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(54) **Titre : METHODES FAVORISANT LA CICATRISATION DES PLAIES**
(54) **Title: METHODS FOR WOUND HEALING**

(57) **Abrégé/Abstract:**
The invention relates to plasmids capable of expressing a protein targeting immune cells when transformed into a lactic acid bacterial cell, wherein the protein is chosen from the group consisting of murine and human CXCL12 1α; CXCL17 and Ym1. The invention further relates to lactic acid bacteria transformed with a said plasmid, as well as the use of said lactic acid bacteria for wound healing in humans and animals.

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(54) **Title**: METHODS FOR WOUND HEALING

(57) **Abstract**: The invention relates to plasmids capable of expressing a protein targeting immune cells when transformed into a lactic acid bacterial cell, wherein the protein is chosen from the group consisting of murine and human CXCL12 1α; CXCL17 and Ym1. The invention further relates to lactic acid bacteria transformed with a said plasmid, as well as the use of said lactic acid bacteria for wound healing in humans and animals.

Methods for wound healing

The invention relates generally to recombinant plasmids, and in particular to plasmids capable of expressing a recombinant protein targeting immune cells when transformed into a lactic acid bacterial cell, wherein the said protein is chosen from the group consisting of murine and human CXCL12 1α, CXCL17 and Ym1. The invention further relates to lactic acid bacteria transformed with a said plasmid, as well as the use of said lactic acid bacteria for wound healing in humans and animals.

The process of wound healing has overlapping phases (coagulation phase, inflammatory phase and proliferative/remodelling phase) where constituents of the local microenvironment change over time and distinct cell types play different roles. Key cell players in the healing process are platelets, keratinocytes/epithelial cells, fibroblasts/myofibroblasts, different immune cells and endothelial cells. All tissues in the body can be injured and the healing process is somewhat specific to the organ, however the initial signals elicited by the damaged cells are similar. The most studied form of wound healing is in skin.

Tissue injury disrupts homeostasis, which initiates the coagulation process and activates the sympathetic nervous system. The platelets forming the blood clots release signals, mainly PDGF (platelet derived growth factor) and TGF (transforming growth factor) changing the local environment (Ref. 1). Injured and stressed cells release alarm signals that initiate the recruitment of immune cells such as neutrophils and monocytes. Within the wounded tissue, the immune cells secrete various chemokines, growth factors like VEGF-A, FGF, and EGF (vascular endothelial growth factor A, fibroblast growth factor, epidermal growth factor), ROS (reactive oxygen species) and matrix digestive enzymes, which change the microenvironment and allow the healing process to enter the proliferative phase where failing and dead tissue is removed by macrophages. Cells from the wound edges, such as fibroblasts and keratinocytes, migrate inwards to the wound centre and cover the wound surface with a layer of collagen and extracellular matrix. The fibroblasts within the wound are then transformed into myofibroblasts expressing contractile α-SMA (α-smooth muscle actin) allowing the wound to contract and finally close. The transition from fibroblasts into myofibroblasts is dependent on signals from the microenvironment, some of which originate from immune cells, mainly macrophages. During this process, blood vessels are growing into the newly formed tissue, the granulation tissue. Blood flow to the adjacent area is normally increased during this phase to increase the availability of oxygen and nutrients, in addition to immune cell recruitment and migration to the afflicted site.

Following wound closure, the afflicted site becomes re-epithelialized by keratinocytes/epithelial cells whereby the integrity of the organ barrier is regained. Even after wound closure, some tissue remodelling occurs to normalize the matrix structure and the majority of involved immune cells either die or leave the site. Also at this stage dead or dying cells are ingested and cleared (phagocytosed) by the remaining tissue macrophages (Ref. 1). Faster wound healing reduces complications and discomfort to the patient,

Impaired or delayed cutaneous or mucosal wound healing is a worldwide clinical problem causing pain, direct exposure to pathogens, loss of tissue function and loss of temperature and fluid balance regulation. There are several conditions where the tightly regulated wound healing process is impaired and the cutaneous or mucosal wounds remain unhealed for longer time periods than normal, which in worst case become chronic.

Reduced blood flow to the skin, especially in extremities, significantly reduces the efficiency of the healing process. There are several clinical conditions where the skin perfusion is either reduced or the function of the vasculature is impaired such as PAD (peripheral artery disease), intermittent claudication, vein insufficiency or vessel obstruction by arteriosclerotic plaques. Impaired blood flow to the wound area results in shortage of oxygen and nutrients and the cells aiding in the tissue remodeling either die from necrosis or are unable to perform their tasks on site. Also the surrounding tissue will if not sufficiently supplied lose functionality and ultimately start to die. Tissues are during the remodeling phase very metabolically active and have high oxygen consumption.

Another factor impairing cutaneous wound healing is hyperglycemia and diabetes mellitus. During hyperglycemic conditions cell signaling and immune system functions are impaired. Complications resulting from diabetes include microvascular changes and damage to peripheral neurons. As a result, diabetic patients often develop chronic wounds on their feet, commonly called diabetic foot. The available treatment for these patients today is removal of dead tissue using surgical debridement or collagenase together with systemic antibiotic treatment and closed wound dressing. There are experimental studies where growth factors and biomaterials have been applied to chronic wounds (Ref. 2).

The stromal cell-derived factor 1 (SDF-1) also known as C-X-C motif chemokine 12 (CXCL12) is a chemokine protein that in humans is encoded by the CXCL12 gene. WO 2009/079451 discloses a method for promoting wound healing in a subject, comprising administering directly to the wound or an area proximate the wound an amount of SDF-1 effective to promote healing of the wound of the subject.

Certain probiotics (*Lactobacillus reuteri* ATCC PTA 6475) have been shown to facilitate wound healing if supplemented in the drinking water during the healing process (Ref. 9), i.e. the bacteria were ingested. Further, supernatants from culture of *Lactobacillus plantarum* have been demonstrated to inhibit biofilm production by *Pseudomonas aeruginosa*, commonly infecting chronic wounds (Ref. 10).

It has surprisingly been found that lactic acid bacteria which are modified, according to the present invention, to express specific proteins, such as cytokines, are useful for promoting wound healing. Lactic acid bacteria are sparsely present on the human skin (Ref. 13) and are not the expected choice of bacteria to use for any intervention on the skin. *Lactobacilli* are difficult to work with since they grow relatively slowly and require special medium and conditions in comparison with more commonly used bacteria like *E. coli* and *S. aureus.* Further, *Lactobacilli* have limited intracellular machinery for transcription, translation and protein folding. For this reason, nucleotide sequences coding for heterologous proteins have to be optimized to fit the specific bacterial strain.

The different phases of wound healing comprise distinct key events that could be altered to change the healing process. Vascular remodeling during the healing process is highly dependent on induction of hypoxia inducible factor 1α (HIF-1α) that regulates the expression of VEGF-A (vascular endothelial growth factor A) and a range of chemokines, such as CXCL12 (also known as SDF-1; SEQ ID NO: 3 and 6). CXCL12 is constitutively expressed in tissues and acts through the receptor CXCR4 found on leukocytes and endothelial cells inducing multiple cellular actions (Ref. 3). CXCL12 is found in high levels in macrophages specialized in tissue remodeling (Ref. 4). Dermal overexpression of CXCL12 using lentiviral vectors improves wound healing in diabetic mice (Ref. 5).

Another recently discovered chemokine is CXCL17 (SEQ ID NO: 9 and 12), which has similar effects on the phenotype of tissue macrophages as CXCL12. In similarity with CXCL12, CXCL17 is co-regulated with VEGF-A measured in cell culture (Ref. 6). CXCL17 is found mainly in mucosal tissues and have been reported to be directly antimicrobial to pathogenic bacteria that are also found on skin whilst showing no effect on survival of *Lactobacillus casei* (Ref. 7).

A further protein of interest is Ym1 (SEQ ID NO: 15 and 18), which is a chitinase-like protein. Chitin is a common polysaccharide in bacterial biofilm. Ym1 both counteracts biofilm production and induces macrophage functions important for tissue remodeling and wound healing and is specific to macrophages since it is not taken up by either vascular cells or epithelial cells (Ref. 8).

Consequently, in a first aspect the invention provides a recombinant plasmid which is capable of expressing a protein in lactic acid bacteria (i.e. when transformed into a lactic acid bacterial cell), wherein the said protein is useful for improving wound healing, such as cutaneous or mucosal wound healing, in a human or animal subject. Preferably, the said protein is useful for wound healing due to its capability of targeting immune cells such as macrophages and their precursors. Preferably, the said protein is a cytokine or chemokine. Most preferably, the said protein is chosen from the group consisting of murine CXCL12, in particular murine CXCL12-1α (SEQ ID NO: 3); human CXCL12, in particular human CXCL12-1α (SEQ ID NO: 6); murine CXCL17 (SEQ ID NO: 9); human CXCL17 (SEQ ID NO: 12); murine Ym1 (SEQ ID NO: 15); and human Ym1 (SEQ ID NO: 18).

This first aspect of the invention more particularly provides a plasmid which is capable of expressing a recombinant protein in lactic acid bacteria (i.e. when transformed into a lactic acid bacterial cell), wherein said plasmid comprises a nucleotide sequence encoding a protein selected from CXCL12, CXCL17 and Ym1.

More specifically the nucleotide sequence may encode murine CXCL12, in particular murine CXCL12-1α; human CXCL12, in particular human CXCL12-1α; murine CXCL17; human CXCL17; murine Ym1; or human Ym1.

In one embodiment, the plasmid comprises a nucleotide sequence encoding a protein selected from murine CXCL12-1α having an amino acid sequence as shown in SEQ ID NO: 3 or 2, or an amino acid sequence with at least 80% sequence identity thereto; human CXCL12-1α having an amino acid sequence as shown in SEQ ID NO: 6 or 5, or an amino acid sequence with at least 80% sequence identity thereto; murine CXCL17 having an amino acid sequence as shown in SEQ ID NO: 9 or 8, or an amino acid sequence with at least 80% sequence identity thereto; human CXCL17 having an amino acid sequence as shown in SEQ ID NO: 12 or 11, or an amino acid sequence with at least 80% sequence identity thereto; murine Ym1 having an amino acid sequence as shown in SEQ ID NO: 15 or 14, or an amino acid sequence with at least 80% sequence identity thereto; and human Ym1 as shown in SEQ ID NO: 18 or 17 or an amino acid sequence with at least 80% sequence identity thereto.

More particularly, the plasmid is for use in expressing a protein in lactic acid bacteria and is accordingly provided, or adapted, for such use (e.g. it is designed, selected, adapted or modified for specific or particular use in lactic acid bacteria). Thus in one embodiment the plasmid is for specific expression in lactic acid bacteria, as compared to bacteria or microorganisms generally. The plasmid may be adapted for expression in lactic acid bacteria by means of its regulatory elements (regulatory

sequences) and/or coding sequences, e.g. which are selected or modified for expression in lactic acid bacteria.

Accordingly, in a more particular aspect the plasmid comprises one or more regulatory (i.e. expression control) sequences which permit expression, or which are specific for expression, in lactic acid bacteria. Thus, the plasmid may contain expression control sequences derived from, or suitable for, or specific for, expression in lactic acid bacteria. Appropriate expression control sequences include for example translational (e.g. start and stop codons, ribosomal binding sites) and transcriptional control elements (e.g. promoter-operator regions, termination stop sequences), linked in matching reading frame with the nucleotide sequence(s) which encode the protein(s) to be expressed. The regulatory sequences(s) are operably linked to a nucleotide sequence encoding said protein, such that they drive, or control, expression of the protein. The plasmid may be introduced into a lactic acid bacterial cell. Suitable transformation techniques are well described in the literature. The bacterial cell may be cultured or otherwise maintained under conditions permitting expression of said protein from the plasmid. This may include conditions in a wound in a subject.

In one embodiment the promoter in the plasmid which controls expression of the protein is a promoter which permits, or which is specific for, expression in lactic acid bacteria. Thus the plasmid may comprise a nucleotide sequence(s) encoding the protein(s), under the control of (or operably linked to) a promoter capable of expressing the protein in lactic acid bacteria. In a particular preferred embodiment the plasmid comprises a lactic acid bacteria promoter, that is the promoter which controls expression of the protein(s) is a promoter which is derived from a lactic acid bacterium, or more particularly which is obtained or derived from a gene expressed in a lactic acid bacterium.

In some embodiments, in addition to a lactic acid bacterial promoter, the plasmid may also contain other regulatory elements or sequences obtained or derived from lactic acid bacteria to control expression of the protein(s). Thus for example such other lactic acid bacterial expression control elements or sequences may include enhancers, terminators and/or translational control elements or sequences as discussed above. In some embodiments the plasmid may also contain regulatory elements or sequences which control or regulate expression from the promoter e.g. operator sequences etc. or one or more regulatory genes, as discussed further below.

Alternatively or additionally the plasmid may be adapted (or modified etc.) for use in lactic acid bacteria by virtue of the nucleotide sequences encoding the protein(s) being codon-optimised for expression in lactic acid bacteria.

In a preferred embodiment the promoter for expression of the protein is a regulated (regulatable) or inducible promoter. Thus, expression of the protein may be controlled or regulated (e.g. initiated, for example at a desired or appropriate time) by providing or contacting the bacteria with a regulatory molecule or inducer which activates or turns on (induces) the promoter. This is advantageous in the context of delivery of the protein to a wound.

Accordingly, a further aspect of the invention provides an expression system for use in expressing a protein in lactic acid bacteria, said expression system comprising (i) a plasmid as defined herein, wherein said plasmid comprises a nucleotide sequence encoding a said protein under the control of an inducible promoter capable of expressing the protein in lactic acid bacteria; and (ii) an inducer (or regulatory molecule) for the promoter. The expression system may conveniently be provided in the form of a kit comprising components (i) and (ii) above.

A still further aspect of the present invention is a bacterium, or bacteria, (i.e. a bacterial cell or strain) transformed with (i.e. comprising) a plasmid of the invention, as defined herein. Particularly, the bacterium is a lactic acid bacterium and the invention accordingly provides lactic acid bacteria (or a lactic acid bacterium) comprising a plasmid of the invention, as defined herein. Alternatively expressed, this aspect of the invention provides a bacterium (or bacterial cell) into which a plasmid of the invention has been introduced.

As described further herein, the plasmids and bacteria of the invention are useful for promoting healing, and thus have particular utility in promoting healing of wounds, which are defined herein to include injured tissue generally (see further below). Accordingly further aspects of the invention provide such plasmids and bacteria for use in therapy, and more particularly for use in wound healing.

The bacteria may be provided for administration to a wound in a subject to be treated in the form of a pharmaceutical composition. Accordingly a still further aspect of the invention provides a pharmaceutical composition comprising bacteria of the invention as defined herein, together with at least one pharmaceutically acceptable carrier or excipient.

More generally, the invention provides a probiotic product comprising the bacteria of the invention.

Such a product, or pharmaceutical composition, may conveniently take the form of a wound dressing comprising the bacteria of the invention. Thus, in a further aspect the invention provides a wound dressing comprising bacteria of the invention as hereinbefore defined, together with at least one dressing material.

A yet further aspect of the invention provides use of a plasmid or of bacteria of the invention as defined herein for the manufacture of a medicament (or a probiotic product) for use in wound healing.

Also provided is a method of treating a subject to heal a wound, said method comprising administering to said subject, or to the wound in said subject, an amount of bacteria of the invention as defined herein effective to promote healing of the wound.

Another aspect of the invention provides a kit for healing wounds, said kit comprising:

(i) lactic acid bacteria comprising a plasmid of the invention as defined herein, wherein said plasmid comprises a nucleotide sequence encoding a said protein under the control of an inducible promoter capable of expressing the protein in lactic acid bacteria; and

(ii) an inducer (or regulatory molecule) for the promoter.

A still further aspect of the invention comprises a pharmaceutical product (e.g. a kit or combination product) comprising;

(i) lactic acid bacteria comprising a plasmid of the invention as defined herein, wherein said plasmid comprises a nucleotide sequence encoding a said protein under the control of an inducible promoter capable of expressing the protein in lactic acid bacteria; and

(ii) an inducer (or regulatory molecule) for the promoter.,

as a combined preparation for separate, sequential or simultaneous use in wound healing (or for treating a wound in a subject).

The term "wound healing" is used broadly herein to include any aspect of promoting or improving the healing of a wound. Thus, the various aspects of the invention set out above may alternatively be defined with respect to a utility of the plasmids or bacteria in promoting or enhancing or improving wound healing or simply promoting or enhancing healing.

Wound healing may accordingly include or encompass any effect which results in faster wound healing, or more complete healing of a wound or indeed any amelioration or improvement in the healing of a wound, e.g. reduced healing time, for example reduced time to achieve partial or complete closure of a wound, improved wound appearance (e.g. the appearance of a healed or healing wound), reduced or improved scar formation, the promotion of healing of a chronic or recalcitrant wound etc. (i.e. the application of the bacteria of the invention to a wound may induce, or cause, or start, the healing of a wound which has up to now not healed or shown any signs of healing). Wounds are discussed in more detail below.

The subject having a wound to be treated may be any human or animal subject, including for example domestic animals, livestock animals, laboratory animals, sports animals or zoo animals. The animal is preferably a mammalian animal, but other animals, e.g. birds are included. Thus the animal may be a primate, a rodent (e.g. a mouse or rat), or a horse, dog or cat. Most preferably the subject is a human.

Lactic acid bacteria (LAB) or *Lactobacillales* are a clade of Gram-positive, low-GC, acid-tolerant, generally nonsporulating, nonrespiring, either rod-shaped (bacillus), or spherical (coccus) bacteria which share common metabolic and physiological characteristics. These bacteria produce lactic acid as the major metabolic end product of carbohydrate fermentation and are characterized by an increased tolerance to acidity (low pH range). These characteristics of LAB allow them to outcompete other bacteria in a natural fermentation because LAB can withstand the increased acidity from organic acid production (e.g. lactic acid). Thus LAB play an important role in food fermentations, as acidification inhibits the growth of spoilage agents. Several LAB strains also produce proteinaceous bacteriocins which further inhibit spoilage and growth of pathogenic microorganisms. LAB have generally recognized as safe (GRAS) status and are amongst the most important groups of microorganisms used in the food industry.

The core genera that comprise the lactic acid bacteria clade are *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus,* and *Streptococcus*, as well as the more peripheral *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus,* and *Weissella*. Any lactic acid bacteria from these genera are included within the scope of the present invention, but particularly bacteria from the genera *Lactobacillus* or *Lactococcus*.

The plasmid may encode one or more of said proteins. Thus it may encode a combination of a CXCL12, CXCL17 and/or a Ym1 protein (e.g. 2 or more of CXCL12, CXCL17 or Ym1). Alternatively, it may encode 2 or more types of a CXCL12, CXCL17 and/or Ym1 protein (e.g. both murine and human CXCL12 etc.). Where more than one protein is encoded, the protein may be expressed from a nucleotide sequence encoding the proteins under the control of a single promoter, or more than one promoter may be used. For example, each protein may be expressed from a separate promoter, which may be the same or different. Techniques for expression of 2 or more proteins together from the same plasmid are well known in the art and include for example translational coupling techniques etc., means for achieving this are known and available in the art. For example multiple transgenes can be expressed simultaneously under one promoter using P2A and T2A sequences.

The CXCL12, CXCL17 or Ym1 protein may be a native or natural protein (i.e. the nucleotide sequence may encode a protein having an amino acid sequence as found in nature) and may be from any species in which these proteins occur. Generally the protein will be a mammalian protein and as indicated above human and murine proteins are preferred. However, the native nucleotide sequences or protein sequences may be modified, for example by one or more amino acid additions, insertions, deletions and/or substitutions, as long as the function or activity of the protein is not substantially or significantly altered, e.g. as long as the activity of the protein is substantially retained. The protein may be a fragment or truncated variant of a natural protein. For example, a sequence-modified variant protein may exhibit at least 80, 85, 90 or 95% of the activity of the parent protein from which it is derived. This may be assessed according to tests known in the art for activity of the protein in question. For example, activity can be measured in systems of receptor phosphorylation or calcium flux upon ligation in culture cells treated with the protein, in systems of cell chemotaxis *in vitro* or *in vivo* in models of cell recruitment to the infected protein. An *in vitro* assay based on chemotaxis is described in Refs. 22 and 32. Ref. 33 describes a further *in vitro* chemokine activity test which might be used. The terms "CXCL12", "CXCL17" or "Ym1" thus include not only the native proteins but also functionally equivalent variants or derivatives thereof. The proteins may thus be synthetic or sequence-modified variants, or may comprise one or more other modifications, e.g. post-translational modifications etc.

As mentioned above, the encoded proteins may have the amino acid sequences indicated above for the native human or murine proteins, namely SEQ ID NOS. 3 and 6 for murine and human CXCL12 respectively, 9 and 12 for murine and human CXCL17 respectively, and 15 and 18 for murine and human Ym1 respectively, or an amino acid sequence having at least 80% sequence identity to any aforesaid sequence. Advantageously, as further indicated above, the nucleotide sequences encoding these native proteins may be codon-optimised for expression in lactic acid bacteria. This may result in a modified amino acid sequence of the protein encoded. For example codon optimised sequences may encode sequences such as secretion sequences suitable, (or better suited) for lactic acid bacteria. Thus the "optimized" protein encoded by a codon-optimised nucleotide sequence may include an altered or substituted leader or signal sequence (e.g. secretory sequence) as compared to the native protein. In a preferred embodiment the mature or cleaved form of the protein encoded by the codon optimised sequence is identical to the native protein. Proteins encoded by codon-optimised nucleotide sequences may have an amino acid sequence as shown in SEQ ID NOS. 2, 5, 8, 11, 14, or 17 as listed in Table IV below. Thus, the

protein encoded by the plasmid may have an amino acid sequence as shown in SEQ ID NOS. 2 and 5 for murine and human CXCL12 respectively, 8 and 11 for murine and human CXCL17 respectively, and 14 and 17 for murine and human Ym1 respectively, or an amino acid sequence having at least 80% sequence identity to any aforesaid sequence.

In other embodiments the encoded protein(s) may have an amino acid sequence which has at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% 90%, 91% 92% 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with any aforesaid amino acid sequence.

Sequence identity may readily be determined by methods and software known and readily available in the art. Thus, sequence identity may be assessed by any convenient method. However, for determining the degree of sequence identity between sequences, computer programs that make multiple alignments of sequences are useful, for instance Clustal W (Ref. 24). Programs that compare and align pairs of sequences, like ALIGN (Ref. 25), FASTA (Ref. 26 and Ref. 27), BLAST and gapped BLAST (Ref. 28) are also useful for this purpose, and may be used using default settings. Furthermore, the Dali server at the European Bioinformatics institute offers structure-based alignments of protein sequences (Ref. 29, Ref. 30 and Ref. 31). Multiple sequence alignments and percent identity calculations may be determined using the standard BLAST parameters, (e.g. using sequences from all organisms available, matrix Blosum 62, gap costs: existence 11, extension 1). Alternatively, the following program and parameters may be used: Program: Align Plus 4, version 4.10 (Sci Ed Central Clone Manager Professional Suite). DNA comparison: Global comparison, Standard Linear Scoring matrix, Mismatch penalty = 2, Open gap penalty = 4, Extend gap penalty = 1. Amino acid comparison: Global comparison, BLOSUM 62 Scoring matrix.

Variants of the naturally occurring polypeptide sequences as defined herein can be generated synthetically e.g. by using standard molecular biology techniques that are known in the art, for example standard mutagenesis techniques such as site-directed or random mutagenesis (e.g. using gene shuffling or error prone PCR).

Derivatives of the proteins as defined herein may also be encoded. By derivative is meant a protein as described above or a variant thereof in which the amino acid is chemically modified e.g. by glycosylation and such like etc.

Where a protein comprises an amino acid substitution relative to the sequence of the native protein, the substitution may preferably be a conservative substitution. The term “a conservative amino acid substitution” refers to any amino acid substitution in which an amino acid is replaced (substituted) with an amino acid having similar

physicochemical properties, i.e. an amino acid of the same class/group. For instance, small residues Glycine (G), Alanine (A) Serine (S) or Threonine (T); hydrophobic or aliphatic residues Leucine (L), Isoleucine (I); Valine (V) or Methionine (M); hydrophilic residues Asparagine (N) and Glutamine (Q); acidic residues Aspartic acid (D) and Glutamic acid (E); positively-charged (basic) residues Arginine (R), Lysine (K) or Histidine (H); or aromatic residues Phenylalanine (F), Tyrosine (Y) and Tryptophan (W), may be substituted interchangeably without substantially altering the function or activity of the protein.

As indicated above, it is preferred to use an inducible promoter for expression of the protein. By "inducible" is meant any promoter whose function (i.e. activity, or effect in allowing or causing transcription of the coding nucleotide sequence) can be regulated or controlled. The term "inducible" is thus synonymous, and may be used interchangeably with "regulatable" (or "regulated"). Thus, there is not constitutive expression of the protein. Accordingly, expression of the protein may be induced, or turned on (or more particularly turned on and off). More particularly, expression may be induced, or turned on for a finite or defined time. This may be because expression ceases after a period of time, and/or because the bacterial cells die.

In some embodiments there may be no expression (transcription) from the promoter until the promoter is induced (or alternatively termed, activated). However, as with any biological system, lack of activity may not be absolute and there may be some basal promoter activity in the absence of promoter activation or induction. However, in a preferred embodiment any basal expression of the uninduced promoter is low, minimal, or insignificant, or more preferably *de minimis* or negligible. Thus, expression from the inducible promoter is advantageously measurably or demonstrably increased when the promoter is induced compared to the promoter when it is not induced.

Inducible promoters are well known in the art, including inducible promoters for use in lactic acid bacteria and any appropriate inducible promoter may be used, suitable for expression in lactic acid bacteria.

