
Recombinant probiotic bacteria

The invention is directed to recombinant probiotic bacteria, especially for use in the treatment of an inflammatory skin dysfunction, as well as a method for treating an inflammatory skin dysfunction.

The present application claims priority of the international application PCT/EP2015/052345, the disclosure of which is incorporated herein by reference.

Macrophages are a type of white blood cell that can be found in essentially all tissues. Macrophages play a critical role in non-specific defence and also help initiate specific defence mechanisms by recruiting other immune cells such as lymphocytes.

Macrophages exist as resilient tissue-specific macrophages or are derived from circulating blood monocytes which differentiate into macrophages.

Macrophages display various activation states. Two opposite activation states are known as classically activated macrophages, which are also designated as M1-polarized macrophages, and alternatively activated macrophages, which are also designated as M2-polarized macrophages.

The M1-polarized macrophages comprise immune effector cells with an active inflammatory phenotype. M1-polarized macrophages are characterized by the expression of high levels of pro-inflammatory cytokines, high production of reactive nitrogen and oxygen intermediates, promotion of T_h1 response, and strong microbicidal and tumoricidal activity.

The M2-polarized macrophages are an anti-inflammatory phenotype. M2-polarized macrophages are considered to be involved in parasite containment and promotion of tissue remodelling and tumor progression and to have immunoregulatory functions. M2-macrophages are also characterized by efficient phagocytic activity, high expression of scavenging molecules.

Plasticity and flexibility are features of mononuclear phagocytes of their activation states. For example, the phenotype of M1-polarized or M2-polarized macrophages can be reversed in vitro and in vivo.

Macrophages can change, depending on the stimulus in the micro-environment, the secretion pattern of cytokine and chemokines several times. For example, human primary M1-polarized macrophages can be repolarized by secreted factors from their own counterparts, to M2-polarized macrophages, and vice versa in vitro and in vivo.

In addition to presenting a first line of defence against pathogens, mononuclear phagocytes contribute to remodelling and repair of tissue under homeostatic and damaged conditions.

Moreover, macrophages are essential contributors towards the control of inflammation with M1-polarized macrophages implicated in initiating and sustaining inflammation and M2-polarized macrophages associated with the control of chronic inflammation.

For example, macrophages undergo dynamic changes during different phases of wound healing. M1-polarized macrophages mediate tissue damage and initiate inflammatory responses. Furthermore, during the early stages of repair response of a wound, the skin infiltrating macrophages show an M2-polarized phenotype and support the formation of a highly vascularized, cellular granulation tissue.

Inflammation can be characterized as acute inflammation, in settings such as sepsis, trauma, and wound healing, or as chronic inflammation, for example in diseases such as rheumatoid arthritis, ulcerative colitis, Crohn's disease, etc.. Many other diseases,

such as cancer, diabetes, arteriosclerosis, Alzheimer's and obesity are also associated with deregulated inflammation.

The acute inflammatory response involves a cascade of events, mediated by a large array of cells and molecules that locate invading pathogens or damaged tissue, alert and recruit other cells and molecules, eliminate the offending agents and finally restore the body to equilibrium.

In sepsis and trauma, this response is accompanied by macroscopic manifestations such as fever and elevated heart rate. In other tissues, inflammation manifests as redness, swelling and pain.

A feed-forward-loop of inflammation leading to damage/dysfunction leading to inflammation can result in a persistent, deregulated inflammation that promotes organ dysfunction and death. A well-regulated inflammatory response is necessary for proper tissue healing.

Restoration of skin integrity and homeostasis following injuries requires a complex and dynamic interplay of epithelial and mesenchymal cells together with tissue-resident and recruited hematopoietic cells to accomplish the sequential phases of the repair response: inflammation, tissue formation, and maturation. The early stage of the repair response is dominated by the inflammatory phase, which is characterized by local activation of the innate immune system resulting in an immediate influx of neutrophiles followed by subsequent invasion of blood monocytes, which differentiate into macrophages.

The mid-stage of the repair response includes the phase of tissue formation, which is characterized by the development of granulation tissue that refills the dermal wound space. Granulation tissue formation encompasses the invasion of endothelial cells resulting in angiogenesis, the influx of fibroblasts and the accumulation of additional macrophages.

Deposition of provisional extracellular wound matrix facilitates cell adhesion, migration and proliferation. Furthermore, at the wound edge, complex epidermal-

mesenchymal interactions stimulate keratinocyte proliferation and migration to restore the epidermal barrier.

Granulation tissue formation continues until the wound space is refilled and the overlaying epidermis is restored. Upon completion of the epidermal barrier, the repair response enters the late stage, which is characterized by tissue maturation. During the phase of tissue maturation, granulation tissue transforms into scar tissue.

During skin repair, the innate immune response of resident cells as well as the recruited inflammatory cells combat invading microbes, contribute to the debridement, but may also support the repair process by releasing a spectrum of growth factors.

However, due to the release of proinflammatory and cytotoxic mediators, uncontrolled activity of macrophages may also be detrimental to tissue repair.

An imbalanced inflammation characterized by increased numbers of macrophages is a hallmark of an attenuated repair response in human diseases leading to the formation of non-healing chronic wounds. Additional factors that contribute to non-healing chronic wounds are, for example, diabetes, venal or arterial diseases, infection, and metabolic deficiencies in old age.

Various methods exist to treat chronic wounds, including the use of antibiotics for treating infections, debridement, vacuum-assisted closure, and oxygenation.

Further methods include, for example, the application of growth factors.

For example, becaplermin is a recombinant human platelet-derived growth factor BB. Becaplermin is sold under the trade name Regranex and is indicated for the treatment of lower extremity diabetic neuropathic ulcers that extend into the subcutaneous tissue or beyond and have an adequate blood supply.

Becaplermin is further indicated as an adjunct to, and not as a substitute for, foot ulcer care practices including initial sharp debridement, pressure release and infection control.

However, an increased rate of mortality secondary to malignancy was observed in patients treated with three or more tubes of Regranex gel in a post-marketing retrospective cohort study.

Another growth factor used is a recombinant human epidermal growth factor which is sold under the trade name REGEN-D gel and which is used for treating chronic diabetic foot ulcers.

Furthermore, trafermine is a recombinant form of human fibroblast growth factor 2, which is sold under the trade name “Fiblast Spray”. Trafermine is used for the treatment of pressure ulcers and other skin ulcers including burn ulcers and black ulcers.

After daily application of trafermine or placebo during six weeks period to patients suffering from chronic diabetic neuropathic ulcer of the foot ulcer size was assessed through weekly clinical examination and computerized photographs. The weekly reduction in ulcer perimeter and area was identical in both groups, as was the rate of linear advance from entry to the sixth week of treatment. Moreover, the percentage of the healed area at the end of the study did not differ significantly. According to Richards et al. (1995) topical application of trafermine had no advantage over the placebo for healing chronic neuropathic diabetic ulcer of the foot.

Commercially available are also two dermal substitutes containing human embryonic cells, which secrete various growth factors after application to the diseased wound area.

The dermal substitute Dermagraft is composed of fibroblasts, extracellular matrix and a bioabsorbable scaffold. Dermagraf is manufactured from human fibroblast cells derived from donated newborn foreskin tissue.

During the manufacture process, the human fibroblasts are seeded onto a bioabsorbable polyglactin scaffold.

The commercially available dermal substitute Apligraf contains two cell types derived from neonatal foreskin. Living human keratinocytes and fibroblasts are embedded in a wound type 1 collagen matrix.

The disadvantage of the afore-mentioned dermal substitutes is a comparable high price which results from the manufacturing process. For example, maternal blood is tested for evidence of infection with human viruses, such as immuno deficiency virus type 1 and 2, hepatitis B virus, hepatitis C virus, syphilis, human T-lymphotropic type 1 and 2 as well as Epstein Barr virus.

A further commercially available product is sold under the tradename Procuren. Procuren is a platelet-derived wound healing formula for treating non-healing wounds. Procuren is an autologous platelet-derived product that is prepared from a sample of the patient's blood.

However, there is insufficient evidence regarding the effectiveness of autologous platelet-derived products, including autologous platelet-derived growth factor, for the treatment of chronic non-healing wounds or the treatment of other conditions such as acute surgical wounds.

Furthermore, the amount of growth factors supplied by the above-mentioned dermal substitutes as well as autologous platelet-derived product varies significantly depending on the quality of the starting material.

It is an object of the present invention to enable an alternative treatment of an inflammatory skin dysfunction, especially a chronic inflammatory skin dysfunction, which allows for an easy application of beneficial factors to the, preferably chronic, inflammatory skin dysfunction.

Furthermore, a controlled amount of beneficial factors supporting healing should be provided for treating an inflammatory skin dysfunction.

The object of the present invention is solved by providing recombinant probiotic bacteria according to claim 1 comprising (a) nucleic acid sequence(s) encoding for a first heterologous factor, (a) nucleic acid sequence(s) encoding for a second heterologous factor, and preferably (a) nucleic acid sequence(s) encoding for a third heterologous factor, with the proviso that said first factor, said second factor, and said third factor are functionally different from each other,
wherein said first factor is a growth factor, wherein said second factor is selected from the group consisting of M2-polarizing factors, and wherein preferably said third factor is selected from the group consisting of M2-polarizing factors and growth factors.

Preferably, said recombinant probiotic bacteria according to claim 1 comprise at least one nucleic acid sequence encoding for a first heterologous factor, at least one nucleic acid sequence encoding for a second heterologous factor, and at least one nucleic acid sequence encoding for a third heterologous factor, with the proviso that said first factor, said second factor, and said third factor are functionally different from each other,
wherein said first factor is a growth factor, wherein said second factor is selected from the group consisting of M2-polarizing factors and wherein said third factor is selected from the group consisting of M2-polarizing factors and growth factors.

Preferred embodiments of the recombinant probiotic bacteria are disclosed in dependent claims 2 to 22.

The object of the present invention is further solved by providing recombinant probiotic bacteria according to claim 17 for use in the treatment of an inflammatory skin dysfunction, preferably a chronic inflammatory skin dysfunction, said recombinant probiotic bacteria comprising:
at least one nucleic acid sequence encoding for a first heterologous factor, and at least one nucleic acid sequence encoding for a second heterologous factor,
wherein said first factor is a growth factor and wherein said second factor is selected from the group consisting of M2-polarizing factors.

Preferably, said recombinant probiotic bacteria according to claim 17 comprise at least one nucleic acid sequence encoding for a first heterologous factor, at least one nucleic acid sequence encoding for a second heterologous factor, and at least one nucleic acid sequence encoding for a third heterologous factor, with the proviso that said first factor, said second factor, and said third factor are functionally different from each other,
wherein said first factor is a growth factor, wherein said second factor is selected from the group consisting of M2-polarizing factors and wherein said third factor is selected from the group consisting of M2-polarizing factors and growth factors.

The object of the present invention is further solved by providing a method for treating an inflammatory skin dysfunction, preferably a chronic inflammatory skin dysfunction, according to claim 23, wherein said method comprises the step of administering recombinant probiotic bacteria according to any one of claims 1 to 22 to an individual suffering from said skin dysfunction.

The inventors surprisingly found that recombinant probiotic bacteria comprising the above-mentioned nucleic acid sequences could be used in the treatment of an inflammatory skin dysfunction, preferably a chronic inflammatory skin dysfunction.

Preferred embodiments of the present invention are disclosed in dependent claims.

According to the invention the term “functionally different factor” or “functionally different from each other” means that the respective factors preferably bind to different receptors and/or activate different second messengers in a target cell. Second messengers are intracellular signalling molecules released by the cell to trigger physiological changes.

According to the invention the term “functional analog” of a factor means an agent that binds to identical receptor(s) as the respective factor and preferably activates identical second messengers in a target cell.

For example, a functional analog of nisin binds to NisK, which acts as a receptor for the mature nisin molecule or a functional analog thereof, and, preferably, leads to a

subsequent phosphorylation of NisR. Phosphorylated NisR induces transcription from the respective nisin promoter.

Preferably, “a functional analog” of said first, second, and third heterologous factor has a sequence identity of the amino acid sequence of at least 80%, further preferably of at least 90%, further preferably of at least 93%, further preferably of at least 95%, further preferably of at least 97%.

According to the invention the term “heterologous factor” means a factor, preferably a protein, which is not naturally occurring in or expressed by said probiotic bacteria used.

A “functional analog” can also be designated as biosimilar.

When referring to “heterologous factor(s)” in general or when referring to specific “heterologous factor(s)” such as, e.g. FGF-2, IL-4, CSF-1, etc., it is intended that this term includes also functional analog(s) thereof.

The recombinant probiotic bacteria comprising the above-mentioned nucleic acid sequences are able to constantly and/or upon induction produce a unique combination of the respective first, second, and, preferably third, heterologous factor by transcribing and translating the respective nucleic acid sequences.

This unique combination of factors comprises at least one growth factor, and at least one M2-polarizing factor.

Preferably, this unique combination of factors comprises a growth factor, a first M2-polarizing factor, and a second M2-polarizing factor different from said first M2-polarizing factor. Alternatively, this unique combination of factors comprises a first growth factor, a M2-polarizing factor, and a second growth factor different from said first growth factor.

The recombinant probiotic bacteria preferably deliver a variety of at least two, preferably at least three, heterologous factors as recited in claim 1 to diseased tissue, thus modulating local immune system and enabling healing.

Preferably, the recombinant probiotic bacteria release the respective first heterologous factor and second heterologous factor, and preferably third heterologous factor, after application to said inflammatory skin dysfunction, preferably said chronic inflammatory skin dysfunction.

Furthermore, the recombinant probiotic bacteria used according to the present invention preferably provide for a constant release of respective first heterologous factor and second heterologous factor, and preferably third heterologous factor, after application to the site of the inflammatory skin dysfunction, preferably chronic inflammatory skin dysfunction.

Thereby, a much-improved, safer, and more cost-effective treatment option for subjects suffering from said inflammatory skin dysfunction, preferably said chronic inflammatory skin dysfunction, is available.

Preferably, the respective first, second, and third heterologous factor, after release from the bacteria, exert a biological active function supporting healing of said inflammatory skin dysfunction, preferably chronic inflammatory skin dysfunction.

The first heterologous factor is a growth factor.

Preferably, said growth factor is selected from the group consisting of fibroblast growth factors (FGF), vascular endothelial growth factors (VEGF), epidermal growth factors (EGF), insulin-like growth factors (IGF), platelet-derived growth factors (PDGF), transforming growth factor beta (TGF-beta), nerve growth factor (NGF), activins, functional analogues thereof, biosimilars thereof, and mixtures thereof.

Of course, functional analogues of the aforementioned or below mentioned factors or biosimilars thereof can also be used within the scope of the invention as claimed.

Fibroblast growth factors are a family of growth factors, which are involved in angiogenesis, wound healing, and various endocrine signalling pathways.

In humans, 22 members of the FGF family have been identified, FGF-1 to FGF-14 and FGF-16 to FGF-23, which can be used in the present invention.

FGF-1 through FGF-10 bind the fibroblast growth factor receptors (FGFRs).

Fibroblast growth factor 1 is also known as acidic fibroblast growth factor. Fibroblast growth factor 2 is also known as basic fibroblast growth factor. Furthermore, fibroblast growth factor 7 is also known as keratinocyte growth factor (KGF) and fibroblast growth factor 10 is also known a keratinocyte growth factor 2 (KGF-2).

In a preferred embodiment, the fibroblast growth factors are selected from the group consisting of FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, and mixtures thereof, further preferably FGF-1, FGF-2, FGF-7, FGF-10, and mixtures thereof, further preferably, FGF-2, FGF-7, functional analogues thereof, biosimilars thereof, and mixtures thereof, further preferably FGF-2.

For example, FGF-1 and FGF-2 can stimulate angiogenesis and are mitogenic for several cell types present at the site of an inflammatory skin dysfunction, including fibroblasts and keratinocytes. Furthermore, FGF-7 can stimulate wound reepithelization in a paracrine manner.

Fibroblast growth factor 2 (FGF-2), preferably human fibroblast growth factor 2 (hFGF-2), is implicated in diverse biological processes, including wound healing and tumor growth.

The mRNA for this gene contains multiple polyadenylation sites and is alternatively translated from non-AUG and AUG initiation codons, resulting in five different isoforms with distinct properties. The non-AUG isoforms are localized in the nucleus and are responsible for the intracrine effect whereas the AUG-initiated form is mostly cytosolic and is responsible for the paracrine and autocrine effects of FGF-2.

The nucleic acid sequence of the mRNA of the human fibroblast growth factor 2 (hFGF-2) is available under the NCBI accession number NM_002006.4. The respective amino acid sequence of the AUG-isomer is available under the NCBI accession number NP_001997.5 as well as the UniProt accession number P09038 – version 182 and is also shown in Figure 22a.

The preproprotein includes a propeptide, which spans the amino acids 1 to 142 of the preproprotein, and the mature human fibroblast growth factor 2 peptide, which spans the amino acids 143 to 288 of the preproprotein.

In a preferred embodiment, fibroblast growth factor 2 comprises one or at least one of the amino acid sequences of SEQ IDs 26 to 30, further preferably the amino acid sequence of SEQ ID 28. The amino acid sequence of SEQ ID 28 is depicted in Figure 22b.

The insulin-like growth factors (IGFs) are proteins with a high sequence similarity to insulin. The insulin-like growth factors comprise two proteins IGF-1 and IGF-2, which can be used in the present invention.

The family of epidermal growth factors (EGFs) are proteins with highly similar structural and functional characteristics and comprises the proteins epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), epiregulin (EPR), epigen (EPGN), betacellulin (BTC), neuregulin-1 (NRG1), neuregulin-2 (NRG2), neuregulin-3 (NRG3), and neuregulin-4 (NRG4), preferably epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), epiregulin (EPR), epigen (EPGN), and betacellulin (BTC), further preferably epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), and transforming growth factor-α (TGF-α), which can be used in the present invention.

Epidermal growth factor (EGF), preferably human epidermal growth factor (hEGF), stimulates the growth of various epidermal and epithelial tissues in vivo and in vitro and of some fibroblasts in cell culture. Furthermore, hEGF has a profound effect on

the differentiation of specific cells in vivo and is a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin.

Human epidermal growth factor exists in at least three isoforms produced by alternative splicing.

The amino acid sequence of the human epidermal growth factor isoform 1 preproprotein is available under the NCBI accession number NP_001954.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001963.2.

The preproprotein of isoform 1 includes a signal peptide, which spans the amino acids 1 to 22 of the preproprotein, a propeptide which spans the amino acids 23 to 1207 of the preproprotein and the mature human epidermal growth factor peptide, which spans the amino acids 971 to 1023 of the preproprotein.

The amino acid sequence of the human epidermal growth factor isoform 2 preproprotein is available under the NCBI accession number NP_001171601.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001178130.1. The amino acid sequence of the human epidermal growth factor isoform 3 preproprotein is available under the NCBI accession number NP_001171602.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001178131.1. The amino acid sequences of the human epidermal growth factor isoforms 1 to 3 are also available under UniProt accession number P01133 – version 180 and are also shown in Figures 25a to 25c. The amino acid sequence of the mature human epidermal growth factor is shown in Figure 25d.

Heparin-binding EGF-like growth factor (HB-EGF), preferably human heparin-binding EGF-like growth factor (hHB-EGF), is an important growth factor in the epithelialization required for cutaneous wound healing. hHB-EGF has a mitogenic and migratory effects on keratinocytes and fibroblasts. hHB-EGF further promotes dermal repair and angiogenesis which is necessary for wound healing. hHB-EGF is a major component of wound fluids. hHB-EGF is released by macrophages,

monocytes, and keratinoctyes. Furthermore, hHB-EGF cell surface binding to heparan sulfate proteoglycans enhances mitogen promoting capabilities increasing the rate of skin wound healing, decreasing human skin graft healing times, and promotes rapid healing of ulcers, burns, and epidermal split thickness wounds.

The amino acid sequence of the human heparin-binding EGF-like growth factor preproprotein is available under the NCBI accession number NP_001936.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001945.2.

The precursor of human heparin-binding EGF-like growth factor includes a signal peptide, which spans the amino acids 1 to 19 of the precursor, proheparin-binding EGF-like growth factor which spans the amino acids 20 to 208 of the precursor and the mature human epidermal growth factor peptide, which spans the amino acids 63 to 148 of the precursor.

The amino acid sequence of human heparin-binding EGF-like growth factor is also available under UniProt accession number Q99075 – version 151 and is also shown in Figures 26a. The amino acid sequence of the mature human heparin-binding EGF-like growth factor is shown in Figure 26b.

Transforming growth factor-α (TGF-α), preferably human transforming growth factor-α (hTGF-α), can be produced in macrophages, brain cells, and keratinocytes. hTGF-α induces epithelial development. hTGF-α and hEGF bind to the same receptor, epidermal growth factor receptor (EGFR; ErbB-1; HER1 in humans). When TGF-α binds to EGFR it can initiate multiple cell proliferation events including wound healing.

Human transforming growth factor-α exists in at least five isoforms produced by alternative splicing.

The amino acid sequence of the human transforming growth factor alpha isoform 1 preproprotein is available under the NCBI accession number NP_003227.1. The

respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_003236.2.

The precursor of human transforming growth factor alpha isoform 1 includes a signal peptide, which spans the amino acids 1 to 23 of the precursor, protransforming growth factor alpha isoform 1 which spans the amino acids 24 to 160 of the precursor and the mature transforming growth factor alpha peptide, which spans the amino acids 40 to 89 of the precursor.

The amino acid sequence of the human transforming growth factor alpha isoform 2 preproprotein is available under the NCBI accession number NP_001093161.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001099691.1.

The amino acid sequence of the human transforming growth factor alpha isoform 3 preproprotein is available under the NCBI accession number NP_001295087.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001308158.1.

The amino acid sequence of the human transforming growth factor alpha isoform 4 preproprotein is available under the NCBI accession number NP_001295088.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001308159.1.

The amino acid sequence of the human transforming growth factor alpha isoform 5 preproprotein is available under the NCBI accession number AAF05090.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number AF149097.1.

The amino acid sequences of the precursors of human transforming growth factor alpha isoforms 1 to 5 are also available under UniProt accession number P01135 – version 168 and are shown in Figures 27a to 27e. The amino acid sequence of the mature human transforming growth factor alpha is shown in Figure 27f.

Amphiregulin (AREG), preferably human amphiregulin (hAREG), is another ligand of the EGF receptor. Human amphiregulin is an autocrine growth factor as well as a mitogen for a broad range of target cells including astrocytes, schwann cells and fibroblasts. Human amphiregulin promotes the growth of epithelial cells.

The amino acid sequence of the human amphiregulin preproprotein is available under the NCBI accession number NP_001648.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001657.3.

The precursor of human amphiregulin preproprotein includes a signal peptide, which spans the amino acids 1 to 19 of the preproprotein, a propeptide which spans the amino acids 20 to 100 of the preproprotein and the mature transforming growth factor alpha peptide, which spans the amino acids 101 to 187 of the preproprotein.

The amino acid sequence of the human amphiregulin preproprotein is also available under UniProt accession number P15514 – version 147 and is also shown in Figure 28a. The amino acid sequence of the mature human amphiregulin is shown in Figure 28b.

Epiregulin (EPR), preferably human epiregulin (hEPR), is a ligand of the EGF receptor which can stimulate cell proliferation and/or angiogenesis.

The amino acid sequence of the human epiregulin preproprotein is available under the NCBI accession number NP_001423.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001432.1.

The preproprotein of human epiregulin includes a signal peptide, which spans the amino acids 1 to 29 of the preproprotein, proepiregulin which spans the amino acids 30 to 169 of the preproprotein and the mature epiregulin, which spans the amino acids 60 to 108 of the preproprotein.

The amino acid sequence of the human epiregulin preproprotein is also available under UniProt accession number O14944 – version 146 and is also shown in Figure

29a. The amino acid sequence of the mature human epiregulin is shown in Figure 29b.

Epigen (EPGN), preferably human epigen (hEPGN), promotes the growth of epithelial cells. Human epigen exists in at least seven isoforms produced by alternative splicing.

