducers) was demonstrated in *L. lactis*. However, application of scaffoldins obtained by the present study requires further analyses on their Coh domains to confirm their functionality. So far, all our attempts to introduce *C. cellulovorans* cellulosomal cellulases (i.e., the exoglucanase ExgS and the endoglucanases EngE, EngH and EngZ) in *L. lactis* (and confirm their ability to form complexes with the synthetic scaffoldins obtained in this study) were unsuccessful (data not shown). An ex vivo approach (i.e., by mixing scaffoldin-displaying *L. lactis* and *C. cellulovorans* cellulosomal subunits produced in another host, e.g., *E. coli*) will be used to test the functionality of the Coh domains present in the synthetic scaffoldins. Different strategies (e.g., engineering of the signal peptide) are currently being performed to enable the expression and secretion of *C. cellulovorans* cellulosomal enzymes in *L. lactis*. In a broader perspective, the present study points to *C. cellulovorans* EngE SLH domains as new potential modules for anchoring proteins to the cell surface of *L. lactis* and possibly other LAB. The display of recombinant proteins on the bacterial (especially LAB) surface is an active research area owing to the large number of biotechnological applications such as the development of bioadsorbents, biosensors, biocatalysts, and oral vaccines.[42] More in detail, LAB displaying heterologous proteins on their surface have already been exploited as therapeutic agents (e.g., mucosal vaccines) and biocatalysts (displaying different enzymes on their surface). Hopefully, the present study will help LAB biotechnology research progress also in these directions.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Loredana Tarraran and Chiara Gandini performed construction of genes for synthetic scaffoldins. Loredana Tarraran transformed these genes in *Lactococcus lactis* and performed most phenotypic characterization of engineered *L. lactis* strains. Anna Luganini contributed to immunofluorescence detection of synthetic scaffoldins. Roberto

Mazzoli conceived the research and supervised experimental work. All the authors contributed to prepare the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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