An inducible promoter may be induced (or activated) in the presence of an inducer or activator molecule, which may act directly or indirectly on the promoter, and which may be added to induce the promoter, or more generally to cause or enable induction or activation of the promoter, and permit expression of the protein, or it may be induced (or activated) by a change in conditions of the bacteria containing the plasmid, e.g. by introducing a change of conditions to the lactic acid bacteria, e.g. starvation or depletion of a particular nutrient. An inducer of the promoter may be encoded by a regulatory gene, which in an embodiment may itself be induced or activated. The term "inducer" is thus used broadly herein to include any regulatory

molecule, or indeed any permissive condition, which may activate or turn on an inducible promoter, or allow or cause an inducible promoter to be induced. Thus, induction of an inducible promoter may comprise the introduction of (e.g. contacting the lactic acid bacteria containing the plasmid with) a regulatory molecule or of a condition permissive to promoter induction (activation). In some embodiments the inducer can be an activation peptide. This may act directly, or indirectly to induce the promoter, for example as described further below.

As noted above, promoters obtained or derived from lactic acid bacteria are preferred. These may be native promoters or modified or mutant promoters. A suitable promoter may for example be identified by growing lactic acid bacteria in a wound, and by determining which genes are expressed, or upregulated in the bacteria in the wound. The promoters from such genes may then be identified. Alternatively a number of different promoters and expression systems in or for use in lactic acid bacteria have been identified and described or available in the art, including expression plasmids containing such promoters or expression systems for use in LAB. Any such known plasmid or expression system may be used as the basis for the recombinant plasmid of the invention.

Various inducible expression systems are known in the art for use with LAB such as *Lactobacilli*. One example includes an auto-inducing system based on the manganese starvation-inducible promoter from the manganese transporter of *L. plantarum* NC8 as described in Ref. 19. This system does not require the addition of external inducers for recombinant protein production.

Duong *et al.* (Ref. 20) describe expression vectors for use with *lactobacilli* based on the broad range pWV01 replicon and containing promoters from operons involved in fructooligosaccharide (FOS), lactose or trehalose metabolism or transport, or in glycolysis. Such promoters may be induced by their specific carbohydrate and repressed by glucose.

More particularly, the inducible expression system may comprise inducible promoters involved in the production of LAB proteins, and in particular bacteriocins. The activity of such promoters may be controlled by a cognate regulatory system based on the bacteriocin regulon, for example a two-component regulatory (signal transduction) system which responds to an externally added activator peptide (i.e. an inducer/regulatory molecule in peptide form) and genes encoding a histidine protein kinase and response regulator necessary to activate this promoter upon induction by an activator peptide.

In an embodiment the expression system may be based on the nisin-controlled expression (NICE) system, based on the combination of the *nis*A promoter and the

*nis*RK regulatory genes. This system is based on the promoters and regulatory genes from the *Lactococcus lactis* nisin gene cluster and has been used to develop regulated gene expression systems for *lactococci*, *lactobacilli* and other Gram-positive bacteria (reviewed briefly in Ref. 15 and Ref. 21). Whilst the NICE systems are efficient and well regulated in *Lactococci*, these systems can exhibit significant basal activity. This can be circumvented by integrating the histidine kinase and response regulator genes in the chromosome, limiting the expression systems to specially designed host strains.

In another embodiment the expression system may be based on the genes and promoter involved in the production of class II bacteriocins sakacin A (*sap* genes) by the sakacin A regulon or sakacin P (*spp* genes) by the sakacin P regulon. Such vectors are known as pSIP vectors and contain a promoter element derived from either the sakacin A or the sakacin P structural gene with an engineered *Nco*I site for translational fusion cloning. A variety of such vectors containing different promoters from the regulons and/or different replicons are described in Ref. 21 and Ref. 15 and any of these vectors could be used as the basis for the recombinant plasmid of the invention.

In a representative embodiment the promoter may be the P_{sapA}, P_{sppA} or P_{orfX} promoter from the sakacin A or P regulon, together with its associated or cognate regulatory genes.

In a particularly preferred embodiment the plasmid contains the pSH71 replicon, the P_{orfX} promoter from the sakacin P regulon and the cognate regulatory genes, based on the vector pSIP411 depicted in FIG 12 and described in Ref. 21. Plasmid pSIP411 is designated pLAB112 in the present application. The inducer for use in such an embodiment is preferably an activation peptide based on the peptide SppIP, e.g. an activation peptide having the sequence of SEQ ID NO.19, or an amino acid sequence with at least 80% (or more particularly at least 85, 90 or 95) sequence identity thereto. In a preferred embodiment the said recombinant plasmid is derived from the plasmid designated pLAB112 having the nucleotide sequence shown in SEQ ID NO: 20.

The use of an inducible promoter (or inducible expression system) may provide the advantage of a more controlled, and in particular prolonged expression of the protein in the wound setting i.e. when the bacteria are administered to the subject or to the wound. For a better effect in promoting wound healing it is advantageous for the protein to be expressed by the bacteria for a period of time at the site of the wound (e.g. at the wound surface), e.g. for at least 40, 45, 50, 55 or 60 minutes, notably for at least one hour, or more. Thus the protein may be expressed for a finite, a defined or a prolonged period of time. Results presented in the Examples below show that using plasmids and bacteria according to the present invention, the protein may be

expressed for a period of about an hour at the wound surface. The plasmids and bacteria may in some embodiments be optimised to allow expression of the protein (e.g. in a wound) for 2, 3 or 4 hours or more.

Continuous expression and delivery of the protein is thus desirable and this may be afforded by using the transformed bacteria of the invention. By "continuous" or "prolonged" is meant that there is expression, and hence delivery, of the protein over a period of time e.g. over a period of at least an hour (or so, as discussed above). In particular this allows the protein to be effective over a period of time which is increased as compared to administration of the protein directly (i.e. as a protein product rather than by expression by the bacteria).

As discussed above, the nucleotide sequences encoding the protein(s) may be codon optimised for expression in LAB. Accordingly, in preferred embodiments the nucleotide sequences (or inserts) in the recombinant plasmids, which encode the proteins, may be selected from the codon-optimised nucleotide sequences shown in SEQ ID NOS. 1, 4, 7, 10, 13 and 16 which encode murine CXCL12, human CXCL12, murine CXCL17, human CXCL17, murine Ym1 and human Ym1 respectively, or a nucleotide sequence having at least 80% sequence identity therewith.

Thus in representative embodiments the recombinant plasmid may be chosen from the group consisting of the plasmids designated mLrCK1, comprising a nucleotide sequence as shown in SEQ ID NO: 1; mLrCK1.4, comprising a nucleotide sequence as shown in SEQ ID NO: 1; mLrCK1.7, comprising a nucleotide sequence as shown in SEQ ID NO: 1;, hLrCK1, comprising a nucleotide sequence as shown in SEQ ID NO: 4; mLrCK2, comprising a nucleotide sequence as shown in SEQ ID NO: 7; hLrCK2, comprising a nucleotide sequence as shown in SEQ ID NO: 10; hLrMP1, comprising a nucleotide sequence as shown in SEQ ID NO: 13; and mLrMP2, comprising a nucleotide sequence as shown in SEQ ID NO: 16.

In some embodiments the plasmid of the invention comprises a nucleotide sequence which has at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% 90%, 91% 92% 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to a nucleotide sequence of the following codon optimized inserts mLrCK1 (i.e., to the nucleotide sequence of SEQ ID NO: 1), mLrCK1.4 (i.e., to the nucleotide sequence of SEQ ID NO: 1), mLrCK1.7 (i.e., to the nucleotide sequence of SEQ ID NO: 1), hLrCK1 (i.e., to the nucleotide sequence of SEQ ID NO: 4), mLrCK2 (i.e., to the nucleotide sequence of SEQ ID NO: 7), hLrCK2 (i.e., to the nucleotide sequence of SEQ ID NO: 10), hLrMP1 (i.e., to the nucleotide sequence of SEQ ID NO: 13), and mLrMP2 (i.e., to the nucleotide sequence of SEQ ID NO: 16).

Sequence identity of nucleotide molecules may be determined by methods and software known and widely available in the art, for example by FASTA Search using GCG packages, with default values and a variable pamfactor, and gap creation penalty set at 12.0 and gap extension penalty set at 4.0 with a window of 6 nucleotides.

Such sequence identity related nucleotide sequences may be functionally equivalent to the nucleotide sequence which is set forth in SEQ ID NO: 1, 4, 10, 13 or 16. Such nucleotide sequences may be considered functionally equivalent if they encode polypeptides which would be considered functional equivalents to the respective CXCL12, CXCL17 or Ym1 proteins. Preferred functional equivalents are those which encode the preferred proteins as set out above.

In another aspect, the invention provides a bacterial strain transformed with the recombinant plasmid described above. The said bacterial strain is preferably a lactic acid bacteria strain such as a *Lactobacillus* strain or a *Lactococcus* (e.g. *Lactococcus lactis*) strain. More preferably, the bacterial strain is a *Lactobacillus reuteri* strain such as *Lactobacillus reuteri* R2LC or *Lactobacillus reuteri* DSM20016. The said strains (*Lactobacillus reuteri* R2LC/DSM20016 and *Lactococcus lactis*) are not found on human skin as determined by phylogenetic analysis of the forearm skin biota of six subjects (Ref. 13).

As well as the plasmids, expression systems, bacteria and kits, further products of the invention include pharmaceutical compositions and medical devices containing the bacteria. Such compositions and devices may include in particular wound dressings, packing materials, swabs, implants etc., or indeed any wholly or partially in-dwelling medical device which may be introduced or present at the site of a wound (e.g. at a surgical wound site), for example a line or catheter or implant. Also included are probiotic products, that is products containing the bacteria for administration to a subject, e.g. for oral administration, for example for consumption or ingestion, or for topical application to a wound or direct administration to a wound site, e.g. during surgery, or rectally, vaginally, etc.

Accordingly, the products (e.g. plasmids, bacterial strains, probiotics and wound dressings etc.) according to the invention are useful in medical therapy, in particular for promoting wound healing in human or animal subjects. As used herein, the term "promoting wound healing" means augmenting, improving, increasing, or inducing closure, healing, or repair of a wound. In preferred aspects of the invention, the human or animal subject is in need of wound healing due to an underlying medical condition leading to impaired wound healing, such as reduced peripheral blood perfusion (peripheral artery disease), hyperglycemia or neuropathy, or the subject may be immunocompromised for any reason, e.g. due to an underlying disease (whether

acquired or inherited) or as an effect of medical treatment. In particular the subject may be suffering from diabetes.

The wound to be healed can include any injury, trauma or damage to any portion of the body of a subject. Examples of wounds that can be treated by the method include acute conditions or wounds; such as thermal burns (hot or cold), chemical burns, radiation burns, electrical burns, burns caused by excess exposure to ultraviolet radiation (e.g. sunburn); damage to bodily tissues, such as the perineum as a result of labor and childbirth; injuries sustained during medical procedures, such as episiotomies, trauma- induced injuries including cuts, incisions, excoriations; injuries sustained from accidents; post-surgical injuries, as well as chronic conditions; such as pressure sores, bedsores, ulcers, conditions related to diabetes and poor circulation, and all types of acne. In addition, the wound can include dermatitis, wounds following dental surgery; periodontal disease; wounds following trauma; and tumor associated wounds. Further examples are gastrointestinal wounds occurring during for instance gastritis or inflammatory bowel disease.

Thus the term "wound" is used broadly herein to include any breach of the integrity of a tissue or any damage or injury to a tissue. Thus the term includes any damage, trauma or injury to tissue or any lesion, howsoever caused (e.g. due to accidental injury or trauma, surgical or other intended or purposeful injury or disease). The trauma may include any physical or mechanical injury or any damage caused by an external agent including pathogens or biological or chemical agents. Tissue damage may also be caused by hypoxia, ischemia or reperfusion. Wounds may include any type of burn. The wound may be acute or chronic. A chronic wound may be described as any wound stalled in a healing stage, e.g. in the inflammatory phase, or any wound that has not healed in 30, 40, 50 or 60 days or more. The wound may be present in or on an internal or external surface or tissue of the body.

In a particular embodiment the wound is on an external surface or tissue of the body, e.g. it is a skin (i.e. cutaneous) wound or a mucosal wound, in particular a wound in an external mucosal tissue or surface of the body (e.g. in the eye, ear or nose etc.) In another embodiment it is a gastrointestinal wound. In a different embodiment it is not a gastrointestinal wound (i.e. it is a wound other than a gastrointestinal wound).

The bacteria may be administered in any convenient or desired way, e.g. orally, or topically, or by direct administration to a wound site e.g. by direct injection or infusion or application or introduction of a pharmaceutical composition or dressing or device containing the bacteria. In other embodiments it may be administered to the oral cavity, or intranasally or by inhalation, rectally or vaginally. The bacteria may thus be

administered to, or via, any orifice of the body. For administration to a gastrointestinal wound the bacteria may be administered perorally.

The bacteria may be formulated or prepared in any convenient or desired way for administration by any of the above routes, according to procedures and using means well known and routine in the art. Thus, as well as pharmaceutical compositions, medical devices and dressings etc., the probiotic products of the invention may be formulated and provided as or in nutritional supplements or foods, e.g. functional foods.

Oral administration forms include powders, tablets, capsules and liquids etc. For topical administration, the product may be formulated as a liquid e.g. a suspension, or a spray or aerosol (powder or liquid), gel, cream, lotion, paste, ointment or salve etc. or as any form of dressing, e.g. bandage, plaster, pad, strip, swab, sponge, mat etc., with or without a solid support or substrate. Further the bacteria may be provided on (e.g. coated on) the surface of a medical device such as an implant (e.g. a prosthetic implant), tube, line or catheter etc.

The bacteria may be provided in any convenient or desired form, e.g. as an active or growing culture or in lyophilized or freeze-dried form.

The bacterial strains according to the invention can be formulated for topical or oral administration to treat surface wounds of skin or mucosa. Consequently, the invention further provides a probiotic product comprising a bacterial strain according to the invention. The said probiotic product is e.g. a pharmaceutical composition, preferably for oral administration. Alternatively, for topical application, the probiotic product is e.g. a lotion or a lotion-soaked wound dressing, comprising a bacterial strain according to the invention.

The product of the invention (i.e. the pharmaceutical composition or device or dressing etc.) may also contain the inducer (where an inducible promoter is used). This may be provided as part of the product (e.g. incorporated into or included in a dressing) or separately, e.g. as part of a kit or combination product, as defined above.

When co-formulated together in a product (e.g. a dressing or device) the bacteria and the inducer may be provided in a format in which the bacteria are separated from the inducer and are brought together (or contacted) in use. For example, the bacteria and inducer may be in separate compartments which are brought together (e.g. contacted or mixed), or may be separated by a barrier (e.g. a membrane or other partition) which may be broken or disrupted or opened in use.

Alternatively, the inducer may be formulated and provided separately (e.g. in a kit also containing the bacteria, or a product containing the bacteria), and the inducer and bacteria (or product containing the bacteria) may be brought together (e.g

contacted) during use. This may be before, during or after administration to the subject. For example, a product comprising the bacteria may be administered first and then the inducer may be added or applied to the bacteria. In another embodiment the bacteria and inducer may be premixed before administration, e.g. just before or immediately before, or during administration.

Where bacteria are provided in lyophilized or freeze-dried form, it may be desirable to reconstitute, or resuspend, them prior to administration e.g. prior to or during use. This may depend on the wound and the format of the product which is used. For example, in the case of some wounds there may be sufficient liquid present to allow for the bacteria to be reconstituted/resuspended and become active. However, in other embodiments it may be desirable to provide a liquid for reconstitution (or alternatively expressed, for suspension or resuspension) of the bacteria. This may be provided in a separate vessel or container (e.g. as part of a kit or combination product) or in a separate compartment of a container, or vessel or device. The liquid may comprise or contain the inducer, or the inducer, when present, may be provided in a separate vessel or container or compartment. The liquid may be any suitable liquid for reconstitution or suspension of freeze-dried bacteria, e.g. water, or an aqueous solution, or buffer or growth or culture medium.

Thus, by way of example a two compartment system (e.g. in a dressing or device or container or vessel (e.g. a bottle)) may comprise freeze-dried bacteria in one compartment and a liquid in another. The liquid may optionally contain an inducer. In use, or prior to use, the two compartments may be mixed or brought into contact, and applied to the wound. In a more particular embodiment, the bacteria may be administered to a wound in liquid form, and a separate dressing may then be applied. It will be seen therefore that in one simple embodiment, a kit may simply contain a first vessel or container comprising the freeze-dried bacteria and a second vessel or container containing a liquid for reconstitution of the bacteria. Optionally the kit may also contain an inducer, which is also contained in the second vessel or in separate third vessel or container.

Hence, for example a said probiotic product preferably comprises an activation peptide capable of activating expression of the protein to be expressed in the lactic acid bacteria strain. The said activation peptide is preferably the peptide SppIP (i.e. a peptide comprising the amino acid sequence of SEQ ID NO: 19, or a sequence with at least 80% sequence identity thereto).

For cutaneous wounds, the said wound dressing can comprise freeze-dried bacteria in one compartment and an activation peptide in another compartment. When applied to the wound, the two compartments are brought together so that the bacteria

are brought into contact with the activation peptide. Alternatively, bacteria can be contained in a gel-like structure on the adhesive side of a waterproof plaster or the side of the dressing in contact with the exudate. At the time of use, activation peptide is manually applied to the bacteria and the plaster or dressing is applied to the wound area.

Viable bacteria may also be comprised in a hydrocolloid, for example a natural gelatin. The bacteria can be incorporated by crosslinking into hydrocolloid e.g. gelatin films, plasticised and dried, retaining viability during storage until hydration. Viable bacteria may also be encapsulated within cross-linked electrospun hydrogel fibers. In this format the bacteria need not be freeze-dried.

For wounds in the gastrointestinal tract, a tablet is designed comprising at least two separate compartments, wherein one compartment comprises freeze-dried bacteria and the other compartment comprises liquid and an activation peptide. The tablet is squeezed before ingestion so that an inner membrane, separating the two compartments, is broken and the contents are mixed together. For wounds in the mouth (e.g. on the gums), bacteria according to the invention can be administered in a high viscous paste.

Specifically, formulations for topical administration to the skin can include ointments, creams, gels, and pastes to be administered in a pharmaceutically acceptable carrier. Topical formulations can be prepared using oleaginous or water-soluble ointment bases, as is well known to those in the art. For example, these formulations may include vegetable oils, animal fats, and more preferably semisolid hydrocarbons obtained from petroleum. Particular components used may include white ointment, yellow ointment, acetyl esters wax, oleic acid, olive oil, paraffin, petrolatum, white petrolatum, spermaceti, starch glycerite, white wax, yellow wax, lanolin, anhydrous lanolin, and glyceryl monostearate. Various water-soluble ointment bases may also be used including, for example, glycol ethers and derivatives, polyethylene glycols, polyoxyl 40 stearate, and polysorbates.

The bacterial strain can be provided in and/or on a substrate, solid support, and/or wound dressing for delivery of active substances to the wound. The solid support or substrate may be a medical device or a part thereof. As used herein, the term “substrate” or “solid support” and “wound dressing” refer broadly to any substrate when prepared for, and applied to, a wound for protection, absorbance, drainage, etc.

In an embodiment the invention provides a wound healing material or dressing attached to or comprising the transformed bacterial strain i.e. the dressing is a vehicle for administering the transformed bacteria of the invention. Alternatively the vehicle may be a plaster or bandage. The present invention may include any one of the

numerous types of substrates and/or backings that are commercially available, the choice of wound healing material will depend on the nature of the wound to be treated. The most commonly used wound dressings are described briefly below.

Transparent film dressings are made of e.g. polyurethane, polyamide, or gelatin. These synthetic films are permeable to water vapor oxygen and other gases but impermeable to water and bacteria, have low absorbency and are suitable for wounds with low exudate), hydrocolloids (hydrophilic colloidal particles bound to polyurethane foam), hydrogels (cross-linked polymers containing about at least 60% water have higher absorbency and eliminate toxic components from the wound bed and maintain the moisture level and temperature in the wound area), foams (hydrophilic or hydrophobic e.g. polymeric foam dressings produced through the modification of polyurethane foam have good absorbency and are permeable to water vapour), calcium alginates (non-woven composites of fibers from calcium alginate from the phycocolloid group, alginates have a very high absorbent capacity. They also promote autolytic debridement because ion-exchange between the alginate and the exudate converts the insoluble calcium alginate into soluble sodium alginate, providing the wound bed with a moist, intact surface ideal for wound healing), and cellophane (cellulose with a plasticizer). The shape and size of a wound may be determined and the wound dressing customized for the exact site based on the measurements provided for the wound. As wound sites can vary in terms of mechanical strength, thickness, sensitivity, etc., the substrate can be molded to specifically address the mechanical and/or other needs of the site. For example, the thickness of the substrate may be minimized for locations that are highly innervated, e.g. the fingertips. Other wound sites, e.g. fingers, ankles, knees, elbows and the like, may be exposed to higher mechanical stress and require multiple layers of the substrate.

In yet a further aspect, the invention provides a method for wound healing in a human or animal subject, comprising administering to a human or animal subject in need thereof a bacterial strain according to the invention. The said bacterial strain is preferably comprised in a pharmaceutical composition or wound dressing as hereinbefore described. In such methods, the human or animal subject is preferably in need of wound healing due to an underlying medical condition leading to impaired wound healing, such as reduced peripheral blood perfusion (peripheral artery disease), hyperglycemia or neuropathy.

Results obtained and included in the Examples below demonstrate the advantages of the invention. In particular, improved wound healing (e.g. in terms of better or faster wound closure) may be obtained by using the protein-expressing transformed bacteria of the invention, as compared to, for example, a protein

preparation directly (i.e. just the protein, no bacteria) or just bacteria alone (bacteria which are not modified to express the protein, e.g. not containing the recombinant plasmid). Further, an improved effect may be seen when bacteria are administered to wound, compared to administration of a supernatant obtained from a transformed bacterial culture. It is thus advantageous to deliver the protein to the wound by means of a lactic acid bacterial host expressing the protein. It is believed that there may be synergistic effect. In other words there may be a synergy between the effect of the bacteria and the effect of the protein on wound healing. Accordingly, in some embodiments there may be a greater than cumulative effect of the transformed bacteria on wound healing, relative to the effect of corresponding untransformed bacteria (i.e. not containing the plasmid) and the effect of the protein when provided as a protein (i.e. not expressed from bacteria *in situ*).

It is believed in this respect that the effect of the bacteria in lowering pH e.g. in the site of the wound may assist in augmenting or enhancing or promoting the activity of the protein. Whilst not wishing to be bound by theory, it is further believed that administration of the transformed bacteria according to the invention may have a beneficial effect in promoting macrophage activity at the site of the wound. For example, the number of macrophages may be increased.

The effect of the transformed bacteria on wound healing may or may not be immediate, and may take some time to be seen (e.g. 1, 2, 3, 4, 5 or 6 or more hours to be seen, or longer, e.g. 8, 10, 12, 15, 18, 20 or 24 hours or more, or 1, 2, 3, 4, 5 or 6 or more days to be seen, or longer e.g. 8, 10, 12, 15, 18, 20 or 24 days or more, before improved wound healing can be observed). For chronic wounds in elderly humans it may take longer to see a difference between the treatment group and control group for example it may take around 12 weeks.

A particular and important utility of the present invention lies in the treatment of chronic wounds, particularly ulcers and in particular in the treatment of diabetic foot ulcers.

The prevalence of chronic foot ulcers in persons with diabetes is about 18%. In 2013, the European population reached 742.5 million, which translates into 32.7 million with diabetes, of which 2.9-5.8 million have chronic foot ulcers. Mean duration of an ulcer of this type is in the range of months where less than 25 % of the wounds are healed within 12 weeks when standard care is given. The end stage of this condition is amputation of the affected limb. Today the treatment of people having chronic foot ulcers is divided between primary care, home care, nursing homes, relatives, self-management and visits to hospital wound clinics. The current treatment relies on off-loading, removal of dead tissue using surgical debridement, repeated changes of

wound dressings, systemic antibiotics and in special cases treatment with living larvae or collagenase and at a few locations in Sweden hyperbaric oxygen treatment can be offered. If an underlying cause also includes obstructions of larger arteries, this can be corrected surgically by bypassing vein graft. Today the wounds are treated every second to third day. Treatment with the suggested modified lactic acid bacteria in any of the suggested forms or formulations would not disrupt this practical routine. Improved healing of such wounds by the treatments of the present invention would thus be of considerable economic benefit, as well as of personal benefit to the patient.

The bacteria are active and produce and deliver the encoded proteins to the wound surface for a period of time (e.g. about one hour) *in vivo*. They may then become inactive and die. Slow or dead lactic acid bacteria can with no risk be in the wound/dressing environment until the dressing is changed as normal.

The biotherapeutic according to the present invention will have significantly lower production cost compared to protein drug compounds. This is because the biotherapeutics produces the active protein itself directly in the wound.

Open wounds such as diabetic foot ulcers, together with loss of function in the foot, cause considerable discomfort, and even disability to the patient, and can have a significant negative impact on quality of life, including significant risk or infection and therefore prolonged use of antibiotics, and ultimately amputation. Improved healing would thus be of tremendous personal benefit to the patient and would also have the benefit of reducing antibiotic use (and consequently the spread of antibiotic resistance). It is believed that treating such chronic wounds according to the invention may amplify endogenous alarm signals in the wound, and kick start the healing process in stalled or chronic wounds, and accelerate healing time.