The amino acid sequence of the human epigen isoform 1 precursor is available under the NCBI accession number NP_001257918.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001270989.1.

The human epigen isoform 1 precursor includes a signal peptide, which spans the amino acids 1 to 22 of the precursor and the mature epiregulin, which spans the amino acids 23 to 154 of the precursor.

The amino acid sequence of the human epigen isoforms 1 to 7 precursors is also available under UniProt accession number Q6UW88 – version 101 and is also shown in Figures 30a to 30g. The amino acid sequence of the mature human epigen is shown in Figure 30h.

Betacellulin (BTC), preferably human betacellulin (hBTC), is a growth factor that also binds to epidermal growth factor receptor and that is synthesized by a wide range of adult tissues and in many cultured cells, including smooth muscle cells and epithelial cells. The amino acid sequence of the human probetacellulin precursor is available under the NCBI accession number NP_001720.1. The respective nucleic acid sequence of the mRNA is available under the NCBI accession number NM_001729.1.

The precursor of human probetacellulin includes a signal peptide, which spans the amino acids 1 to 31 of the precursor, probetacellulin, which spans the amino acids 32 to 178 of the precursor, and the mature betacellulin, which spans the amino acids 32 to 111 of the precursor.

The amino acid sequence of the human probetacellulin precursor is also available under UniProt accession number P35070 – version 139 and is also shown in Figure 31a. The amino acid sequence of the mature human betacellulin is shown in Figure 31b.

Insulin-like growth factor 1 (IGF-1) is also called somatomedin C. The nucleic acid sequence of the mRNA of the human IGF-1 is available under the NCBI accession number NM_000618.2. The respective amino acid sequence is available under NCBI accession number NP_000609.1 as well as the UniProt accession number P05019 – version 178.

The preproprotein includes a signal peptide, which spans the amino acids 1 to 21 of the preproprotein, and the human insulin-like growth factor 1 peptide, which spans the amino acids 49 to 118 of the preproprotein.

The nucleic acid sequence of the mRNA of the human insulin-like growth factor 2 (hIGF-2) is available under the NCBI accession number NM_000612.4. The respective amino acid sequence of the human insulin-like growth factor 2 preproprotein is available under the NCBI accession number NP_000603.1 as well as the UniProt accession number P01344 – version 192.

The preproprotein includes a signal peptide, which spans the amino acids 1 to 24 of the preproprotein, and the mature chain of the insulin-like growth factor 2, which spans the amino acids 25 to 91 of the preproprotein.

The family of vascular endothelial growth factors (VEGF) is a group of growth factors which include VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PGF), which can be used in the present invention.

In a preferred embodiment, the vascular endothelial growth factor is the vascular endothelial growth factor A (VEGF-A).

VEGF-A can induce angiogenesis, vasculogenesis and endothelial cell growth.

The nucleic acid sequence of the mRNA of the human vascular endothelial growth factor A (hVEGF-A) is available under the NCBI accession number NM_001025366.1. The respective amino acid sequence of the human vascular endothelial growth factor A is available under the NCBI accession number NP_001020537.2 as well as the UniProt accession number P15692 – version 197.

The precursor protein of the human vascular endothelial growth factor A includes a signal peptide, which spans the amino acids 1 to 26 of the precursor protein, as well as the mature human vascular endothelial growth factor A, which spans the amino acids 27 to 232 of the precursor protein.

Platelet-derived growth factor (PDGF) regulates cell growth and division. Human platelet-derived growth factor (hPDGF) has four subunits, PDGF-A, PDGF-B, PDGF-C and PDGF-D, which form either homo- or heterodimers of the respective subunits, which can be used in the present invention.

Preferably, the platelet-derived growth factor is PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, PDGF-DD, or a mixture thereof.

Further preferably, the platelet-derived growth factor is a dimeric protein composed of two PDGF-A subunits, a dimeric glycoprotein composed of two PDGF-B subunits, a dimeric glycoprotein composed of a PDGF-A subunit and a PDGF-B subunit, or a mixture thereof.

The nucleic acid sequence of the mRNA of the human platelet-derived growth factor subunit A (hPDGF-A) is available under the NCBI accession number NM_002607.4. The respective amino acid sequence is available under the NCBI accession number NP_002598.4 as well as the UniProt accession number P04085 – version 159.

The respective preproprotein of the human PDGF-A subunit encodes a signal peptide, which spans the amino acids 1 to 20 of the preproprotein, and the mature human platelet-derived growth factor subunit A which spans the amino acids 87 to 211 of the preproprotein.

The nucleic acid sequence of the mRNA of the human platelet-derived growth factor subunit B (hPDGF) is available under the NCBI accession number NM_002608.1. The respective amino acid sequence of the human platelet-derived growth factor subunit preproprotein is available under the NCBI accession number NP_002599.1 as well as the UniProt accession number P01127 – version 181.

The preproprotein of the human PDGF-B subunit encodes a signal peptide, spanning the amino acids 1 to 20 of the preproprotein, and the mature form of the human platelet-derived growth factor subunit B, which spans the amino acids 82 to 190 of the preproprotein.

Hepatocyte growth factor (HGF) is a growth factor which is secreted by mesenchymal cells and acts primarily upon epithelial cells and endothelial cells but also on hemapoeitic progenitor cells and can be used in the present invention.

HGF is secreted as a single preproprotein and is cleaved by serine proteinase into a 69-kGa alpha chain and a 34-kDa beta chain. The amino acid sequence of the preproprotein as well as the respective alpha and beta chain are depicted in Figures 23a to 23 c.

The nucleic acid sequence of the mRNA of the human hepatocyte growth factor (hHGF) is available under the NCBI accession number NM_000601.3. The respective amino acid sequence of the human hepatocyte growth factor preproprotein is available under the NCBI accession number NP_000592.3 as well as the UniProt accession number P14210 – version 186.

The preproprotein includes a signal peptide, which spans the amino acids 1 to 31 of the preproprotein, the human hepatocyte growth factor alpha chain, which spans the amino acids 32 to 494 of the preproprotein, and the human hepatocyte growth factor beta chain, which spans the amino acids 495 to 728 of the preproprotein.

Transforming growth factor β (TGF-β), preferably human transforming growth factor β (hTGF-β), is a cytokine which is secreted by many cell types, including macrophages.

TGF-β exists in at least three isoforms, TGF-β1, TFG-β2 and TGF-β3, which can be used in the present invention. The amino acid sequences of the respective precursor proteins and of the mature proteins is depicted in Figures 21a to 21g.

Human transforming growth factor β 1 is a secreted protein that is cleaved into a latency-associated peptide (LAP) and a mature TGF-β1 peptide. The mature peptide may either form TGF-β1 homodimers or heterodimers with other TGF-β family members.

The nucleic acid sequence of the mRNA of the human transforming growth factor β1 precursor can be obtained by the NCBI accession number NM_000660.4. The respective amino acid sequence is available under the NCBI accession number NP_000651.3 or the UniProt accession number P01137 – version 199.

The amino acid sequence of the human TGF-β1 precursor includes a signal peptide, which spans the amino acids 1 to 29 of the precursor protein, the latency-associated peptide, which spans the amino acid 30 to 278 of the precursor protein, and the mature transforming growth factor β1, which spans the amino acids 279 to 390 of the precursor protein.

Transforming growth factor β2 (TGF-β2), preferably human transforming growth factor β2 (hTGF-β2), is a multifunctional cytokine that regulates proliferation, differentiation, adhesion, and migration of many cell types.

Alternatively spliced transcript variants of the human transforming growth factor β2 gene have been identified, which encode two different isoforms.

The nucleic acid sequence of the mRNA of the human human transforming growth factor beta 2 isoform 1 precursor is available under the NCBI accession number NM_001135599.3. The respective amino acid sequence is available under the NCBI accession number NP_001129071.1.

The human transforming growth factor beta 2 isoform 1 precursor includes a signal peptide spanning the amino acids 1 to 20 of the precursor protein, a latency-

associated peptide, which spans the amino acids 21 to 302 of the precursor protein, and the mature transforming growth factor beta 2, which spans the amino acids 303 to 414 of the precursor protein.

The nucleic acid sequence of the mRNA of the human transforming growth factor β2 isoform 2 precursor is available under the NCBI accession number NM_003238.3. The respective amino acid sequence is available under the NCBI accession number NP_003229.1. The precursor protein includes a signal peptide, which spans the amino acids 1 to 20 of the precursor protein, a latency associated peptide, which spans the amino acids 21 to 302 of the precursor protein, and the mature TGF-β2 peptide, which spans the amino acids 303 to 414 of the precursor protein.

The amino acid sequence of transforming growth factor β2 is further available under the UniProt accession number P61812 – version 128.

Transforming growth factor β3 (TGF-β3), preferably human transforming growth factor β3 (hTGF-β3), is a secreted cytokine that is involved in embryogenesis and cell differentiation.

The nucleic acid sequence of the mRNA of the human transforming growth factor β3 precursor protein is available under the NCBI accession number NM_003239.3. The corresponding amino acid sequence is available under the NCBI accession number NP_003230.1 as well as the UniProt accession number P10600 – version 170.

The precursor protein includes a signal peptide, which includes the amino acids 1 to 23 of the precursor protein, a latency associated peptide, which includes the amino acids 24 to 30 of the precursor protein, and the mature transforming growth factor β3 peptide, which spans the amino acids 301 to 412 of the precursor protein.

Preferably, transforming growth factor β comprises one or at least one of the amino acid sequences of SEQ IDs 19 to 25

Activins are disulfide-linked dimeric proteins originally purified from gonadal fluids as proteins that stimulated pituitary follicle stimulating hormone (FSH) release. Activin

proteins have a wide range of biological activities, including mesoderm induction, neural cell differentiation, bone remodeling, hematopoiesis and roles in reproductive physiology.

Activins are homodimers or heterodimers of the various beta subunit isoforms, while inhibins are heterodimers of a unique alpha subunit and one of the various beta subunits.

Four beta subunits are known, beta A, beta B, beta C, and beta E.

The second heterologous factor is selected from the group consisting of M2-polarizing factors.

Preferably, M2-polarzing factors act on unpolarized macrophages, M1-polarized macrophages as well as undifferentiated monocytes, and other macrophage progenitor cells.

Further preferably, said M2-polarzing factors induce M2-polarization of unpolarized macrophages, M1-polarized macrophages as well as undifferentiated monocytes, and other macrophage progenitor cells.

It is known to the skilled person that Macrophages can undergo specific differentiation depending on the local tissue environment. Similar to the T-helper type 1 (T_H1) and T-helper type 2 (T_H2) polarization, two distinct states of polarization of macrophages have been defined: the classically activated (M1- polarized) macrophage phenotype and the alternatively activated (M2- polarized) macrophage phenotype.

The M2-polarized macrophage phenotype can be further divided into subsets: M2a-polarized, M2b-polarized and M2c-polarized phenotype based on gene expression profiles.

M1-polarized and M2-polarized macrophages have distinct chemokines and chemokine receptor profiles.

M1-polarized macrophages secrete preferably the T_H1 cell-attracting chemokines chemokine (C-X-C motif) ligand 9 (CXCL9) and C-X-C motif chemokine 10 (CXCL10). M2-polarized macrophages secrete preferably the chemokines chemokine (C-C motif) ligand 17 (CCL17), C-C motif chemokine 22 (CCL22), and chemokine (C-C motif) ligand 24 (CCL24).

An M2-polarising effect of a heterologous factor can, for example, be determined by contacting unpolarized macrophages, M1-polarized macrophages, or macrophage progenitor cells, preferably monocytes, with at least one M2-polarizing factor, and, subsequently, determining the expression and/or secretion of M2-polarization markers.

For example, a murine unpolarized macrophage cell line, a murine M1-polarized macrophage cell line, or a murine macrophage progenitor cell line, preferably a murine monocyte cell line, can be contacted with at least one M2-polarizing factor generating a murine M2-polarized macrophage cell line. M2-polarization of murine macrophage cell lines can, for example, be detected by determining expression of the following factors: arginase 1 (Arg1), interleukin 10 (IL-10), mannose receptor C type 1 (Mrc1) and Ym1, which is also called T-lymphocyte-derived eosinophil chemotactic factor (ECF-L) or Chitinase-like protein 3 (Chil3). M1 polarization of murine macrophage cell lines can, for example, be detected by determining expression and/or release of the following factors: tumor necrosis factor alpha (TNFalpha, TNFα), interleukin 6 (IL-6), chemokine (C-C motif) ligand 2 (CCL2), and chemokine (C-C motif) ligand 4 (CCL4).

Preferably human M2-polarized macrophages are derived from human monocytes, which are incubated with at least one M2-polarzing factor. M2-polarization of macrophages derived from human monocytes can, for example, be detected by determining expression and/or release of the following factors: interleukin 1 receptor antagonist (IL1RA),, prostaglandin E2 (PGE2), interleukin 10 (IL-10), and transforming growth factor beta (TGF-β).

It has recently been demonstrated that, in vitro, macrophages are capable of reverse polarization from M2 to M1, and vice versa, in response to changes in the cytokine environment (Davis et al.(2013)). Furthermore, the change in polarization is rapid and occurs at the level of gene expression, protein, metabolite, and microbicidal activity.

Furthermore, macrophages are one of the major populations of infiltrating leukocytes associated with solid tumors. Tumor associated macrophages (TAMs) play an important role in tumor immunity and show similar functions to M2-polarization, also designated M2d TAM polarization.

The respective factors necessary for stimulation and/or activation of the respective polarization (M1, M2a, M2b, M2c, and M2d TAM) are known to the skilled person and, for example, are disclosed in Hao et al. (2012) or Duluc et al. (2007).

Preferably, the respective second heterologous factors and, optionally, third heterologous factor induce M2-polarization after expression in said recombinant probiotic bacteria and release from the bacteria into the site of the inflammatory, preferably chronic inflammatory, skin dysfunction.

Anti-inflammatory M2-polarized macrophages then preferably promote angiogenesis, connective tissue deposition, and wound repair, which leads to an improval, preferably curing, of said inflammatory, preferably chronic inflammatory, skin dysfunction to be treated.

In a preferred embodiment, said M2 polarizing factor is selected from the group consisting of M2-polarizing cytokines, M2-polarizing chemokines, and mixtures thereof. Preferably, said M2 polarizing factor induces M2c polarization.

Further preferably, said M2-polarizing factor is selected from the group consisting of interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), colony stimulating factor-1 (CSF1), interleukin 34 (IL34), functional analogues thereof, biosimilars thereof, and mixtures thereof, preferably interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), colony stimulating factor-1 (CSF1), interleukin 34 (IL34), and mixtures thereof, further preferably colony stimulating factor-1 (CSF1), interleukin 34

(IL34) interleukin 4 (IL-4), interleukin 13 (IL-13), functional analogues thereof, biosimilars thereof, and mixtures thereof, further preferably colony stimulating factor-1 (CSF1), interleukin 34 (IL34) interleukin 4 (IL-4), interleukin 13 (IL-13), and mixtures thereof.

Interleukin 4 (IL-4), preferably human interleukin 4 (hIL-4), is a pleiotropic cytokine. Interleukin 4 is a ligand for the interleukin 4 receptor. The interleukin 4 receptor also binds to interleukin 13 (IL-13), which may contribute to many overlapping functions of interleukin 4 and interleukin 13.

Interleukin 4 (IL-4) has also shown to have proliferation inducing properties. Furthermore, interleukin 4 (IL-4) induces the production of collagen.

The nucleic acid sequence of the mRNA of the human interleukin 4 isoform 1 precursor is available under the NCPI-accession number NM_000589.3. The respective amino acid sequence is available under the NCBI accession number NP_000580.1 and is depicted in Figure 18a.

The nucleic acid sequence of the mRNA of the human interleukin 4 isoform 2 precursor is available under the NCBI accession number NM_172348.2. The respective amino acid sequence is available under the NCBI accession number NP_758858.1 and is depicted in Figure 18b.

The amino acid sequence of human interleukin 4 is also available under the UniProt accession number P05112 – version 178.

The amino acid sequences comprise a signal peptide which spans the amino acids 1 to 24 of the interleukin 4 isoform 1 and isoform 2 precursors.

Preferably, human interleukin 4 comprises one or at least one of the amino acid sequences of SEQ IDs 10 to 14.

Interleukin 13 (IL-13), preferably human interleukin 13 (hIL-13), is an immuno-regulatory cytokine produced primarily by activated T_H2 cells.

The nucleic acid sequence of the mRNA of the human interleukin 13 precursor is available under the NCBI accession number NM_002188.2. The respective amino acid sequence is available under the NCBI accession number NP_002179.2 as well as the UniProt accession number P35225 – version 157 and is depicted in Figure 20a.

The interleukin 13 precursor comprises a signal peptide which spans the amino acids 1 to 24 of the interleukin 13 precursor protein deposited under UniProt accession number P35225 – version 157.

Preferably, interleukin 13 comprises one or at least one of the amino acid sequence of SEQ IDs 17 and 18.

Interleukin 10 (IL-10), preferably human interleukin 10 (hIL-10), is a cytokine produced primarily by monocytes and to a lesser extend by lymphocytes. Interleukin 10 has pleiotropic effects in immunoregulation and inflammation. For example interleukin 10 down-regulates the expression of T_H1 cytokines.

The nucleic acid sequence of the mRNA of the human interleukin 10 precursor can be found under the NCBI accession number NM_000572.2. The respective amino acid sequence can be found under the NCBI accession number NP_000563.1 as well as the UniProt accession number P22301 – version 156 and is depicted in Figure 19a.

The amino acid sequence of the human interleukin 10 precursor includes a signal peptide spanning the amino acids 1 to 18 of the human interleukin 10 precursor protein.

Preferably, interleukin 10 comprises one or at least one of the amino acid sequences of SEQ IDs 15 to 16.

Colony stimulating factor-1 (CSF-1), which is also known as macrophage colony-stimulating factor (M-CSF), is a cytokine that controls the production, differentiation, and function of macrophages.

Due to alternative splicing, the human CSF-1 exists in different isoforms, which can be used in the present invention.

The nucleic acid sequence of the mRNA of the human CSF-1 isoform 1, which is also designated as macrophage colony-stimulating factor 1 isoform A precursor, is available under the NCBI accession number NM_000757.5. The corresponding amino acid sequence is available under the NCBI accession number NP_000748.3 and is depicted in Figure 16a.

The nucleic acid sequence of the mRNA of the human CSF-1 isoform 2 precursor, which is also designated human macrophage colony-stimulating factor 1 isoform B precursor, is available under the NCBI accession number NM_172210.2. The corresponding amino acid sequence is available under the NCBI accession number NP_757349.1 and is depicted in Figure 16b.

The nucleic acid sequence of the mRNA of the human CSF-1 isoform 3 precursor, which is also designated as macrophage colony-stimulating factor 1 isoform C precursor, is available under the NCBI accession number NM_172211.3. The corresponding amino acid sequence is available under the NCBI accession number NP_757350.1 and is depicted in Figure 16c.

The respective amino acid sequences are also available under the UniProt accession number P09603 – version 158. Isoform 1 has been chosen as canonical UniProt sequence.

The respective protein sequences of the human CSF-1 precursor isoforms 1 to 3 include an N-terminal signal peptide, which spans the amino acid number 1 to amino acid number 32 of the respective amino acid sequences.

The active form of human CSF-1 can be found extracellularly as a disulfide-linked homodimer. The active form is produced by proteolytic cleavage of a membrane-bound precursor resulting in the loss of the N-terminal signal peptide.

Preferably, colony stimulating factor 1 comprises one or at least one of the amino acid sequences of SEQ IDs 1 to 6.

Interleukin 34 (IL-34) is a cytokine that also promotes the differentiation and viability of monocytes and macrophages.

Due to alternative splicing, human interleukin 34 exists in two isoforms, which can be used in the present invention.

The nucleic acid sequence of the mRNA of the human interleukin 34 isoform 1 precursor is available under the NCBI accession number NM_001172772.1. The corresponding amino acid sequence is available under the NCBI accession number NP_001166243.1 and is depicted in Figure 17a.

The nucleic acid sequence of the mRNA of the human interleukin 34 isoform 2 precursor is available under the NCBI accession number NM_001172771.1. The corresponding amino acid sequence is available under the NCBI accession number NP_001166242.1 and is depicted in Figure 17b.

The respective amino acid sequence is also available under the UniProt accession number Q6ZMJ4 – version 80.

The human interleukin 34 precursor includes a signal peptide, which spans the amino acids 1 to 20 of the respective amino acid sequences of the precursor proteins.

Preferably, interleukin 34 comprises one or at least of the amino acid sequences of SEQ IDs 7 to 9.

In a preferred embodiment, said M2-polarizing factor promotes the differentiation and viability of monocytes and macrophages through binding to the colony stimulating factor-1 receptor.

Colony stimulating factor-1 receptor (CSF1R), which is also known as macrophage colony stimulating-factor receptor, is a tyrosine protein-kinase that acts as a cell surface receptor and plays an essential role at the regulation of survival, proliferation and differentiation of macrophages and monocytes.

CSF1R promotes, for example, the release of pro-inflammatory chemokines in response to the binding of CSF1R ligand and thereby plays an important role at innate immunity and in inflammatory processes.

Preferably said M2-polarizing factor is at least one colony stimulating factor-1 receptor (CSF1R) ligand.

CSF1R ligands are known to the skilled person and include interleukin 34 (IL-34) and colony stimulating-factor 1 (CSF-1). Preferably, said colony stimulating factor-1 receptor ligand is selected from the group consisting of colony stimulating factor-1 (CSF-1), interleukin 34 (IL-34), functional analogs thereof, and biosimilars thereof.

Further preferably, said colony stimulating factor-1 receptor ligand is a human colony stimulating factor-1 receptor ligand, further preferably selected from the group consisting of human colony stimulating factor-1 (hCSF-1), human interleukin 34 (hIL-34), functional analogs thereof, and biosimilars thereof.

In a further preferred embodiment, said colony stimulating factor-1 receptor ligand is a protein having one or at least one of the amino acid sequences of SEQ IDs No. 1 to 9.

In a preferred embodiment, said first, second, and/or third heterologous factor is expressed with a secretory signal sequence, preferably N-terminal signal peptide or propeptide. After expression of the respective factor, the secretory signal sequence, preferably N-terminal signal peptide or propeptide, can be removed by

posttranslational modification. Alternatively, said first, second, and/or third heterologous factor might be expressed in mature form, preferably without a secretory signal sequence, preferably N-terminal signal peptide or propeptide.

In a preferred embodiment, said recombinant probiotic bacteria according to claim 1 comprise at least one nucleic acid sequence encoding for a first heterologous factor, at least one nucleic acid sequence encoding for a second heterologous factor, and at least one nucleic acid sequence encoding for a third heterologous factor, with the proviso that said first factor, said second factor, and said third factor are functionally different from each other,
wherein said first factor is a growth factor, wherein said second factor is selected from the group consisting of M2-polarizing factors and wherein said third factor is selected from the group consisting of M2-polarizing factors and growth factors.

In a preferred embodiment, said second heterologous factor and said third heterologous factor are selected from the group consisting of M2-polarizing factors, wherein said second factor and said third factor are functionally different M2-polarizing factors.

This is to say, said second heterologous factor is a first M2-polarizing factor and said third heterologous factor is a second M2-polarizing factor functionally different from said first M2-polarizing factor.

Preferably, said first M2-polarizing factor is a M2-polarizing factor selected from the group consisting of colony stimulating factor-1 (CSF-1), interleukin 34 (IL-34), interleukin 4 (IL-4), and interleukin 13 (IL-13), and said second M2-polarizing factor is a M2-polarizing factor selected from the group consisting of colony stimulating factor-1 (CSF-1), interleukin 34 (IL-34), interleukin 4 (IL-4), interleukin 10 (IL-10), and interleukin 13 (IL-13), with the proviso that said second M2-polarizing factor is functionally different from said first M2-polarizing factor.

Further preferably, said first M2-polarizing factor is a colony stimulating factor-1 receptor (CSF1R) ligand and said second M2-polarizing factor is a M2-polarizing factor selected from the group consisting of interleukin 4 (IL-4), interleukin 10 (IL-10),

interleukin 13 (IL-13), functional analogs thereof, biosimilars thereof, and mixtures thereof.

Further preferred combinations of M2-polarizing factors are:

- colony stimulating factor-1 and interleukin 4,
- colony stimulating factor-1 and interleukin 13,
- colony stimulating factor-1 and interleukin 10,
- interleukin 34 and interleukin 4,
- interleukin 34 and interleukin 13,
- interleukin 34 and interleukin 10,
- interleukin 4 and interleukin 10, or
- interleukin 13 and interleukin 10.

The above mentioned further preferred combinations of M2-polarizing factors are combined with at least one of the above mentioned growth factors, preferably with a growth factor selected from the group consisting of fibroblast growth factor 1, fibroblast growth factor 2, fibroblast growth factor 7, fibroblast growth factor 10, hepatocyte growth factor, transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), and platelet derived growth factor BB.

Preferably, said first, second, and third heterologous factor is a combination of

- fibroblast growth factor 2, colony stimulating factor-1 and interleukin 4,
- fibroblast growth factor 2, interleukin 34 and interleukin 4,
- fibroblast growth factor 2, colony stimulating factor-1 and interleukin 13,
- fibroblast growth factor 2, interleukin 34 and interleukin 13,
- fibroblast growth factor 2, colony stimulating factor-1 and interleukin 10,
- fibroblast growth factor 2, interleukin 34 and interleukin 10,
- fibroblast growth factor 7, colony stimulating factor-1 and interleukin 4,
- fibroblast growth factor 7, interleukin 34 and interleukin 4,
- fibroblast growth factor 7, colony stimulating factor-1 and interleukin 13,
- fibroblast growth factor 7, interleukin 34 and interleukin 13,
- fibroblast growth factor 7, colony stimulating factor-1 and interleukin 10,
- fibroblast growth factor 7, interleukin 34 and interleukin 10,

- transforming growth factor beta, colony stimulating factor-1 and interleukin 4,
- transforming growth factor beta, interleukin 34 and interleukin 4,
- transforming growth factor beta, colony stimulating factor-1 and interleukin 13,
- transforming growth factor beta, interleukin 34 and interleukin 13
- transforming growth factor beta, colony stimulating factor-1 and interleukin 10,
- transforming growth factor beta, interleukin 34 and interleukin 10,
- transforming growth factor alpha, colony stimulating factor-1 and interleukin 4,
- transforming growth factor alpha, interleukin 34 and interleukin 4,
- transforming growth factor alpha, colony stimulating factor-1 and interleukin 13,
- transforming growth factor alpha, interleukin 34 and interleukin 13
- transforming growth factor alpha, colony stimulating factor-1 and interleukin 10,
- transforming growth factor alpha, interleukin 34 and interleukin 10
- platelet derived growth factor BB, colony stimulating factor-1 and interleukin 4,
- platelet derived growth factor BB, interleukin 34 and interleukin 4,
- platelet derived growth factor BB, colony stimulating factor-1 and interleukin 13,
- platelet derived growth factor BB, interleukin 34 and interleukin 13
- platelet derived growth factor BB, colony stimulating factor-1 and interleukin 10, or
- platelet derived growth factor BB, interleukin 34 and interleukin 10.

Further preferably, said first, second, and third heterologous factor is a combination of fibroblast growth factor 2, colony stimulating factor-1 and interleukin 4, functional analogs thereof, and biosimilars thereof.

Preferably, by release of two or more M2-polarizing factors from the probiotic bacteria of the present invention M2-polarization of unpolarized macrophages, M1-polarized macrophages as well as undifferentiated monocytes, and other macrophage progenitor cells is further fostered.

In an alternative embodiment, said first factor is a first growth factor selected from the group consisting from the above-mentioned growth factors, and said third factor is a

second growth factor selected from the group consisting from the above-mentioned growth factors and which is functionally different from said first growth factor. Preferably, said second growth factor is transforming growth factor beta (TGF-beta).

Further preferred combinations of growth factors are:

- fibroblast growth factor 1 and transforming growth factor beta,
- fibroblast growth factor 2 and transforming growth factor beta,
- fibroblast growth factor 7 and transforming growth factor beta,
- fibroblast growth factor 10 and transforming growth factor beta,
- platelet derived growth factor BB and transforming growth factor beta,
- transforming growth factor alpha and transforming growth factor beta,
- epidermal growth factor and transforming growth factor beta,
- heparin-binding EGF-like growth factor and transforming growth factor beta,
- hepatocyte growth factor and transforming growth factor beta, or
- vascular endothelial growth factor A and transforming growth factor beta.

The above mentioned further preferred combinations of growth factors are preferably combined with a M2-polarizing factor selected from the group consisting of colony stimulating factor-1 (CSF-1), interleukin 34 (IL-34), interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), and mixtures thereof, preferably colony stimulating factor-1 (CSF-1), interleukin 34 (IL-34), interleukin 4 (IL-4), interleukin 13 (IL-13), and mixtures thereof.

The recombinant probiotic bacteria of the invention are to be used in the treatment of an inflammatory skin dysfunction, preferably a chronic inflammatory skin dysfunction.

In a preferred embodiment, said inflammatory skin dysfunction, preferably said chronic inflammatory skin dysfunction, is an inflammatory skin disease, preferably a chronic inflammatory skin disease, or an inflammatory connective tissue disease, preferably a chronic inflammatory connective tissue disease. Said inflammatory skin disease can be frostbite, eczema, psoriasis, dermatitis, ulcer, wound, lupus eritematosus, neurodermitis, and combinations thereof, preferably frostbites, dermatitis, ulcer, wound, and combinations thereof, further preferably wound, ulcer, and combinations thereof, further preferably ulcer.

Said inflammatory, preferably chronic inflammatory, skin disease can also include an inflammatory, preferably a chronic inflammatory, skin trauma, which may progress into a chronic inflammatory state.

Frostbite is the medical condition in which localized damage is caused to skin and other tissues due to freezing and can involve tissue destruction. Said frostbite can also be chilblains (perniones), which are superficial ulcers of the skin that result from exposure to cold and humidity. Damage to capillary beds in the skin causes redness, itching, inflammation, and sometimes blisters. Further preferably, said frostbites are chilblains.

Said wound can be a burn wound, a chemical wound, a radiation induced wound, an ischemic wound, or a mechanical wound.

Said ulcer can be a decubitus ulcer or an ulcus cruris. Said ulcer can also be a venous ulcer, an arterial ulcer, a diabetic ulcer, or a pressure ulcer.

Said ulcer can also be a preulceration stage of the above-mentioned ulcers without any visible sign of open skin wound. Without medical intervention, the preulceration stage may progress to ulceration.

Said wound can also be an acute wound or a chronic wound, preferably a chronic wound.

Chronic wounds are known to the skilled person and include, for example, chronic venous ulcers, chronic arterial ulcers, chronic diabetic ulcers, and chronic pressure ulcers. Chronic wounds may also be caused by radiation poisoning or ischemia. Preferably said chronic wound is at least one of a chronic venous ulcer, a chronic arterial ulcer, a chronic pressure ulcer and a chronic preulceration stage thereof, preferably a chronic venous ulcer, a chronic arterial ulcer and a chronic pressure ulcer.

Chronic venous ulcers usually may occur in the legs and account for about 70% to 90% of chronic wounds, mostly affecting elderly patients.

Another major cause of chronic wounds is diabetes. Patients suffering from diabetes have a 15% higher risk for amputation than the general population due to chronic ulcers. Diabetes causes neuropathy, which inhibits nociception and the perception of pain. Thus, patients may not notice small wounds to legs and feet and may therefore fail to prevent infection or repeated injury.

A further problem is that diabetes causes immune compromise and damage to small blood vessels resulting in a reduced oxygenation of tissue. Preventing adequate oxygenation of tissue significantly increase the prevalence for chronic wounds.

Pressure ulcers, which are also known as decubitus ulcers or bed sore, may occur with our without a diabetic condition. Pressure ulcers are localized injuries to the skin and/or underlying tissue that can occur over a bony prominence as a result of pressure, or pressure in combination with shear or friction.

Diabetes is a main endocrine metabolic disorder with an expanding proportion that has approached to a worldwide pandemic status. Lower extremity ulceration, for example diabetic foot ulcers, is one of the serious long-term complications associated with diabetes that may be sustained and amplified by the underlying failure of tissue regeneration.

There are approximately 13 million chronic wound patients in the USA, EU, and Japan, of which approximately 2.8 million suffer from lower extremity ulceration such as diabetic foot ulcers.

Lower extremity ulceration, for example diabetic foot ulcers, are challenging to treat with no definite conventional, non-invasive therapy existing. Lower extremity ulceration need a lot of medical attention and are extremely debilitating for the patients associated with substantial loss of quality of life.

Approximately, 24 % of patients suffering from lower extremity ulceration will require amputation leading to long-term disability.

The annual healthcare cost for patients suffering from lower extremity ulceration is in the range of $ 100 billion USD. Furthermore, the five year mortality rate of patients suffering from lower extremity ulceration is about 45 %, which is higher than that of many cancers.

Currently, the standard therapeutic management of chronic wounds including diabetic wounds such as lower extremity ulceration is focussed primarily on controlling infection and promoting revascularisation. Despite these strategies, the rate of amputation remains unacceptably high in patients suffering from lower extremity ulceration.

Furthermore, when an underlying disease condition or cause of an ulceration, such as diabetes mellitus or chronic venous insufficiency, is improved and/or treated, for example by controlling blood sugar levels or administering blood pressure medication, respectively, existing ulcers may still take a very long time to heal.

Thus, in order to overcome the lack of definitive, non-invasive treatments of chronic wounds including diabetic wounds such as lower extremity ulceration new strategies to reactivate and promote wound healing in patients suffering from chronic wounds are urgently needed.

Preferably, in case of a chronic wound, the unique combination of factors, preferably released from said probiotic bacteria, allows for a reprogramming of said chronic wound into an acute wound, which subsequently undergoes wound closure.

In a preferred embodiment, the recombinant probiotic bacteria are to be administered topically and/or by subcutaneous injection, further preferably topically.

The recombinant probiotic bacteria preferably are to be administered topically, to the inflammatory, preferably chronic inflammatory, skin dysfunction to be treated.

The recombinant probiotic bacteria can be administered topically to the inflammatory, preferably chronic inflammatory, skin dysfunction and/or by subcutaneous injection into the vicinity of the skin dysfunction, preferably into the edges or cavity of the inflammatory, preferably chronic inflammatory, skin dysfunction.

After application of the recombinant probiotic bacteria of the present invention, the bacteria express said first and second heterologous factor, preferably said first, second and third heterologous factor.

Furthermore, by topical application or subcutaneous injection of the recombinant probiotic bacteria of the present invention to and/or into the site of the preulceration a progression of the preulceration to an open wound can be avoided.

The recombinant probiotic bacteria are preferably obtained by transforming probiotic bacteria with at least one nucleic acid sequence encoding for said first heterologous factor and at least one nucleic acid sequence encoding for said second heterologous factor and, preferably with at least one nucleic acid sequence encoding for said third heterologous factor.

Suitable probiotic bacteria for obtaining the recombinant probiotic bacteria of the present invention are non-pathogenic bacteria. Preferably, the non-pathogenic bacteria are non-invasive bacteria. In a further preferred embodiment, the recombinant probiotic bacteria are gram-positive bacteria, preferably gram-positive non-sporulating bacteria. Further preferably, said probiotic bacteria are non-colonizing bacteria lacking the ability to multiply in human gastrointestinal tract.

In a further preferred embodiment, the recombinant probiotic bacteria comprise a gram-positive food grade bacterial strain.

According to a preferred embodiment of the present invention, the recombinant probiotic bacteria can be from the same bacterial strain or a mixture of different bacterial strains.

In another embodiment, the probiotic bacteria are classified as “generally recognized as safe” (GRAS) by the United States Food and Drug Administration (FDA).

In another embodiment, the probiotic bacteria have a “qualified presumption of safety” (QPS-status) as defined by the European Food Safety Authority (EFSA). An introduction of the qualified presumption of safety (QPS) approach for the assessment of selected microorganisms is described in the EFSA Journal, Vol. 587, 2007, pages 1-16.

In a further preferred embodiment, the probiotic bacteria are non-pathogenic bacteria belonging to the phylum firmicutes or actinobacteria. Preferably, the probiotic bacteria are non-pathogenic bacteria from at least one genus selected form the group consisting of bifidobacterium, corynebacterium, enterococcus, lactobacillus, lactococcus, leuconostoc, pediococcus, propionibacterium, and streptococcus.

In another preferred embodiment, the probiotic bacteria are lactic acid bacteria (LAB). Lactic acid bacteria are a clade of gram-positive, acid-tolerant, generally non-sporulating, non-respiring bacteria that share common metabolic and physiological characteristics. These bacteria produce lactic acid as a major metabolic end product of carbohydrate formation.

Lactic acid bacteria are long known and are used, for example, in food fermentation. Furthermore, proteinaceous bacteriocins are produced by several lactic acid bacteria strains.

Furthermore, lactic acid bacteria have a generally recognized as safe (GRAS) status due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces.

In a further preferred embodiment, the probiotic bacteria exclude pathogenic and/or opportunistic bacteria.

In another embodiment, the probiotic bacteria are from the genus Bifidobacterium sp., including but not limited to, Bifidobacterium actinocoloniiforme, Bifidobacterium

adolescentis, Bifidobacterium aesculapii, Bifidobacterium angulatum, Bifidobacterium Bifidobacterium animalis, for example Bifidobacterium animalis subsp. animalis or Bifidobacterium animalis subsp. lactis, Bifidobacterium asteroides, Bifidobacterium biavatii, Bifidobacterium bifidum, Bifidobacterium bohemicum, Bifidobacterium bombi, Bifidobacterium boum, Bifidobacterium breve, Bifidobacterium callitrichos, Bifidobacterium catenulatum, Bifidobacterium choerinum, Bifidobacterium coryneforme, Bifidobacterium crudilactis, Bifidobacterium cuniculi, Bifidobacterium denticolens, Bifidobacterium dentium, Bifidobacterium faecale, Bifidobacterium gallicum, Bifidobacterium gallinarum, Bifidobacterium globosum, Bifidobacterium indicum, Bifidobacterium infantis, Bifidobacterium inopinatum, Bifidobacterium kashiwanohense, Bifidobacterium lactis, Bifidobacterium longum, for example Bifidobacterium longum subsp. infantis, Bifidobacterium longum subsp. longum, or Bifidobacterium longum subsp. suis, Bifidobacterium magnum, Bifidobacterium merycicum, Bifidobacterium minimum, Bifidobacterium mongoliense, Bifidobacterium moukalabense, Bifidobacterium pseudocatenulatum, Bifidobacterium pseudolongum, for example Bifidobacterium pseudolongum subsp. globosum or Bifidobacterium pseudolongum subsp. pseudolongum, Bifidobacterium psychraerophilum, Bifidobacterium pullorum, Bifidobacterium reuteri, Bifidobacterium ruminantium, Bifidobacterium saeculare, Bifidobacterium saguini, Bifidobacterium scardovii, Bifidobacterium stellenboschense, Bifidobacterium subtile, Bifidobacterium stercoris, Bifidobacterium suis, Bifidobacterium thermacidophilum, for example Bifidobacterium thermacidophilum subsp. porcinum or Bifidobacterium thermacidophilum subsp. thermacidophilum, Bifidobacterium thermophilum, or Bifidobacterium tsurumiense.

Preferably, the probiotic bacteria are not Bifidobacterium dentium.

Preferably, the probiotic bacteria are bacteria having a “Qualified Presumption of Safety” (QPS) status in the genus Bifidobacterium sp., including but not limited to, Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium longum, Bifidobacterium breve, or Bifidobacterium bifidum.

In one embodiment, the probiotic bacteria are from the genus Corynebacterium sp., including but not limited to, Corynebacterium accolens, Corynebacterium afermentans, for example Corynebacterium afermentans subsp. afermentans or

Corynebacterium afermentans subsp. lipophilum, Corynebacterium ammoniagenes, Corynebacterium amycolatum, Corynebacterium appendices, Corynebacterium aquatimens, Corynebacterium aquilae, Corynebacterium argentoratense, Corynebacterium atypicum, Corynebacterium aurimucosum, Corynebacterium auris, Corynebacterium auriscanis, Corynebacterium betae, Corynebacterium beticola, Corynebacterium bovis, Corynebacterium callunae, Corynebacterium camporealensis, Corynebacterium canis, Corynebacterium capitovis, Corynebacterium casei, Corynebacterium caspium, Corynebacterium ciconiae, Corynebacterium confusum, Corynebacterium coyleae, Corynebacterium cystitidis, Corynebacterium deserti, Corynebacterium diphtheriae, Corynebacterium doosanense, Corynebacterium durum, Corynebacterium efficiens, Corynebacterium epidermidicanis, Corynebacterium equi, Corynebacterium falsenii, Corynebacterium fascians, Corynebacterium felinum, Corynebacterium flaccumfaciens, for example Corynebacterium flaccumfaciens subsp. betae, Corynebacterium flaccumfaciens subsp. flaccumfaciens, Corynebacterium flaccumfaciens subsp. oortii, or Corynebacterium flaccumfaciens subsp. poinsettiae, Corynebacterium flavescens, Corynebacterium frankenforstense, Corynebacterium freiburgense, Corynebacterium freneyi, Corynebacterium glaucum, Corynebacterium glucuronolyticum, Corynebacterium glutamicum, Corynebacterium halotolerans, Corynebacterium hansenii, Corynebacterium hoagie, Corynebacterium humireducens, Corynebacterium ilicis, Corynebacterium imitans, Corynebacterium insidiosum, Corynebacterium iranicum, Corynebacterium jeikeium, Corynebacterium kroppenstedtii, Corynebacterium kutscheri, Corynebacterium lactis, Corynebacterium lilium, Corynebacterium lipophiloflavum, Corynebacterium liquefaciens, Corynebacterium lubricantis, Corynebacterium macginleyi, Corynebacterium marinum, Corynebacterium maris, Corynebacterium massiliense, Corynebacterium mastitidis, Corynebacterium matruchotii, Corynebacterium michiganense, for example Corynebacterium michiganense subsp. insidiosum, Corynebacterium michiganense subsp. michiganense, Corynebacterium michiganense subsp. nebraskense, Corynebacterium michiganense subsp. sepedonicum, or Corynebacterium michiganense subsp. tessellarius, Corynebacterium minutissimum, Corynebacterium mooreparkense, Corynebacterium mucifaciens, Corynebacterium mustelae, Corynebacterium mycetoides, Corynebacterium nebraskense, Corynebacterium nigricans, Corynebacterium nuruki, Corynebacterium oortii,

Corynebacterium paurometabolum, Corynebacterium phocae, Corynebacterium pilbarense, Corynebacterium pilosum, Corynebacterium poinsettiae, Corynebacterium propinquum, Corynebacterium pseudodiphtheriticum, Corynebacterium pseudotuberculosis, Corynebacterium pyogenes, Corynebacterium pyruviciproducens, Corynebacterium rathayi, Corynebacterium renale, Corynebacterium resistens, Corynebacterium riegelii, Corynebacterium seminale, Corynebacterium sepedonicum, Corynebacterium simulans, Corynebacterium singulare, Corynebacterium sphenisci, Corynebacterium spheniscorum, Corynebacterium sputi, Corynebacterium stationis, Corynebacterium striatum, Corynebacterium suicordis, Corynebacterium sundsvallense, Corynebacterium terpenotabidum, Corynebacterium testudinoris, Corynebacterium thomssenii, Corynebacterium timonense, Corynebacterium tritici, Corynebacterium tuberculostearicum, Corynebacterium tuscaniense, Corynebacterium ulcerans, Corynebacterium ulceribovis, Corynebacterium urealyticum, Corynebacterium ureicelerivorans, Corynebacterium uterequi, Corynebacterium variabile, Corynebacterium vitaeruminis, or Corynebacterium xerosis.

Preferably, the probiotic bacteria are not Corynebacterium diphtheriae, Corynebacterium amicolatum, Corynebacterium striatum, Corynebacterium jeikeium, Corynebacterium urealyticum, Corynebacterium xerosis, Corynebacterium pseudotuberculosis, Corynebacterium tenuis, Corynebacterium striatum, or Corynebacterium minutissimum.

Preferably, the probiotic bacteria are bacteria classified as "generally regarded as safe" (GRAS) in the Corynebacterium genus, including but not limited to Corynebacterium ammoniagenes, Corynebacterium casei, Corynebacterium flavescens, or Corynebacterium variabile.

In another embodiment, the bacteria are from the genus Enterococcus sp., including but not limited to, Enterococcus alcedinis, Enterococcus aquimarinus, Enterococcus asini, Enterococcus avium, Enterococcus caccae, Enterococcus camelliae, Enterococcus canintestini, Enterococcus canis, Enterococcus casseliflavus, Enterococcus cecorum, Enterococcus columbae, Enterococcus devriesei, Enterococcus diestrammenae, Enterococcus dispar, Enterococcus durans,

Enterococcus eurekensis, Enterococcus faecalis, Enterococcus faecium, Enterococcus flavescens, Enterococcus gallinarum, Enterococcus gilvus, Enterococcus haemoperoxidus, Enterococcus hermanniensis, Enterococcus hirae, Enterococcus italicus, Enterococcus lactis, Enterococcus lemanii, Enterococcus malodoratus, Enterococcus moraviensis, Enterococcus mundtii, Enterococcus olivae, Enterococcus pallens, Enterococcus phoeniculicola, Enterococcus plantarum, Enterococcus porcinus, Enterococcus pseudoavium, Enterococcus quebecensis, Enterococcus raffinosus, Enterococcus ratti, Enterococcus rivorum, Enterococcus rotai, Enterococcus saccharolyticus, for example Enterococcus saccharolyticus subsp. saccharolyticus or Enterococcus saccharolyticus subsp. taiwanensis, Enterococcus saccharominimus, Enterococcus seriolicida, Enterococcus silesiacus, Enterococcus solitarius, Enterococcus sulfureus, Enterococcus termitis, Enterococcus thailandicus, Enterococcus ureilyticus, Enterococcus viikkiensis, Enterococcus villorum, or Enterococcus xiangfangensis.

Preferably, the probiotic bacteria are bacteria classified as "generally regarded as safe" (GRAS) in the Enterococcus genus, including but not limited to Enterococcus durans, Enterococcus faecalis, or Enterococcus faecium.