The present invention as claimed relates to:

[1] Lactic acid bacteria transformed with a plasmid which comprises a nucleotide sequence encoding a protein selected from the group consisting of CXCL12, CXCL17 and Ym1, wherein the bacteria are *Lactobacillus reuteri,* and wherein the nucleotide sequence is codon-optimised for expression in lactic acid bacteria;

[2] The bacteria of [1], wherein said plasmid comprises a nucleotide sequence encoding a protein selected from: (i) murine CXCL12-1α having an amino acid sequence as shown in SEQ ID NO: 3 or 2, or an amino acid sequence with at least 80% sequence identity thereto; (ii) human CXCL12-1α having an amino acid sequence as shown in SEQ ID NO: 6 or 5, or an amino acid sequence with at least 80% sequence identity thereto; (iii) murine CXCL17 having an amino acid sequence as shown in SEQ ID NO: 9 or 8, or an amino acid sequence with at least 80% sequence identity thereto; (iv) human CXCL17 having an amino acid sequence as shown in SEQ ID NO: 12 or 11, or an amino acid sequence with at least 80% sequence identity

thereto; (v) murine Ym1 having an amino acid sequence as shown in SEQ ID NO: 15 or 14, or an amino acid sequence with at least 80% sequence identity thereto; and (vi) human Ym1 as shown in SEQ ID NO: 18 or 17 or an amino acid sequence with at least 80% sequence identity thereto;

[3] The bacteria according to [1] or [2], wherein the plasmid comprises one or more regulatory sequences which permit expression in lactic acid bacteria, wherein the regulatory sequences are obtained or derived from lactic acid bacteria;

[4] The bacteria according to any one of [1] to [3], wherein expression of said protein is regulatable;

[5] The bacteria according to any one of [1] to [4], wherein the plasmid comprises one or more nucleotide sequences encoding one or more of said proteins under the control of an inducible promoter;

[6] The bacteria according to any one of [1] to [5] wherein the plasmid comprises an inducible promoter and regulatory elements from the nisin regulon, the sakacin A regulon or the sakacin P regulon of a lactic acid bacterium;

[7] The bacteria according to [5] or [6], wherein the inducible promoter is the PorfX promoter from the sakacin P regulon;

[8] The bacteria according to any one of [1] to [7], wherein the plasmid is derived from the plasmid designated pSIP411, wherein pSIP411 has the sequence of SEQ ID NO: 20.

[9] The bacteria according to any one of [1] to [8], which comprises one or more nucleotide sequences selected from the group consisting of: a nucleotide sequence comprising the sequence of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, SEQ ID NO: 10, SEQ ID NO: 13, and SEQ ID NO: 16, or a nucleotide sequence having at least 80% sequence identity to any aforesaid sequence;

[10] The bacteria according to any one of [1] to [9], wherein the bacteria are *Lactobacillus reuteri* strain R2LC;

[11] A wound dressing comprising the bacteria according to any one of [1] to [10];

[12] A pharmaceutical composition comprising the bacteria according to any one of [1] to [10] and at least one pharmaceutically acceptable carrier or excipient;

[13] The pharmaceutical composition of [12], wherein the composition is a topical composition for administration to the skin;

[14] Use of the bacteria according to any one of [1] to [10] for wound healing in a human or animal subject;

[15] Use of the bacteria according to any one of [1] to [10] in the manufacture of a medicament for wound healing in a human or animal subject;

[16] The bacteria according to any one of [1] to [10] for use in wound healing in a human or animal subject;

[17] The use of [14] or [15], or the bacteria for use according to [16], wherein the wound is a cutaneous wound;

[18] The use of [14] or [15], or the bacteria for use according to [16], wherein the wound is a mucosal wound;

[19] The use of any one of [14], [15], [17] or [18], or the bacteria for use according to [16], wherein the bacteria are for administration directly to the wound site;

[20] A kit for use in healing wounds, said kit comprising: (i) lactic acid bacteria as defined in any one of [1] to [10], wherein said plasmid comprises a nucleotide sequence encoding said protein under the control of an inducible promoter capable of expressing the protein in lactic acid bacteria; and (ii) an inducer for the promoter;

[21] A medical device comprising the bacteria according to any one of [1] to [10];

[22] The bacteria, wound dressing, pharmaceutical composition, use, kit or medical device of any one of [1] to [21] wherein the bacteria are freeze-dried;

[23] The kit of [22], wherein the kit further comprises a liquid for resuspending the freeze-dried bacteria;

[24] The kit of [23], wherein the liquid comprises the inducer;

[25] The kit of any one of [20], or [22] to [24], wherein the kit comprises a wound dressing comprising the bacteria; and

[26] The kit of any one of [20], or [22] to [24], wherein the kit further comprises a wound dressing.

Further, the invention may have advantages in flexibility and ease of use by medical staff.

Representative methods and preferred embodiments according to the present invention will be further described with reference to the following non-limiting Examples and Figures in which:

Figure 1. Growth (A) and pH (B) over time in mLrCK1 *Lactococcus lactis* re-inoculated from overnight culture at start OD 0.285 and 0.51 with addition of 10 or 50 ng/ml promoter activation peptide SppIP.

Figure 2. Expression of pLAB112_Luc in *Lactobacillus reuteri* R2LC re-inoculated from overnight culture at start OD 0.5 *in vitro* measured by bioimaging over time. A baseline image at time 0 was acquired. Promoter activation peptide SppIP (50 ng/ml) and substrate D-Luciferin (150 µg/ml) were added immediately after. The plate

was imaged at 5 minutes and then every 30 minutes for 1400 minutes. Media used in all samples is MRS. Peptide is promoter activation peptide SpplP. Each group consists of eight samples.

Figure 3. Expression of pLAB112_Luc in *Lactobacillus reuteri* R2LC re-inoculated from overnight culture to start OD 0.5 applied on 1 day old cutaneous full thickness wounds. *In vivo* expression measured by non-invasive bioimaging over time. A baseline image at time 0 was acquired on 5 anesthetized mice with 1 day old cutaneous full thickness wounds. Then 25 µl *Lactobacillus reuteri* R2LC_ pLAB112_Luc activated with promoter activation peptide SpplP (50 ng/ml) and substrate D-Luciferin (150 µg/ml) was added to the middle of the wounds and mice were imaged at 5 minutes and then every 15 minutes for 270 minutes.

Figure 4. Time to wound healing in healthy mice. Time to 50 % (A), 75 % (B) or complete (100 %) (C) healed wound surface, n=5 all groups. A, B, C, One-way ANOVA, Bonferroni compare all columns.

Figure 5. Wound size (A) and wound exposure (B) over time in healthy mice. Wound size measured daily from images with a scale included, n=5 all groups. A, Two-way ANOVA, Bonferroni compare all columns, d0-d5 analyzed. Change due to time and treatment. Decreased wound size by R2LC_pLAB112_LrCK1.4 at d1 and d2 compared to Controls. B, One-way ANOVA, Bonferroni compare all columns, all days analyzed. Decreased wound exposure by R2LC_pLAB112_LrCK1.4 for the whole healing process.

Figure 6. Ischemia induction by femoral artery ligation prior to wound induction, n=4 in all groups. Cutaneous blood flow measured in ischemic limb (A) and the contralatheral corresponding unaffected limb (B) of anesthetized mice over time using Laser Speckle Contrast Analysis. Data is expressed in perfusion unites (PFU). A and B, Two-way ANOVA, Bonferroni compare all columns, d0-d7 analyzed. No change is observed due to time or treatment.

Figure 7. Time to wound healing in mice with ischemia at the time of wound induction. Time to 50 % (A), 75 % (B) or complete (100 %) (C) healed wound surface, n=4 all groups. A, B, C, One-way ANOVA, Bonferroni compare all columns.

Figure 8. Wound size and wound exposure over time in mice with local ischemia at the time and location of wound induction. Wound size measured daily from images with a scale included, n=4 all groups. A, Two-way ANOVA, Bonferroni compare all columns, d0-d7 analyzed. Change due to time and treatment. Decreased wound size R2LC_pLAB112_LrCK1.4 at d1 and d2 compared to Controls. B, One-way ANOVA, Bonferroni compare all columns, all days analyzed. Decreased wound exposure by R2LC_pLAB112_LrCK1.4 for the whole healing process.

Figure 9. Body weight (A) and blood glucose (B) following induction of diabetes using a single i.v. injection of alloxan monohydrate. Diabetic Controls, n=4, Diabetic R2LC_pLAB112_Luc, n=5, Diabetic R2LC_pLAB_LrCK1.4, n=4. A and B, Two-way ANOVA, Bonferroni compare all columns, d0-d6 analyzed. No change was observed due to time or treatment.

Figure 10. Time to wound healing in mice with diabetes at wound induction. Time to 50 % (A), 75 % (B) or complete (100 %) (C) healed wound surface, Diabetic Controls, n=4, Diabetic R2LC_pLAB112_Luc, n=5, Diabetic R2LC_pLAB_LrCK1.4, n=4. A, B, C, One-way ANOVA, Bonferroni compare all columns.

Figure 11. Wound size and wound exposure over time in mice with diabetes at wound induction. Wound size measured daily from images with a scale included, Diabetic Controls, n=4, Diabetic R2LC_pLAB112_Luc, n=5, Diabetic R2LC_pLAB_LrCK1.4, n=4. A, Two-way ANOVA, Bonferroni compare all columns, d0-d6 analyzed. Change due to time. B, One-way ANOVA, Bonferroni compare all columns, all days analyzed. No diff. (p=0.08).

Figure 12. The pSIP411 plasmid.

Figure 13. Quantification of plasmid expression in dermis in wound edge (40 µg DNA) using detection of luminescent signal by non-invasive bioimaging (IVIS Spectrum) over 11 days (n=10).

Figure 14. Time to wound healing in healthy mice. Time to 50 % (A), 75 % (B) or complete (100 %) (C) healed wound surface (n=8 pCTR, n=9 pCXCL12). A, B, C, Students unpaired two-tailed t-test.

Figure 15. Wound size (A) and wound exposure (B) over time in healthy mice. Wound size measured daily from images with a scale included (n=8 pCTR, n=9 pCXCL12). (A) Two-way ANOVA, Bonferroni compare all columns, d0-d7 analyzed. Change due to time. (B) Students two-tailed unpaired t-test. Tendency (p=0.08) to decreased wound exposure by pCXCL12 for the whole healing process.

Figure 16. Measurements of bacterial concentrations for *Lactobacillus reuteri* R2LC expressed as optical density (OD) and colony forming units per ml (CFU/ml).

Figure 17. Wound size (A) and wound exposure (B) over time in healthy mice treated with different concentrations of *Lactobacillus reuteri* R2LC_pLAB112_LrCK1.4. Wound size measured daily from images with a scale included. A, Two-way ANOVA, Bonferroni compare all columns, d0-d2 analyzed. Change due to time and treatment. (A) Two way ANOVA Bonferroni compare all columns, (B) One Way ANOVA Bonferroni compare all columns ($p<0.05$). Decreased wound exposure by treatment with *Lactobacillus reuteri* R2LC__pLAB112_LrCK1.4 at OD 0.2, 0.5, 1.0 and 1.25 as

compared to wound receiving no treatment. (Control, n=15; OD 0.2, n=4; 0.5, n=10, OD 1.0, n=4; OD 1.25, n=5).

Figure 18. Wound size (A) and wound exposure (B) over time in healthy mice treated with different concentrations of murine CXCL12 1α at one time point per day for two days. Wound size measured daily from images with a scale included. A, Two-way ANOVA, Bonferroni compare all columns, d0-d2 analyzed. Change due to time. B, wound exposure the first two days. (Control, n=15; 0.2 µg CXCL12 1α, n=4; 0.6 µg CXCL12 1α, n=5, 1.0 µg CXCL12 1α, n=4).

Figure 19. Wound size (A) and wound exposure (B) over time in healthy mice treated with 0.2 µg recombinant protein every 10th minute for one hour every day. Wound size measured daily from images with a scale included. A, Two-way ANOVA, Bonferroni compare all columns, d0-d2 analyzed. Change due to time. B, wound exposure the first two days. (No treatment, n=15; CXCL12 1α, n=6; CXCL17, n=9, Ym1, n=9).

Figure 20. Re-epithelialization measured in human skin epidermal punch biopsy wounds. Panel A shows pH measured in culture medium after 24 hours of culturing skin discs with epidermal wounds with no treatment or treatment with LB_Luc or LB_LrCK1. Panel B shows length of the newly formed epidermis sleeve growing from the wound edge over the exposed dermis after 14 days of culture. * indicates difference, One Way ANOVA Bonferroni compare selected columns ($p<0.05$).

Figure 21. *In vitro* expression of pLAB112_Luc in *Lactobacillus reuteri* R2LC immediately after revival from freeze-dried state measured *in vitro* by bioimaging over time. A baseline image at time 0 was acquired. Then promoter activation peptide SppIP (50 ng/ml) and substrate D-Luciferin (150 µg/ml) was added immediately after. The plate was imaged at 5 minutes and then every 5-15 minutes for 930 minutes. Media used in all samples is MRS. Peptide is promoter activation peptide SppIP. Each group consists of four samples.

Figure 22. *In vivo* expression of pLAB112_Luc in *Lactobacillus reuteri* R2LC immediately after revival from freeze-dried state and application on 1 day old cutaneous full thickness wounds measured *in vivo* by bioimaging over time. A baseline image at time 0 was acquired on three anesthetized mice with two separate 1 day old cutaneous full thickness wounds. Then 25 µl *Lactobacillus reuteri* R2LC_ pLAB112_Luc activated with promoter activation peptide SppIP (50 ng/ml) and substrate D-Luciferin (150 µg/ml) was added to the middle of the wounds and mice were imaged at 5 minutes and then every 15 minutes for 270 minutes.

Figure 23. Wound size (A) and wound exposure (B) over time in healthy mice treated with freeze-dried, revived and induced *Lactobacillus reuteri*

R2LC_pLAB112_LrCK1.4. Wound size measured daily from images with a scale included. (A) Two-way ANOVA, Bonferroni compare all columns, d0-d2 analyzed. Change due to time and treatment. (B) One Way ANOVA Bonferroni compare all columns ($p<0.05$). Decreased wound size was observed following treatment with *Lactobacillus reuteri* R2LC_pLAB112_LrCK1.4 compared to *Lactobacillus reuteri* R2LC_pLAB112_Luc also when the bacteria had been freeze-dried and directly revived, induced and applied to wounds (R2LC_pLAB112_Luc, n=4, R2LC_pLAB112_LrCK1.4, n=5).

Figure 24. Wound size (A) and wound exposure (B) over time in healthy mice. Wound size was measured daily from images with a scale included. The change is due to time and treatment and there is a trend towards decreased wound size by CXCL12 1α in pH of 6.35 compared to suspension with pH 7.35 ($p=0.07$) (pH 7.35; n=8, pH 6.35; n=5, pH 5.35; n=4). One-way ANOVA, Bonferroni compare all columns.

Figure 25. Wound size (A) and wound exposure (B) over time in healthy mice. Wound size measured daily from images with a scale included. The observed change was only due to time and did not differ between the two different bacterial suspensions (R2LC_pLAB112_Luc; n=4, R2LC_pLAB112_LrCK1; n=5). Student's two-tailed unpaired t-test.

Figure 26. Measurements of CXCL12 1α levels sections of the skin just next to the wound two days post wound induction in dermis (A), epidermis (B) and hair follicles (C) where the wounds were treated with *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 at OD 0.5, 1.0, and OD 1.25. One-way ANOVA, Bonferroni compare all columns.

Figure 27. Measurements of density of $F4/80^+$ macrophages in dermis (A) and epidermis (B) in the skin next to the wound two days following wound induction in control wounds and wounds treated with *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 at OD 0.5, 1.0 and OD 1.25. (Control, n=15; 0.5, n=10, OD 1.0, n=4; OD 1.25, n=5). One-way ANOVA, Bonferroni compare all columns.

Figure 28. Time to wound healing in healthy mice. Wounds were treated with *Lactococcus Lactis* transformed with pLAB112 (L.L_pLAB112_LrCK1) or control *Lactococcus Lactis*. Time to 50 % (A), 75 % (B) or complete (100 %) (C) healed wound surface, n=5 both groups. Student's two-tailed unpaired t-test.

Figure 29. Wound size (A) and wound exposure (B) over time in healthy mice. Wound size measured daily from images with a scale included, n=5 both groups. The change is due to time and treatment and wound size is decreased by L.L_pLAB112_LrCK1 at d1 to d4 compared to control *Lactococcus Lactis*. Student's two-tailed unpaired t-test.

Figure 30. Time to wound healing in healthy mice treated with recombinant chemokines for one hour. Time to 50 % (A), 75 % (B) or complete (100 %) (C) healed wound surface (Control; n=11, mCXCL12 1α; n=6, mCXCL17; n=8, mYm1; n=9). One-way ANOVA, Bonferroni compare all columns.

Figure 31. Wound size (A) and wound exposure (B) over time in healthy mice treated with recombinant chemokines for one hour. Wound size measured daily from images with a scale included (Control; n=11, mCXCL12 1α; n=6, mCXCL17; n=8, mYm1; n=9). The change is due to time and treatment and wound size is decreased by CXCL12 1α, CXCL17 and Ym1 compared to Control. One-way ANOVA, Bonferroni compare all columns.

Figure 32. Wound closure during the 24 first hours in healthy mice with no or different treatments. (No treatment, n=15; 0.2 µg CXCL12 1α, n=4; 0.6 µg CXCL12 1α, n=5; 1.0 µg CXCL12 1α, n=4; 0.2 µg CXCL12 1α 1hr, n=6; 0.2 µg CXCL17 1hr, n=9, 0.2 µg Ym1 1hr, n=9; R2LC_pLAB112_Luc OD 0.5, n=4; R2LC_ pLAB112_LrCK1.4 OD 0.2, n=4; R2LC_pLAB112_LrCK1.4 OD 0.5, n=10, R2LC_pLAB112_LrCK1.4 OD 1.0, n=4; R2LC_pLAB112_LrCK1.4 OD 1.25; n=5, Freeze-dried R2LC_pLAB112_Luc, n=4, Freeze-dried R2LC_pLAB112_LrCK1.4, n=5, R2LC_pLAB112_Luc supernatant; n=4, R2LC_pLAB112_LrCK1.4 supernatant, n=5, pCTR n=8; pCXCL12, n=9). No statistical analyses have been performed on this dataset.

Figure 33. Assessment of DSS-induced disease activity daily (A) and total disease burden, day 1-7 (B). Similar amelioration of DSS-induced colitis disease activity by treatment with *Lactobacillus reuteri* pLAB112_Luc and pLAB112_LrCK1.4 (DSS+Vehicle; n=5, DSS+R2LC_pLAB112_Luc; n=6, DSS+R2LC_pLAB112_LrCK1.4; n=7) as compared to the control group treated with vehicle, One-way ANOVA, Bonferroni compare all columns.

Figure 34. Assessment of DSS-induced disease activity daily (A) and total disease burden, day 1-8 (B). Disease activity was assessed measuring relevant clinical symptoms as described earlier (Ref. 16). Arrow indicates start of treatment. Amelioration of DSS-induced colitis disease activity by treatment with *Lactobacillus reuteri* pLAB112_LrCK1.4 compared to treatment with pLAB112_Luc (DSS+R2LC_pLAB112_Luc; n=6, DSS+R2LC_pLAB112_LrCK1.4; n=6), Student's two-tailed unpaired t-test.

Figure 35. Representative images of full thickness skin wounds (5 mm diameter) induced in healthy mice at time 0 and after 24 hours with no treatment, with R2LC Luc or R2LC LrCK1. Images are taken with a scale included in anesthetized mice.

EXAMPLES

MATERIALS AND METHODS

Gene construct design and production

The plasmid backbone pLAB112 (equal to pSIP411; Refs. 11 and 15; Table I) was provided by Professor Lars Axelsson (Norwegian Food Research Institute). *Lactococcus lactis* MG1363 bacteria was transformed with pLAB112 and expanded for 24 hours. The plasmid was then purified and the DNA product was verified on a gel.

Table I: Main features of pSIP411/pLAB112

Feature	Positions (SEQ ID NO: 20)
Replication determinant (replicon region)	260-2010
ermB (erythromycin resistance marker)	2342-2840
$P_{spp}IP$ (inducible promoter)	3139-3290
sppK (histidine protein kinase)	3305-4647
sppR (response regulator)	4653-5396
gusA (beta-glucuronidase)	5853-7658
$P_{orf}X$ (inducible promoter)	5689-5835
Transcriptional terminators	129-155; 5428-5460; 5602-5624
Multicloning sites	1-35; 5851-5856; 7662-7673

The sequence for murine CXCL12-1α was optimized for translation in *Lactobacillus reuteri* by Stefan Roos at the Swedish University of Agricultural Sciences (SLU) using DNA2.0 (Menlo Park, CA, USA). The optimized sequence (SEQ ID NO: 1) was synthesized by DNA 2.0 in plasmid vector pJ204. The sequences for human CXCL12-1α, murine CXCL17, human CXCL17, murine Ym1 and human Ym1 were optimized for translation in *Lactobacillus reuteri* by Stefan Roos at SLU using GenScript (Piscataway, NJ, USA). The optimized sequences are shown as SEQ ID NO: 4 (human CXCL12-1α); SEQ ID NO: 7 (murine CXCL17); SEQ ID NO: 10 (human CXCL17); SEQ ID NO: 13 (murine Ym1); and SEQ ID NO: 16 (human Ym1).

Primers were designed to detect the insert (hCXCL12opt), 171 bp in pLAB112:
5' GCAGCCTTAACAGTCGGCACCT3' (SEQ ID NO: 22);
5'ACGTGCAACAATCTGCAAAGCAC3' (SEQ ID NO: 23).

The ends of the insert were also optimized for continuing the molecular processing so the insert would fit in the new vector pLAB112. The optimized mCXCL12opt sequence was delivered in a plasmid PJ204. *E. coli* PK401 was

transformed with pJ204. Plasmids (pLAB112 and pJ204) were cleaved with the restriction enzymes *Xho*I and *Nco*I in NEB2 buffer. The fragment mCXCL12opt was then purified on a gel. The mCXCL12opt insert was then ligated into the pLAB112 vector using T4 DNA ligase, resulting in the construct mLrCK1. The insert construct in the pLAB112 vector was verified by PCR. The construct was then verified by sequence analysis (Macrogen). Finally *Lactobacillus reuteri* strain R2LC and DSM 20016 was transformed with mLrCK1 and two R2LC clones (4 and 7) positive for the construct were collected and the plasmid mLrCK1 (now mLrCK1.4 and mLrCK1.7) from these colonies were again verified by sequence analysis (Macrogen).

The plasmids hLrCK1, mLrCK2, hLrCK2, mLrMP1 and hLrMP2 were produced in an analogous way following the same protocol and procedure (See Table II below).

Table II: Overview of plasmids

Plasmid	**Description**
pLAB112	Identical with pSIP411 (Ref. 15 and SEQ ID NO: 20)
mLrCK1	pLAB112 with optimized mCXCL12-1α insert
mLrCK1.4	mLrCK1 from transformed *Lactobacillus reuteri* R2LC clone 4
mLrCK1.7	mLrCK1 from transformed *Lactobacillus reuteri* R2LC clone 7
hLrCK1	pLAB112 with optimized hCXCL12-1α insert
mLrCK2	pLAB112 with optimized mCXCL17 insert
hLrCK2	pLAB112 with optimized hCXCL17 insert
hLrMP1	pLAB112 with optimized human Ym1 insert
mLrMP2	pLAB112 with optimized murine Ym1 insert
pLAB112_Luc	pLAB112 with luciferase insert

In vitro analysis of plasmid expression

Lactobacillus reuteri R2LC pLAB112_Luc cultured overnight, re-inoculated and grown to OD 0.5 were plated (200 µl/well) on a 96 well plate or immediately resuspended from freeze-dried formulation. Luminescence intensity was determined using non-invasive bioimaging (IVIS Spectrum, Perkin Elmer). A baseline image at time 0 was acquired. Then activation peptide SppIP (50 ng/ml) and D-Luciferin (150 µg/ml) was added immediately after. The plate was then imaged at 5 minutes and then every 30th minute for 1400 minutes. Data was quantified using Living Image 3.1 software

(Perkin Elmer) and imaging parameters were maintained for comparative analysis. Radiance was considered proportional to plasmid expression.

Animals

Experiments were approved by Uppsala Regional Laboratory Animal Ethical Committee. Mice, C57Bl/6 (Taconic) and CX3CR1$^{+/GFP}$ on C57Bl/6 background (originally from The Jackson Laboratory) were used. Animals had free access to water and chow throughout experiments.

Wound induction

Mice were anesthetized (1-3 % isoflurane, 200 ml/min) and hair was removed on the hind limb by shaving and then by 1 min application of hair removal cream (Veet®) that were rinsed off with water. A sterile punch biopsy needle (5 mm diameter) was used to induce full-thickness (epidermis, dermis and subcutis) wounds. Local topical analgesic (Embla cream) was applied daily for the first 5 days.

Topical wound treatments

Wounds were treated daily with either 25 µl saline, Lactobacillus reuteri R2LC pLAB112_Luc or R2LC pLAB112_LrCK1. Bacteria was cultured overnight, re-inoculated and grown to OD 0.5, preactivated 5 min prior to application with activation peptide SppIP (50 ng/ml) and added topically to the middle of the wound surface. For dosing experiments wounds were treated daily for two days with either 25 µl saline or Lactobacillus reuteri R2LC pLAB112_LrCK1 re-inoculated from overnight culture and grown to OD 0.5, preactivated 5 min prior to application with activation peptide SppIP (50 ng/ml) and added topically to the middle of the wound surface at concentrations of OD 0.2, 0.5, 1.0 or 1.25. For comparative experiments with the respective proteins wounds were treated daily with either 10 µl saline or murine CXCL12, CXCL17 or Ym1 (total of 200 ng protein in 60 µl saline given in 10 min intervals for one hour). For a dose escalation study of CXCL12 200 ng, 600 ng or 1µg was added to the wound in 10 µl saline at one time point once per day.

In vivo analysis of plasmid expression

Lactobacillus reuteri R2LC pLAB112_Luc were cultured overnight, re-inoculated and grown to OD 0.5. Luminescence intensity was determined using non-invasive bioimaging (IVIS Spectrum, Perkin Elmer). A baseline image at time 0 was acquired. Then 25 µl *Lactobacillus reuteri* R2LC pLAB112_Luc was added in the middle of the wound. Bacteria was preactivated 5 min prior to application with activation peptide SppIP (50 ng/ml) and D-Luciferin (150 µg/ml). Mice were the imaged every 15th minute for 270 minutes. Data was quantified using Living Image 3.1 software (Perkin Elmer) and imaging parameters were maintained for comparative analysis. Radiance was considered proportional to plasmid expression.

Wound size and appearance monitoring

The size and appearance of the wounds were monitored daily in anesthetized mice (1-3% isoflurane, 200 ml/min) by acquisition of conventional photos. A scale was included in the image at acquisition and wound size was analyzed using ImageJ (Free software from NIH). Wounds were considered healed when <0.5 mm^2 in size.

Cutaneous blood flow monitoring

Blood flow in the whole hind limb with the healing wound was measured in anesthetized (1-3% isoflurane, 200 ml/min) mice using noninvasive Laser Speckle Contrast Analysis and data was analyzed, PIMSoft 3 (Perimed). Limbs (Frame 1.4 x1.4 cm) were imaged for 2 minutes at 10 images/s with averaging by 20. Data is expressed in perfusion units (PFU).

Reduction of perfusion

Mice were anesthetized (1-3 % isoflurane, 200 ml/min) and hind limb ischemia was induced by ligation and excision of the femoral artery above the superficial epigastric artery branch.

Induction of hyperglycemia

A single dose of alloxan monohydrate (8 mg/ml, 1µl/g body weight) immediately dissolved in sterile saline was injected in the tail vein. Blood glucose and body weight was monitored daily throughout the experiment. Hyperglycemia was defined as blood glucose >16.7 mmol/l.

Statistical analysis

Data are presented as mean ± SEM. Two-Way ANOVA with Bonferroni compare all columns post hoc test was used analyzing the healing process over time. One-Way ANOVA with Bonferroni compare all columns post hoc test was used analyzing the healing process at one time point in groups of n>2 and Students two-tailed unpaired t-test was used analyzing the healing process at one time point when n=2. $p<0.05$ was considered statistically significant.

EXAMPLE 1: Growth of bacteria transformed with plasmid LrCK1

Lactococcus lactis with mLrCK1 cultured overnight, re-inoculated and grown to OD 0.3 or 0.5 showed no growth impairment when the activation peptide SppIP (SEQ ID NO: 19) were added at either 10 or 50 ng/ml. During these growth experiments pH was measured and the lowering was most accentuated in the growth phase and then stabilized around pH 6.7 when grown is Mes-medium (Figure 1). (pH of skin=5,5, pH in wounds=7.15-8.9 where alkaline pH correlates with lower healing rate (Ref. 14))

EXAMPLE 2: Expression of plasmid pLAB112_Luc

In vitro expression of plasmid pLAB112_Luc in *Lactobacillus reuteri* R2LC re-inoculated and grown for 2 hours from overnight culture remained high for more than 600 minutes (10 h.). There was no leakage/expression from plasmids not activated with activation peptide SppIP (Figure 2).

When *Lactobacillus reuteri* R2LC with pLAB112_Luc re-inoculated and grown for 2 hours from overnight culture were placed in 1 day old cutaneous full thickness wounds of anesthetized mice, bacteria was restricted to the wound site and plasmid expression was high for the first hour but signal was detected for more than 4 hours (Figure 3).

EXAMPLE 3: Improved wound healing in healthy mice

Wounds were monitored daily during the healing process. In healthy mice daily single application of *Lactobacillus reuteri* R2LC_pLAB112_mLrCK1.4 reduced time to both 75 % wound surface closure and to complete (100 %) wound closure compared to control mice where nothing was applied to the wound and to mice where control *Lactobacillus reuteri* R2LC (pLAB112_Luc) was applied daily (Figure 4). The effect of *Lactobacillus reuteri* R2LC_pLAB112_mLrCK1.4 on wound healing was most prominent during the first days post wound induction. Wound size was then further reduced by daily application (one and two days post wound induction) of *Lactobacillus reuteri* R2LC_pLAB112_mLrCK1.4 when compared to control mice where nothing was applied to the wound. The total wound exposure measured as area under curve was also reduced in this group compared to control mice where nothing was applied to the wound (Figure 5). Figure 35 shows representative images of full thickness skin wounds (5 mm diameter) induced in healthy mice at time 0 and after 24 hours with no treatment, with R2LC Luc or R2LC LrCK1.

EXAMPLE 4: Improved wound healing in healthy mice having impaired tissue perfusion

Cutaneous perfusion was reduced by 50% at the day of wound induction by ligation of the femoral artery in the limb where the wound was induced (Figure 6 and Table III). In mice with ischemia, daily single application of *Lactobacillus reuteri* R2LC_pLAB112_mLrCK1.4 resulted in reduced time to both 50% and 75% wound surface closure compared to control mice where nothing was applied to the wound as well as to mice where control *Lactobacillus reuteri* R2LC (pLAB112_Luc) was applied daily (Figure 7). Also in mice with reduced cutaneous perfusion the effect of *Lactobacillus reuteri* R2LC_pLAB112_mLrCK1.4 on wound healing was most prominent during the first days post wound induction, and wound size were reduced by

daily application of *Lactobacillus reuteri* R2LC_pLAB112_mLrCK1.4 at one and two days post wound induction compared to control mice where nothing was applied to the wound. The total wound exposure was also reduced in this group compared to control mice where nothing was applied to the wound (Figure 8).

Table III: Basal skin perfusion measured by Laser Speckle Contrast Analysis in anesthetized mice. Data is expressed as Mean±SEM in perfusion units (PFU), n=4 all groups.

	Healthy	Ischemic	Reduction (%)
Control	62.5±4.3	34.0±1.8	46
R2LC_pLAB112_Luc	57.3±2.7	31.3±1.1	46
R2LC_pLAB112_LrCK1.4	65.0±7.2	30.8±0.4	52

EXAMPLE 5: Improved wound healing in hyperglycemic mice

Mice were rendered diabetic using alloxan, where after they remained hyperglycemic (>16.7 mmol/l) during the process of wound healing and did not lose weight (Figure 9). In mice with diabetes, daily single application of *Lactobacillus reuteri* R2LC_pLAB112_LrCK1.4 reduced time to 75 % wound surface closure compared to control mice where nothing was applied to the wound and to mice where control *Lactobacillus reuteri* R2LC (pLAB112_Luc) was applied daily (Figure 10). There was a trend (p=0.08) towards reduced wound exposure in diabetic mice by daily application of *Lactobacillus reuteri* R2LC_pLAB112_mLrCK1.4 compared to daily application of *Lactobacillus reuteri* R2LC with Luc and control mice where nothing was applied to the wound (Figure 11).

EXAMPLE 6: CXCL12 dermal overexpression in the wound edge dermis transfection with plasmid encoding CXCL12

Plasmids were constructed on the pVAX1 backbone with CMV promoter (SEQ ID NO: 24) (V260-20, Invitrogen, Waltham, MA, USA), and either insert -copGFP-T2A-Luc2- referred to as pCTR (SEQ ID NO: 25) or -CXCL12-P2A-copGFP-T2A-Luc2- referred to as pCXCL12 (SEQ ID NO:26) was introduced as previously described (Ref. 18). The secretion sequence for CXCL12 was substituted for the murine IgG secretory sequence. Thus, pCTR plasmids encode GFP (Green Fluorescent Protein) and

luciferase but no chemokines. Plasmids (40 µg in a total volume of 100 µl saline) were injected in the dermis in four locations in the wound edge. Transgene expression was measured over time based on luciferase activity following intraperitoneal injection of D-Luciferin (150 mg/kg, #122796, Perkin Elmer, Waltham, MA, USA) 10 min prior to anesthesia and image acquisition using a bioimaging device (IVIS Spectrum, Perkin Elmer). Data was quantified using Living Image 3.1 software (Perkin Elmer) and imaging parameters were maintained for comparative analysis. Settings were also maintained selecting region of interest where the contralateral reference area was subtracted. Radiance was considered proportional to plasmid expression.

Plasmid expression from the dermis in the wound edge was measured using non-invasive bioimaging and correlated to light produced by the luciferase enzyme encoded by the plasmids equivalent to the expression of CXCL12. Expression peaked on day 2 and then declined as the wound was closing and the dermis reconstituted (Figure 13). Overexpression of CXCL12 did not result in accelerated complete wound healing but lead to shorter time to closure of 75 % of the wound surface as compared to pCTR (Figure 14). Wound surface was decreased by pCXCL12 dermal expression as compared to pCTR day 4-6 post wound induction and dermis transfection (Figure 15). These results demonstrate that with CXCL12 delivered to the wound with this system there is not a dramatic effect the 24 first hours but rather a smaller effect at the later time points.

EXAMPLE 7: Dose-response *Lactobacillus reuteri* of topical treatment with Luc and LrCK1.

Lactobacillus was reinoculated from overnight culture and grown to OD 0.5 and then diluted or concentrated to OD 0.2, 0.5, 1.0 and 1.25 in MRS. The four different concentrations were diluted tenfold to 10^{-9} and 10 µl of every sample was plated on MRS agar with erythromycin and cultured in an anaerobic chamber overnight in at 37°C, 5% carbon dioxide overnight. Colonies on the plates were counted and concentration expressed as colony forming units per ml (CFU/ml).

For dosing experiments wounds were treated daily for two days with either 25 µl saline *or Lactobacillus reuteri* R2LC pLAB112_LrCK1.4 re-inoculated from overnight culture and grown to OD 0.5, preactivated 5 min prior to application with activation peptide SppIP (50 ng/ml) and added topically to the middle of the wound surface at concentrations of OD 0.2, 0.5, 1.0 or 1.25. In 25 µl OD of 0.5 there are $5x10^7$ bacteria ($2x10^9$ cfu/ml) meaning a dose span of 1000 times.

Bacterial concentration was measured by optical density and colony forming units per ml are displayed in Figure 16. The lowest dose (OD 0.2 equals $2x10^7$

bacteria) of R2LC__pLAB112_LrCK1.4 cultured and activated as before administered to the wound resulted in the smallest wound size after 24 hours and all four different concentrations resulted in significantly accelerated wound closure at 24 and 48 hours post wound induction (Figure 17 A) and thus resulted in decreased wound exposure to the first 48 hours as compare to wounds receiving no treatment (Figure 17 B). These results indicate that administration of a dose that is 10^3 higher (OD 1.25 equals $1x10^{10}$ bacteria) than the lowest dose giving the greatest effect also significantly accelerates wound healing the first 48 hours as compared to wounds receiving no treatment and to the same extent as the dose giving maximal wound closure. No signs of induced inflammation or other negative side effects were observed for wounds given the highest dose. The data show that even a low dose of *Lactobacillus reuteri* R2LC_LrCK1 accelerates wound healing.

EXAMPLE 8: Dose escalation of mCXCL12 1α protein as a topical treatment.

To investigate the effects of the dose of the mCXCL12 1α administered to the wound surface 0.2 µg, 0.6 µg or 1µg mCXCL12 1α (RnD Systems) was delivered to the wounds daily for two days in 10 µl saline. The administration was once per day.

Delivery of the mCXCL12 1α daily at one single time point per day did not accelerate wound healing for the first two days as compared to no treatment (Figure 18 A, B). These data shows that it is the continuous delivery of the CXCL12 1α that causes the accelerated wound healing since a total 0.2 µg mCXCL12 1α given every day for one hour in 10 min intervals accelerated healing the first 48 hours (Figure 18 and 19).

EXAMPLE 9: Re-epithelialization assay in human skin biopsies.

Sterile normal human skin was obtained from healthy white women having routine breast reduction at Uppsala University Hospital giving consent for donation. Samples were covered with physiological DMEM supplemented with 2 % bovine calf serum (Hyclone®, HyClone Laboratories, Logan USA) and transported to the laboratory under sterile conditions.

As previously described (Ref. 17), the subcutis was removed and remaining dermis and epidermis was cut using a 6 mm skin biopsy punch (Integra Miltex, York, PA, USA) and sterile scissors. In the center of each 6 mm diameter skin disc the epidermis was removed using a 3 mm skin biopsy punch and sterile scissors. Samples were then placed one by one in a sterile 24 well plate with the epidermal side up. All culture media (DMEM) was supplemented with BSA, 2 or 10 % and antibiotics (erythromycin Sigma Aldrich, Buchs, Swizerland at 10µg/ml). To maintain the nutrients

on the dermal side i.e. nutrients at the highest concentration on the dermal side of the skin, 0.5 ml medium was added to each well and medium was changed daily. At the same time as the change of medium 10^6 in 10 µl MRS *Lactobacilus reuteri* R2LC_Luc or *Lactobacilus reuteri* R2LC _LrCK1 were placed in the middle of the epidermal wound in the floating skin discs. The bacteria was inoculated and grown in MRS for 2-4 hours to be in the exponential phase. Samples were incubated at 37°C, 5% carbon dioxide, and 95% humidity for 14 days.

The specimens were cut through the middle and one half was fixated overnight in 4% formaldehyde, pH 7.38 and dehydrated through an ethanol-xylene series to finally be embedded in paraffin. Cross-sections (10 µm) starting from the part being at the center of the specimens, were mounted, deparaffinized, rehydrated, and stained with hematoxylin and eosin. Images were captured using Leica Leits Dmrb with a Leica DFC420 C camera and Plan Fluot 40x0.7 NA objective. Re-epitelialization or epidermis sleeve length was measured in images using ImageJ (NIH).

Adding *Lactobacillus* to the skin discs in culture lowered the pH of the culture medium when measured after 24 hours (Figure 20A). The epidermis on the edges of the induced wound in the skin discs was proliferating to cover the exposed dermis when 10 % FCS was present in the culture medium and there was almost no proliferation when skin discs were cultured in medium supplied with 2 % FCS after 14 days in culture (Figure 20B). No detrimental effects were macroscopically detected in the skin discs treated with R2LC pLAB112 Luc or R2LC pLAB112_LrCK1 and increased re-epithelialization was measured on wounds where the skin discs were treated with R2LC pLAB112_LrCK1 for 14 days (Figure 20B).

EXAMPLE 10: Functionality of bacteria after freeze-drying and revival

Different protocols and 35 different formulations for freeze-drying were tested and viability was measured for up to two months. Also a larger batch of freeze-dried *Lactobacillus reuteri* was produced in settings identical to large scale industrialized production and in accordance with good manufacturing practice. The freeze-dried samples from this batch have been analyzed for viability after storing for up to two months in temperatures ranging from -20 to 40 °C. Freeze-dried bacteria were revived by adding equivalent volume of water or MRS medium with SppIP (50ng/ml) and then analyzed immediately for expression *in vitro* and *in vivo* by plating them in a 96 well plate or applying them directly on 1 day old wounds as described above.

With the most promising formulation, viability was stable from directly after freeze-drying to analysis at two months measured on samples stored at +4°C. The viability was well within range of what is acceptable of freeze-dried bacteria currently

being sold as dietary supplements. Measuring the plasmid expression in freeze-dried *Lactobacillus reuteri* R2LC_pLAB112_Luc directly after resuscitation showed immediate induction of expression, which peaked at 450 minutes and then declined (Figure 21). After 24 hours (1440 minutes) there was no expression and no alive bacteria. When freeze-dried *Lactobacillus reuteri* R2LC_pLAB112_Luc were revived, induced with 50 ng/ml (SppIP) and immediately placed on cutaneous wounds of mice ($5x10^7$ per 25 µl), expression directly increased and was high for about one hour (Figure 22) in a similar pattern as was seen when adding fresh bacteria in growth phase in solution (Figure 3).

The effect on wound healing was tested where the freeze-dried bacteria ($5x10^7$ per 25 µl) was again revived, induced and immediately placed on cutaneous wounds of mice. The wounds were monitored every day for two days and the wounds treated with *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 showed accelerated healing compared to wounds treated with *Lactobacillus reuteri* R2LC_pLAB112_Luc (Figure 23) even with this protocol. These data show that the *Lactobacillus reuteri* R2LC_pLAB112 does not have to be precultured to the exponential growth phase in order to produce and deliver enough CXCL12 to accelerate wound healing *in vivo*.

EXAMPLE 11: pH dependent effects of chemokine signaling

Chemokines can appear as monomers, dimers or multimers either with itself or interacting with other chemokines (Ref. 22.). The different combinations and conformations induce different receptor signaling and thus different cell responses (Ref. 34). This is a new and unexplored area and the combination of possibilities is dependent on the local tissue microenvironment. Also local pH impacts on local macrophage function (Ref. 23).

For studies of pH dependent effects of chemokine potency, 0.2 µg CXCL12 1α was applied to wounds in 10 µl saline with pH 7.35, 6.35 or 5.35 daily for two days.

Altering the pH in the buffer containing the chemokines had an effect on the healing pattern of the treated wounds and there was a trend towards smaller wound size one day post wound induction when the CXCL12 were suspended in saline with pH of 6.35 compared to when the CXCL12 were suspended in saline with pH of 7.35 ($p=0.07$) (Figure 24). These data indicate that a pH of 6.35 potentiates the effect of recombinant CXCL12 applied to the wound surface in the aspect of inducing accelerated wound healing.

EXAMPLE 12: Importance of bacterial on site chemokine delivery to the wound surface for effect

For wound treatment with fresh supernatants *Lactobacillus reuteri* R2LC_pLAB112_Luc and R2LC_pLAB112_LrCK1 were inoculated in 10 ml MRS in 37 °C and grown to OD 0.5, centrifuged (>2000 rpm, 5 minutes), resuspended in 1 ml MRS, activated (SppIP, 50 ng/ml) and grown for 4 hours. Samples were then centrifuged (>2000 rpm, 5 minutes) and the supernatant was saved. 25 µl of this supernatant was then applied to wounds once daily for two days.

The importance of bacterial delivery of CXCL12 1α directly to the wound surface by the *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 was demonstrated in a model where fresh supernatants from induced *Lactobacillus reuteri* were added to the wounds following wound induction every day for two days. There was no difference in wound size or total wound exposure (p=0.2595) of treatment with fresh supernatants from *Lactobacillus reuteri* R2LC_pLAB112_Luc or R2LC_pLAB112_LrCK1 (Figure 25).

EXAMPLE 13: *Lactobacillus* delivered CXCL12 increases levels of CXCL12 in the skin surrounding the wound

For quantitative analysis the skin surrounding the wound (0-100 µm from the wound) was removed on the last day of experiments and snap frozen in liquid nitrogen and sectioned (10 µm). After fixation in ice cold methanol (10 min) and permeabilization in 0.5 % Triton-X (15 min) tissues were incubated with antibodies targeting CXCL12 1α (polyclonal, Abcam) and macrophage antigen F4/80 (clone BM8, eBioscience) washed and incubated with matching secondary antibodies conjugated to Alexa Fluor488 and Nordic Lights 557 (Invitrogen). Tissues were finally washed and mounted (Fluoromount, #0100-10, Southern Biotech, Birmingham, AL, USA) before imaging using a line-scanning confocal microscope (Zeiss LSM 5 Live, with a piezo motor-controlled WPlanApo 40x/1.0 with 0.5 optical zoom, Zeiss, Oberkochen, Germany). Protein levels and macrophages were quantified in images using ImageJ (NIH) and IMARIS software 8.2 (Bitplane, Zurich, Switzerland). Microscope settings were maintained during acquisition to allow comparison. Values for CXCL12 1α measurements are presented as mean fluorescent intensity (MFI).

Treatment of wounds once daily for two days with *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 in different doses resulted in increased skin tissue levels of CXCL12 1α in the skin just next to the wound compared to in the skin next to wounds receiving no treatment (Figure 26) and this was true for both dermis, epidermis and in hair follicles.

EXAMPLE 14: *Lactobacillus* delivered CXCL12 increases macrophages in the skin surrounding the wound

Treatment of wounds once daily for two days with *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 in different doses resulted in increased density of F4/80+ macrophages in dermis just next to the wound two days post wound induction when *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 at OD 0.2 and OD 0.5 were applied to the wound compared to the dermis next to wounds receiving no treatment (Figure 27A). F4/80+ macrophages were increased in the epidermis next to the wound two days post wound induction when *Lactobacillus reuteri* R2LC_pLAB112_LrCK1 were given to the wound surface at OD 1.25 as compared to the epidermis next to wounds receiving no treatment (Figure 27B).

EXAMPLE 15: Verification of effect on acceleration of wound healing using *Lactococcus Lactis*

To show that the local and continuous delivery of the specific chemokine produced by the bacteria is important for the mechanism irrespectively of bacterial strain, another strain was used to produce and deliver the chemokine directly to the wound surface, *Lactococcus Lactis* was transformed with pLAB112 (mLrCK1). Bacteria were applied once daily to full thickness wounds in healthy mice following the same protocol as described for treatment with using *Lactobacuillus reuteri*.

There is a clear trend that mCXCL12 1α delivery accelerates wound closure (Figure 28) and reduces wound size and exposure in this model (Figure 29).

EXAMPLE 16: Moderate effects on time to wound closure by treatment with mCXCL12 1α, mCXCL17 and mYm1 delivered as recombinant proteins

To show that the mode of delivery and continuous protein production enabled by the lactic acid bacteria is important for the mechanism, murine recombinant mCXCL12 1α, mCXCL17, mYm1 (total of 200ng in 60 µl, all RnD Systems) or saline (10µl) as control was delivered to the wound once daily every 10th minute for one hour.

For mCXCL12 1α, delivery of 30 ng into the peritoneal cavity induces significantly increased recruitment of immune cells in 3 hours, why 200 ng to an area of 25 μm^2 is to be considered a high dose.

It is likely that the high protease activity in the wound degrades the chemokines when given as recombinant protein at one single time point, and thus the de novo production by the bacteria is required for the protein to enhance wound closure. In addition, the lactic acid bacteria might also provide a beneficial local environment for wound healing (Figures 1B, 4, 5, 24, 30 and 31).

EXAMPLE 17: Comparison of the effects of different treatments on wound closure in healthy mice.

Wound closure during the 24 first hours in healthy mice was analyzed for all the different treatments performed (Figure 32). It is clear that treatment with *Lactobacillus reuteri* R2LC_Luc or low single doses of CXCL12 1α administered to the wound at one time point affects the healing during the 24 first hours. Though there is a trend that CXCL12 1α, CXCL17 and Ym1 delivered to the wound surface every 10th minute for one hour accelerates the wound closure during the first 24 hours and this effect is even more clear when the CXCL12 1α is delivered continuously for one hour to the wound surface by *Lactobacillus reuteri* R2LC_LrCK1. Delivering the CXCL12 via dermal overexpression rather have a detrimental effect on 24 hour wound closure.

EXAMPLE 18: Acceleration of wound healing also on mucosal surfaces by *Lactobacillus reuteri* with pLAB112_mLrCK1.4

To test if the local continuous delivery of CXCL12 to a wounded surface works through a global mechanism on both skin epithelium and intestinal epithelium, two experimental protocols of DSS-induced colitis was used. DSS (dextran sulfate sodium) is known to induce wounds in the mucosal surface of the colon (Ref. 16).

For the first protocol, mice were treated with *Lactobacillus reuteri* by gavage (1ml OD 0.5 spun and resuspended in 0.1 ml) once daily for 14 days while DSS was given in the drinking water day 7-14. Since this strain of *Lactobacillus reuteri* colonizes in the colon using this protocol the aim is to assess if presence of *Lactobacillus reuteri* pLAB112_mLrCK1 in the colon is beneficial as compared to *Lactobacillus reuteri* pLAB112_Luc when colitis is induced.

The second protocol aimed at treating manifest colitis, and mice were given DSS in the drinking water day 1-8 while receiving *Lactobacillus reuteri* by gavage three times daily at day 5-8.

The severity of colitis was assessed daily on the basis of clinical parameters including weight loss, stool consistency and blood content, and presented as Disease Activity Index (DAI), a scoring method described in detail by Cooper and coworkers (Ref. 16).

There was similar amelioration of DSS-induced colitis disease activity by pretreatment with *Lactobacillus reuteri* pLAB112_Luc and pLAB112_LrCK1.4 (Figure 33) indicating the effect is only due to the *Lactobacillus reuteri*.

In contrast, disease development was ameliorated when *Lactobacillus reuteri* pLAB112_LrCK1.4 was administered to colitic mice which was not observed for treatment with pLAB112_Luc (Figure 34) indicating effect of the delivered chemokine.

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5

SEQUENCES

Table IV: Summary of Sequence Listing

SEQ ID NO:	Description
1.	mLrCK1_opt DNA
2.	mLrCK1_opt protein
3.	mCXCL12 native protein
4.	hLrCK1_opt DNA
5.	hLrCK1_opt protein
6.	hCXCL12 native protein
7.	mLrCK2_opt DNA
8.	mLrCK2_opt protein
9.	mCXCL17 native protein
10.	hLrCK2_opt DNA
11.	hLrCK2_opt protein
12.	hCXCL17 native protein
13.	mYm1_opt DNA
14.	mYm1 protein
15.	mYm1 native protein
16.	hYm1_opt DNA
17.	hYm1 protein
18.	hYm1 native protein
19.	SppIP; activation peptide
20.	pSIP411 DNA
21.	pSIP411 protein
22.	PCR primer
23.	PCR primer
24.	pVAX1 DNA
25.	pCTR DNA insert
26.	pCXCL12 DNA insert

CLAIMS:

1. Lactic acid bacteria transformed with a plasmid which comprises a nucleotide sequence encoding a protein selected from the group consisting of CXCL12, CXCL17 and Ym1, wherein the bacteria are *Lactobacillus reuteri,* and wherein the nucleotide sequence is codon-optimised for expression in lactic acid bacteria.