In another embodiment, the probiotic bacteria are from the genus Lactobacillus sp., including but not limited to, Lactobacillus acetotolerans, Lactobacillus acidifarinae, Lactobacillus acidipiscis, Lactobacillus acidophilus, Lactobacillus agilis, Lactobacillus algidus, Lactobacillus alimentarius, Lactobacillus amylolyticus, Lactobacillus amylophilus, Lactobacillus amylotrophicus, Lactobacillus amylovorus, Lactobacillus animalis, Lactobacillus antri, Lactobacillus apinorum, Lactobacillus apis, Lactobacillus apodemi, Lactobacillus aquaticus, Lactobacillus arizonensis, Lactobacillus aviaries, for example Lactobacillus aviarius subsp. araffinosus or Lactobacillus aviarius subsp. aviarius, Lactobacillus backii, Lactobacillus bavaricus, Lactobacillus bifermentans, Lactobacillus bobalius, Lactobacillus bombi, Lactobacillus brantae, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus bulgaricus, Lactobacillus cacaonum, Lactobacillus camelliae, Lactobacillus capillatus, Lactobacillus carnis, Lactobacillus casei, for example Lactobacillus casei subsp. alactosus, Lactobacillus casei subsp. casei, Lactobacillus casei subsp. pseudoplantarum, Lactobacillus casei subsp. rhamnosus, or Lactobacillus casei

subsp. tolerans, Lactobacillus catenaformis, Lactobacillus cellobiosus, Lactobacillus ceti, Lactobacillus coleohominis, Lactobacillus collinoides, Lactobacillus composti, Lactobacillus concavus, Lactobacillus confusus, Lactobacillus coryniformis, for example Lactobacillus coryniformis subsp. coryniformis or Lactobacillus coryniformis subsp. torquens, Lactobacillus crispatus, Lactobacillus crustorum, Lactobacillus curieae, Lactobacillus curvatus, for example Lactobacillus curvatus subsp. curvatus or Lactobacillus curvatus subsp. melibiosus, Lactobacillus cypricasei, Lactobacillus delbrueckii, for example Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus delbrueckii subsp. delbrueckii, Lactobacillus delbrueckii subsp. indicus, Lactobacillus delbrueckii subsp. jakobsenii, Lactobacillus delbrueckii subsp. lactis, or Lactobacillus delbrueckii subsp. sunkii, Lactobacillus dextrinicus, Lactobacillus diolivorans, Lactobacillus divergens, Lactobacillus durianis, Lactobacillus equi, Lactobacillus equicursoris, Lactobacillus equigenerosi, Lactobacillus fabifermentans, Lactobacillus faecis, Lactobacillus farciminis, Lactobacillus farraginis, Lactobacillus ferintoshensis, Lactobacillus fermentum, Lactobacillus floricola, Lactobacillus florum, Lactobacillus fornicalis, Lactobacillus fructivorans, Lactobacillus fructosus, Lactobacillus frumenti, Lactobacillus fuchuensis, Lactobacillus furfuricola, Lactobacillus futsaii, Lactobacillus gallinarum, Lactobacillus gasseri, Lactobacillus gastricus, Lactobacillus ghanensis, Lactobacillus gigeriorum, Lactobacillus graminis, Lactobacillus halotolerans, Lactobacillus hammesii, Lactobacillus hamsteri, Lactobacillus harbinensis, Lactobacillus hayakitensis, Lactobacillus heilongjiangensis, Lactobacillus helsingborgensis, Lactobacillus helveticus, Lactobacillus heterohiochii, Lactobacillus hilgardii, Lactobacillus hokkaidonensis, Lactobacillus hominis, Lactobacillus homohiochii, Lactobacillus hordei, Lactobacillus iners, Lactobacillus ingluviei, Lactobacillus intestinalis, Lactobacillus iwatensis, Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus kalixensis, Lactobacillus kandleri, Lactobacillus kefiranofaciens, for example Lactobacillus kefiranofaciens subsp. kefiranofaciens or Lactobacillus kefiranofaciens subsp. kefirgranum, Lactobacillus kefiri, Lactobacillus kefirgranum, Lactobacillus kimbladii, Lactobacillus kimchicus, Lactobacillus kimchiensis, Lactobacillus kimchii, Lactobacillus kisonensis, Lactobacillus kitasatonis, Lactobacillus koreensis, Lactobacillus kullabergensis, Lactobacillus kunkeei, Lactobacillus lactis, Lactobacillus leichmannii, Lactobacillus lindneri, Lactobacillus malefermentans, Lactobacillus mali, Lactobacillus maltaromicus, Lactobacillus manihotivorans, Lactobacillus mellifer, Lactobacillus mellis, Lactobacillus melliventris,

Lactobacillus mindensis, Lactobacillus minor, Lactobacillus minutus, Lactobacillus mucosae, Lactobacillus murinus, Lactobacillus nagelii, Lactobacillus namurensis, Lactobacillus nantensis, Lactobacillus nasuensis, Lactobacillus nenjiangensis, Lactobacillus nodensis, Lactobacillus odoratitofui, Lactobacillus oeni, Lactobacillus oligofermentans, Lactobacillus oris, Lactobacillus oryzae, Lactobacillus otakiensis, Lactobacillus ozensis, Lactobacillus panis, Lactobacillus pantheris, Lactobacillus parabrevis, Lactobacillus parabuchneri, Lactobacillus paracasei, for example Lactobacillus paracasei subsp. paracasei or Lactobacillus paracasei subsp. tolerans, Lactobacillus paracollinoides, Lactobacillus parafarraginis, Lactobacillus parakefiri, Lactobacillus paralimentarius, Lactobacillus paraplantarum, Lactobacillus pasteurii, Lactobacillus paucivorans, Lactobacillus pentosus, Lactobacillus perolens, Lactobacillus piscicola, Lactobacillus plantarum, for example Lactobacillus plantarum subsp. argentoratensis or Lactobacillus plantarum subsp. plantarum, Lactobacillus pobuzihii, Lactobacillus pontis, Lactobacillus porcinae, Lactobacillus psittaci, Lactobacillus rapi, Lactobacillus rennini, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus rimae, Lactobacillus rodentium, Lactobacillus rogosae, Lactobacillus rossiae, Lactobacillus ruminis, Lactobacillus saerimneri, Lactobacillus sakei, for example Lactobacillus sakei subsp. carnosus or Lactobacillus sakei subsp. sakei, Lactobacillus salivarius, for example Lactobacillus salivarius subsp. salicinius or Lactobacillus salivarius subsp. salivarius, Lactobacillus sanfranciscensis, Lactobacillus saniviri, Lactobacillus satsumensis, Lactobacillus secaliphilus, Lactobacillus selangorensis, Lactobacillus senioris, Lactobacillus senmaizukei, Lactobacillus sharpeae, Lactobacillus shenzhenensis, Lactobacillus sicerae, Lactobacillus silagei, Lactobacillus siliginis, Lactobacillus similes, Lactobacillus sobrius, Lactobacillus songhuajiangensis, Lactobacillus spicheri, Lactobacillus sucicola, Lactobacillus suebicus, Lactobacillus sunkii, Lactobacillus suntoryeus, Lactobacillus taiwanensis, Lactobacillus thailandensis, Lactobacillus thermotolerans, Lactobacillus trichodes, Lactobacillus tucceti, Lactobacillus uli, Lactobacillus ultunensis, Lactobacillus uvarum, Lactobacillus vaccinostercus, Lactobacillus vaginalis, Lactobacillus versmoldensis, Lactobacillus vini, Lactobacillus viridescens, Lactobacillus vitulinus, Lactobacillus xiangfangensis, Lactobacillus xylosus, Lactobacillus yamanashiensis, for example Lactobacillus yamanashiensis subsp. mali or Lactobacillus yamanashiensis subsp. yamanashiensis, Lactobacillus yonginensis, Lactobacillus zeae, or Lactobacillus zymae.

Prefereably, the probiotic bacteria are bacteria classified as "generally regarded as safe" (GRAS) in the genus Lactobacillus sp., including but not limited to, Lactobacillus acidophilus strain NP 28, Lactobacillus acidophilus strain NP51 , Lactobacillus subsp. lactis strain NP7, Lactobacillus reuteri strain NCIMB 30242, Lactobacillus casei strain Shirota, Lactobacillus reuteri strain DSM 17938, Lactobacillus reuteri strain NCIMB 30242, Lactobacillus acidophilus strain NCFM, Lactobacillus rhamnosus strain HN001 , Lactobacillus rhamnosus strain HN001, Lactobacillus reuteri strain DSM 17938, Lactobacillus casei subsp. rhamnosus strain GG, Lactobacillus acidophilus, Lactobacillus lactis, Lactobacillus acetotolerans, Lactobacillus acidifarinae, Lactobacillus acidipiscis, Lactobacillus acidophilus, Lactobacillus alimentarius, Lactobacillus amylolyticus, Lactobacillus amylovorus, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus cacaonum, Lactobacillus casei subsp. casei, Lactobacillus collinoides, Lactobacillus composti, Lactobacillus coryniformis subsp. coryniformis, Lactobacillus crispatus, Lactobacillus crustorum, Lactobacillus curvatus subps. curvatus, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus delbrueckii subsp. delbrueckii, Lactobacillus delbrueckii subsp. lactis, Lactobacillus dextrinicus, Lactobacillus diolivorans, Lactobacillus fabifermentans, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus fructivorans, Lactobacillus frumenti, Lactobacillus gasseri, Lactobacillus ghanensis, Lactobacillus hammesii, Lactobacillus harbinensis, Lactobacillus helveticus, Lactobacillus hilgardii, Lactobacillus homohiochii, Lactobacillus hordei, Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus kefiri, Lactobacillus kefiranofadens subsp. kefiranofaciens, Lactobacillus kefiranofadens subsp. kefirgranum, Lactobacillus kimchii, Lactobacillus kisonensis, Lactobacillus mail, Lactobacillus manihotivorans, Lactobacillus mindensis, Lactobacillus mucosae, Lactobacillus nagelii, Lactobacillus namurensis, Lactobacillus nantensis, Lactobacillus nodensis, Lactobacillus oeni, Lactobacillus otakiensis, Lactobacillus panis, Lactobacillus parabrevis, Lactobacillus parabuchneri, Lactobacillus paracasei subsp. paracasei, Lactobacillus parakefiri, Lactobacillus paralimentarius, Lactobacillus paraplantarum, Lactobacillus pentosus, Lactobacillus perolens, Lactobacillus plantarum subsp. plantarum, Lactobacillus pobuzihii, Lactobacillus pontis, Lactobacillus rapi, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus rossiae, Lactobacillus sakei subsp carnosus, Lactobacillus sakei subsp. sakei, Lactobacillus sali varius subsp. salivarius, Lactobacillus

sanfranciscensis, Lactobacillus satsumensis, Lactobacillus secaliphilus, Lactobacillus senmaizukei, Lactobacillus siliginis, Lactobacillus spicheri, Lactobacillus suebicus, Lactobacillus sunkii, Lactobacillus tucceti, Lactobacillus vaccinostercus, Lactobacillus versmoldensis, or Lactobacillus yamanashiensis.

Preferably, the probiotic bacteria are bacteria having a “Qualified Presumption of Safety” (QPS) status in the genus Lactobacillus sp., including but not limited to, Lactobacillus acidophilus, Lactobacillus amylolyticus, Lactobacillus amylovorus, Lactobacillus alimentarius, Lactobacillus aviaries, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus gallinarum, Lactobacillus gasseri, Lactobacillus helveticus, Lactobacillus hilgardii, Lactobacillus johnsonii, Lactobacillus kefiranofaciens, Lactobacillus kefiri, Lactobacillus mucosae, Lactobacillus panis, Lactobacillus paracasei, Lactobacillus paraplantarum, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus pontis, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus sakei, Lactobacillus salivarius, Lactobacillus sanfranciscensis, or Lactobacillus zeae.

In another embodiment, the probiotic bacteria are from the genus Lactococcus sp., including but not limited to Lactococcus chungangensis, Lactococcus formosensis, Lactococcus fujiensis, Lactococcus garvieae, Lactococcus lactis, for example Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. hordniae, Lactococcus lactis subsp. lactis, or Lactococcus lactis subsp. tructae, Lactococcus piscium, Lactococcus plantarum, Lactococcus raffinolactis, or Lactococcus taiwanensis.

Preferably, the probiotic bacteria are bacteria classified as "generally regarded as safe" (GRAS) in the genus Lactococcus, including but not limited to, Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. lactis, and Lactococcus raffinolactis.

Preferably, the probiotic bacteria are Lactococcus lactis, preferably Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. lactis biovariant diacetylactis, or Lactococcus lactis subsp. cremoris, further preferably Lactococcus lactis subsp. cremoris.

In another embodiment, the probiotic bacteria are from the genus Leuconostoc sp., including but not limited to, Leuconostoc amelibiosum, Leuconostoc argentinum, Leuconostoc carnosum, Leuconostoc citreum, Leuconostoc cremoris, Leuconostoc dextranicum, Leuconostoc durionis, Leuconostoc fallax, Leuconostoc ficulneum, Leuconostoc fructosum, Leuconostoc gasicomitatum, Leuconostoc gelidum, for example Leuconostoc gelidum subsp. aenigmaticum, Leuconostoc gelidum subsp. gasicomitatum, or Leuconostoc gelidum subsp. gelidum, Leuconostoc holzapfelii, Leuconostoc inhae, Leuconostoc kimchii, Leuconostoc lactis, Leuconostoc mesenteroides, for example Leuconostoc mesenteroides subsp. cremoris, Leuconostoc mesenteroides subsp. dextranicums, Leuconostoc mesenteroides subsp. mesenteroides, or Leuconostoc mesenteroides subsp. suionicum, Leuconostoc miyukkimchii, Leuconostoc oeni, Leuconostoc paramesenteroides, Leuconostoc pseudoficulneum, or Leuconostoc pseudomesenteroides.

Preferably, the bacteria are bacteria classified as "generally regarded as safe" (GRAS) in the genus Leuconostoc sp., including but not limited to, Leuconostoc carnosum, Leuconostoc citreum, Leuconostoc fallax, Leuconostoc holzapfelii, Leuconostoc inhae, Leuconostoc kimchii, Leuconostoc lactis, Leuconostoc mesenteroides subsp. cremoris, Leuconostoc mesenteroides subsp. dextranicums, Leuconostoc mesenteroides subsp. mesenteroides, Leuconostoc palmae, or Leuconostoc pseudomesenteroides.

Preferably, the probiotic bacteria are bacteria having a “Qualified Presumption of Safety” (QPS) status in the genus Leuconostoc sp., including but not limited to, Leuconostoc citreum, Leuconostoc lactis, Leuconostoc mesenteroides subsp. cremoris, Leuconostoc mesenteroides subsp. dextranicum, or Leuconostoc mesenteroides subsp. mesenteroides.

In another embodiment, the bacteria are from the genus of Pediococcus sp., including but not limited to, Pediococcus acidilactici, Pediococcus argentinicus, Pediococcus cellicola, Pediococcus claussenii, Pediococcus damnosus, Pediococcus dextrinicus. Pediococcus ethanolidurans, Pediococcus halophilus, Pediococcus

inopinatus, Pediococcus lolii, Pediococcus parvulus, Pediococcus pentosaceus, Pediococcus siamensis, Pediococcus stilesii, or Pediococcus urinaeequi

Preferably, the probiotic bacteria are bacteria having a “Qualified Presumption of Safety” (QPS) status in the genus Pediococcus sp., including but not limited to, Pediococcus acidilactici, Pediococcus dextrinicus, or Pediococcus pentosaceus.

In another embodiment, the probiotic bacteria are from the genus Propionibacterium sp., including but not limited to, Propionibacterium acidifaciens, Propionibacterium acidipropionici, Propionibacterium acnes, Propionibacterium australiense, Propionibacterium avidum, Propionibacterium cyclohexanicum, Propionibacterium damnosum, Propionibacterium freudenreichii, for example Propionibacterium freudenreichii subsp. freudenreichii or Propionibacterium freudenreichii subsp. shermanii, Propionibacterium granulosum, Propionibacterium innocuum, Propionibacterium jensenii, Propionibacterium lymphophilum, Propionibacterium microaerophilum, Propionibacterium olivae, Propionibacterium propionicum, or Propionibacterium thoenii.

According to an embodiment, the probiotic bacteria are not Propionibacterium acnes.

Further preferably, the probiotic bacteria are bacteria classified as "generally regarded as safe" (GRAS) in the genus Propionibacterium sp., including but not limited to Propionibacterium acidipropionici, Propionibacterium freudenreichii subsp. Freudenreichii, Propionibacterium freudenreichii subsp. shermanii, Propionibacterium jensenii, or Propionibacterium thoenii.

Further preferably, the probiotic bacteria are Propionibacterium freudenreichii, further preferably Propionibacterium freudenreichii subsp. freudenreichii or Propionibacterium freudenreichii subsp. shermanii.

In another embodiment, the probiotic bacteria are from the genus Streptococcus sp., including but not limited to, Streptococcus acidominimus, Streptococcus adjacens, Streptococcus agalactiae, Streptococcus alactolyticus, Streptococcus anginosus, Streptococcus australis, Streptococcus bovis, Streptococcus caballi, Streptococcus

canis, Streptococcus caprinus, Streptococcus castoreus, Streptococcus cecorum, Streptococcus constellatus, for example Streptococcus constellatus subsp. constellatus, Streptococcus constellatus subsp. pharynges, or Streptococcus constellatus subsp. viborgensis, Streptococcus cremoris, Streptococcus criceti, Streptococcus cristatus, Streptococcus cuniculi, Streptococcus danieliae, Streptococcus defectivus, Streptococcus dentapri, Streptococcus dentirousetti, Streptococcus dentasini, Streptococcus dentisani, Streptococcus devriesei, Streptococcus didelphis, Streptococcus difficilis, Streptococcus downei, Streptococcus durans, Streptococcus dysgalactiae, for example Streptococcus dysgalactiae subsp. dysgalactiae or Streptococcus dysgalactiae subsp. equisimilis, Streptococcus entericus, Streptococcus equi, for example Streptococcus equi subsp. equi, Streptococcus equi subsp. ruminatorum, or Streptococcus equi subsp. zooepidemicus, Streptococcus equinus, Streptococcus faecalis, Streptococcus faecium, Streptococcus ferus, Streptococcus gallinaceus, Streptococcus gallinarum, Streptococcus gallolyticus, for example Streptococcus gallolyticus subsp. gallolyticus, Streptococcus gallolyticus subsp. macedonicus, or Streptococcus gallolyticus subsp. pasteurianus, Streptococcus garvieae, Streptococcus gordonii, Streptococcus halichoeri, Streptococcus hansenii, Streptococcus henryi, Streptococcus hongkongensis, Streptococcus hyointestinalis, Streptococcus hyovaginalis, Streptococcus ictaluri, Streptococcus infantarius, for example Streptococcus infantarius subsp. coli or Streptococcus infantarius subsp. infantarius, Streptococcus infantis, Streptococcus iniae, Streptococcus intermedius, Streptococcus intestinalis, Streptococcus lactarius, Streptococcus lactis, for example Streptococcus lactis subsp. cremoris, Streptococcus lactis subsp. diacetilactis, or Streptococcus lactis subsp. lactis, Streptococcus loxodontisalivarius, Streptococcus lutetiensis, Streptococcus macacae, Streptococcus macedonicus, Streptococcus marimammalium, Streptococcus massiliensis, Streptococcus merionis, Streptococcus minor, Streptococcus mitis, Streptococcus morbillorum, Streptococcus moroccensis, Streptococcus mutans, Streptococcus oligofermentans, Streptococcus oralis, Streptococcus orisasini, Streptococcus orisuis, Streptococcus ovis, Streptococcus parasanguinis, Streptococcus parauberis, Streptococcus parvulus, Streptococcus pasteurianus, Streptococcus peroris, Streptococcus phocae, for example Streptococcus phocae subsp. phocae or Streptococcus phocae subsp. salmonis, Streptococcus plantarum, Streptococcus pleomorphus, Streptococcus pluranimalium,

Streptococcus plurextorum, Streptococcus pneumonia, Streptococcus porci, Streptococcus porcinus, Streptococcus porcorum, Streptococcus pseudopneumoniae, Streptococcus pseudoporcinus, Streptococcus pyogenes, Streptococcus raffinolactis, Streptococcus ratti, Streptococcus rifensis, Streptococcus rubneri, Streptococcus rupicaprae, Streptococcus saccharolyticus, Streptococcus salivarius, for example Streptococcus salivarius subsp. salivarius or Streptococcus salivarius subsp. thermophilus, Streptococcus saliviloxodontae, Streptococcus sanguinis, Streptococcus shiloi, Streptococcus sinensis, Streptococcus sobrinus, Streptococcus suis, Streptococcus thermophilus, Streptococcus thoraltensis, Streptococcus tigurinus, Streptococcus troglodytae, Streptococcus uberis, Streptococcus urinalis, Streptococcus ursoris, Streptococcus vestibularis, or Streptococcus waius.

Preferably, the probiotic bacteria are a bacteria classified as "generally regarded as safe" (GRAS) in the genus Streptococcus sp., including but not limited to, Streptococcus thermophilus strain Th4, Streptococcus gallolyticus subsp. macedonicus, Streptococcus salivarius subsp. salivarius, or Streptococrus salivarius subsp. thermophilus.

Preferably, the probiotic bacteria are bacteria having a “Qualified Presumption of Safety” (QPS) status in the genus Streptococcus sp., including but not limited to, Streptococcus thermophilus.

In a preferred embodiment, said probiotic bacteria produce no endotoxins or other potentially toxic substances. Thereby, said probiotic bacteria are safe to use and are not harmful to a subject after application.

Preferably, said probiotic bacteria produce no spores. Bacterial spores are not part of a sexual cycle but are resistant structures used for survival under unfavourable conditions. Since said probiotic bacteria preferably produce no spores, said recombinant probiotic bacteria can not survive, for example, without nutrients or if an auxotrophic factor is missing.

Preferably, said probiotic bacteria produce no inclusion bodies. Inclusion bodies often contain over-expressed proteins and aggregation of said over-expressed proteins in inclusion bodies can be irreversible.

Since said probiotic bacteria preferably produce no inclusion bodies, the amount of said first, second, and third heterologous factor after transcribing and translating the respective nucleic acid sequences is not diminished by intracellular accumulation in inclusion bodies.

Further preferably, said probiotic bacteria produce also no extracellular proteinases. Extracellular proteinases may be secreted from bacteria to destroy extracellular structures, such as proteins, to generate nutrients, such as carbon, nitrogen, or sulfur. Extracellular proteinases may also act as an exotoxin and be an example of a virulence factor in bacterial pathogenesis.

Due to the absence of extracellular proteinases the safety of said probiotic bacteria after application to a subject is preferably increased. Furthermore, said probiotic bacteria preferably do not degrade the respective first, second, and third heterologous factor after release from said recombinant probiotic bacteria.

Preferably, said probiotic bacteria are devoid of all naturally occurring plasmids. Due to the absence of all naturally occurring plasmids, said probiotic bacteria preferably possess no antibiotic resistance.

Further preferably, said probiotic bacteria lack host factors required for conjugative transposition.

Naturally occurring plasmids can be broadly classified into conjugative plasmids and non-conjugative plasmids. Conjugative plasmids contain a set of transfer or tra genes which promote sexual conjugation between different cells. In the complex process of conjugation, plasmid may be transferred from one bacterium to another via sex pili encoded by some of the tra genes. Non-conjugative plasmids are incapable of initiating conjugation, hence they can be transferred only with the assistance of host factors required for conjugative transposition, such as conjugative plasmids.

Due to the absence of host factors required for conjugative transposition, gene transfer of said nucleic acid(s) encoding the respective first, second, and third heterologous factor from said probiotic bacteria to other microorganisms is highly unlikely, preferably gene transfer is suppressed.

In a further preferred embodiment, said recombinant probiotic bacteria are lactic acid bacteria, preferably a lactobacillus or a lactococcus species. In a further preferred embodiment, said lactococcus species is lactococcus lactis subsp. cremoris.

Lactic acid bacteria further release lactic acid as the major metabolic end-product of carbohydrate fermentation. Lactic acid is known to also stimulate endothelial growth and proliferation. Furthermore, lactic acid has an antibacterial effect which reduces the probability of a bacterial infection at the site of the skin dysfunction.

Techniques for transforming the above-mentioned probiotic bacteria are known to the skilled person, and, for example, are described in Green and Sambrook (2012): “Molecular cloning: a laboratory manual”, fourth edition, Cold Spring Harbour Laboratory Press (Cold Spring Harbor, NY, USA).

After transformation the recombinant probiotic bacteria comprise at least one nucleic acid sequence encoding for said first heterologous factor and at least one nucleic acid sequence encoding for said second heterologous factor, and preferably at least one nucleic acid sequence encoding for said third heterologous factor.

In an embodiment, the at least one nucleic acid sequence encoding for said first heterologous factor and the at least one nucleic acid sequence encoding said second heterologous factor, preferably and the at least one nucleic acid sequence encoding for said third heterologous factor, are located in different sub-populations of the recombinant probiotic bacteria.

For example, the respective nucleic acid sequences are each located in separate sub-populations of the probiotic bacteria, which are combined to obtain the recombinant probiotic bacteria of the present invention.

The respective individual sub-population comprising the respective nucleic acid sequences can, for example, be from the same species or from different species of the above mentioned probiotic bacteria.

For example, different species of recombinant probiotic bacteria expressing different heterologous factors can be used to adapt the expression and, preferably the release, of the respective heterologous factors from the probiotic bacteria.

In a preferred embodiment of the present invention, the respective nucleic acid sequences are present in the recombinant probiotic bacteria in various numbers of copies. For example, the recombinant probiotic bacteria comprise at least one copy of the respective nucleic acid sequences encoding for said first, said second, and/or preferably said third factor.