2. The bacteria of claim 1, wherein said plasmid comprises a nucleotide sequence encoding a protein selected from:

(i) murine CXCL12-1α having an amino acid sequence as shown in SEQ ID NO: 3 or 2, or an amino acid sequence with at least 80% sequence identity thereto;

(ii) human CXCL12-1α having an amino acid sequence as shown in SEQ ID NO: 6 or 5, or an amino acid sequence with at least 80% sequence identity thereto;

(iii) murine CXCL17 having an amino acid sequence as shown in SEQ ID NO: 9 or 8, or an amino acid sequence with at least 80% sequence identity thereto;

(iv) human CXCL17 having an amino acid sequence as shown in SEQ ID NO: 12 or 11, or an amino acid sequence with at least 80% sequence identity thereto;

(v) murine Ym1 having an amino acid sequence as shown in SEQ ID NO: 15 or 14, or an amino acid sequence with at least 80% sequence identity thereto; and

(vi) human Ym1 as shown in SEQ ID NO: 18 or 17 or an amino acid sequence with at least 80% sequence identity thereto.

3. The bacteria according to claim 1 or claim 2, wherein the plasmid comprises one or more regulatory sequences which permit expression in lactic acid bacteria, wherein the regulatory sequences are obtained or derived from lactic acid bacteria.

4. The bacteria according to any one of claims 1 to 3, wherein expression of said protein is regulatable.

5. The bacteria according to any one of claims 1 to 4, wherein the plasmid comprises one or more nucleotide sequences encoding one or more of said proteins under the control of an inducible promoter.

6. The bacteria according to any one of claims 1 to 5 wherein the plasmid comprises an inducible promoter and regulatory elements from the nisin regulon, the sakacin A regulon or the sakacin P regulon of a lactic acid bacterium.

7. The bacteria according to claim 5 or claim 6, wherein the inducible promoter is the PorfX promoter from the sakacin P regulon.

8. The bacteria according to any one of claims 1 to 7, wherein the plasmid is derived from the plasmid designated pSIP411, wherein pSIP411 has the sequence of SEQ ID NO: 20.

9. The bacteria according to any one of claims 1 to 8, which comprises one or more nucleotide sequences selected from the group consisting of: a nucleotide sequence comprising the sequence of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, SEQ ID NO: 10, SEQ ID NO: 13, and SEQ ID NO: 16, or a nucleotide sequence having at least 80% sequence identity to any aforesaid sequence.

10. The bacteria according to any one of claims 1 to 9, wherein the bacteria are *Lactobacillus reuteri* strain R2LC.

11. A wound dressing comprising the bacteria according to any one of claims 1 to 10.

12. A pharmaceutical composition comprising the bacteria according to any one of claims 1 to 10 and at least one pharmaceutically acceptable carrier or excipient.

13. The pharmaceutical composition of claim 12, wherein the composition is a topical composition for administration to the skin.

14. Use of the bacteria according to any one of claims 1 to 10 for wound healing in a human or animal subject.

15. Use of the bacteria according to any one of claims 1 to 10 in the manufacture of a medicament for wound healing in a human or animal subject.

16. The bacteria according to any one of claims 1 to 10 for use in wound healing in a human or animal subject.

17. The use of claim 14 or claim 15, or the bacteria for use according to claim 16, wherein the wound is a cutaneous wound.

18. The use of claim 14 or claim 15, or the bacteria for use according to claim 16, wherein the wound is a mucosal wound.

19. The use of any one of claims 14, 15, 17 or 18, or the bacteria for use according to claim 16, wherein the bacteria are for administration directly to the wound site.

20. A kit for use in healing wounds, said kit comprising:

(i) lactic acid bacteria as defined in any one of claims 1 to 10, wherein said plasmid comprises a nucleotide sequence encoding said protein under the control of an inducible promoter capable of expressing the protein in lactic acid bacteria; and

(ii) an inducer for the promoter.

21. A medical device comprising the bacteria according to any one of claims 1 to 10.

22. The bacteria, wound dressing, pharmaceutical composition, use, kit or medical device of any one of claims 1 to 21 wherein the bacteria are freeze-dried.

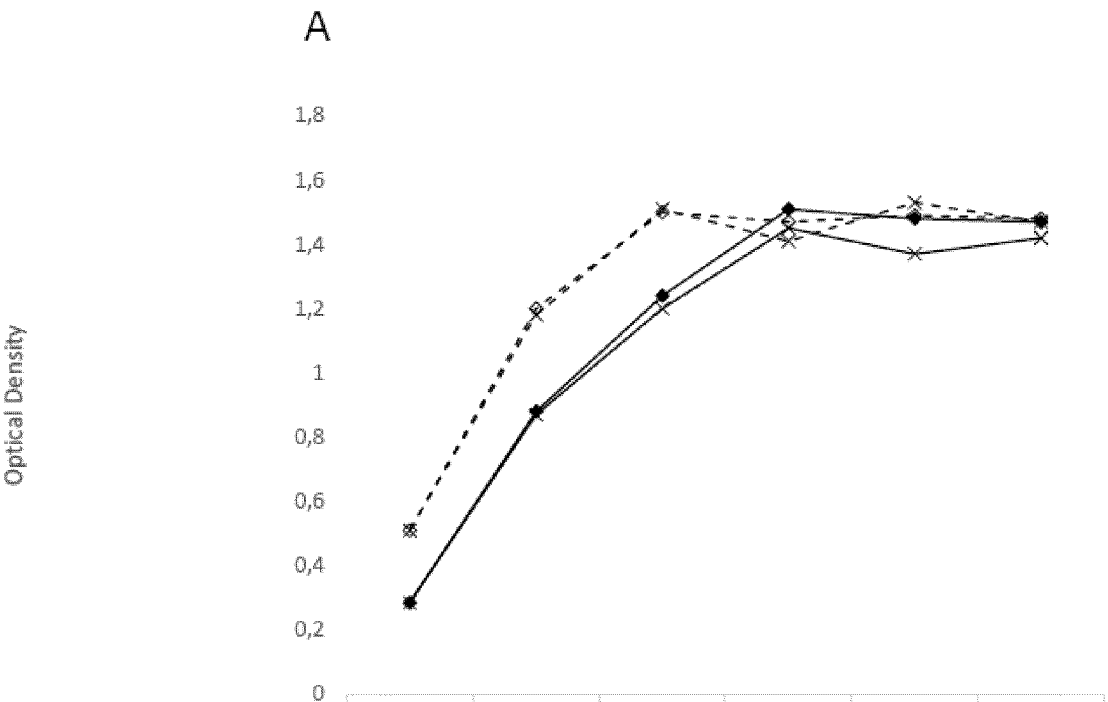
23. The kit of claim 22, wherein the kit further comprises a liquid for resuspending the freeze-dried bacteria.

24. The kit of claim 23, wherein the liquid comprises the inducer.

25. The kit of any one of claims 20, or 22 to 24, wherein the kit comprises a wound dressing comprising the bacteria.

26. The kit of any one of claims 20, or 22 to 24, wherein the kit further comprises a wound dressing.

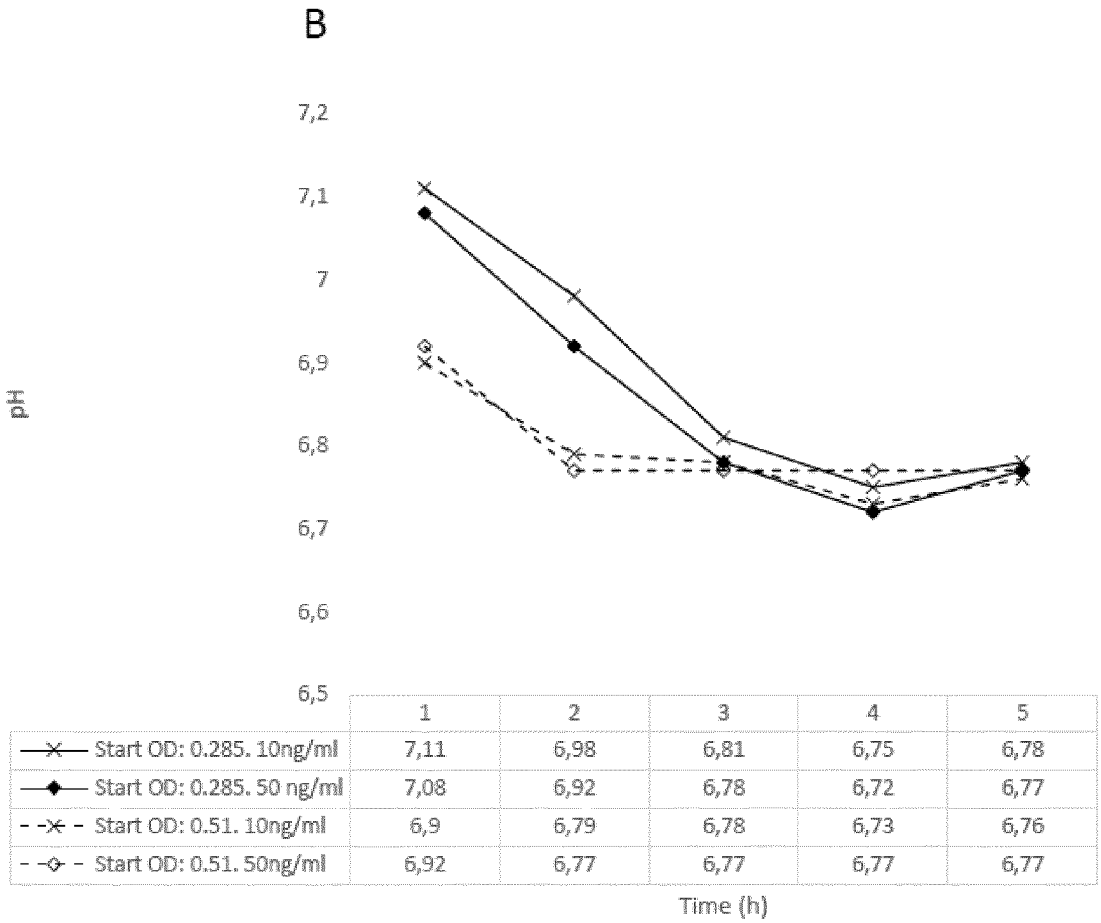
Fig. 1A



	0	1	2	3	4	5
Start OD 0.285. 10ng/ml	0,285	0,87	1,2	1,45	1,37	1,42
Start OD: 0.285. 50 ng/ml	0,285	0,88	1,24	1,51	1,48	1,47
Start OD: 0.51. 10ng/ml	0,51	1,18	1,51	1,41	1,53	1,47
Start OD: 0.51. 50ng/ml	0,51	1,2	1,5	1,47	1,49	1,48

Time (h)

Fig. 1B



	1	2	3	4	5
Start OD: 0.285. 10ng/ml	7,11	6,98	6,81	6,75	6,78
Start OD: 0.285. 50 ng/ml	7,08	6,92	6,78	6,72	6,77
Start OD: 0.51. 10ng/ml	6,9	6,79	6,78	6,73	6,76
Start OD: 0.51. 50ng/ml	6,92	6,77	6,77	6,77	6,77

Fig. 2

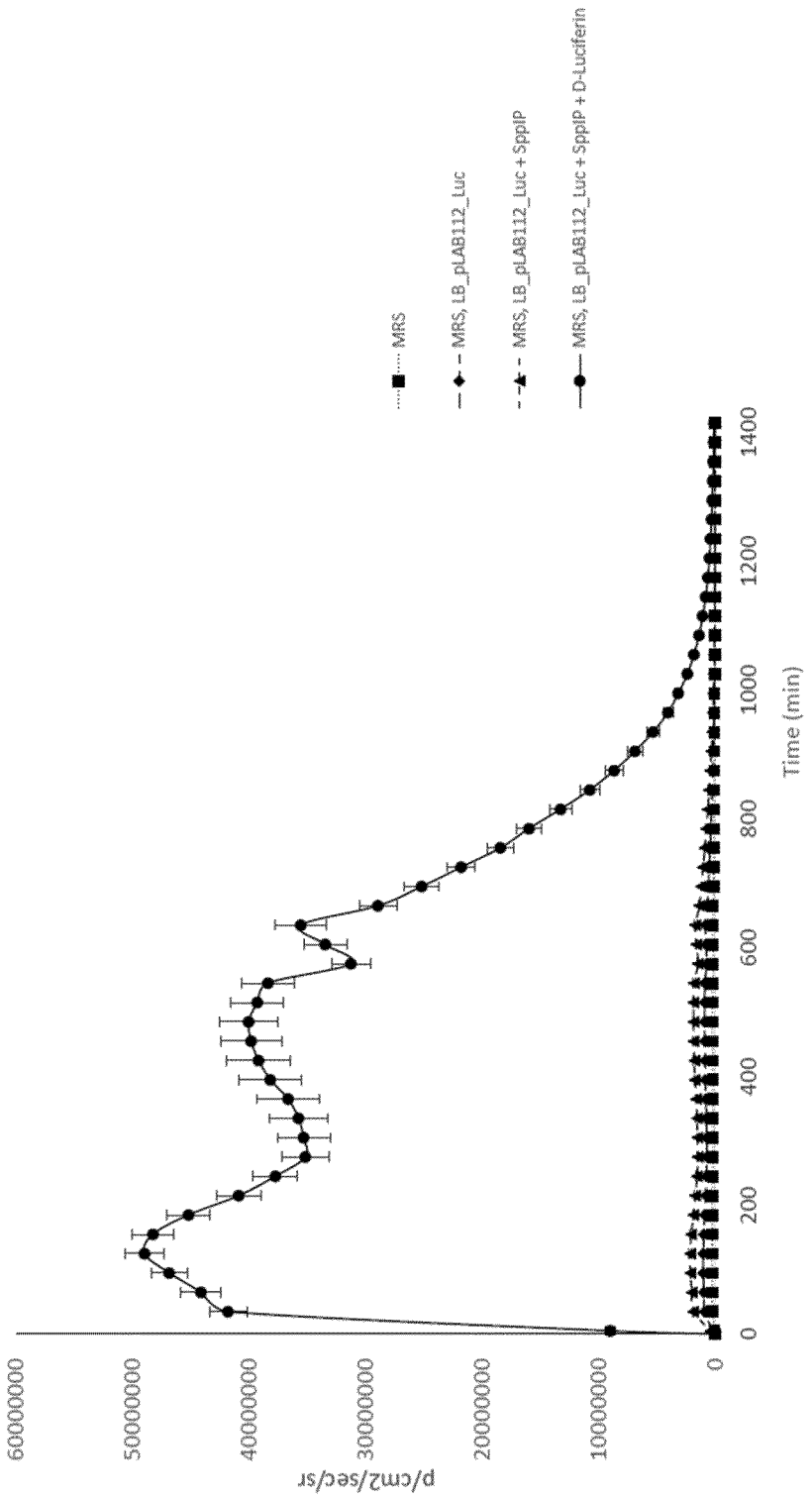

MRS
MRS, LB_pLAB112_Luc
MRS, LB_pLAB112_Luc + SppIP
MRS, LB_pLAB112_Luc + SppIP + D-Luciferin
p/cm2/sec/sr
60000000
50000000
40000000
30000000
20000000
10000000
0
0
200
400
600
800
1000
1200
1400
Time (min)

Fig. 3

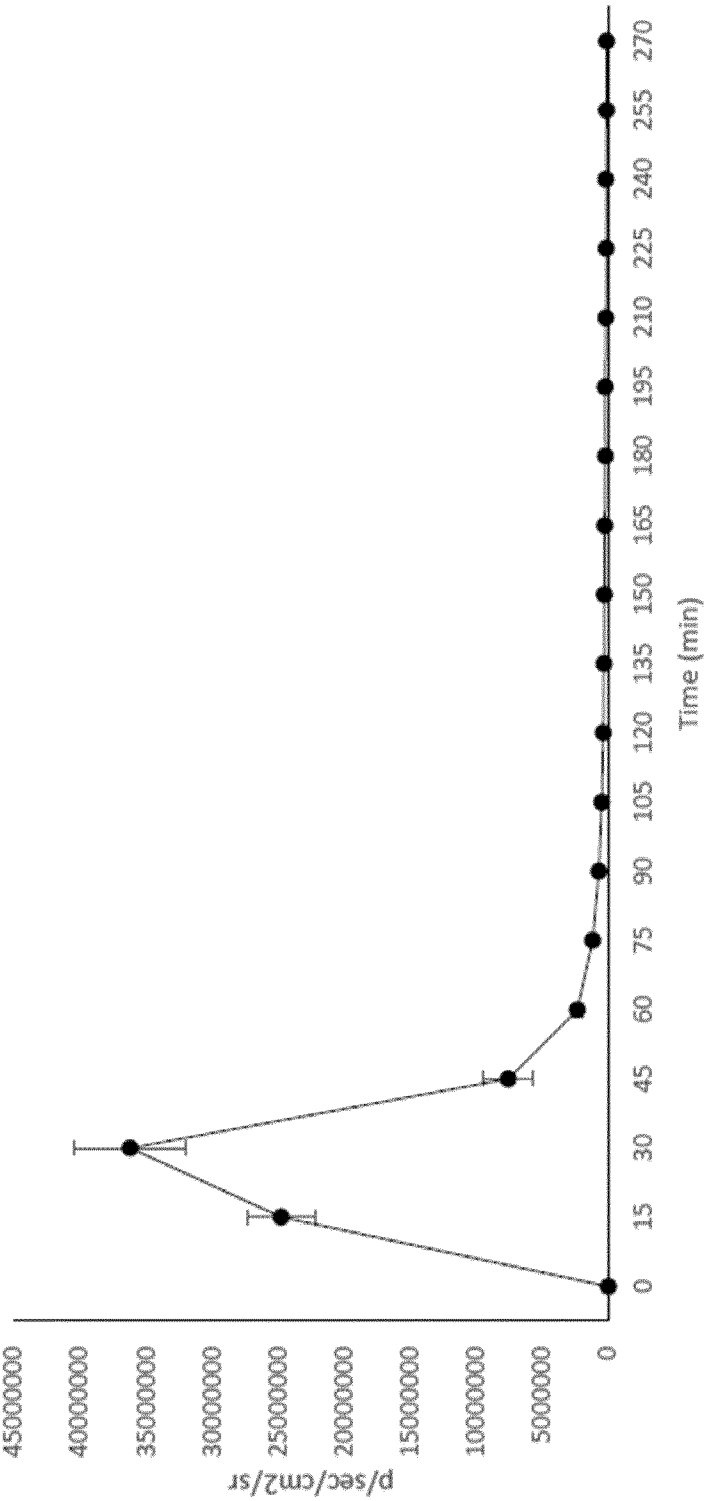

p/sec/cm2/sr
45000000
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
Time (min)
0
15
30
45
60
75
90
105
120
135
150
165
180
195
210
225
240
255
270

Fig. 4

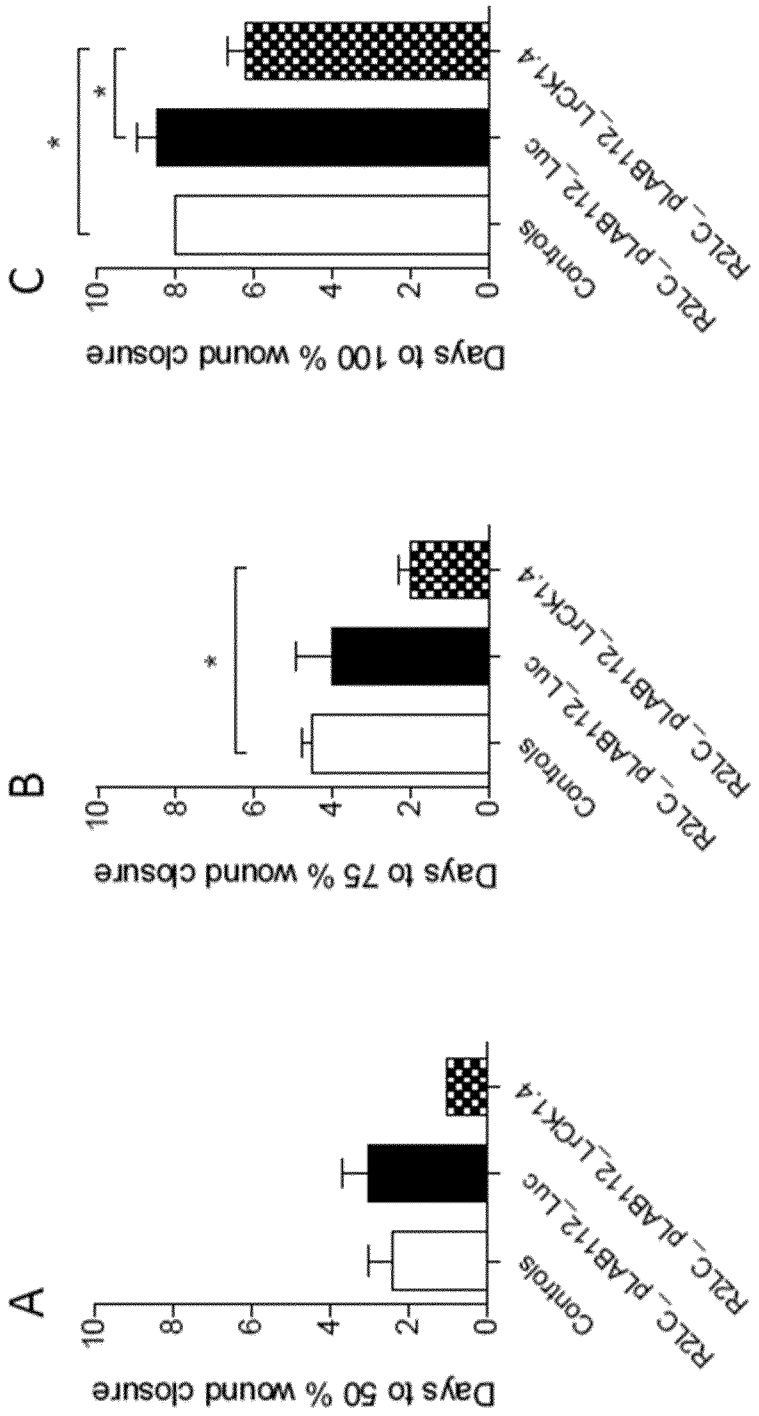

A
Days to 50 % wound closure
0
2
4
6
8
10
Controls
R2LC_pLAB112_Luc
R2LC_pLAB112_LrCK1.4
B
Days to 75 % wound closure
0
2
4
6
8
10
*
Controls
R2LC_pLAB112_Luc
R2LC_pLAB112_LrCK1.4
C
Days to 100 % wound closure
0
2
4
6
8
10
*
*
Controls
R2LC_pLAB112_Luc
R2LC_pLAB112_LrCK1.4

Fig. 5

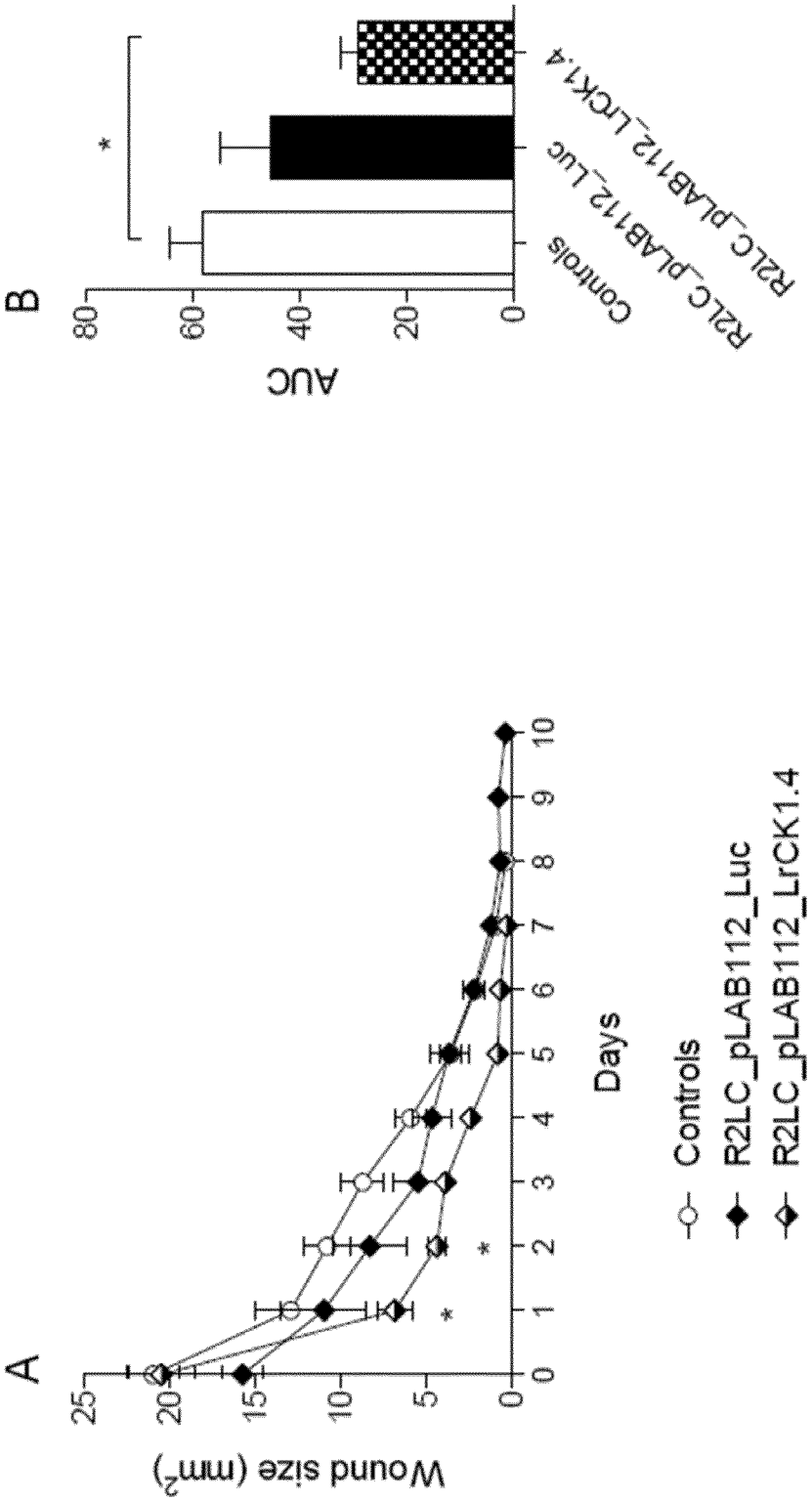

A
Wound size (mm²)
Days
Controls
R2LC_pLAB112_Luc
R2LC_pLAB112_LrCK1.4
B
AUC
Controls
R2LC_pLAB112_Luc
R2LC_pLAB112_LrCK1.4

Fig. 6

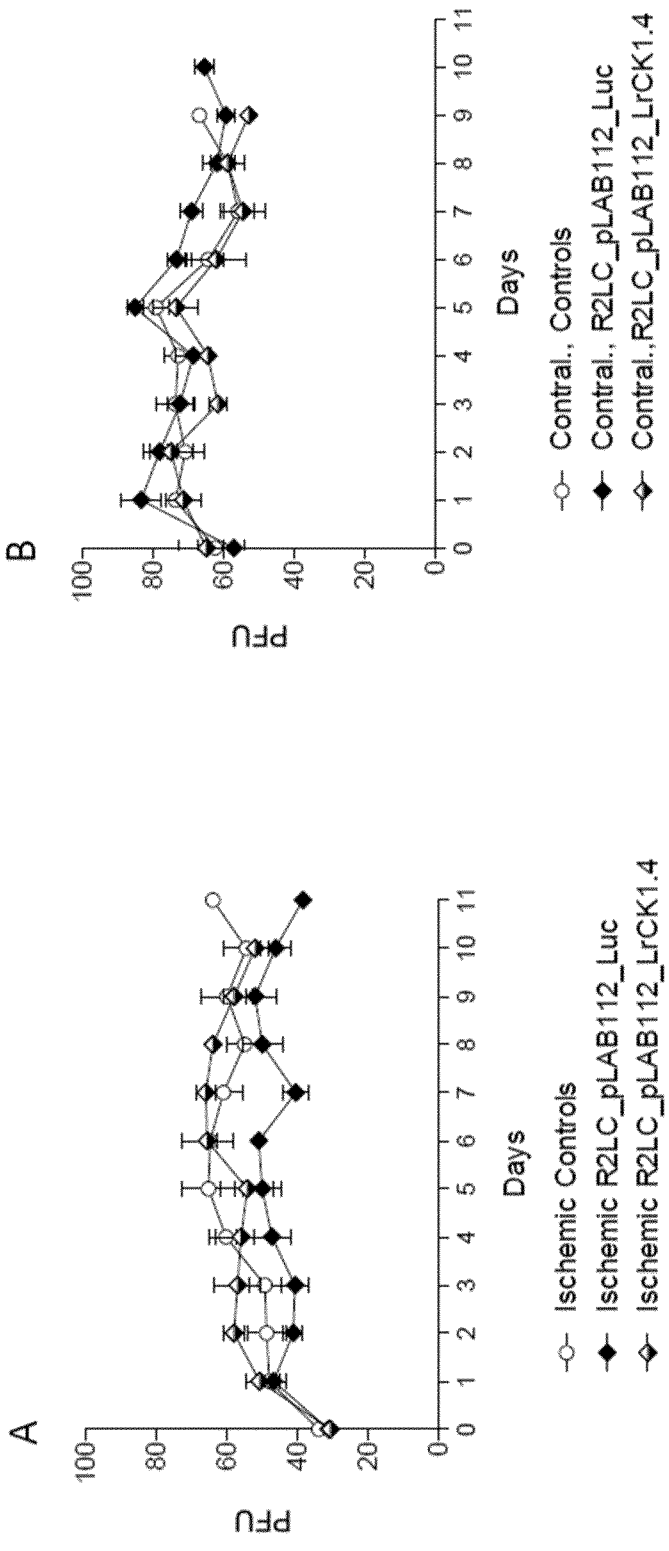

B
PFU
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11
Days
Contral., Controls
Contral., R2LC_pLAB112_Luc
Contral.,R2LC_pLAB112_LrCK1.4
A
PFU
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11
Days
Ischemic Controls
Ischemic R2LC_pLAB112_Luc
Ischemic R2LC_pLAB112_LrCK1.4

Fig. 7

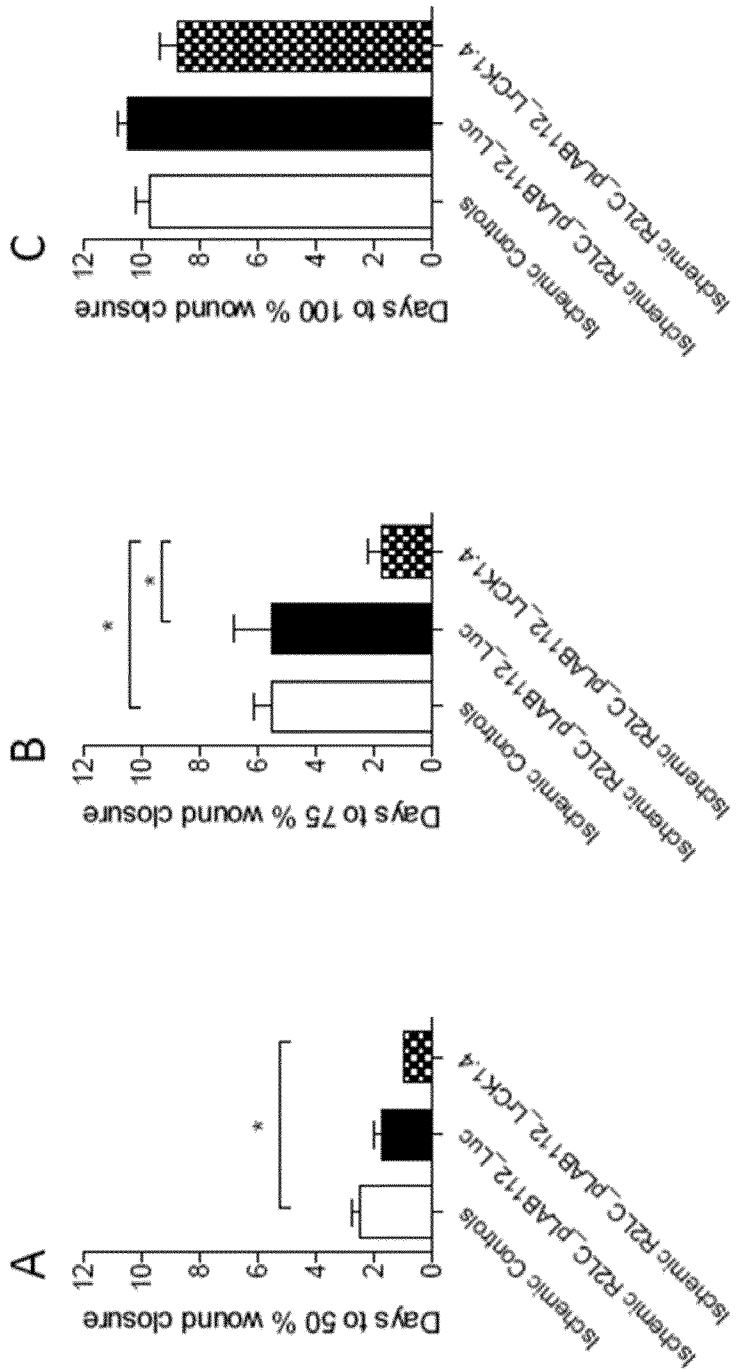

A
Days to 50 % wound closure
B
Days to 75 % wound closure
C
Days to 100 % wound closure
0
2
4
6
8
10
12
*
Ischemic Control
Ischemic R2LC_pLAB112_Luc
Ischemic R2LC_pLAB112_LrCK1.4

Fig. 8

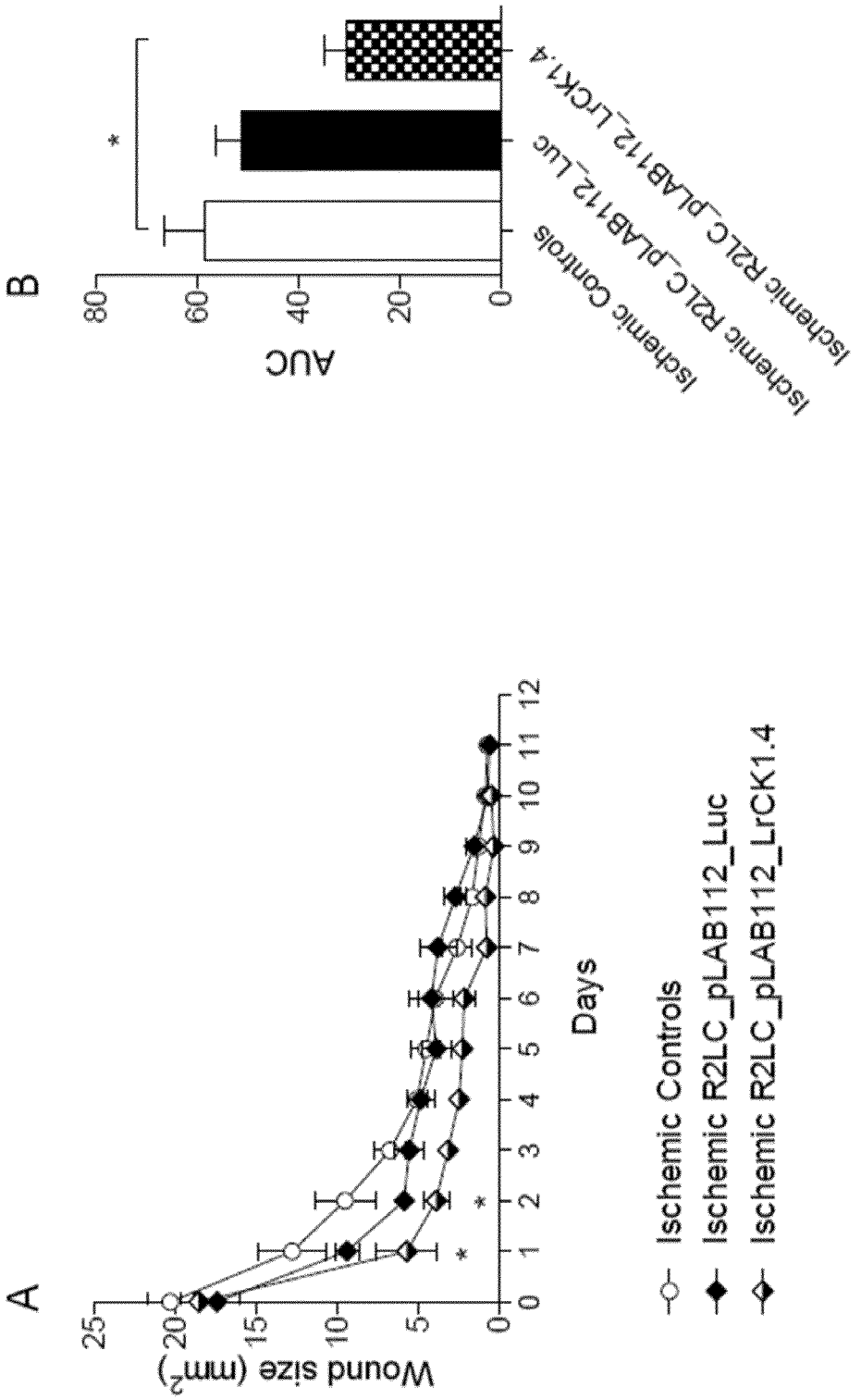

A
Wound size (mm²)
Days
Ischemic Controls
Ischemic R2LC_pLAB112_Luc
Ischemic R2LC_pLAB112_LrCK1.4
B
AUC
Ischemic Controls
Ischemic R2LC_pLAB112_Luc
Ischemic R2LC_pLAB112_LrCK1.4

Fig. 9

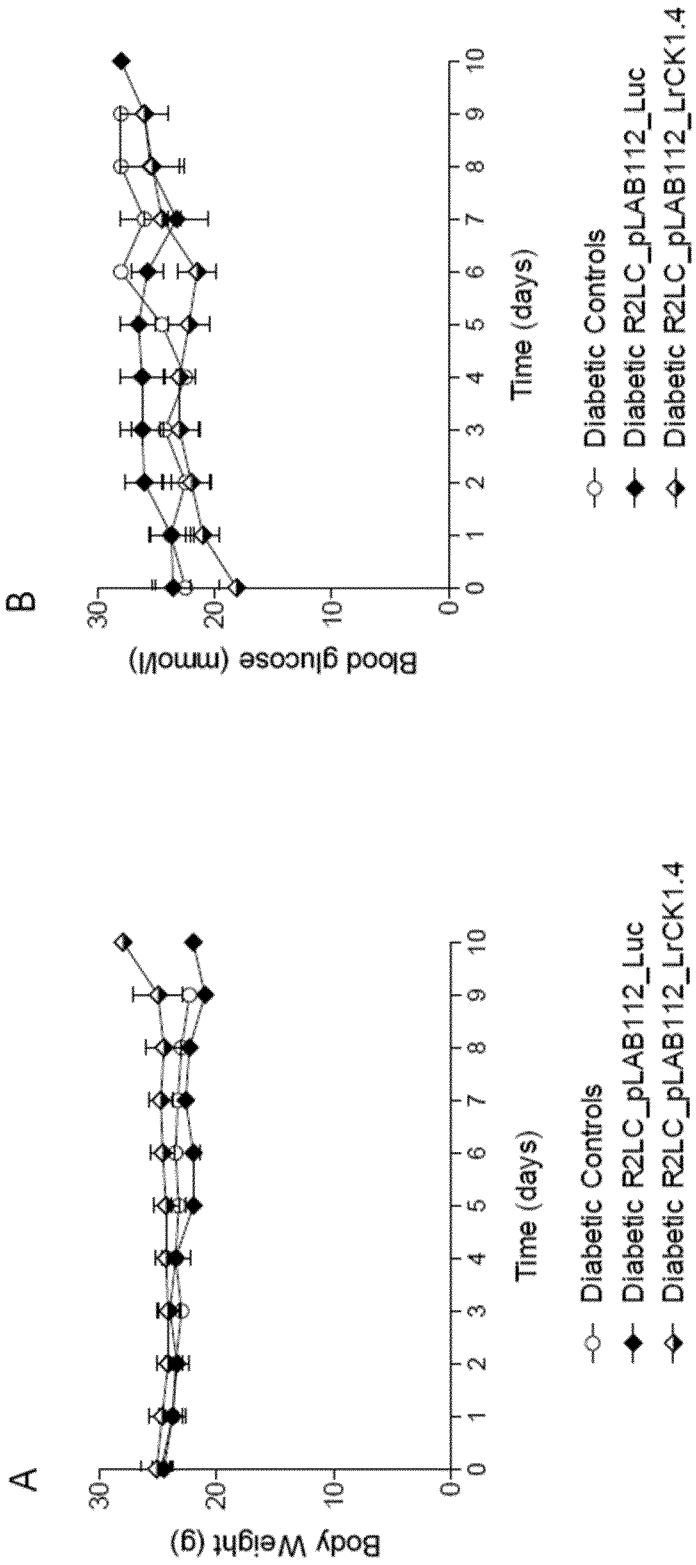

A
Body Weight (g)
0
10
20
30
0 1 2 3 4 5 6 7 8 9 10
Time (days)
Diabetic Controls
Diabetic R2LC_pLAB112_Luc
Diabetic R2LC_pLAB112_LrCK1.4
B
Blood glucose (mmol/l)
0
10
20
30
0 1 2 3 4 5 6 7 8 9 10
Time (days)
Diabetic Controls
Diabetic R2LC_pLAB112_Luc
Diabetic R2LC_pLAB112_LrCK1.4

Fig. 10

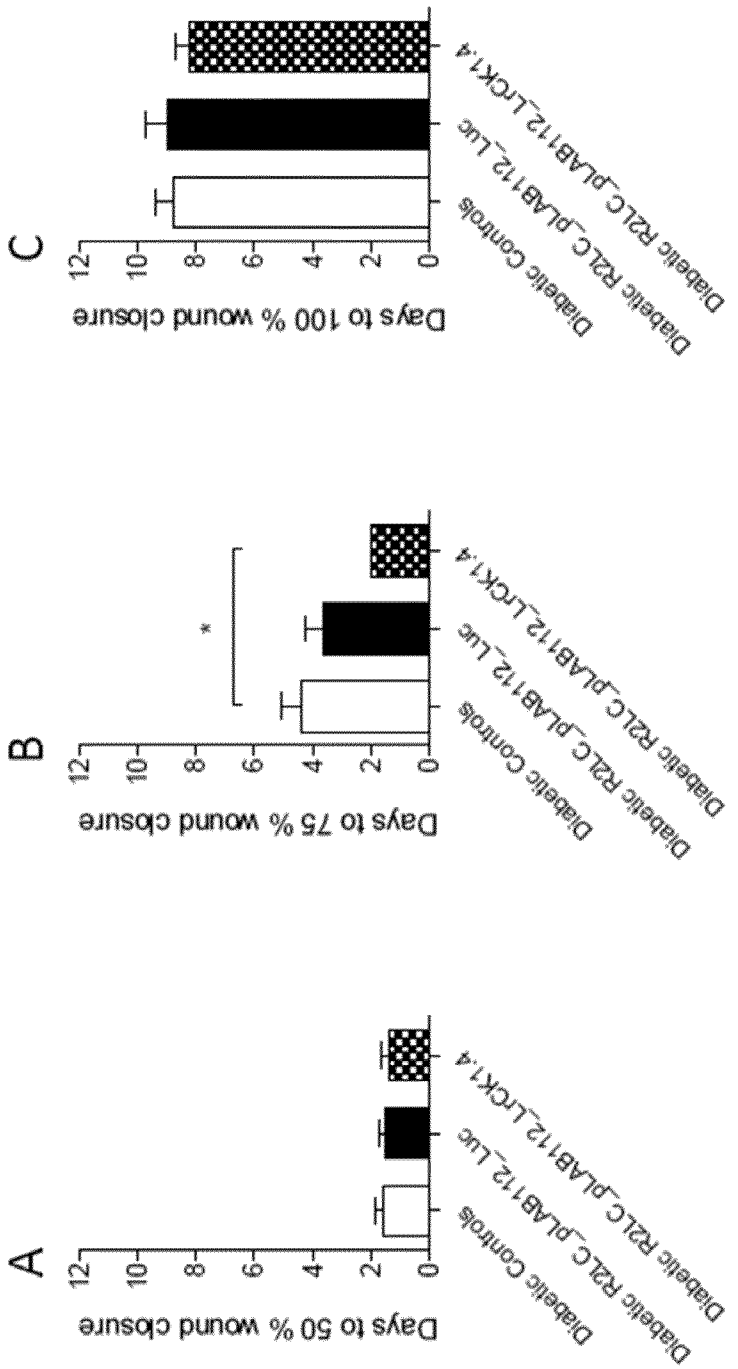

A
Days to 50 % wound closure
0
2
4
6
8
10
12
Diabetic Controls
Diabetic R2LC_pLAB112_Luc
Diabetic R2LC_pLAB112_LrCK1.4
B
Days to 75 % wound closure
*
C
Days to 100 % wound closure

Fig. 11

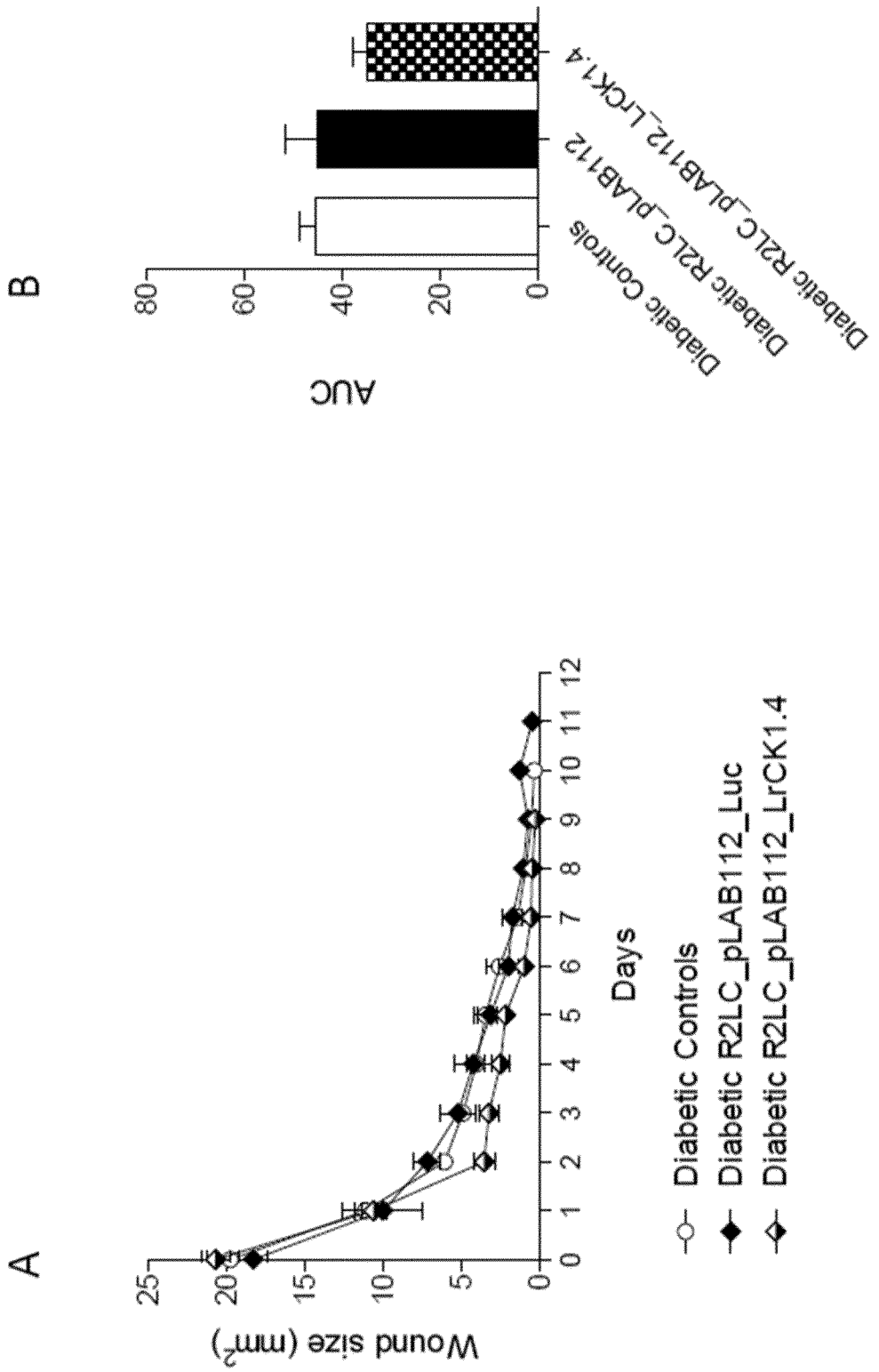

B
AUC
0
20
40
60
80
Diabetic Controls
Diabetic R2LC_pLAB112
Diabetic R2LC_pLAB112_LrCK1.4
A
Wound size (mm²)
0
5
10
15
20
25
0 1 2 3 4 5 6 7 8 9 10 11 12
Days
Diabetic Controls
Diabetic R2LC_pLAB112_Luc
Diabetic R2LC_pLAB112_LrCK1.4

Fig. 12

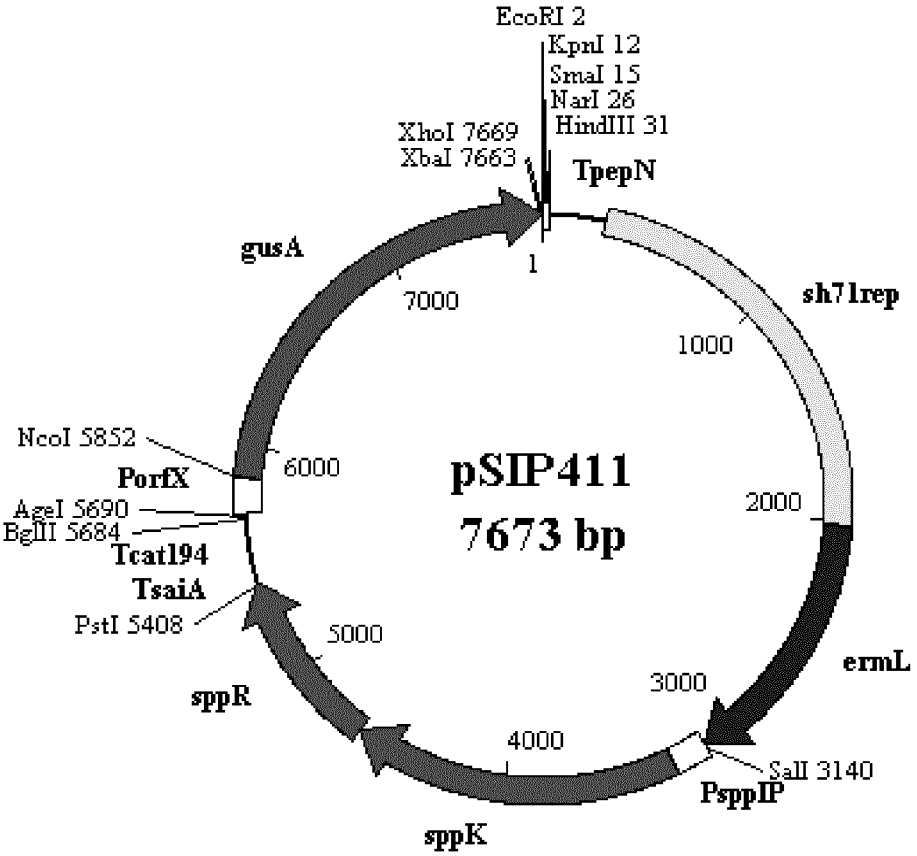

EcoRI 2
KpnI 12
SmaI 15
NarI 26
HindIII 31
XhoI 7669
XbaI 7663
TpepN
gusA
1
7000
1000
sh71rep
NcoI 5852
PorfX
6000
pSIP411
7673 bp
2000
AgeI 5690
BglII 5684
Tcat194
TsaiA
PstI 5408
5000
ermL
3000
sppR
4000
SalI 3140
PsppIP
sppK