The numbers of copies of the respective nucleic acid sequence can be increased in order to enhance the expression of the respective heterologous factor.

Preferably, the ratio of copies of the respective nucleic acid sequences encoding for said first, said second, and/or said third factor is 1 : 1 : 1.

The translation efficiency can be enhanced by providing additional copies of a nucleic acid sequence to be transcribed by the recombinant probiotic bacteria.

In a further embodiment of the present invention, a sub-population of the recombinant probiotic bacteria comprises at least one nucleic acid sequence encoding for two of the three heterologous factors wherein preferably the third heterologous factor is encoded by a nucleic acid sequence located in a second sub-population of the recombinant probiotic bacteria.

In a preferred embodiment, the nucleic acid sequences encoding for all three heterologous factors are located in one population of the recombinant probiotic bacteria.

In a further preferred embodiment, the respective nucleic acid sequences are located on at least one of a chromosome and a plasmid of said recombinant probiotic bacteria.

Locating nucleic acid sequence on a chromosome of the recombinant probiotic bacteria or on a plasmid of said recombinant probiotic bacteria determines inter alia the amount of protein translated by the bacteria, since expression levels on a plasmid are higher compared to expression levels on a chromosome.

In a further preferred embodiment of the present invention, at least one nucleic acid sequence encoding for the respective heterologous factor is provided on a chromosome of the bacteria and at least one nucleic acid sequence encoding for another heterologous factor is provided on a plasmid of said recombinant probiotic bacteria.

The respective nucleic acid sequence encoding for the third factor might then either be provided on a chromosome or on a plasmid of the recombinant probiotic bacteria.

In an alternative embodiment of the present invention, the respective nucleic acid sequences are either provided on individual portions of the chromosome of the recombinant probiotic bacteria or on different plasmids of said recombinant probiotic bacteria.

In particular preferred embodiment of the invention, all nucleic acid sequences encoding for said heterologous factors are provided on a single plasmid, for example, each controlled and functionally coupled to a distinct promoter.

In another particular preferred embodiment of the invention, the respective nucleic acid sequences encoding for the respective heterologous factors are located in a single operon which is operatively linked to and controlled by a single promoter.

An operon is a functioning unit of DNA containing a cluster of genes under the control of a single promoter. The genes are transcribed together in mRNA strands and are

either translated together in the cytoplasm, or undergo trans-splicing to create monocistronic mRNAs that are translated separately.

Preferably, the respective nucleic acid sequences encoding for the respective heterologous factors located in said single operon are provided with a nucleic acid sequences encoding a ribosome binding site for translational initiation.

Preferably, the nucleic acid sequences encoding a ribosome binding site is located upstream of the start codon, which is towards the 5' region of the same strand which is to be transcribed. The sequence is preferably complementary to the 3' end of the 16S ribosomal RNA.

Preferably, the respective nucleic acid sequences encoding for the respective heterologous factors located in said single operon are provided with a nucleic acid sequence encoding for a secretory signal sequence at the 5'-end of the open reading frame (ORF) of the heterologous factor generating a fusion protein comprising the secretory signal sequence and the heterologous factor.

In a further preferred embodiment, the nucleic acid sequences are controlled by and functionally coupled to a constitutive promoter or an inducible promoter, preferably to an inducible promoter.

For example, the nucleic acid sequences encoding for the respective heterologous factors can be controlled by and functionally coupled to individual promoters, which could either be a constitutive promoter or an inducible promoter, further preferably an inducible promoter.

A constitutive promoter is active in a cell in all circumstances, while an inducible promoter becomes active in response to specific inducer.

A promoter is a nucleic acid sequence that initiates transcription of a particular gene. Promoters are located near the transcription start side of the gene, on the same strand and upstream of the DNA, which is towards the 5' region of the same strand which is to be transcribed.

Preferably, the respective promoter used is an autologous promoter from the probiotic bacteria, which expresses the respective nucleic acid sequence.

Alternatively, the respective promoter is a heterologous promoter, preferably a prokaryotic promoter, further preferably a promoter from bacteria.

In a further preferred embodiment, the expression of at least one nucleic acid sequence is controlled by an inducible promoter, said at least one nucleic acid sequence being expressible in the presence of at least one inducer.

Preferably, said inducible promoter, which is inducible by inducer, is a promoter for at least one microbial gene which encodes for a lantibiotic peptide.

Lantibiotic peptides are known to the skilled person. Lantibiotics are a class of peptides that contain the characteristic polycyclic thioether amino acid lanthionine as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid.

Lanthionine, for example, is a monosulfide analogue of a cysteine and is composed of two alanine residues that are cross-linked on their beta-carbon atoms by a thioether linkage.

Lantibiotics are produced by a large number of gram-positive bacteria.

In a preferred embodiment, said inducible promoter is a nisin promoter from lactococcus lactis, a bisin promoter from bifidobacterium longum, an optiline promoter from bacillus subtilis, a salvarizine promoter from streptococcus salivarius, an epidermin promoter from staphylococcus epidermidis, a gallidermin promoter from staphylococcus gallinarum, a mutacin promoter from streptococcus mutans, a streptin promoter from streptococcus pyogenes, a streptococcinum promoter from streptococcus pyogenes, a lacticin promoter from lactococcus lactis, an epidermin promoter from staphylococcis epidermidis, a epilanzine promoter from staphylococcus epidermidis, a pep5 promoter from staphylococcus epidermidis, a lactocin-s promoter from lactobacillus sakei, a salvaricin promoter from streptococcus

salivarius, or streptococcus pyogenes, a plantarizine promoter from lactobacillus plantarum, a thermophilin promoter from streptococcus, a bovicine promoter from streptococcus or combinations thereof.

Further preferably, said inducible promoter is PnisA, PnisZ, PnisQ, PnisF, PnisU or combinations thereof of lactococcus lactis.

In a preferred embodiment, the inducer is at least one lantibiotic peptide or functional analogue thereof, preferably selected from the group consisting of nisin A, nisin Z, nisin Q, nisin F, nisin U, functional analogues thereof and mixtures thereof.

A nisin controlled gene expression system is, for example, commercially available from MoBiTec GmbH (Göttingen, Germany) under the trade name NICE®, which was developed at NIZO Food Research (Ede, Netherlands).

Nisin, which is well known to the skilled person, is a 34 amino acid lantibiotic peptide with a broad host spectrum. For example, nisin from Lactococcus lactis is commercially available from Sigma-Aldrich Chemie GmbH (Munich, Germany).

Nisin is widely used as a preservative in food. Initially, nisin is ribosomally synthesized as precursor. After subsequent enzymatic modifications, the modified molecule is translocated across the cytoplasmic membrane and processed into its mature form.

Nisin induces its own expression. Expression of a nucleic acid sequence of interest can be induced by addition of a sub-inhibitory amount of nisin, which is preferably of from 0.01 to 50 ng/ml culture medium, further preferably from 0.1 to 5 ng/ml culture medium, when the gene of interest is subsequently based behind the inducible promoters of the nisin system.

Furthermore, the NICE system has been transferred to other gram-positive bacteria, for example, leuconostoc lactis, lactobacillus brevis, lactobacillus helveticus, lactobacillus plantarum, streptococcus pyogenes, streptococcus agalactiae,

streptococcus pneumoniae, streptococcus zooepidemicus, enterococcus faecalis or lactobacillus subtilis.

An inducible nisin promoter, which preferably is selected from PnisA, PnisZ, PnisQ, PnisF, PnisU, and combinations thereof, can be integrated into a chromosome of the probiotic bacteria used together with at least one nucleic acid sequence encoding for said first heterologous factor, said second heterologous factor, and/or preferably said third heterologous factor.

Preferably, said inducible promoter is PnisA.

Alternatively, the respective promoters together with at least one nucleic acid sequence are provided on a plasmid which is then transformed into the probiotic bacteria.

The respective nucleic acid sequence is expressible in the presence of nisin.

Suitable plasmids are commercially available, for example, from MoBiTec GmbH, (Göttingen, Germany) such as pNZ8008, pNZ8148, pNZ8149, and pNZ8150.

In a further preferred embodiment, the genes NisR and NisK are also provided on either a chromosome or a plasmid of the probiotic bacteria used. The respective protein NisR and NisK belong to the family of bacterial two-component signal transduction systems. NisK is a histidine-protein kinase that resides in the cytoplasmic membrane where it preferably acts as a receptor for the mature nisin molecule. Upon binding of nisin to NisK, it autophosphorylates and transfers the phosphate group to NisR, which is the response regulator that becomes activated upon phosphorylation by NisK.

Activated NisR induces transcription from the PnisA promoter, the PnisF promoter, the PnisZ promoter, PnisQ promoter, and/or the PnisU promoter.

The respective regulators of the expression of the nisin genes NisR and NisK can, for example, be provided on a chromosome of the probiotic bacteria and/or on a plasmid.

Suitable plasmids are commercially available and include, for example, the plasmid pNZ9530 (MoBiTec GmbH, Göttingen, Germany). The plasmid pNZ9530 carries the NisR and NisK genes and is preferably used for cloning in lactococcus strains and in strains of other lactic acid bacteria genera that do not have the regulatory genes integrated into the chromosome. For example, for nisin induced expression in enterococcus faecalis, a plasmid pMSP3535 has been described by Brian et al. (2000) and which is, for example, available from AddGene, a non-profit plasmid repository (Plasmid No. 46886).

The nucleic acid sequence of the plasmid pMSP3535 is available under the NCBI accession number AY303239.1.

In a further preferred embodiment, probiotic bacteria are used, which contain the regulatory genes for nisin controlled gene expression NisR and NisK.

Suitable strains are commercially available, for example, from MoBiTec GmbH (Göttingen, Germany). Suitable strains are, for example, lactococcus lactis strain NZ9000, lactococcus lactis strain NZ9100, lactococcus lactis strain NZ3900.

A suitable strain is also lactococcus lactis strain NZ3000, which is commercially available, for example, from MoBiTec GmbH. Lactococcus lactis strain NZ3000 is a strain without NisR and NisK. Lactococcus lactis strain NZ3000 can be used, when expression of said nucleic acid sequence(s) is/are controlled by a constitutive promoter.

In a further preferred embodiment the probiotic bacteria are lactococcus lactis strain NZ3900, which is a food grade strain. Lactococcus lactis strain NZ3900 includes a plasmid selection system which is not based on antibiotic resistance but on the ability to grow on lactose.

Lactococcus lactis strain NZ3900 produces no endotoxins or other potentially toxic substances. Furthermore, lactococcus lactis strain NZ3900 produces no inclusion bodies and no spores. Moreover, lactococcus lactis strain NZ3900 produces no extracellular proteinases.

In a further preferred embodiment, the nucleic acid sequences encoding for the respective heterologous factors are modified in order to increase expression, secretion and/or stability of the encoded heterologous factor.

Suitable modifications are known to the skilled person and include, for example, adapting the codon usage of the at least one nucleic acid sequence to the probiotic bacteria used.

For example, the at least one nucleic acid sequence can be designed to fit the codon usage pattern of the probiotic bacteria used. Furthermore, in addition to a general codon optimization, specific codon tables can be used, such as a codon table for the highly expressed ribosomal protein genes of the respective probiotic bacteria, to further increase translation of the encoded factors.

Suitable modifications include also, for example, the incorporation of a nucleic acid sequence encoding for a secretory signal sequence at the 5'-end of the open reading frame (ORF) of the heterologous factor generating a fusion protein comprising the secretory signal sequence and the heterologous factor. The secretory signal sequence is preferably arranged at the N-terminal side of the respective heterologous factor.

The secretory signal sequence preferably directs the newly synthesized protein to the plasma membrane. At the end of the secretory signal sequence, preferably signal peptide or propeptide, there is preferably a stretch of amino acids that is recognized and cleaved by a signal peptidase in order to generate a free secretory signal sequence, preferably signal peptide or propeptide, and the mature heterologous factor, which is secreted extracellularly.

In bacteria, two major pathways exist to secrete proteins across the cytoplasmic membrane. The general secretion route, termed Sec-pathway, catalyses the transmembrane translocation of proteins in their unfolded conformation, whereupon they fold into their native structure at the transside of the membrane.

The twin-argenine translocation pathway, termed Tat-pathway, catalyses translocation of secretory proteins in their folded state.

Suitable secretory signal sequences include, for example, a signal peptide.

Preferably, the secretory signal sequence is the native signal peptide or propeptide of the respective heterologous factor encoded by the nucleic acid sequence, which preferably is a precursor and/or preproprotein of the respective factor.

In a further preferred embodiment, said secretory signal sequence is a homologous secretory sequence from said recombinant probiotic bacteria, preferably said secretory signal sequence is the ubiquitin specific peptidase 45 (Usp 45) signal sequence of Lactococcus sp.. The Usp 45 secretion signal has very high secretion efficiency. Other secretion signals are known to the skilled person and include, for example, SP310, SPEXP4 and AL9 of Lactococcus sp..

For example, the native signal peptide of the heterologous factor can be replaced by a homologous secretory signal sequence from the probiotic bacteria used to express said heterologous factor.

A secretory signal sequence preferably improves the secretion efficiency of the respective heterologous factor. For example, in lactococcus lactis, most proteins are secreted via the Sec-pathway. Proteins are synthesized as precursors containing the mature moiety of the proteins with an N-terminal signal peptide. The signal peptide targets the protein to the cyctoplasmic membrane. Following cleavage of the signal peptide, the mature protein is released extracellularly.

Further preferably, the secretory signal comprises the amino acid sequence of SEQ ID No. 34.

In a further preferred embodiment, the respective heterologous factor is expressed as a propeptide with or without a secretion enhancing factor. The propeptide can be cleaved by proteases to release the mature heterologous factor.

In a further preferred embodiment, the recombinant probiotic bacteria comprise at least one inactivated gene encoding for an essential protein necessary for viability of said probiotic bacteria.

In a preferred embodiment, the essential protein necessary for viability of said probiotic bacteria is a protein, further preferably an enzyme, necessary for synthesis of an organic compound necessary for viability of said probiotic bacteria.

After inactivation of said essential protein, preferably enzyme, the respective organic compound is an auxotrophic factor required for the viability of the recombinant probiotic bacteria and which has to be supplemented in order to sustain the viability of the recombinant probiotic bacteria.

Preferably, said auxotrophic factor is a vitamin, amino acid, nucleic acid, and/or a fatty acid.

For example, amino acids and nucleotides are biologically important organic compounds, which are precursors to proteins and nucleic acids, respectively.

In a further preferred embodiment, the at least one gene necessary for viability of the probiotic bacteria is selected from the group consisting of alanine racemase (alaR), thymidylate synthase (thyA), asparagine synthase (asnH), CTP synthase (pyrG), tryptophan synthase (trbBA), and combinations thereof.

For example, alanine racemase is an enzyme that catalyzes the conversion of L-alanine into D-alanine. D-alanine produced by alanine racemase is used for peptidoglycan synthesis. Peptidoglycan is found in the cell walls of all bacteria.

It is known to the skilled person that inactivating the alanine racemase (alaR) gene in bacteria results in the need of the bacteria to use an external source of D-alanine in order to maintain cell wall integrity.

It is known to the skilled person that, for example, lactococcus lactis and lactobacillus plantarum contain a single alaR gene. Inactivation of the gene effects the incorporation of D-alanine into the cell wall.

Lactococcus lactis bacteria, for example, with an inactivated alanine racemase (alaR) gene are dependent on external supply of D-alanine to be able to synthesize peptidoglycan and to incorporate D-alanine in the lipoteichoic acid (LTA). These bacteria lyses rapidly when D-alanine is removed, for example, at mid-exponential growth.

Alanine racemase deficient strains of lactic acid bacteria could, for example, be generated by methods known in the art.

Thymidylate synthase is an enzyme that catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidylate monophosphate (dTMP). dTPM is one of the three nucleotides that form thymine (dTMP, dTDP, and dTTP). Thymidine is a nucleic acid in DNA.

It is known to the skilled person that thymidylate synthase plays a crucial role in the early stage of DNA biosynthesis. For example, DNA damage or deletion occurs on a daily basis as a result of endogenous and environmental factors.

Furthermore, for proliferation of the probiotic bacteria it is necessary to synthesize DNA.

Inactivation of the thymidylate synthase (thyA) gene in bacteria results in the need of the bacteria to use an external source of thymidine in order to maintain DNA integrity.

Asparagine synthetase is an enzyme that generates the amino acid asparagine from aspartate. Inactivation of the asparagines synthetase (asnH) gene results in an

inability to synthesize the respective amino acid, which becomes an auxotrophic factor.

CTP-synthase is an enzyme involved in pyrimidine synthesis. CTP synthase interconverts uridine-5'-triphosphate (UTP) and cytidine triphosphate (CTP). Inactivation of the CTP-synthase (pyrG) gene renders the bacteria unable to synthesize cytosine nucleotides from both the de novo synthesis as well as the uridine cell wall pathway.

CTP becomes an auxotrophic factor, which has to be supplemented for the synthesis of RNA and DNA.

Tryptophan synthase is an enzyme that catalyzes the final two steps in the biosynthesis of the amino acid tryptophan.

Inactivation of the tryptophan synthase (trpBA) gene renders the bacteria unable to synthesize the respective amino acid, which becomes an auxotrophic factor.

Methods for inactivation of said gene necessary for the viability of the probiotic bacteria are known to the skilled person and include deletion of the gene, mutation of the gene, epigenetic modification of said gene, RNA interference (RNAi) mediated gene silencing of said gene, translational inhibition of said gene, or combinations thereof.

In a further preferred embodiment, the respective auxotrophic factor is provided together with said probiotic bacteria.

In a further preferred embodiment of the invention, said inactivated gene necessary for viability of the probiotic bacteria is used for environmental containment.

After application of the recombinant probiotic bacteria comprising at least one inactivated gene necessary for viability of said probiotic bacteria, the respective auxotrophic factor has to be provided externally, for example, with an application medium or a growth medium.

In case the recombinant probiotic bacteria are released into the environment, the auxotrophic factor is missing and, preferably, the recombinant probiotic bacteria die.

Furthermore, application of the externally supplemented auxotrophic factor enables for a control of the biosynthesis and release of the respective heterologous factors.

Preferably, at least one of said first factor, said second factor and said third factor is releasable from said recombinant probiotic bacteria. Further preferably, at least one of said first factor, said second factor and said third factor is secreted from said recombinant probiotic bacteria.

For example, in gram-positive bacteria, such as lactic acid bacteria, proteins to be secreted from the bacteria are preferably synthesized as a precursor containing an N-terminal signal peptide and the mature moiety of the protein. Precursors are recognized by the secretion machinery of the bacteria and are translocated across the cytoplasmic membrane. The signal peptide is then cleaved and degraded and the mature protein is secreted from the bacteria, for example, into the culture supernatant or into the area of an inflammatory skin dysfunction.

For example, lactococcus lactis is able to secrete proteins ranging from low molecular mass, for example smaller than 10 kDa, to high molecular mass, for example molecular weight above 160 kDa, through a Sec-dependent pathway.

Alternatively, at least one of said first factor, said second factor, and said third factor is released from said recombinant probiotic bacteria through a leaky cytoplasmic membrane.

For example, when using nisin as an inducer for protein synthesis in said recombinant probiotic bacteria, nisin also forms pores within the cytoplasmic membrane through which at least one of said first factor, said second factor, and said third factor is released from the probiotic bacteria.

Alternatively, the cytoplasmic membrane of said recombinant probiotic bacteria becomes leaky due to an impaired synthesis of cell wall components.

For example, said recombinant probiotic bacteria comprise an inactivated alanine racemase (alaR) gene. In the absence of D-alanine, said recombinant probiotic bacteria cannot maintain the integrity of the cytoplasmic membrane and, subsequently, at least one of said first factor, said second factor and said third factor is released from said recombinant probiotic bacteria.

During the healing process of said inflammatory skin dysfunction, the released first heterologous factor, second heterologous factor and/or third heterologous factor can undergo degradation, for example by protease cleavage, which may lead to a loss of the biological activity of said heterologous factors.

The degradation or depletion of said heterologous factors can be compensated by a sustained release of said heterologous factors from said recombinant probiotic bacteria. Preferably, said recombinant probiotic bacteria express at least one of said first heterologous factor, said second heterologous factor and said third heterologous factor in a constant manner. Preferably, said recombinant probiotic bacteria release said heterologous factors constantly.

In a preferred embodiment, said probiotic bacteria are provided in a pharmaceutical composition for use in the treatment of said inflammatory skin dysfunction.

Preferably, said pharmaceutical composition comprises at least one pharmaceutically acceptable carrier or excipient which, further preferably, is suitable for the intended route of administration.

Preferably, said pharmaceutically acceptable carrier or said pharmaceutically acceptable excipient comprises at least one polymeric carrier, preferably selected from the group consisting of polysaccharides, polyesters, polymethacrylamide, and mixtures thereof. For example, said pharmaceutically acceptable carrier is a hydrogel, which, for example, provide additionally optimal moisture environment for promoting wound healing. Furthermore, the pharmaceutically acceptable carrier

assists in the adhesion of said recombinant probiotic bacteria to the site of said inflammatory skin dysfunction after application.

Preferably, said pharmaceutical composition further comprises at least one nutrient for said recombinant probiotic bacteria, preferably selected from the group consisting of carbohydrates, vitamins, minerals, amino acids, trace elements and mixtures thereof.

Further preferably, said pharmaceutical composition further comprises at least one component for stabilizing at least one of said first factor, said second factor and said third factor.

Said stabilizing compound is preferably selected from the group consisting of anti-microbial agents, cryoprotectants, protease inhibitors, reducing agents, metal chelators, and mixtures thereof.

In a further preferred embodiment, said recombinant probiotic bacteria are in a solution, frozen or dried, preferably lyophilized or spray dried.

Preferably, said recombinant probiotic bacteria are to be applied in a solution, for example, in form of said pharmaceutical composition.

In an alternative embodiment, said recombinant probiotic bacteria are frozen or dried, preferably lyophilized or spray dried. The recombinant probiotic bacteria might be reconstituted prior to use.

After application, the recombinant probiotic bacteria might be contacted with said at least one inducer, preferably to induce expression of at least one of said first factor, said second factor, and said third factor.

Further preferably, after induction of the expression, at least one of said first factor, said second factor and said third factor is released from said bacteria.

Literature

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Sequences

SEQ ID Nos. 1 to 3 correspond to human CSF-1 isoform 1 to 3 precursor, respectively, including a signal peptide.
SEQ ID No. 4 corresponds to the mature human CSF-1.

SEQ ID No. 5 corresponds to the synthetic construct of CSF-1 used in Example 1, including the Usp45 secretion signal from Lactococcus lactis, which spans the amino acids 1 to 27 of the protein, and mature human CSF-1, which spans the amino acids 28 to 185 of the protein.

SEQ ID No. 6 corresponds to the synthetic construct of CSF-1 generated after secretion from Lactococcus lactis expressing pCSF1.

SEQ ID Nos. 7 and 8 correspond to human IL-34 isoform 1 and 2 precursor, respectively, including a signal peptide.

SEQ ID No. 9 corresponds to the mature human IL-34.

SEQ ID Nos. 10 and 11 correspond to human IL-4 isoform 1 and 2 precursor, respectively, including a signal peptide.

SEQ ID No. 12 corresponds to the mature human IL-4.

SEQ ID No. 13 corresponds to the synthetic construct of human IL-4 used in Example 1, including the Usp45 secretion signal from Lactococcus lactis, which spans the amino acids 1 to 27 of the protein, and mature human IL-4, which spans the amino acids 28 to 185 of the protein.

SEQ ID No. 14 corresponds to the synthetic construct of IL-4 generated after secretion from Lactococcus lactis expressing pIL4.

SEQ ID No. 15 corresponds to human IL-10 precursor including a signal peptide.

SEQ ID No. 16 corresponds to the mature human IL-10.

SEQ ID No. 17 corresponds to human IL-13 precursor including a signal peptide.

SEQ ID No. 18 corresponds to the mature human IL-13.

SEQ ID No. 19 corresponds to human TGF-β1 precursor including a signal peptide.

SEQ ID No. 20 corresponds to the mature human TGF-β1.