Fig. 13

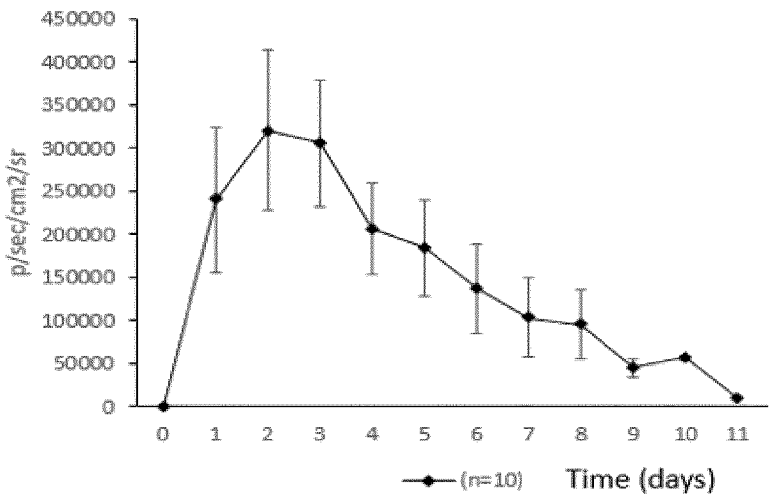

450000
400000
350000
300000
250000
200000
150000
100000
50000
0
p/sec/cm2/sr
0 1 2 3 4 5 6 7 8 9 10 11
(n=10)
Time (days)

Fig. 14

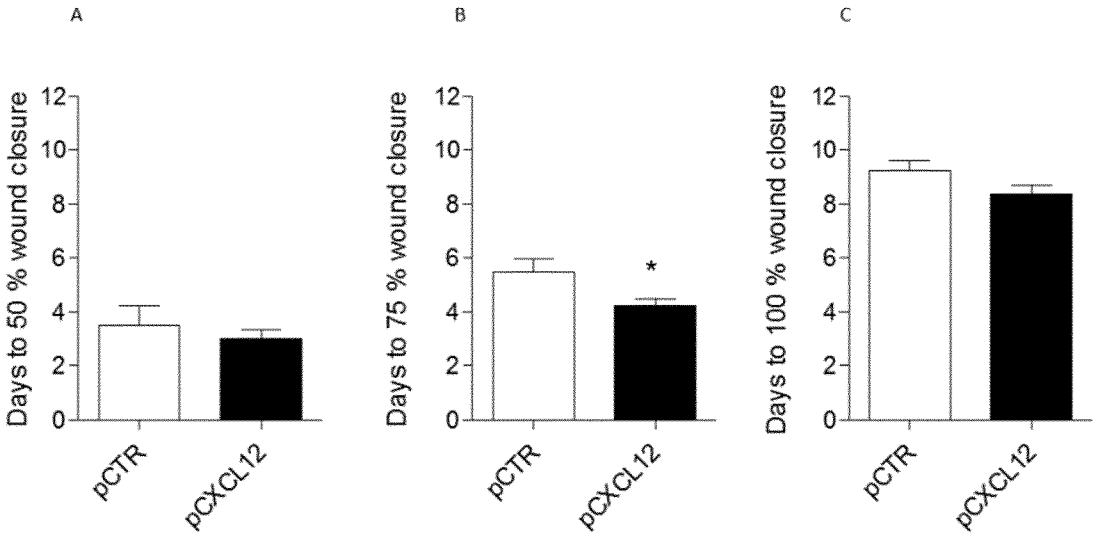

A
B
C
Days to 50 % wound closure
Days to 75 % wound closure
Days to 100 % wound closure
0
2
4
6
8
10
12
*
pCTR
pCXCL12

Fig. 15

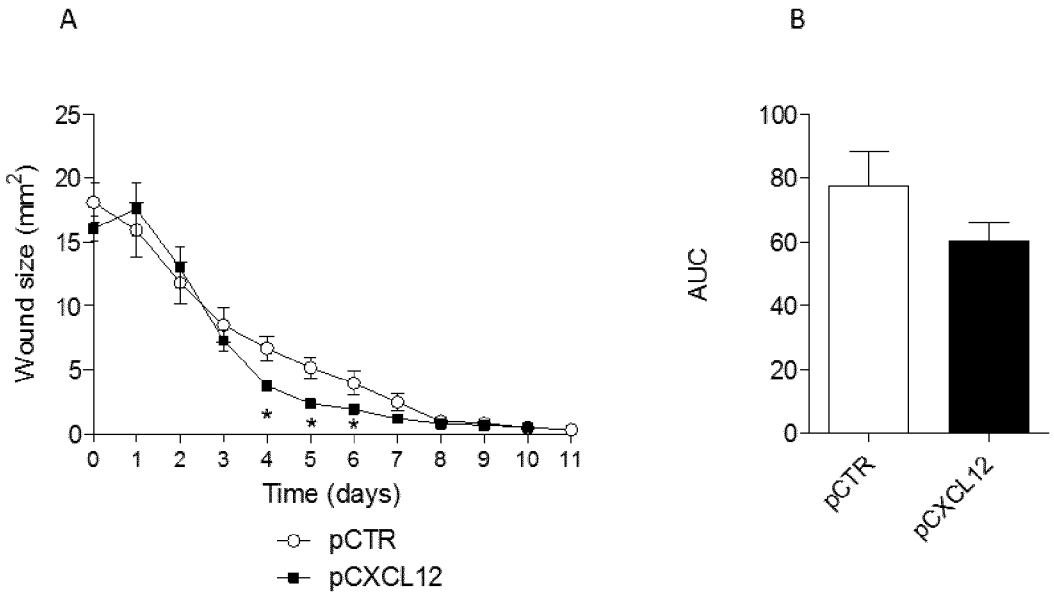

A
B
Wound size (mm2)
Time (days)
0
5
10
15
20
25
0 1 2 3 4 5 6 7 8 9 10 11
*
pCTR
pCXCL12
AUC
0
20
40
60
80
100
pCTR
pCXCL12

Fig. 16

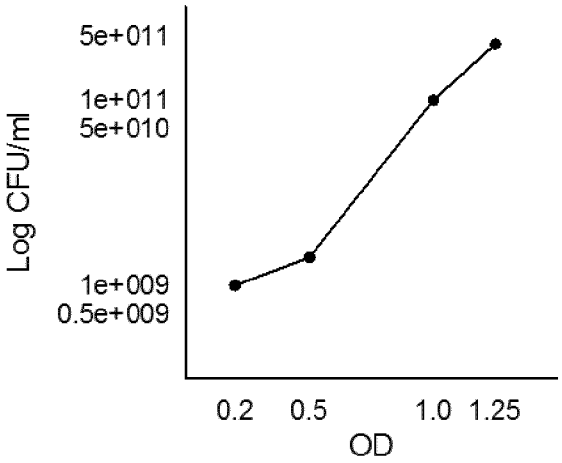

5e+011
1e+011
5e+010
1e+009
0.5e+009
Log CFU/ml
0.2
0.5
1.0
1.25
OD

Fig. 17

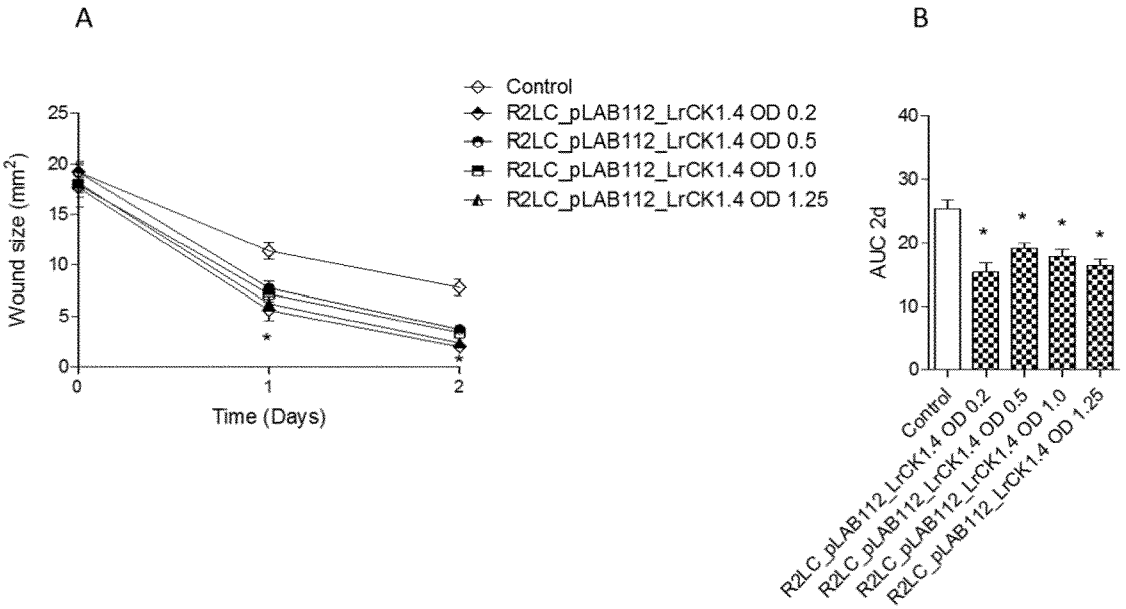

A
B
Control
R2LC_pLAB112_LrCK1.4 OD 0.2
R2LC_pLAB112_LrCK1.4 OD 0.5
R2LC_pLAB112_LrCK1.4 OD 1.0
R2LC_pLAB112_LrCK1.4 OD 1.25
Wound size (mm2)
Time (Days)
AUC 2d

Fig. 18

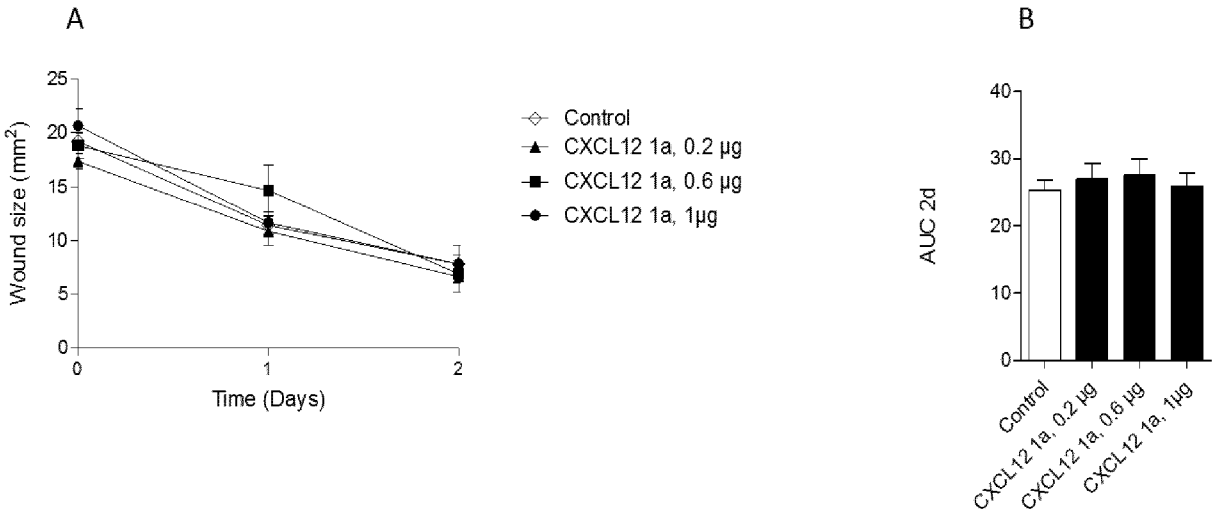

A
B
Wound size (mm2)
Time (Days)
Control
CXCL12 1a, 0.2 µg
CXCL12 1a, 0.6 µg
CXCL12 1a, 1µg
AUC 2d
Control
CXCL12 1a, 0.2 µg
CXCL12 1a, 0.6 µg
CXCL12 1a, 1µg

Fig. 19

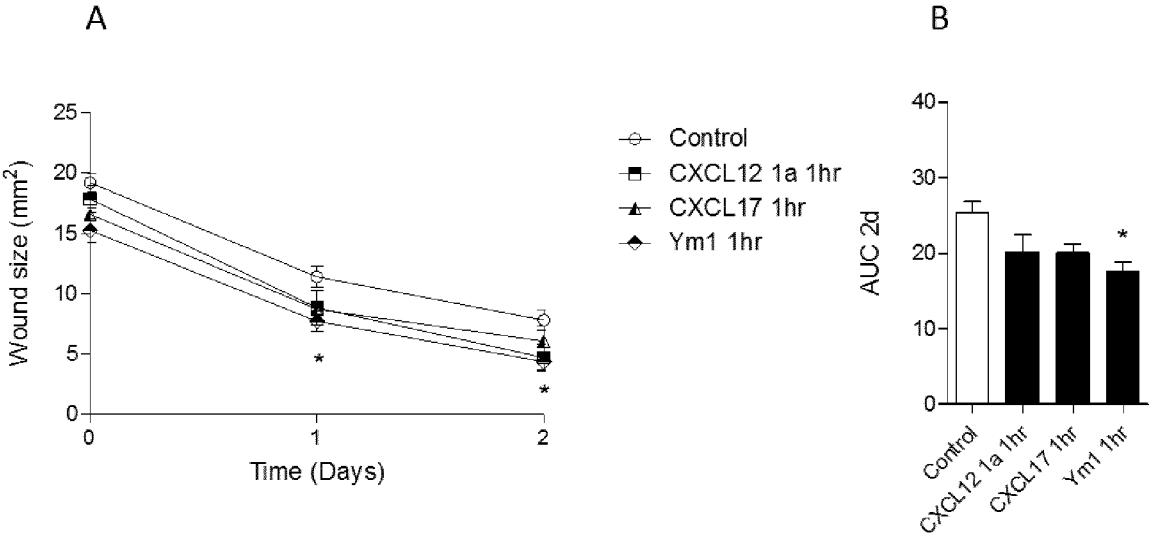

A
B
Wound size (mm2)
Time (Days)
Control
CXCL12 1a 1hr
CXCL17 1hr
Ym1 1hr
AUC 2d
Control
CXCL12 1a 1hr
CXCL17 1hr
Ym1 1hr

Fig. 20

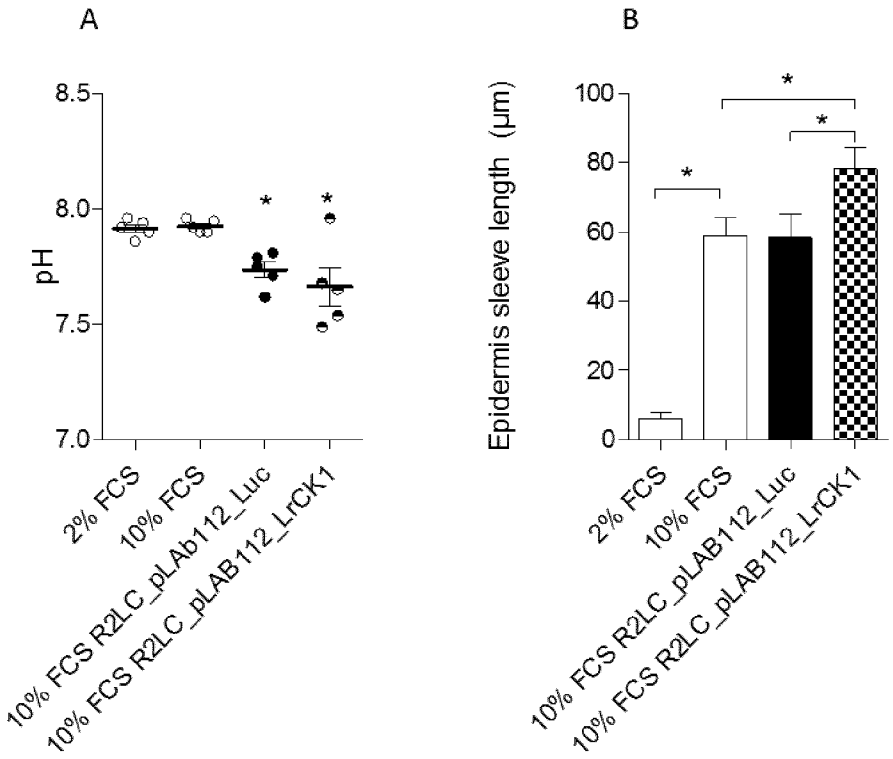

A
B
pH
8.5
8.0
7.5
7.0
Epidermis sleeve length (µm)
100
80
60
40
20
0
*
2% FCS
10% FCS
10% FCS R2LC_pLAb112_Luc
10% FCS R2LC_pLAB112_LrCK1

Fig. 21

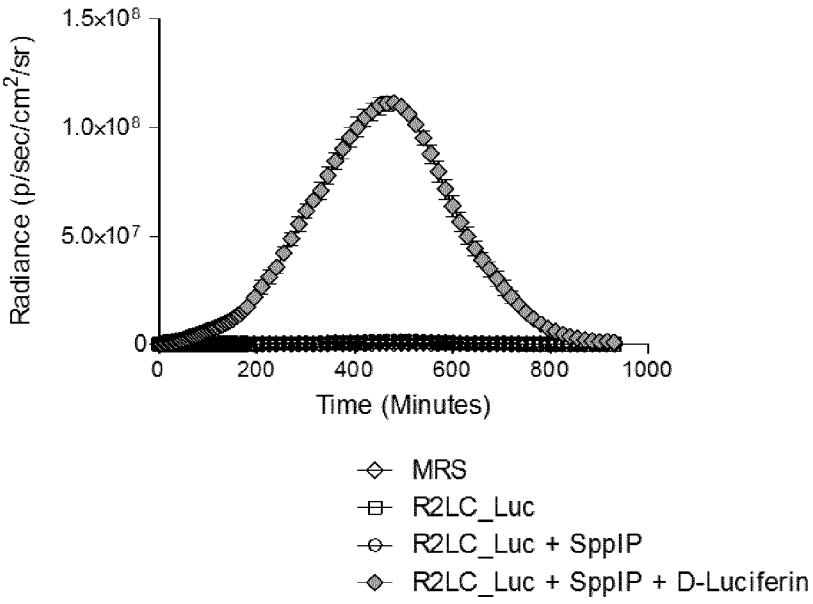

Radiance (p/sec/cm²/sr)
1.5x10⁸
1.0x10⁸
5.0x10⁷
0
0
200
400
600
800
1000
Time (Minutes)
MRS
R2LC_Luc
R2LC_Luc + SppIP
R2LC_Luc + SppIP + D-Luciferin

Fig. 22

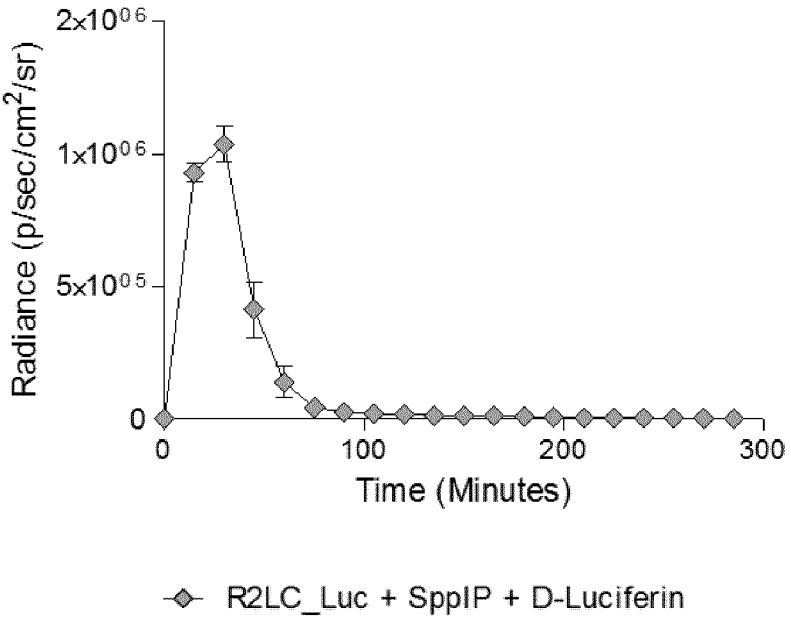

2x10^06
1x10^06
5x10^05
0
Radiance (p/sec/cm2/sr)
0
100
200
300
Time (Minutes)
R2LC_Luc + SppIP + D-Luciferin

Fig. 23

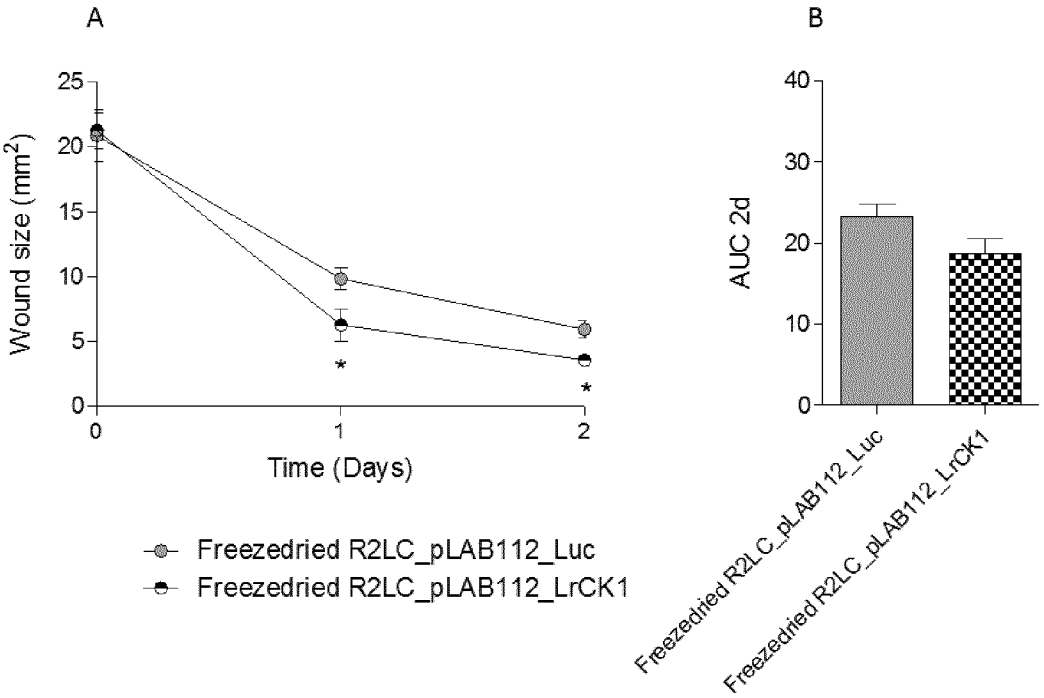

A
B
25
20
15
10
5
0
Wound size (mm2)
0
1
2
Time (Days)
*
*
Freezedried R2LC_pLAB112_Luc
Freezedried R2LC_pLAB112_LrCK1
40
30
20
10
0
AUC 2d
Freezedried R2LC_pLAB112_Luc
Freezedried R2LC_pLAB112_LrCK1

Fig. 24

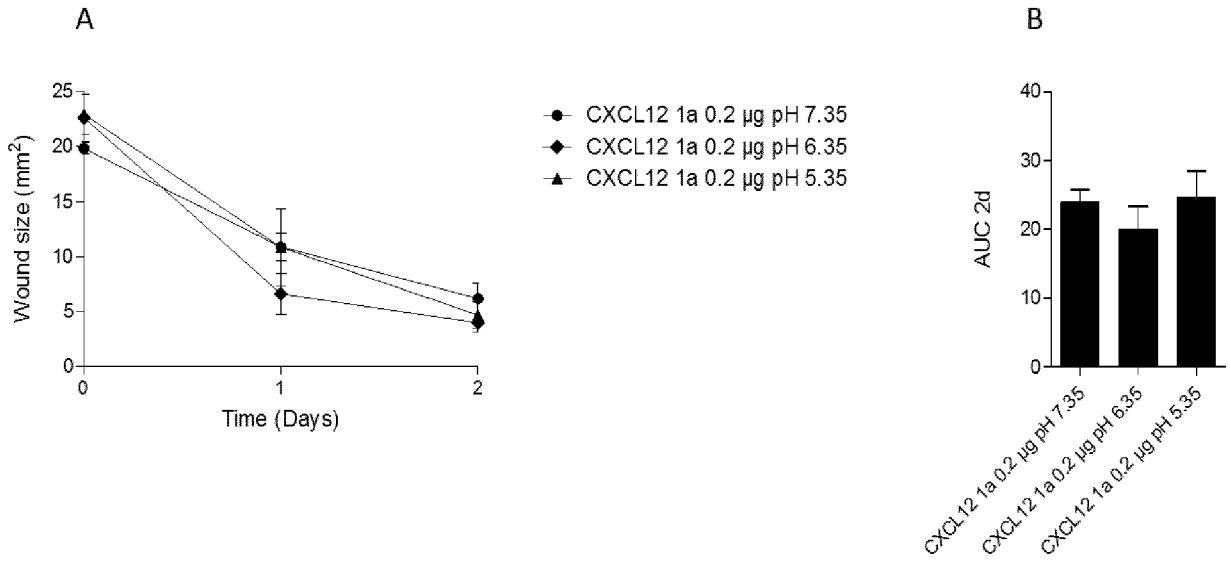

A
B
Wound size (mm2)
Time (Days)
CXCL12 1a 0.2 µg pH 7.35
CXCL12 1a 0.2 µg pH 6.35
CXCL12 1a 0.2 µg pH 5.35
AUC 2d