SEQ ID Nos. 21 and 22 correspond to human TGF-β2 isoform 1 and 2 precursor, respectively, including a signal peptide.

SEQ ID No. 23 corresponds to the mature human TGF-β2.

SEQ ID No. 24 corresponds to human TGF-β3 preproprotein including a signal peptide.

SEQ ID No. 25 corresponds to the mature human TGF-β3.

SEQ ID No. 26 corresponds to human FGF-2 preproprotein, including a propeptide.

SEQ ID No. 27 corresponds to the mature human FGF-2 after secretion (hFGF2-155).

SEQ ID No. 28 corresponds to the partial sequence of the mature human FGF-2.

SEQ ID No. 29 corresponds to the synthetic construct of human FGF-2 used in Example 1, including the Usp45 secretion signal from Lactococcus lactis, which spans the amino acids 1 to 27 of the protein, and mature human FGF-2, which corresponds to the amino acids 136 to 288 of SEQ ID No. 26.

SEQ ID No. 30 corresponds to the human FGF-2 variant hFGF2-153 used in Example 1 after secretion from Lactococcus lactis expressing pFGF.

SEQ ID No. 31 corresponds to human HGF.

SEQ ID No. 32 corresponds to human HGF alpha chain.

SEQ ID No. 33 corresponds to human HGF beta chain.

SEQ ID No. 34 corresponds to the Usp45 secretion signal from Lactococcus lactis.

SEQ ID No. 35 corresponds to the nucleic acid sequence of the plasmid pNZ8149.

SEQ ID Nos. 36 to 43 correspond to primers used in the Examples.

SEQ ID No. 44 corresponds to the nucleic acid sequence of nisin A promoter of Lactococcus lactis.

SEQ ID No. 45 corresponds to the nucleic acid sequence of the synthetic construct ssUsp45-FGF2-153 containing the nisinA promoter, the Usp45 signal sequence and the hFGF2-153 gene.

SEQ ID No. 46 corresponds to the nucleic acid sequence of the plasmid pFGF2.

SEQ ID No. 47 corresponds to the nucleic acid sequence of the synthetic construct ssUsp45-hIL4 containing the nisinA promoter, the Usp45 signal sequence and the hIL4 gene.
SEQ ID No. 48 corresponds to the nucleic acid sequence of the plasmid pIL4.

SEQ ID No. 49 corresponds to the nucleic acid sequence of the synthetic construct ssUsp45-hCSF1 containing the nisinA promoter, the Usp45 signal sequence and the hCSF1 gene.
SEQ ID No. 50 corresponds to the nucleic acid sequence of the plasmid pCSF1.

SEQ ID No. 51 to SEQ ID No. 53 correspond to nucleic acid sequences providing the respective ribosome binding sites including the start codon (ATG) of the respective open reading frame used in the Examples.

SEQ ID No. 54 corresponds to the nucleic acid sequence of the 3′ end of 16S ribosomal RNA (rRNA) of Lactococcus lactis.

SEQ ID No. 55 corresponds to the nucleic acid sequence of the synthetic construct CSF-RBS1-FGF-RBS2-IL4 depicted in Figure 24b. SEQ ID No. 56 corresponds to the nucleic acid sequence of the synthetic construct FGF-RBS1-IL4-RBS2-CSF depicted in Figure 24c. SEQ ID No. 57 corresponds to the nucleic acid sequence of the synthetic construct IL4-RBS1-CSF-RBS2-FGF depicted in Figure 24d.

SEQ ID No. 58 corresponds to the amino acid sequence of epidermal growth factor isoform 1 preproprotein from Homo sapiens. SEQ ID No. 59 corresponds to the amino acid sequence of epidermal growth factor isoform 2 preproprotein from Homo sapiens. SEQ ID No. 60 corresponds to the amino acid sequence of epidermal growth factor isoform 3 preproprotein from Homo sapiens. SEQ ID No. 61 corresponds to the amino acid sequence of the mature epidermal growth factor from Homo sapiens. SEQ ID No. 62 corresponds to the amino acid sequence of the heparin-binding EGF-like growth factor precursor from Homo sapiens. SEQ ID No. 63 corresponds to the amino acid sequence of the mature heparin-binding EGF-like growth factor from Homo sapiens.

SEQ ID No. 64 corresponds to the amino acid sequence of transforming growth factor alpha isoform 1 preproprotein from Homo sapiens. SEQ ID No. 65 corresponds to the amino acid sequence of transforming growth factor alpha isoform 2 preproprotein from Homo sapiens. SEQ ID No. 66 corresponds to the amino acid sequence of transforming growth factor alpha isoform 3 preproprotein from Homo sapiens. SEQ ID No. 67 corresponds to the amino acid sequence of transforming growth factor alpha isoform 4 preproprotein from Homo sapiens. SEQ ID No. 68 corresponds to the amino acid sequence of transforming growth factor alpha isoform 5 preproprotein from Homo sapiens. SEQ ID No. 69 corresponds to the amino acid sequence of the mature transforming growth factor alpha from Homo sapiens.

SEQ ID No. 70 corresponds to the amino acid sequence of amphiregulin preproprotein from Homo sapiens. SEQ ID No. 71 corresponds to the amino acid sequence of the mature amphiregulin from Homo sapiens.

SEQ ID No. 72 corresponds to the amino acid sequence of epiregulin preproprotein from Homo sapiens. SEQ ID No. 73 corresponds to the amino acid sequence of the mature epiregulin from Homo sapiens.

SEQ ID No. 74 corresponds to the amino acid sequence of the epigen isoform 1 precursor from Homo sapiens. SEQ ID No. 75 corresponds to the amino acid sequence of the epigen isoform 2 precursor from Homo sapiens. SEQ ID No. 76 corresponds to the amino acid sequence of the epigen isoform 3 precursor from Homo sapiens. SEQ ID No. 77 corresponds to the amino acid sequence of the epigen isoform 4 precursor from Homo sapiens. SEQ ID No. 78 corresponds to the amino acid sequence of the epigen isoform 5 precursor from Homo sapiens. SEQ ID No. 79 corresponds to the amino acid sequence of the epigen isoform 6 precursor from Homo sapiens. SEQ ID No. 80 corresponds to the amino acid sequence of the epigen isoform 7 precursor from Homo sapiens. SEQ ID No. 81 corresponds to the amino acid sequence of the mature epigen from Homo sapiens.

SEQ ID No. 82 corresponds to the amino acid sequence of the betacellulin precursor from Homo sapiens. SEQ ID No. 83 corresponds to the amino acid sequence of the mature betacellulin from Homo sapiens.

The following Figures and Examples are given for illustrative purpose only. The invention is not to be construed to be limited to the following examples:

Figures:

Figure 1 shows a SDS-PAGE of a human FGF2 reference protein.

Figure 2 shows a plasmid map of expression vector pNZ8149. “lacF” is a selection marker for growth of the host strain NZ3900 on lactose; “Pnis” is the nisin A promoter of the nisin operon of Lactococcus lactis; “T” is a terminator; “repC” and “repA” are replication genes. The nucleic acid sequence is also shown in SEQ ID No. 35.

Figure 3 shows the nucleic acid sequence of the nisinA promoter of L. lactis, which is also shown in SEQ ID No. 44.

Figures 4a, 4b, and 4c show readouts of SignalP 4.1 obtained with the amino acid sequence depicted in SEQ ID No. 29, SEQ ID No. 13, and SEQ ID No.5, respectively.

Figures 5a, 5b, and 5c show the synthesized inserts used in Example 1 as well as the corresponding amino acid sequence. “FGF2-153” denotes the human FGF-2 coding insert. “hIL4” denotes the human IL4 coding insert. “hCSF1” denotes the human CSF1 coding insert. “PnisA” denotes the nisinA promoter of the Lactococcus lactis nisin operon. “ssUsp45” denotes the signal sequence of the usp45 gene of Lactococcus lactis. An asterisk denotes a stop codon. The respective nucleic acid sequences are also depicted in SEQ ID No. 45, SEQ ID No. 47, and SEQ ID No. 49.

Figures 6a, 6b, and 6c show the results of colony PCR analysis of potential colonies of Lactococcus lactis NZ3900 with pFGF2, pIL4, and pCSF1, respectively.

Figures 7a, 7b, and 7c show plasmid maps as well as the nucleic acid sequence of the plasmids pFGF2, pIL4, and pCSF1, respectively, for the expression and secretion of hFGF2, hIL4, and hCSF1, respectively, in Lactococcus lactis. “Pnis” is the nisin A

promoter of the nisin operon of Lactococcus lactis; "T" is a terminator; "repC" and "repA" are replication genes. The nucleic acid sequences of the plasmids pFGF2, pIL4, and pCSF1, are depicted in SEQ ID No. 46, SEQ ID No. 48, and SEQ ID No. 50, respectively.

Figure 8 shows a Western blot analysis of fermentation samples of Lactococcus expressing NZ3900(pFGF2) under different induction conditions. "0.5/5" denotes induction at OD 600 = 0.5 with 5 ng/mL nisin; "3/5" denotes induction at OD 600 = 3 with 5 ng/mL nisin, "T" denotes the time point, at which a sample was taken from the fermentation.

Figure 9 shows a SDS-PAGE analysis of supernatant (Sup) samples from controlled fermentation runs after Coomassie (Coom) staining and a Western blot (WB) analysis of fermentation samples of Lactococcus expressing NZ3900(pIL4). 0.5/5" denotes induction at OD 600 = 0.5 with 5 ng/mL nisin; "3/5" denotes induction at OD 600 = 3 with 5 ng/mL nisin, "T" denotes the time point, at which a sample was taken from the fermentation.

Figure 10 shows a SDS-PAGE analysis of supernatant (Sup) samples from controlled fermentation runs after Coomassie (Coom) staining of fermentation samples of Lactococcus expressing NZ3900(pCSF1). 0.5/5" denotes induction at OD 600 = 0.5 with 5 ng/mL nisin; "3/5" denotes induction at OD 600 = 3 with 5 ng/mL nisin, "T" denotes the time point, at which a sample was taken from the fermentation.

Figure 11 shows a Western blot (WB) analysis of fermentation samples of Lactococcus expressing NZ3900(pCSF1). 0.5/5" denotes induction at OD 600 = 0.5 with 5 ng/mL nisin; "3/5" denotes induction at OD 600 = 3 with 5 ng/mL nisin, "T" denotes the time point, at which a sample was taken from the fermentation.

Figure 12 shows the growth of acidifying cultures for induction experiment under conditions that resemble wound healing circumstances.

Figure 13 shows SDS-PAGE and Western blot analysis of hFGF2 production in the supernatant after induction at pH 5 or at OD 600 = 3.

Figure 14a shows the result of a human fibroblast migration assay described in Example 4a after 72 h exposure with recombinant probiotic bacteria expressing hFGF2 or the commercially available product Fiblast.

Figure 14b shows the result of a human fibroblast migration assay described in Example 4a after 72 h exposure with recombinant probiotic bacteria expressing hIL4 or recombinant human IL4.

Figure 14c shows the result of the induction of ERK 1+2 phosphorylation in NHDF cells by AUP-10 culture medium containing 0.4-80 ng/ml FGF2 and by rhFGF2.

Figure 14d shows the result of the induction of ERK 1+2 phosphorylation in M-NFS-60 cells by AUP-14 culture medium containing 0.2-40 ng/ml of CSF1 and by rhCSF1.

Figure 14e shows the inhibition of LPS-induced TNFalpha mRNA production in human THP-1 cells described in Example 4c.

Figure 14f shows the induction of RNA expression of M2 genes for the macrophage mannose receptor 1 (Mrc1) and Kruppel-like factor 4 (KLF4) in THP1-derived macrophages as described in Example 4d.

Figure 14g shows the STAT-6 transcriptional Activity of AUP-12 and rhIL-4 in Human Glial Hybrid Cell Line MO3.13 as described in Example 4e.

Figure 14h shows the STAT-6 Transcriptional Activity of AUP-12 and rhIL-4 in Human Embryonic Kidney 293T Cells as described in Example 4e.

Figure 15a shows results of wound healing experiments described in Example 5.

Figure 15b shows a comparison of wound-healing status of control, positive control and AUP-16 treated tissue after the indicated time points described in Example 5.

Figure 15c shows a comparison of the remaining wound area after day 8 and day 12 of the positive control (treatment group 17) and AUP-16 treated tissue (treatment group 18) described in Example 5.

Figure 16a shows the amino acid sequence of macrophage colony-stimulating factor 1 isoform a precursor from Homo sapiens. Figure 16b shows the amino acid sequence of macrophage colony-stimulating factor 1 isoform b precursor from Homo sapiens. Figure 16c shows the amino acid sequence of macrophage colony-stimulating factor 1 isoform c precursor from Homo sapiens. Figure 16d shows the amino acid sequence of the mature human macrophage colony-stimulating factor 1.

Figure 17a shows the amino acid sequence of interleukin-34 isoform 1 precursor from Homo sapiens. Figure 17b shows the amino acid sequence of interleukin-34 isoform 2 precursor from Homo sapiens. Figure 17c shows the amino acid sequence of interleukin-34 from Homo sapiens.

Figure 18a shows the amino acid sequence of interleukin-4 isoform 1 precursor from Homo sapiens. Figure 18b shows the amino acid sequence of interleukin-4 isoform 2 precursor from Homo sapiens. Figure 18c shows the amino acid sequence of interleukin-4 from Homo sapiens.

Figure 19a shows the amino acid sequence of interleukin-10 precursor from Homo sapiens. Figure 19b shows the amino acid sequence of interleukin-10 from Homo sapiens.

Figure 20a shows the amino acid sequence of interleukin-13 precursor from Homo sapiens. Figure 20b shows the amino acid sequence of interleukin-13 from Homo sapiens.

Figure 21a shows the amino acid sequence of transforming growth factor beta-1 precursor from Homo sapiens. Figure 21b shows the amino acid sequence of transforming growth factor beta-1 from Homo sapiens. Figure 21c shows the amino acid sequence of transforming growth factor beta-2 isoform 1 precursor from Homo sapiens. Figure 21d shows the amino acid sequence of transforming growth factor

beta-2 isoform 2 precursor from Homo sapiens. Figure 21e shows the amino acid sequence of transforming growth factor beta-2 from Homo sapiens. Figure 21f shows the amino acid sequence of transforming growth factor beta-3 preproprotein from Homo sapiens. Figure 21g shows the amino acid sequence of transforming growth factor beta-3 from Homo sapiens.

Figure 22a shows the amino acid sequence of fibroblast growth factor 2 precursor from Homo sapiens. Figure 22b shows the amino acid sequence of a fragment of fibroblast growth factor 2 from Homo sapiens.

Figure 23a: Amino acid sequence of hepatocyte growth factor from Homo sapiens. Figure 23b: Amino acid sequence of hepatocyte growth factor alpha chain from Homo sapiens. Figure 23c: Amino acid sequence of hepatocyte growth factor beta chain from Homo sapiens.

Figure 24a shows an overview of the three ribosome binding sites used in Example 2d. The respective sequences of the synthesized DNA sequences CSF-RBS1-FGF-RBS2-IL4, FGF-RBS1-IL4-RBS2-CSF, and IL4-RBS-CSF-RBS2-FGF used in Example 2d are depicted in Figures 24b to 24d, respectively, as well as in SEQ ID No. 55, SEQ ID No. 56, and SEQ ID No. 57, respectively. Figures 24e to 24g show respective SDS-PAGE gels as well as Western blots obtained with the single operon constructs used in Example 2d).

Figure 25a shows the amino acid sequence of epidermal growth factor isoform 1 preproprotein from Homo sapiens. Figure 25b shows the amino acid sequence of epidermal growth factor isoform 2 preproprotein from Homo sapiens. Figure 25c shows the amino acid sequence of epidermal growth factor isoform 3 preproprotein from Homo sapiens. Figure 25d shows the amino acid sequence of the mature epidermal growth factor from Homo sapiens.

Figure 26a shows the amino acid sequence of the heparin-binding EGF-like growth factor precursor from Homo sapiens. Figure 26b shows the amino acid sequence of the mature heparin-binding EGF-like growth factor from Homo sapiens.

Figure 27a shows the amino acid sequence of transforming growth factor alpha isoform 1 preproprotein from Homo sapiens. Figure 27b shows the amino acid sequence of transforming growth factor alpha isoform 2 preproprotein from Homo sapiens. Figure 27c shows the amino acid sequence of transforming growth factor alpha isoform 3 preproprotein from Homo sapiens. Figure 27d shows the amino acid sequence of transforming growth factor alpha isoform 4 preproprotein from Homo sapiens. Figure 27e shows the amino acid sequence of transforming growth factor alpha isoform 5 preproprotein from Homo sapiens. Figure 27f shows the amino acid sequence of the mature transforming growth factor alpha from Homo sapiens.

Figure 28a shows the amino acid sequence of amphiregulin preproprotein from Homo sapiens. Figure 28b shows the amino acid sequence of the mature amphiregulin from Homo sapiens.

Figure 29a shows the amino acid sequence of epiregulin preproprotein from Homo sapiens. Figure 29b shows the amino acid sequence of the mature epiregulin from Homo sapiens.

Figure 30a shows the amino acid sequence of the human epigen isoform 1 precursor from Homo sapiens. Figure 30b shows the amino acid sequence of the human epigen isoform 2 precursor from Homo sapiens. Figure 30c shows the amino acid sequence of the human epigen isoform 3 precursor from Homo sapiens. Figure 30d shows the amino acid sequence of the human epigen isoform 4 precursor from Homo sapiens. Figure 30e shows the amino acid sequence of the human epigen isoform 5 precursor from Homo sapiens. Figure 30f shows the amino acid sequence of the human epigen isoform 6 precursor from Homo sapiens. Figure 30g shows the amino acid sequence of the human epigen isoform 7 precursor from Homo sapiens. Figure 30h shows the amino acid sequence of the mature epigen from Homo sapiens.

Figure 31a shows the amino acid sequence of the betacellulin precursor from Homo sapiens. Figure 31b shows the amino acid sequence of the mature betacellulin from Homo sapiens.

Figure 32a shows the amino acid sequence of the mature fibroblast growth factor 2 from Homo sapiens. Figure 32b shows the amino acid sequence of the fibroblast growth factor 2 variant hFGF2-153 used in the Examples.

Figure 33 shows the amino acid sequence of the human interleukin 4 (hIL-4) variant used in the Examples.

Figure 34 shows the amino acid sequence of the human colony stimulating factor 1 (hCSF-1) variant used in the Examples.

Figure 35 shows the amino acid sequence of the secretion signal of the Lactococcus protein Usp45 used in the Examples.

Examples:

1. General experimental procedures:

Unless otherwise stated, Examples were carried out following the protocol of the manufacturer of the analytical systems. Unless otherwise stated, indicated chemicals were commercially obtained from Sigma-Aldrich Chemie Gmbh (Munich, DE), Merck KGaA (Darmstadt, DE), Thermo Fisher Scientific Inc.(Waltham, MA, USA) or Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

1.1 Growth media

For different purposes cells were grown in different media. For general cloning procedures M17 medium (Oxoid Deutschland GmbH, Wesel, DE) was used containing 0.5% glucose or lactose.

In order to monitor food grade selection the Elliker medium was used, on which lactococcal colonies colour yellow when they grow on lactose. The yellow colouring is a pH effect that changes the colour of the indicator dye bromocresol purple in the vicinity of a growing colony and of the colony itself.

The recipe of the Elliker medium is as follows:

20 g/L	tryptone
5 g/L	yeast extract
4 g/L	NaCl
1.5 g/L	Na-acetate (water free)
0.5 g/L	L(+)Ascorbic acid
15 g/L	agar
pH 6.8	

Sterilization: 15 min at 121 °C

After sterilization and cooling 1% lactose was added from 20% heat- or filter-sterilized stock and 0.004% bromocresol purple (0.4% stock solution, filter sterilized).

For fermentations and other functional growth experiments the IM1 medium was used, which is free from animal derived ingredients. Lactose, the only remaining animal derived ingredient, can be obtained in pharmaceutical quality.

The recipe for the IM1 medium is as follows:

5%	Lactose monohydrate (Scharlab S.L., Barcelona, ES)
1.5%	Soy peptone
1%	Yeast extract
1 mM	$MgSO_4 \times 7\ H_2O$
0.1 mM	$MnSO_4 \times 4\ H_2O$
pH 6.7	

Sterilisation: 110 °C 15 min

During fermentation no buffer is added since the pH was automatically regulated using 2.5 M NaOH. For acidifying test tube cultivations 2% Na-beta-glycerophosphate was added to the medium.

This buffer neutralizes the produced lactate and allows the culture to reach a cell density of OD_{600} = 4 – 5.

Stocks of the final constructs NZ3900(pFGF2), NZ3900(pIL4), and NZ3900(pCSF1) were prepared after growth in IM1 medium with lactose and Na-beta-glycerophosphate by the addition of glycerol to a final concentration of 20%. Stocks were stored at -80°C.

1.2 Induction of protein production using the NICE system

The NICE system was induced using nisin concentrations between 0.1 – 10 ng/ml nisin. Nisin from Lactococcus lactis was obtained from Sigma (product number N5764).

1.3 Molecular cloning techniques

For molecular cloning standard techniques were used as, for example, described in Green and Sambrook (2012): “Molecular cloning: a laboratory manual”, fourth edition, Cold Spring Harbour Laboratory Press (Cold Spring Harbor, NY, USA).

Nucleotide sequencing, gene synthesis and primer synthesis was outsourced to BaseClear (Leiden, NL).

1.4 Fermentations

Fermentations were carried out in a 0.5-L Multifors 6-fermenter array (Infors Benelux, Velp, NL). Inoculation was carried out with 1% pre-culture in IM-1 medium; stirrer speed was 200 rpm and the incubation temperature was 30 °C. During the runs pH was controlled using 2.5 M NaOH. pH, temperature and the addition of NaOH were continuously monitored.

Induction with nisin was carried out at different time points of the growth cycle with different amounts of nisin as indicated in the results section. Samples were taken also as indicated in the results section.

1.5 Cinac fermentations

For the monitoring of acidifying cultures the Cinac pH monitoring unit (AMS France, Frepillion, FR) was used for continuous measurement of pH changes. In order to ensure homogeneous pH the cultures were mixed with a magnetic stirrer at 200 rpm.

1.6 Sample treatment

For SDS-PAGE and Western blotting, samples were processed to produce three fractions: (i) supernatant fraction, (ii) total cell extract (containing cell free extract, cell wall fragments and other particulate matter) and (iii) cell free extract.

The supernatant fraction was prepared by mixing 1 mL fermentation supernatant with 150 µL TCA and incubated at 4 °C for at least 1 h. The precipitate was collected by centrifugation at 20,000 x g. The pellet was either used immediately or stored at -20°C; after an additional centrifugation step to remove traces of TCA it was re-suspended in 15 µL 5 mM Tris-HCl pH 7.5.

Subsequently, 5 µL 4x sample buffer (containing 75mM DTT) was added and the suspension was heated for 20 min at 70 °C. In all cases, volumes of 20 µL were applied to the gel.

Total cell extracts were prepared as follows: the pellet of 10 mL of sample of a batch culture or 1 ml of a fermentation culture was dissolved in 1 mL 5 mM Tris-HCl pH7.5 and transferred to a bead beating tube with 500 mg zirconium beads and frozen at -20 °C until further processing. After thawing, this suspension was shaken with a FastPrep (MP Biomedicals LLP, Santa Ana, CA, USA) bead-beater 4 x 30 seconds at speed 4 with intermittent cooling for 1 min on ice. After beads had settled for 1 min 15 µL supernatant were mixed with 5 µL loading buffer and treated as mentioned above.

Cell free extract was prepared by centrifuging the total cell extract for 1 min at 20,000 x g. Further preparation for SDS-PAGE was carried out as described above.

1.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out with the commercially available NuPage system (Life Technologies GmbH, Darmstadt, DE) following the protocol of the manufacturer.

Human FGF2 reference protein was obtained from Sigma Aldrich.50 µg were dissolved in 25 µl of 5 mM Tris pH 7.5 to give a final concentration of 2 µg/µl. In a test run 5 and 10 µg were applied to the SDS-PAGE gel after mixing with 4 x concentrated loading buffer resulting in 1 x loading buffer concentration.

As depicted in Figure 1, the gel shows monomers, a small amount of dimer and a very small amount of trimer of hFGF2. Lanes 1 and 4 = SeeBlue marker; lane = 2 5 µg FGF2 and lane 3 = 10 µg hFGF2. This gel was run without the addition of DTT to the loading buffer and the gel buffer.

Human IL4 reference protein was obtained from R&D systems (Minneapolis, MN, USA). Human IL4 was dissolved in PBS (5.8 mM Na_2HPO_4, 4.2 mM NaH_2PO_4, 145 mM NaCl) to give a final concentration of 100 µg/mL. As reference 1 µg was applied per lane for Coomassie staining and 0.1 µg for silver staining and Western blots.

Human CSF1 reference protein was obtained from Abcam PLC (Cambridge, GB). Human CSF1 was dissolved in sterile water to give a final concentration of 100 µg/mL. As reference 1 µg was applied per lane for Coomassie staining and 0.1 µg for silver staining and Western blots.

1.8 Western blots

Protein blotting was also carried out using the NuPage system (XCell Blot Module) according to the protocol of LifeTechnologies. The blotting membranes were pre-cut nitrocellulose membranes from Thermo Fisher Scientific Inc.. The transfer buffer was NuPage Transfer buffer (20x) from Life Technologies. Transfer of the proteins was carried out at 35 V for 1 h.

For detection of human FGF2 a purified mouse anti-human FGF2 monoclonal antibody (BD Transduction laboratories, Heidelberg, DE) was used at a dilution of

1:250. The second antibody was horseradish peroxidase (HRP)-linked antibody anti-mouse IgG at a dilution of 1:10,000 obtained from Bioké B.V. (Leiden, NL).

For detection of human IL4 a monoclonal mouse anti-human IL4 IgG1 from R&D systems (clone #3007) was used at a dilution of 1:500. The second antibody was HRP-linked antibody anti-mouse IgG at a dilution of 1:10,000 obtained from Bioké B.V. (Leiden, NL).

For detection of human CSF1 a rabbit polyclonal antibody to human MCSF from Abcam PLC (clone ab9693) was used at a dilution of 1:2,000. The second antibody was HRP-linked anti-rabbit IgG antibody at a dilution of 1:2,000 obtained from Bioké B.V. (Leiden, NL).

Western blots were developed with SuperSignal West Pico Chemiluminescent substrate obtained from Thermo Fisher Scientific Inc..

1.9 N-terminal amino acid sequencing

For N-terminal amino-acid sequencing of hFGF2, hIL4, and hCSF1 from the supernatant of controlled fermentation cultures the bands of two lanes of a Coomassie stained SDS-PAGE were cut out and sent to Eurosequence (Groningen, NL).

Example 1: Generation of expression plasmids

1.1 Sequencing of plasmid pNZ8149

Plasmid pNZ8149 was used as vector for the expression of human fibroblast growth factor 2 (hFGF2), human interleukin 4 (hIL4), and human colony stimulating factor 1 (hCSF1) in L. lactis. Plasmid pNZ8149 is selected in the host cells via a food grade mechanism based on growth on lactose.

For reference purposes plasmid DNA was isolated and sequenced (for primers see Table 1). No differences were found in relation to the currently available sequence. The sequence of pNZ8149 is provided in SEQ ID No.35.

1.1.1 human fibroblast growth factor 2 (hFGF-2)

The mature form of hFGF-2 contains 155 amino acids and is designated in the following as hFGF2-155. It has a molecular weight of 17.3 kDa and a pI of 9.85. The molecule contains 4 cysteine residues.

The hFGF-2 variant used for cloning and expression in L. lactis is lacking the first two amino acids methionine and alanine and thus contains 153 amino acids. Thus, this variant is designated in the following as hFGF2-153. hFGF2-153 has a molecular weight of 17.1 kDa and a pI of 9.85. This variant also contains four cysteine residues.

The corresponding amino acid sequence of the human FGF2-155 is shown below, in Figure 32a and in SEQ ID No. 27:

MAAGSITTLP ALPEDGGSGA FPPGHFKDPK RLYCKNGGFF LRIHPDGRVD
GVREKSDPHI KLQLQAEERG VVSIKGVCAN RYLAMKEDGR LLASKCVTDE
CFFFERLESN NYNTYRSRKY TSWYVALKRT GQYKLGSKTG PGQKAILFLP
MSAKS

The underlined amino acids denote the first two amino acids methionine and alanine which are missing in the variant hFGF2-153. The amino acid sequence of the variant hFGF2-153 is depicted in Figure 32b and in SEQ ID No. 30.

The sequence of FGF2-155 is the mature human FGF2 sequence after secretion. In vivo this sequence is further processed. However, various existing recombinant products have used this 155 amino acid sequence with or without the N-terminal methionine residue.

1.1.2 Human interleukin 4 (hIL-4)

The mature protein of hIL-4 contains 129 amino acids, which correspond to the amino acids 25 to 153 of the amino acid sequence available under the NCBI accession number NP_000580.1. The mature protein has a molecular weight of 14.96 kDa and a pI of 9.36. The molecule contains 6 cysteine residues.

The amino acid sequence of the mature human IL-4 is shown below, in Figure 18c, and in SEQ ID No. 12:

HKCDITLQEI IKTLNSLTEQ KTLCTELTVT DIFAASKNTT EKETFCRAAT
VLRQFYSHHE KDTRCLGATA QQFHRHKQLI RFLKRLDRNL WGLAGLNSCP
VKEANQSTLE NFLERLKTIM REKYSKCSS

The hIL-4 variant that is used for cloning and expression in L. lactis contains an additional alanine residue at the N-terminus of the mature protein and thus contains 130 amino acids. It has a molecular weight of 15.03 kDa and a pI of 9.36. This variant also contains 6 cysteine residues.

The corresponding amino acid sequence of the human hIL-4 variant that is used for cloning and expression is shown below, in Figure 33 and in SEQ ID No. 14:

AHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA
TVLRQFYSHH EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC
PVKEANQSTL ENFLERLKTI MREKYSKCSS

The additional alanine residue at the N-terminus is underlined in the above amino acid sequence.

1.1.3 Human colony stimulating factor 1 (hCSF1)

The amino acid sequence used for expression is derived from the 554 amino acid sequence of the human CSF1 precursor available under the NCBI accession number NP_000748.3. The sequence used starts at amino acid 33 (E), which is the first amino acid of the mature hCSF1, and ends at amino acid 181 (Q).

Furthermore an additional 9 amino acids have been added at the C-terminus that occupy in the native protein the positions 480 – 488 (GHERQSEGS). In order to improve the removal of the signal peptide by the signal peptidase an additional alanine residue was added at the N-terminus. The resulting amino acid sequence is shown below, in Figure 34, and in SEQ ID No. 6:

AEEVSEYCSH MIGSGHLQSL QRLIDSQMET SCQITFEFVD QEQLKDPVCY
LKKAFLLVQD IMEDTMRFRD NTPNAIAIVQ LQELSLRLKS CFTKDYEEHD
KACVRTFYET PLQLLEKVKN VFNETKNLLD KDWNIFSKNC NNSFAECSSQ
GHERQSEGS

The additional alanine residue at the N-terminus is underlined in the above amino acid sequence. The additional 9 amino acids added at the C-terminus are depicted in bold. The protein encoded by this sequence contains 159 amino acids, has a molecular weight of 18.5 kDa and an pI of 4.72. The protein contains 7 cysteine residues.

1.2 Construction of a plasmid for the expression of hFGF2, hIL4, and hCSF1 in Lactococcus lactis

For L. lactis the aim was to express and secrete hFGF2, hIL4, and hCSF1, for example, into the supernatant of the growth medium. For that signals for transcription, translation and protein secretion were used. The signals for transcription and translation were present in the NICE expression system of L. lactis and originating from the nisinA promoter of L. lactis. The nisinA promoter of L. lactis is depicted in Figure 3 and SEQ ID No. 44 and is, for example, described in de Ruyter et al. (1996).

Figure 3 further shows the -10 element and the -35 element of the promoter as well as the ribosomal binding site (RBS).The ATG start codon of the respective target protein follows directly the two Cysteine residues at the 3'-end of the sequence.

The secretion signal was derived from the Lactococcus protein Usp45, which is described, for example, in van Asseldonk et al. (1993) and van Asseldonk et al. (1990).

The secretion signal of the Lactococcus protein Usp45 is shown below, in Figure 35, and in SEQ ID No. 34:

MKKKIISAIL MSTVILSAAA PLSGVYA

In the cell a mechanism exists that ensures that after the secretion of a protein this signal sequence is cleaved off from the target protein. This reaction is catalysed by the signal peptidase which has a specific preference for certain amino acids in the positions around the cleavage site.

In order to optimize the cleavage site the web based program SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used.

For the amino acid sequence of hFGF2 this program showed that optimum cleavage occurs when the first two amino acids of hFGF2-155 are removed, leaving an hFGF2-155 protein starting with an Ala residue and a total length of 153 amino acids (Variant hFGF2-153).

The respective amino acid sequence of the hFGF2-153 precursor protein used for analysis with the program SignalP 4.1 is shown below and in SEQ ID No. 29:

MKKKIISAIL MSTVILSAAA PLSGVYAAGS ITTLPALPED GGSGAFPPGH
FKDPKRLYCK NGGFFLRIHP DGRVDGVREK SDPHIKLQLQ AEERGVVSIK
GVCANRYLAM KEDGRLLASK CVTDECFFFE RLESNNYNTY RSRKYTSWYV
ALKRTGQYKL GSKTGPGQKA ILFLPMSAKS

The underlined amino acids denote the secretion signal of the Lactococcus protein Usp45.

For the amino acid sequence of hIL4 this program showed that optimum cleavage occurs when an alanine residue is added to the N-terminus of the mature IL4 protein, resulting in a protein of 130 amino acids.

The respective amino acid sequence of the hIL4 precursor protein used for analysis with the program SignalP 4.1 is shown below and in SEQ ID No. 13:

MKKKIISAIL MSTVILSAAA PLSGVYAAHK CDITLQEIIK TLNSLTEQKT
LCTELTVTDI FAASKNTTEK ETFCRAATVL RQFYSHHEKD TRCLGATAQQ
FHRHKQLIRF LKRLDRNLWG LAGLNSCPVK EANQSTLENF LERLKTIMRE
KYSKCSS

The underlined amino acids denote the secretion signal of the Lactococcus protein Usp45.

For the amino acid sequence of hCSF1 this program showed that optimum cleavage occurs when an alanine residue is added to the N-terminus of the mature hCSF1 protein resulting in a protein of 159 amino acids.

The respective amino acid sequence of the hCSF1 precursor protein used for analysis with the program SignalP 4.1 is shown below and in SEQ ID No. 5:

MKKKIISAIL MSTVILSAAA PLSGVYAAEE VSEYCSHMIG SGHLQSLQRL
IDSQMETSCQ ITFEFVDQEQ LKDPVCYLKK AFLLVQDIME DTMRFRDNTP
NAIAIVQLQE LSLRLKSCFT KDYEEHDKAC VRTFYETPLQ LLEKVKNVFN
ETKNLLDKDW NIFSKNCNNS FAECSSQGHE RQSEGS

The underlined amino acids denote the secretion signal of the Lactococcus protein Usp45.

Figure 4a shows a readout of SignalP 4.1 obtained with the amino acid sequence depicted in SEQ ID No. 29, in which the secretory signal of the Usp45 protein is fused to the N-Terminus of hFGF2-153. The readout shows a clear cleavage site

between amino acids 27 and 28 of the hFGF2-153 precursor and scores for this cleavage site.

Figure 4b shows a readout of SignalP 4.1 obtained with the amino acid sequence depicted in SEQ ID No. 13, in which the secretory signal of the Usp45 protein is fused to the N-Terminus of hIL4. The readout shows a clear cleavage site between amino acids 27 and 28 of the hIL4 precursor and scores for this cleavage site.

Figure 4c shows a readout of SignalP 4.1 obtained with the amino acid sequence depicted in SEQ ID No. 5, in which the secretory signal of the Usp45 protein is fused to the N-Terminus of hCSF1. The readout shows a clear cleavage site between amino acids 27 and 28 of the hCSF1 precursor and scores for this cleavage site.

The codon usage for the respective genes, coding for hFGF2-153, hIL4, and hCSF1 were adapted to the general codon usage of L. lactis. This is a routine procedure of the supplier of the gene synthesis.

Furthermore, the same Usp45 signal sequence was used for all three proteins. Thereby, expression of all three proteins and posttranslational processing of the signal peptide in the same bacterial cell was improved.

For hFGF2 a fragment was synthesized that contained the nisinA promoter, the Usp45 signal sequence and the hFGF2-153 gene. The nucleic sequence of the artificial construct ssUsp45-FGF2-153 is depicted in SEQ ID No. 45 and Figure 5a. The codon usage was optimized to the general codon usage of L. lactis.

Figure 5a shows the synthesized insert as well as the corresponding amino acid sequence obtained after transcription and translation. FGF2-153 denotes the human FGF-2 coding insert. PnisA denotes the nisinA promoter of the Lactococcus lactis nisin operon. ssUsp45 denotes the signal sequence of the usp45 gene of Lactococcus lactis. An asterisk denotes a stop codon. Furthermore, in the nucleic acid sequence, the restriction recognition sites for endonucleases BamHI (position 1 to 6) and XbaI (position 751 to 756) are denoted by underlining.

For hIL4 a fragment was synthesized that contained the nisinA promoter, the Usp45 signal sequence and the hIL4 gene. The nucleic sequence of the artificial construct ssUsp45-hIL4 is depicted in SEQ ID No. 47 and Figure 5b. The codon usage was optimized to the general codon usage of L. lactis.

Figure 5b shows the synthesized insert as well as the corresponding amino acid sequence obtained after transcription and translation. hIL4 denotes the human IL4 coding insert. PnisA denotes the nisinA promoter of the Lactococcus lactis nisin operon. ssUsp45 denotes the signal sequence of the usp45 gene of Lactococcus lactis. An asterisk denotes a stop codon. Furthermore, in the nucleic acid sequence, the restriction recognition sites for endonucleases BamHI (position 1 to 6) and XbaI (position 682 to 687) are denoted by underlining.

For hCSF1 a fragment was synthesized that contained the nisinA promoter, the Usp45 signal sequence and the hCSF1 gene. The nucleic sequence of the artificial construct ssUsp45-hCSF2 is depicted in SEQ ID No. 49 and in Figure 5c. The codon usage was optimized to the general codon usage of L. lactis.

Figure 5c shows the synthesized insert as well as the corresponding amino acid sequence obtained after transcription and translation. hCSF1 denotes the human CSF1 coding insert. PnisA denotes the nisinA promoter of the Lactococcus lactis nisin operon. ssUsp45 denotes the signal sequence of the usp45 gene of Lactococcus lactis. An asterisk denotes a stop codon. Furthermore, in the nucleic acid sequence, the restriction recognition sites for endonucleases BamHI (position 1 to 6) and XbaI (position 769 to 774) are denoted by underlining.

The finished gene synthesis products were obtained from BaseClear each as a fragment cloned in the E. coli vector pUC57.

The fragments were cloned into the plasmid pNZ8149 by digesting the pUC57 plasmids with the respective gene synthesis products with the restriction enzymes BamHI and XbaI. The BamHI and XbaI fragments containing the respective gene synthesis products were isolated and ligated into PsuI and XbaI cut plasmid pNZ8149 to result in the respective plasmids pFGF2, pIL4, and pCSF1. The

respective restriction endonucleases were obtained from Thermo Fisher Scientific Inc..

The respective ligation mix was transformed into strain L. lactis NZ3900. Verification of the plasmid was carried out by colony PCR analysis. The respective nucleic acid sequences of the primers used are depicted in Table 1 below. The results are shown in Figures 6a to 6c.

Figure 6a shows the result of a colony PCR analysis of potential colonies of L. lactis strain NZ3900 with pFGF2. Lane 1: lambda DNA digested with HindIII; lane 2: pNZ8149 (empty vector) amplified with primers nis2 and seqlacF (expected fragment size 391 bp); lanes 3 and 4 isolated colonies amplified with primers nis2 and seqlacF (expected fragment size 907 bp).

Figure 6b shows the result of a colony PCR analysis of potential colonies of L. lactis strain NZ3900 with pIL4. Lane 1: lambda DNA digested with HindIII; lane 2: pNZ8149 (empty vector) amplified with primers nis2 and seqlacF (expected fragment size 391 bp); lanes 11 and 12 isolated colonies amplified with primers nis2 and seqlacF (expected fragment size 838 bp).

Figure 6c shows the result of a colony PCR analysis of potential colonies of L. lactis strain NZ3900 with pCSF1. Lane 1: lambda DNA digested with HindIII; lane 2: pNZ8149 (empty vector) amplified with primers nis2 and seqlacF (expected fragment size 391 bp); lane 3: isolated colony amplified with primers nis2 and seqlacF (expected fragment size 925 bp).

Table 1: Primers used.

Primers	Sequence	SEQ ID No.
seqlacF	5' TGTGATTCATCACCTCATCG 3'	36
seq2F	5' CGTGGCTTTGCAGCGAAGATG 3'	37
seq3F	5' GACTCAATTCCTAATGATTGG 3'	38
seq4F	5' TCAGTGTGGATATAGAGCAAG 3'	39
seq6R	5' CTGTAATTTGTTTAATTGCC 3'	40

seq7R	5' TTGAGCCAGTTGGGATAGAG 3'	41
seq8R	5' GTATCTCAACATGAGCAACTG 3'	42
nis2	5' CAATTGAACGTTTCAAGCCTTGG 3'	43

Figure 7a shows a plasmid map and nucleic acid sequence of the plasmid pFGF2 for the expression and secretion of hFGF2 in Lactococcus lactis. lacF denotes a selection marker for growth of the host strain NZ3900 on lactose; PnisA denotes a nisin A promoter of the nisin operon of Lactococcus lactis; ssUSP45 denotes a signal sequence of the usp45 gene of L. lactis. T denotes a terminator; repC and repA denotes replication genes. The nucleic acid sequence of the plasmid pFGF2 is also depicted in SEQ ID No. 46.

Figure 7b shows a plasmid map and nucleic acid sequence of the plasmid pIL4 for the expression and secretion of hIL4 in Lactococcus lactis. lacF denotes a selection marker for growth of the host strain NZ3900 on lactose; PnisA denotes a nisin A promoter of the nisin operon of Lactococcus lactis; ssUSP45 denotes a signal sequence of the usp45 gene of L. lactis. T denotes a terminator; repC and repA denotes replication genes. The nucleic acid sequence of the plasmid pIL4 is also depicted in SEQ ID No. 48.

Figure 7c shows a plasmid map and nucleic acid sequence of the plasmid pCSF1 for the expression and secretion of hCSF1 in Lactococcus lactis. lacF denotes a selection marker for growth of the host strain NZ3900 on lactose; PnisA denotes a nisin A promoter of the nisin operon of Lactococcus lactis; ssUSP45 denotes a signal sequence of the usp45 gene of L. lactis. T denotes a terminator; repC and repA denotes replication genes. The nucleic acid sequence of the plasmid pCSF1 is also depicted in SEQ ID No. 50.

Example 2: Testing production of recombinant factors in Lactococcus lactis.

In order to test whether the respective recombinant factors hFGF2, hIL4, or hCSF1 are produced in and secreted from Lactococcus lactis, five colonies of the initial transformation were chosen for further analysis. These colonies were first purified by

plating and single colony isolation. The five colonies were individually inoculated into M17 lactose medium and grown to an OD 600 ≈ 0.5 and induced with 1 ng/ml nisin.

The cultures were terminated and harvested 2 h after the addition of nisin. All cultures showed the same behaviour after the addition of nisin and a certain amount of growth retardation (Data not shown).

a) Production of hFGF2 in L. lactis NZ3900(pFGF2) under controlled fermentation conditions

In order to establish the yield of a NZ3900(pFGF2) culture, controlled fermentation runs were carried out.

The following five fermentation runs were carried out:

1. NZ3900(pFGF2) not induced
2. NZ3900(pFGF2) induced at OD 600 = 0.5 with 5 ng/mL nisin
3. NZ3900(pFGF2) induced at OD 600 = 3 with 5 ng/mL nisin
4. NZ3900(pNZ8149) induced at OD 600 = 0.5 with 5 ng/mL nisin
5. NZ3900(pNZ8149) induced at OD 600 = 3 with 5 ng/mL nisin

Samples were taken at t = 0 (before induction), t = 2 hrs, t = 4 hrs and t = 6 hrs after induction with the indicated amount of nisin.

Cultures 4 and 5 were performed to establish whether 5 ng/mL nisin has an effect on the growth of a culture because of the activation of the nisin promoter, which is in this case is not followed by a target gene. No effect on growth was observed (Data not shown).

Western blots have been carried out with samples of both induction conditions. Detection of hFGF2 was performed with the antibodies described above under item 1.8.

Figure 8 shows in the Western blot in lanes 3 and 4 the baseline expression of FGF2 without induction by nisin.

Furthermore, Figure 8 shows that after induction of the culture at OD 600 = 0.5 hFGF2 production is strongly induced and increases up to 6 hours after the moment of induction.

hFGF2 bands from an SDS-PAGE gel (induction at OD 600 = 0.5 with 5 ng/mL nisin, time points t = 4 and t = 6) were isolated and shipped to Eurosequence B.V. (Groningen, NL) for N-terminal amino acid sequencing.

After clean-up of the samples a tentative N-terminal amino acid sequence was determined. The determined sequence is: Ala-Gly-Ser-Ile-Thr-Thr.

This sequence exactly matches the expected sequence after the signal sequence is cleaved off. The expected sequence is: AGSITTLPAL. This means that hFGF2 is expressed, secreted and correctly processed in L. lactis.

The culture supernatant of NZ3900(pFGF2) induced at OD 600 = 0.5 with 5 ng/mL nisin was used in the following Examples. The culture was designated AUP-10.

b) Production of hIL4 in L. lactis NZ3900(pIL4) under controlled fermentation conditions

In order to establish the yield of a NZ3900(pIL4) culture, controlled fermentation runs were carried out with the following fermentations runs:

1. NZ3900(pIL4) not induced
2. NZ3900(pIL4) induced at OD 600 = 0.5 with 5 ng/mL nisin
3. NZ3900(pIL4) induced at OD 600 = 3 with 5 ng/mL nisin

Samples were taken at t = 0 (before induction), t = 2 hrs, t = 4 hrs and t = 6 hrs after induction with the indicated amount of nisin.

The production of hIL4 was analysed by SDS-PAGE of the supernatant fraction and the cell free extract in combination with Western blotting. Detection of hIL4 was performed with the antibodies described above under item 1.8.

During SDS-PAGE DTT was added to the sample buffer and the running buffer in order to facilitate the dissociation of disulphide bridges. These same SDS-PAGE gel were also used to run a Western blot for the specific detection of hIL4.

The effect of the addition of DTT is that hIL4 forms a single protein band in the Coomassie stained gel and in the Western blot (see Figure 9).

Furthermore, an hIL4 reference protein preparation was used without a carrier protein. The reference protein band is at the same height as the protein produced by L. lactis NZ3900(pIL4) cells.

The Western blot shows further detail on the expression of hIL4 in L. lactis NZ3900(pIL4).

Induction takes place when the culture is induced at OD 600 = 0.5 with 5 ng/mL nisin but not when induced at OD 600 = 3 with 5 ng/mL nisin. The production of hIL4 continues up to at least 6 h. In this period the IL4 concentration in the supernatant increases.

hIL4 bands from an SDS-PAGE gel (induction at OD 600 = 0.5 with 5 ng/mL nisin, time points t = 4 and t = 6) were isolated and shipped to Eurosequence B.V. for N-terminal amino acid sequencing.

After clean-up of the samples a tentative N-terminal amino acid sequence was determined. The determined sequence is: Ala-His-Lys-Cys.

This sequence exactly matches the expected sequence after the signal sequence is cleaved off. The expected sequence is: AHKCDITLQE.