Fig. 25

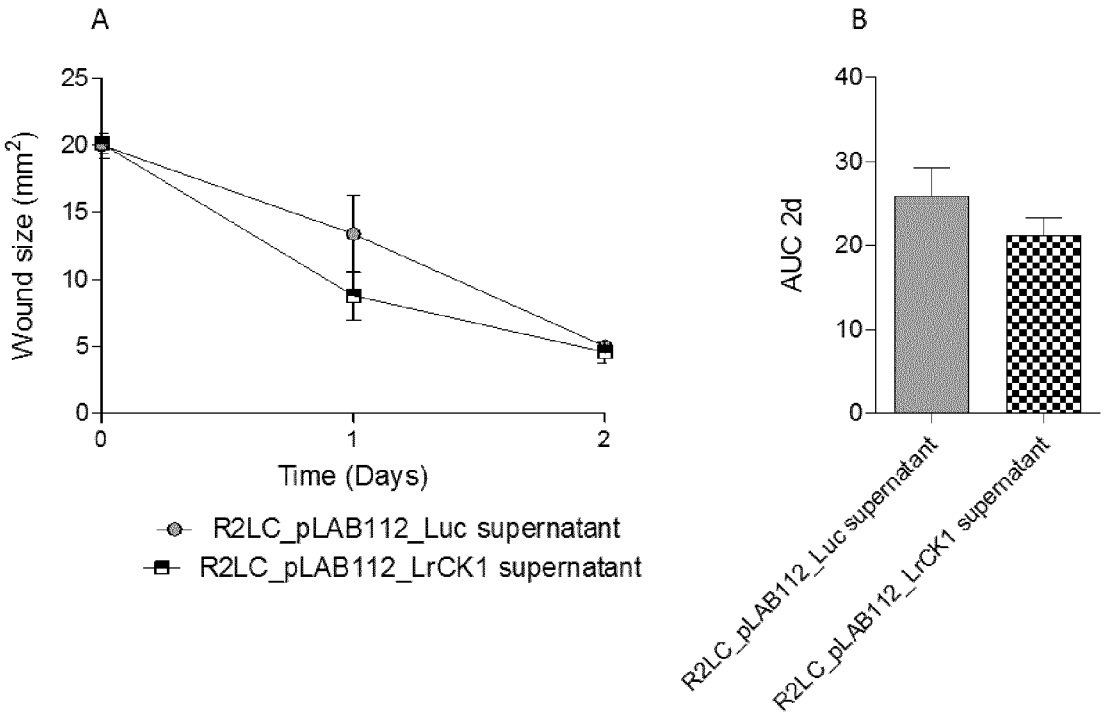

A
B
Wound size (mm2)
Time (Days)
R2LC_pLAB112_Luc supernatant
R2LC_pLAB112_LrCK1 supernatant
AUC 2d

Fig. 26

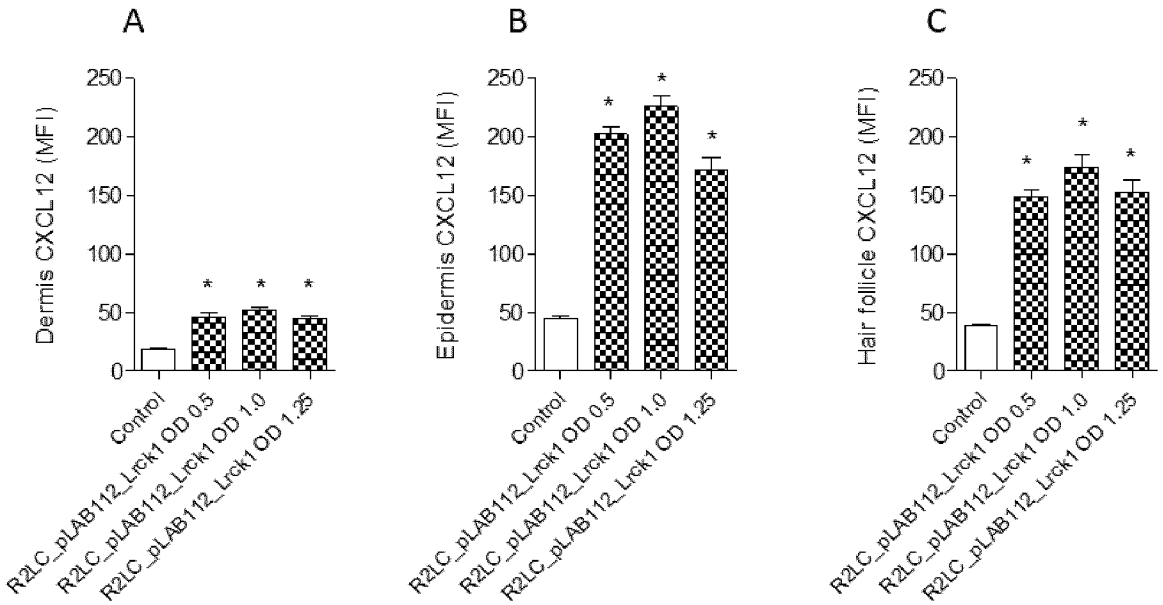

A
B
C
Dermis CXCL12 (MFI)
Epidermis CXCL12 (MFI)
Hair follicle CXCL12 (MFI)
250
200
150
100
50
0
*
Control
R2LC_pLAB112_Lrck1 OD 0.5
R2LC_pLAB112_Lrck1 OD 1.0
R2LC_pLAB112_Lrck1 OD 1.25

Fig 27

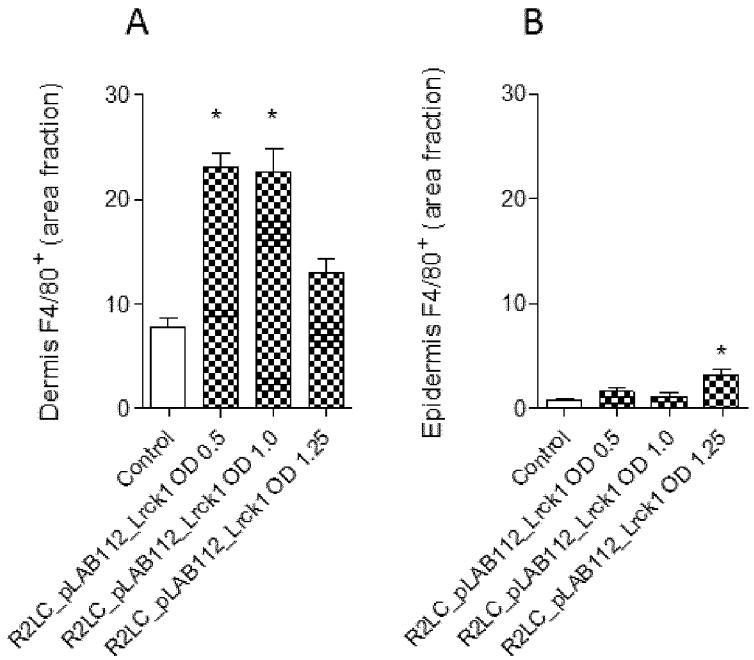

A
B
Dermis F4/80+ (area fraction)
Epidermis F4/80+ (area fraction)
30
20
10
0
*
Control
R2LC_pLAB112_Lrck1 OD 0.5
R2LC_pLAB112_Lrck1 OD 1.0
R2LC_pLAB112_Lrck1 OD 1.25

Fig. 28

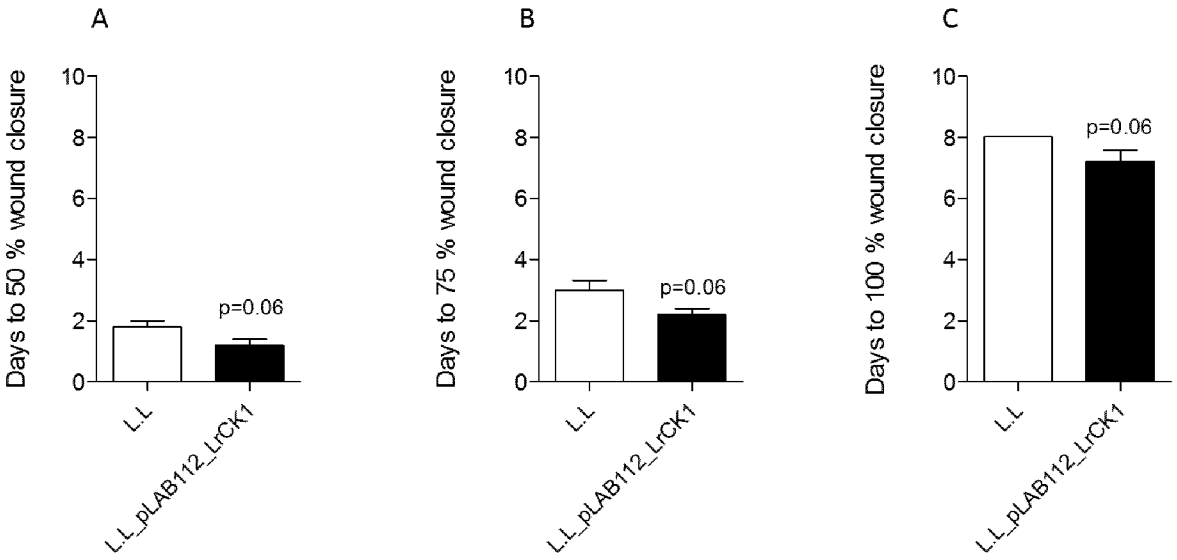

A
B
C
Days to 50 % wound closure
Days to 75 % wound closure
Days to 100 % wound closure
p=0.06
p=0.06
p=0.06
L.L
L.L_pLAB112_LrCK1

Fig. 29

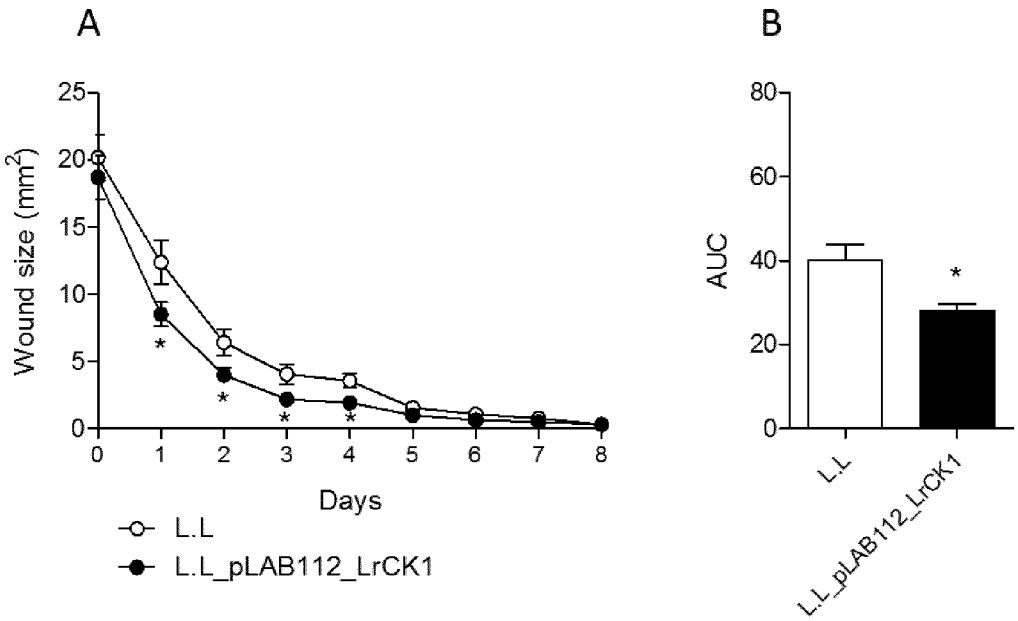

A
B
Wound size (mm2)
Days
AUC
L.L
L.L_pLAB112_LrCK1

Fig. 30

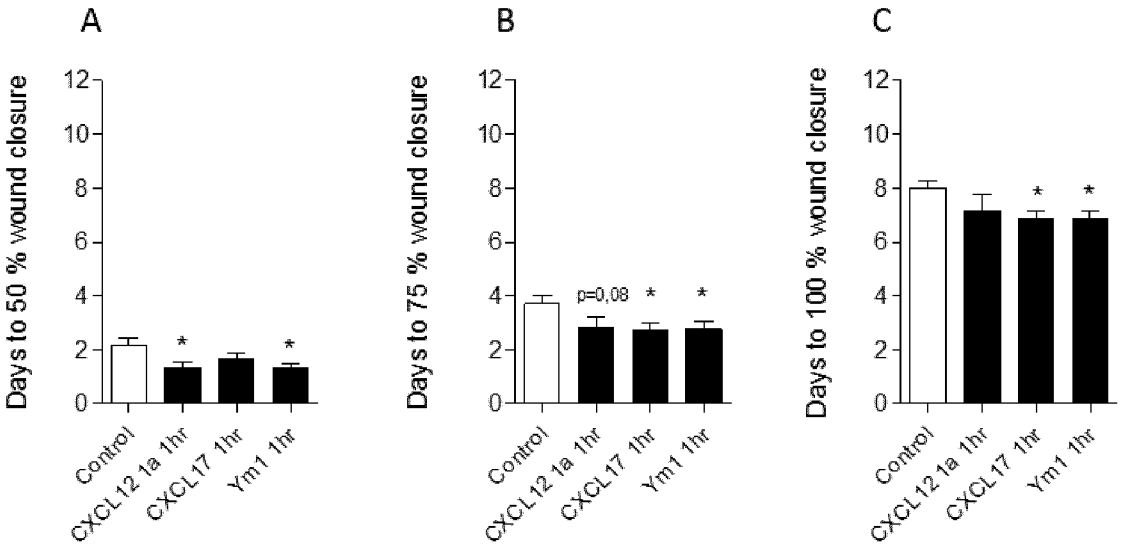

A
B
C
Days to 50 % wound closure
Days to 75 % wound closure
Days to 100 % wound closure
p=0,08
Control
CXCL12 1a 1hr
CXCL17 1hr
Ym1 1hr

Fig. 31

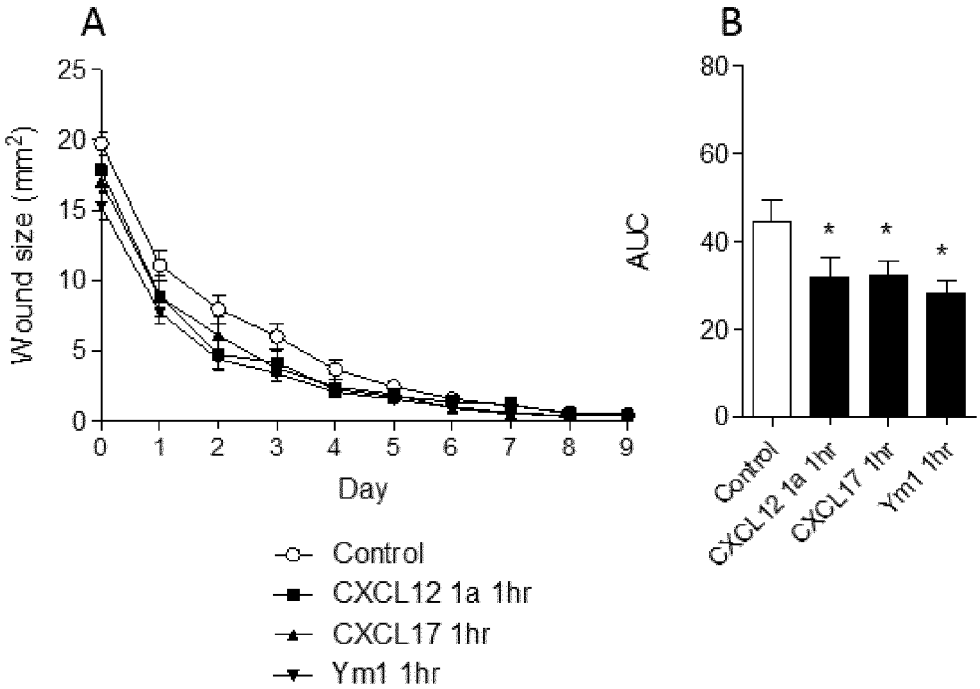

A
B
Wound size (mm²)
Day
AUC
Control
CXCL12 1a 1hr
CXCL17 1hr
Ym1 1hr

Fig. 32

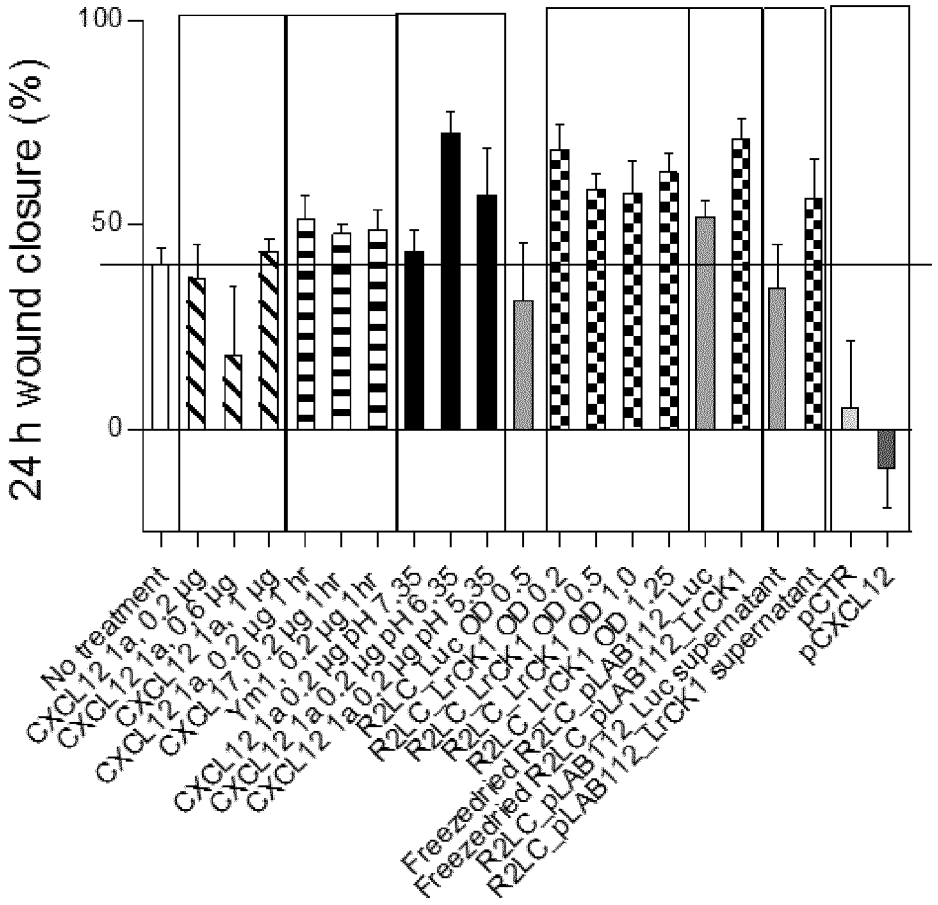

24 h wound closure (%)
100
50
0
No treatment
CXCL12 1a; 0.2 µg
CXCL12 1a; 0.6 µg
CXCL12 1a; 1 µg
CXCL12 1a 0.2 µg 1 hr
CXCL17; 0.2 µg 1hr
Ym1; 0.2 µg 1hr
CXCL12 1a 0.2 µg pH 7.35
CXCL12 1a 0.2 µg pH 6.35
CXCL12 1a 0.2 µg pH 5.35
R2LC_Luc OD 0.5
R2LC_LrCK1 OD 0.2
R2LC_LrCK1 OD 0.5
R2LC_LrCK1 OD 1.0
R2LC_LrCK1 OD 1.25
Freezedried R2LC_pLAB112_Luc
Freezedried R2LC_pLAB112_LrCK1
R2LC_pLAB112_Luc supernatant
R2LC_pLAB112_LrCK1 supernatant
pCTR
pCXCL12

Fig. 33

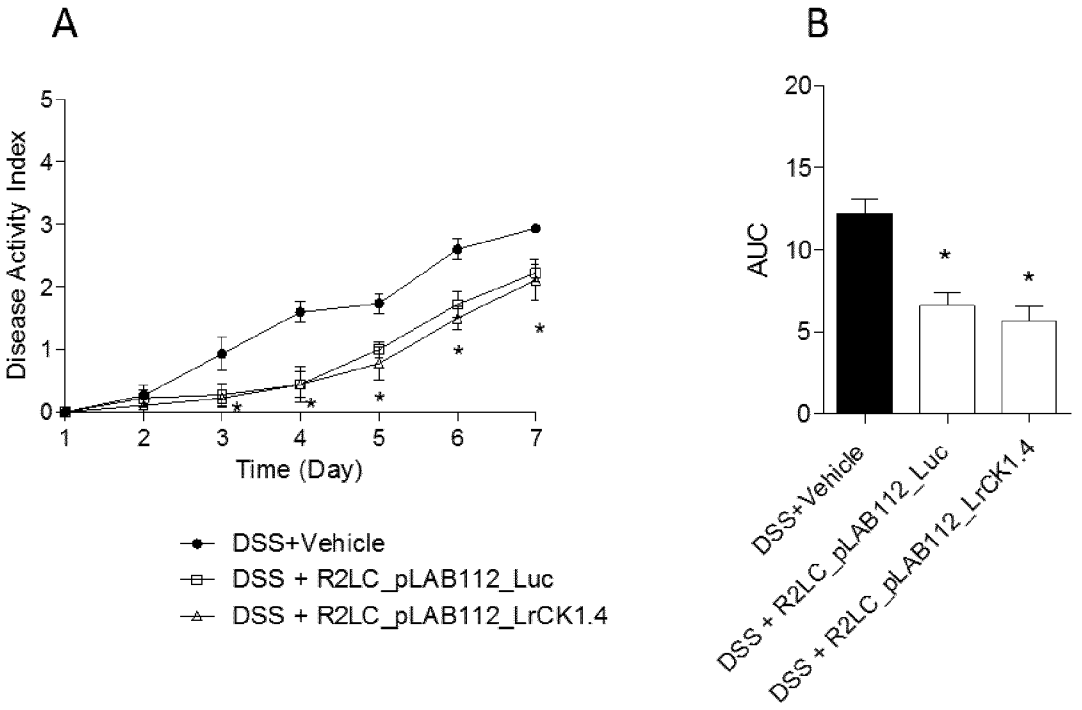

A
B
Disease Activity Index
Time (Day)
AUC
DSS+Vehicle
DSS + R2LC_pLAB112_Luc
DSS + R2LC_pLAB112_LrCK1.4

Fig. 34

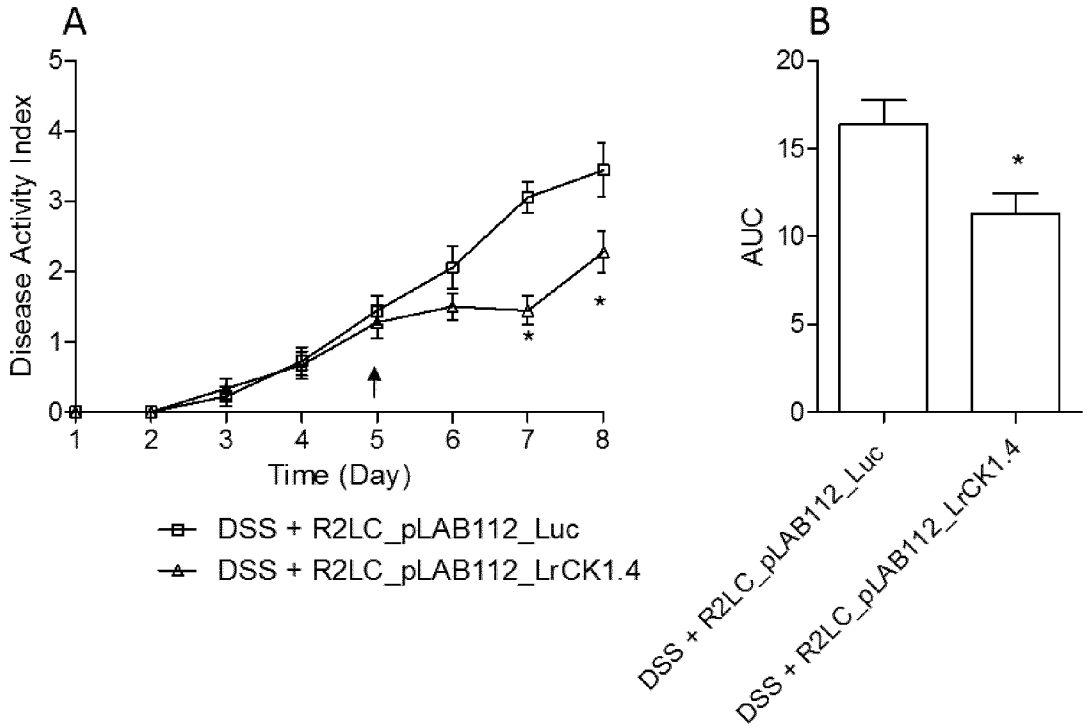

A
B
Disease Activity Index
Time (Day)
AUC
DSS + R2LC_pLAB112_Luc
DSS + R2LC_pLAB112_LrCK1.4

Fig. 35