This means that hIL4 is expressed, secreted and correctly processed in L. lactis. The culture supernatant of NZ3900(pIL4) induced at OD 600 = 0.5 with 5 ng/mL nisin was used in the following Examples. The culture was designated AUP-12.

c) Production of hCSF1 in L. lactis NZ3900(pCSF1) under controlled fermentation conditions

In order to establish the yield of a NZ3900(pCSF1) culture, controlled fermentation runs were carried out. The following three fermentations were carried out.

1. NZ3900(pCSF1) not induced
2. NZ3900(pCSF1) induced at OD 600 = 0.5 with 5 ng/mL nisin
3. NZ3900(pCSF1) induced at OD 600 = 3 with 5 ng/mL nisin

The NZ3900(pCSF1) culture was designated AUP-14.

Samples were taken at t = 0 (before induction), t = 2 hrs, t = 4 hrs and t = 6 hrs after induction with the indicated amount of nisin.

The production of hCSF1 was analysed by SDS-PAGE of the supernatant fraction and the cell free extract in combination with Western blotting. Detection of hCSF1 was performed with the antibodies described above under item 1.8.

During SDS-PAGE DTT was added to the sample buffer and the running buffer in order to facilitate the dissociation of disulphide bridges. These same SDS-PAGE gel were also used to run a Western blot for the specific detection of hCSF1.

In the Western blot (see Figure 11) the lane with the reference protein still shows a small amount of dimer. Furthermore, the Coomassie stained gel (see Figure 10) also shows bands of the same size as the reference protein.

In uninduced cultures hardly any baseline production can be detected. In the cultures induced at OD 600 = 0.5 with 5 ng/mL nisin CSF1 production increases over time up to at least 6 h. Only a small amount of degradation products could be observed.

hCSF1 bands from an SDS-PAGE gel (induction at OD 600 = 0.5 with 5 ng/mL nisin, time points t = 4 and t = 6) were isolated and subjected to N-terminal amino acid sequencing.

After clean-up of the samples a tentative N-terminal amino acid sequence was determined. The determined sequence is: Ala-Glu-Glu-Val-Ser.

This sequence exactly matches the expected sequence after the signal sequence is cleaved off. The expected sequence is: AEEVSEYCSH.

This means that hCSF1 is expressed, secreted and correctly processed in L. lactis.

The culture supernatant of NZ3900(pCSF1) induced at OD 600 = 0.5 with 5 ng/mL nisin was used in the following Examples. The culture was designated AUP-14.

d) Production of hFGF2, hIL4 and hCSF1 in L. lactis NZ3900 from a single operon under controlled fermentation conditions

3 constructs were made with 3 of the 6 permutation of the three genes (FGF2, IL4, CSF1):

a) hFGF2, hIL4, hCSF1,
b) hCSF1, hFGF2, hIL4,
c) hIL4, hCSF1, hFGF2.

For the design of the operons each gene was provided with its own ribosome binding site for translational initiation and its own signal peptide for protein secretion. In relation to the signal peptide it had been decided earlier to use for all three genes the same signal sequence (ssUsp45). In order to avoid large identical nucleotide regions on the plasmid in close proximity (possibility of intra-plasmid recombination/deletions) the same signal peptide was coded by different codons on the basis of codon degeneration and the codon usage of L. lactis.

For each of the first genes of the 3 constructs the ribosome binding site of the Nisin A (nisA) gene was used. For the other two genes of each of the 3 constructs the ribosome binding sites of the ATP synthase subunit gamma (atpG) gene and the galactoside O-acetyltransferase (lacA) gene were chosen on the basis of good fits with the 3'-end of the 16S rRNA of L. lactis, as described in Bolotin et al. (2001) ("The complete genome sequence of the lactic acid bacterium lactococcus lactis ssp. lactis

IL1403", Genome Res. Volume 11, pages 731 to 753). The nucleotide sequence of the L. lactis IL1403 genome is available from NCBI with accession no. AE005176.1.

An overview of all three ribosome binding sites is provided in Figure 24a. Furthermore, nucleic acid sequences providing the respective ribosome binding sites including the start codon (ATG) of the respective open reading frame are also shown in SEQ ID No. 51 to SEQ ID No. 53.

The 3′ end of 16S rRNA of L. lactis (5′ GGAUCACCUCCUUUCU 3′) is shown in SEQ ID No. 54. In Figure 24a the 16S rRNA is shown as 16S rDNA, in which uracil (U) was replaced by thymidin (T).

Figure 24a also shows the six-base consensus sequence of the Shine-Dalgarno sequence (5' AGGAGG 3'). The Shine-Dalgarno sequence is a ribosomal binding site in prokaryotic messenger RNA, generally located around 8 bases upstream of the start codon. The RNA sequence helps recruit the ribosome to the mRNA to initiate protein synthesis by aligning the ribosome with the start codon.

The three operons were designed in such a way that the synthesized sequences can be cloned in one step into plasmid pNZ8149 using the PsuI and NcoI sites of the vector (BamHI and PsuI are compatible). Furthermore, MluI sites were introduced in front of the ribosome binding sites in front of genes two and three. Thereby creation of the remaining three operons was enabled by deletion of the middle gene and cloning of the third gene by PCR using the NcoI and XbaI sites of the plasmid.

The respective sequences of the synthesized nucleic acids are depicted in Figures 24b to 24d and in SEQ ID No. 55 to SEQ ID No. 57. The codon usage of the three genes has been adapted for L. lactis.

Expression from the 3 constructs are each controlled by a single nisinA promoter followed by the above indicated nucleic acid sequences coding for the hFGF2-153 precursor protein, hIL4 precursor protein, and hCSF1 precursor protein obtained in Example 1.

The finished gene synthesis products were obtained from BaseClear each as a fragment cloned in the E. coli vector pUC57.

The fragments were cloned into the plasmid pNZ8149 by digesting the pUC57 plasmids containing the respective gene synthesis products with the restriction enzymes BamHI and NcoI. The BamHI / NcoI fragments containing the respective gene synthesis products were isolated and ligated into PsuI and NcoI cut plasmid pNZ8149 to result in the respective plasmids

- pC-F-I (containing the BamHI / NcoI fragment of the CSF-RBS1-FGF-RBS2-IL4 gene synthesis product),
- pF-I-C (containing the BamHI / NcoI fragment of the FGF-RBS1-IL4-RBS2-CSF gene synthesis product), and
- pI-C-F (containing the BamHI / NcoI fragment of the IL4-RBS-CSF-RBS2-FGF gene synthesis product).

The respective restriction endonucleases used were obtained from Thermo Fisher Scientific Inc..

The respective constructs were transformed L. lactis NZ3900.

Each 5 colonies of the three constructs were grown in pH regulated fermentation runs and induced at OD600 = 0.5 with 5 ng/ml nisin and harvested after 4 hours.

1 ml of the supernatant was precipitated with TCA and applied to one well of the SDS-PAGE gel. One gel was stained with Coomassie blue and the other as Western blots for the three target proteins.

The respective SDS-PAGE gels as well as Western blots were processed as outlined above and are depicted in Figures 24e to 24g.

As can be seen from Figure 24e construct a) pF-I-C (FGF2, hIL4, hCSF1) resulted in the expression and secretion of much FGF2, little IL4 as well as little CSF1.

As can be seen from Figure 24f construct b) pC-F-I (hCSF1, hFGF2, hIL4) resulted in the expression and secretion of much CSF1 and FGF2 as well as medium IL4.

As can be seen from Figure 24g construct c) pI-C-F (hIL4, hCSF1, hFGF2) resulted in the expression and secretion of much IL4 and little less CSF1 and FGF2. The coomassie stained gel showed best production and induction of all three proteins.

The three target proteins can be cloned in and expressed from an operon structure resulting in the release of the respective proteins hIL4, hCSF1, and hFGF2 into the supernatant.

Example 3: Induction of FGF2 production under conditions that resemble wound healing circumstances

The physiological conditions in a wound are different from those in a test tube or fermenter.

Therefore an experiment was set up to test the induction of the NICE system and thus production of hFGF2 under conditions in which strain NZ3900 does not grow or grows only minimally. These conditions are present when the pH of a culture has reached 5.0 and when the culture is going into the stationary phase.

The experiment was carried out in 200 mL bottles with stirring and pH monitoring. As growth medium IM1 medium with 2% weight by volume Na-beta-glycerophosphate and 0.5% weight by volume lactose was used. The following fermentations were carried out:

1. NZ3900(pFGF2) – no induction
2. NZ3900(pFGF2) – induction when culture reaches pH = 5 with 1 ng/mL nisin
3. NZ3900(pFGF2) – induction when culture reaches pH = 5 with 0.1 ng/mL nisin
4. NZ3900(pFGF2) – induction when culture reaches OD 600 = 3 with 1 ng/mL nisin
5. NZ3900(pFGF2) – induction when culture reaches OD 600 = 3 with 0.1 ng/mL nisin.

The cultures were harvested 3 h after induction. The control culture was harvested at the same time as the cultures that were induced at pH 5.0. The results are depicted in Figure 11.

Figure 12 shows that growth and pH development are identical in all five cultures and that induction with nisin has no effect on either growth or pH development.

Formation of hFGF2 was measured in 1 ml culture supernatant using SDS-PAGE and Western blotting. Figure 13 shows that under conditions where the cells do not grow or are in the slowing down phase of growth, nisin does not induce gene expression. In relation to the background production of FGF2 (without induction) there is little or no increase in the amount of FGF2 in the culture supernatant.

Western blot analysis shows that hFGF2 is detectable in the culture supernatant of all five cultures. Furthermore, two breakdown products of hFGF2 are detectable.

This means that all FGF2 with signal sequence that is produced in the cell is processed and secreted.

The gene for human FGF2 has been cloned into a food-grade NICE expression vector for Lactococcus lactis.

The expression of the FGF2 gene can be induced by the addition of nisin. hFGF2 is secreted into the supernatant at about 100 – 200 ng/mL. Determination of the N-terminal amino acid sequence of the secreted FGF2 has shown that the protein is processed correctly

Induction of the production of FGF2 leads to a limited growth retardation, but not to arrest of growth.

In a controlled fermentation FGF2 production has been induced at a wide window in the growth curve (between OD 600 = 0.5 – 3). The highest FGF2 production has been observed after induction at OD 600 = 0.5 with 5 ng/ml nisin.

Plasmid pFGF2 is stable for at least 100 generations in strain NZ3900 also under non-selective conditions, such as growth on glucose.

Example 4a: Human fibroblast migration assay

The biological activity of FGF2 released from Lactococcus lactis NZ3900(pFGF2) and of IL4 released from Lactococcus lactis NZ3900(pIL4) on wound healing was assessed by measuring the migration of normal human dermal fibroblasts (NHDF) into an artificial migration zone. The effect of the test compound was evaluated using an image analysis of the wound recovery.

Lactococcus lactis NZ3900(pFGF2) from Example 2a and Lactococcus lactis NZ3900(pIL4) from Example 2b were each induced at OD 600 = 0.5 with 5 ng/mL nisin. After 6 h fermentation, the respective culture supernatants were isolated, freeze dried and stored at -20°C as described above.

For further testing stock solutions from the freeze-dried supernatants were prepared in a concentration of 1 mg/ml by reconstituting the powder with tris HCl (5 mM, pH 7.6). The AUP-10 stock solution was obtained from the culture supernatant of Lactococcus lactis NZ3900(pFGF2) from Example 2a. The AUP-12 stock solution was obtained from the culture supernatant of Lactococcus lactis NZ3900(pIL4) from Example 2b.

The stock solutions were diluted with assay medium n°1 to the final concentration of 0.5 ng/ml, 1 ng/ml, 2.5 ng/ml, and 5 ng/ml , respectively, for AUP-10 (FGF2) and to the final concentration of 0.1 ng/ml, 1 ng/ml, 5 ng/ml, and 10 ng/ml, respectively, for AUP-12 (IL4).

The indicated concentrations for the stock solutions as well as dilutions refer to the total amount of protein, which is present in respective solution and which mainly corresponds to the amount of FGF2 and IL4 into the culture supernatants of Lactococcus lactis NZ3900(pFGF2) from Example 2a and Lactococcus lactis NZ3900(pIL4) from Example 2b, respectively.

Furthermore, recombinant human bFGF (Fiblast,), which was purchased from Kaken Pharmaceutical Co., Inc (Tokyo, Japan), was diluted with assay medium n°1 to the final concentration of 0.5 ng/ml, 1 ng/ml, 2.5 ng/ml, and 5 ng/ml, , respectively.

Recombinant human IL4, which was purchased from R&D systems (Minneapolis, MN, USA), was diluted with tris HCl (5 mM, pH 7.6) to the final concentration of 0.1 ng/ml, 1 ng/ml, 5 ng/ml, and 10 ng/ml, respectively.
Normal human dermal fibroblasts (NHDF) were obtained from Lonza Group AG (Basel, CH).

Fibroblasts were seeded in 96-well microplates dedicated to migration analysis and incubated for 24 hours.

- Culture conditions: 37°C, 5% CO_2 .
- Culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine 2 mM, penicillin 50 U/ml, streptomycin 50 µg/ml, and fetal calf serum (FCS) 10% by volume.

In these plates, wells were coated with a collagen solution and a cell seeding stopper was placed in the center of the well in order to restrict cell seeding to the outer annular regions of the wells, thus creating an artificial wound (migration zone). The cells were then labeled using calcein-AM obtained from Thermo Fisher Scientific.

Calcein-AM is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells. In live cells the nonfluorescent calcein AM is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases.

After 30 minutes of incubation, the stoppers were removed and culture medium was replaced by assay medium n°1 containing the test compounds (FGF2, Fiblast, IL4 or recombinant hIL4) or only assay medium n°1 (control) or the reference (FCS at 10%). Cells were then incubated for 72 hours. All experimental conditions were performed in n=5.

- Assay medium n°1: DMEM supplemented with L-glutamine 2 mM, penicillin 50 U/ml, and streptomycin 50 µg/ml.

Cell migration into the migration zone was observed after 0, 24, 48 and 72 hours of incubation, using a NIKON Diaphot 300 microscope (lens x 4) and images were captured using a NIKON DX-1200 camera.

The surface of the artificial wound was measured at each time point and compared to the pre-migration area measured at T0 in order to visualize and quantify the wound recovery. The effects of the test compounds on cell migration were compared to the untreated control.

The respective concentrations applied were 0.5 ng/ml, 1.0 ng/ml, 2.5 ng/ml, and 5.0 ng/ml for hFGF2 and 0.1 ng/ml, 1 ng/ml, 5 ng/ml, and 10 ng/ml for hIL4. After 72 h exposure migration of the fibroplasts were compared to fibroblast incubated with growth medium (control).

The %-recovery of the area of the artificial wound is shown in Figures 14a and 14b.

Figure 14a shows a significant increase of migrating fibroblasts after incubation with hFGF2 expressing recombinant probiotic bacteria. Under the experimental conditions of the assay, recombinant human bFGF (Fiblast,) purchased from Kaken Pharmaceutical Co., Inc (Tokyo, Japan), did not modulate NHDF migration and proliferation.

hFGF2 expressed in recombinant probiotic bacteria AUP-10 obtained in Example 2a posses biological activity.

Figure 14b shows that AUP-12 culture medium containing 0.1-10 ng/ml of hIL4 induces NHDF proliferation similar to rhIL4.

hIL4 expressed in recombinant probiotic bacteria AUP-12 obtained in Example 2b posses biological activity.

Example 4b: ERK1- and ERK2-phosphorylation in normal human dermal fibroblasts and in mouse lymphoblasts.

The biological activity of FGF2 released from Lactococcus lactis NZ3900(pFGF2) was further assessed by measuring the phosporylation of extracellular signal-regulated kinase 1 (ERK1) and extracellular signal-regulated kinase 2 (ERK2) in normal human dermal fibroblasts (NHDF).

The biological activity of CSF1 released from Lactococcus lactis NZ3900(pCSF1) was assessed by measuring the phosporylation of extracellular signal-regulated kinase 1 (ERK1) and extracellular signal-regulated kinase 2 (ERK2) in mouse lymphoblasts M-NFS-60 cells.

The AUP-10 stock solution was prepared as described above in Example 4a.

Furthermore, Lactococcus lactis NZ3900(pCSF1) from Example 2c (AUP-14) was induced at OD 600 = 0.5 with 5 ng/mL nisin. After 6 h fermentation, the culture supernatant was isolated, freeze dried and stored at -20°C. For further testing a stock solution from the freeze-dried supernatant was prepared in a concentration of 1 mg/ml total protein by reconstituting the powder with tris HCl (5 mM, pH 7.6) obtaining the AUP-14 stock solution.

The stock solutions were further diluted with the respective serum free medium to a final concentration of 0.4 ng/ml, 4 ng/ml, 40 ng/ml, and 80 ng/ml, respectively, for AUP-10 (FGF2) and to the final concentration of 0.2 ng/ml, 2 ng/ml, 20 ng/ml, and 40 ng/ml, respectively, for AUP-14 (CSF1).

Recombinant human bFGF from Kaken Pharmaceutical Co. was diluted with serum free medium (Assay medium n°1) to a final concentration of 0.5 ng/ml, 5 ng/ml, 50 ng/ml, and 100 ng/ml, respectively.

Recombinant human CSF1, which was purchased from Abcam PLC (Cambridge, GB), was diluted with serum free medium (Assay medium n°2) to a final concentration of 0.2 ng/ml, 2 ng/ml, 20 ng/ml, and 40 ng/ml, respectively.

Normal human dermal fibroblasts (NHDF) were seeded in 96-well microplates and incubated for 24 hours under the conditions described above under Example 4a.

Mouse M-NFS-60 cells (ATCC® CRL-1838™) were obtained from LGC Standards GmbH (Wesel, Germany). The cells were seeded in 96-well microplates and incubated for 24 hours under the following conditions:

- Culture conditions: 37°C.
- Culture medium: RPMI-1640 Medium (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 0.05 mM 2-mercaptoethanol, 62 ng/ml human recombinant macrophage colony stimulating factor (M-CSF), penicillin 50 U/ml, streptomycin 50 µg/ml, and fetal calve serum to a final concentration of 10% by volume.
- Assay medium n°2: RPMI-1640 Medium, supplemented with 0.05 mM 2-mercaptoethanol, penicillin 50 U/ml and streptomycin 50 µg/ml,

Exponentially growing NHDF were incubated in serum free medium (Assay medium n°1) for 24 hours, transferred to eppendorf tubes (0.5 x 10^5 cells/tube) and incubated for 15 min at 37ºC in serum free medium (Assay medium n°1) containing 0.5 ng/ml, 5 ng/ml, 50 ng/ml, and 100 ng/ml of rhFGF2 or dilutions of the AUP-10 stock solution at final concentrations as indicated above. Cells were then washed with PBS and resuspended in lysis buffer.

Exponentially growing Mouse M-NFS-60 cells were incubated in serum free medium (Assay medium n°2) for 24 hours, transferred to eppendorf tubes (2 x 10^6 cells/tube) and incubated with Ki-20227 at a concentration of 1µM for 1 hour. The c-fms inhibitor Ki-20227, which was obtained from Abcam PLC, was included as a control for CSF-1 receptor (CSF-1R) specificity.

After 1 h preincubation with Ki-20227 cells were incubated for 15 min at 37ºC in Assay medium n°2 containing 0.2, 2, 20, and 40 ng/ml of rhCSF1 or dilutions of the AUP-14 stock solution at final concentrations as indicated above. Cells were then washed with PBS and resuspended in lysis buffer.

The recipe for the lysis buffer is as follows: 0.5 % by volume Surfact-Amps NP-40 (Pierce Biotechnology, Rockford, IL, USA), 20 mM Hepes (pH 8.0), 0.35 M NaCl, 0.1 mM EGTA, 0.5 mM EDTA, 1 mM $MgCl_2$, 20 % by volume Glycerol, 1 mM DTT, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin, 0.5 µg/ml apronitin, and 1 mM phenylmethylsulfonyl fluoride.

Cells were each incubated for 15 min in ice, and cellular proteins were obtained by centrifugation. Protein concentration was determined by Bradford assay (Bio-Rad), and 50 µg of proteins were boiled in Laemmli buffer and electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel. Separated proteins were transferred to nitrocellulose membranes (24V) for 30 min. Blots were blocked in Tris-buffered saline solution containing 0.1% Tween 20 and 5% nonfat dry milk, and the expression of phosphorylated ERK 1+2 and total ERK 1+2 were determined by using the following specifics antibodies.

For detection of ERK1 and ERK2 a rabbit anti-human ERK1 and ERK2 polyclonal antibody (whole antiserum, obtained from Sigma-Aldrich Chemie GmbH, Munich, Germany, product no. M5670) was used at a dilution of 1:10000. The second antibody was horseradish peroxidase (HRP)-linked antibody anti-rabbit IgG at a dilution of 1:10,000 obtained from Bioké B.V. (Leiden, NL).

For detection of phosphorylated ERK1 and ERK2 a purified mouse anti-human p-ERK (E-4) monoclonal antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany, product no. sc-7383) was used at a dilution of 1:1000. p-ERK (E-4) is a mouse monoclonal antibody epitope corresponding to a sequence containing Tyr 204 phosphorylated ERK of human origin. The monoclonal antibody detects ERK 1 phosphorylated at Tyr 204 and correspondingly phosphorylated ERK 2 of mouse, rat and human origin.

The second antibody was horseradish peroxidase (HRP)-linked antibody anti-mouse IgG at a dilution of 1:10,000 obtained from Bioké B.V. (Leiden, NL).

The induction of ERK1+2 phosphorylation was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

As can be seen from Figure 14c AUP-10 culture medium containing 0.4-80 ng/ml of FGF2 induce ERK 1+2 phosphorylation in NHDF similar to rhFGF2.

As can be seen from Figure 14d AUP-14 culture medium containing 0.2-40 ng/ml of CSF1 induce ERK 1+2 phosphorylation in mouse M-NFS-60 cells similar to rhCSF1.

Example 4c: Inhibition of Lipopolysaccharide (LPS) induced M1-activation of human peripheral blood mononuclear cells (PBMCs)

Human THP-1 cells (ATCC® TIB-202™) were obtained from LGC Standards GmbH. The cells were seeded in 96-well microplates and incubated for 24 hours under the following conditions:

- Culture conditions: 37°C, 5% CO_2.
- Culture medium: RPMI-1640 Medium (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 0.05 mM 2-mercaptoethanol, penicillin 50 U/ml, streptomycin 50 µg/ml, and fetal calve serum to a final concentration of 10% by volume.
- Assay medium n°2: RPMI-1640 Medium, supplemented with 0.05 mM 2-mercaptoethanol, penicillin 50 U/ml and streptomycin 50 µg/ml,

THP1 is a human monocytic cell line derived from an acute monocytic leukemia patient. THP-1 cells were differentiated to macrophage cells and polarized according to established protocols. Escheria coli-derived lipopolysaccharide (LPS) (serotype 026:B6 – obtained from Sigma-Aldrich) and Interferon gamma (IFN-γ) obtained from U-Cytech (Utrecht, The Netherlands) was used for polarization.

Briefly, 1 million THP1 cells were plated in 6-well plates in 3-mL DMEM media plus 100 ng/mL phorbol-12-myristate-13-acetate (PMA), which was obtained from Sigma-Aldrich, for 24 hours to induce differentiation to unpolarized macrophages (unpolarized THP-1 derived macrophages).

After differentiation, unpolarized THP-1 derived macrophages were treated in DMEM media for 48 hours with LPS (1 µg/ml) and IFN-γ (100U/ml) to induce M1 phenotype resulting in the generation of M1 polarized THP-1 derived macrophages.

For inhibition of M1 polarization, unpolarized THP-1 macrophages were first incubated with dilutions of the AUP-12 stock solution obtained in Example 4a or with dilutions of a stock solution of recombinant human IL4 (rhIL4) for 18 hours and then stimulated as described above with LPS and IFN-γ for an additional 48 hours.

The AUP-12 stock solution, having a total protein concentration of 1 mg/ml, was diluted to concentrations of 0.1%, 1%, 10% and 20% by volume with DMEM medium. The stock solution of rhIL4 was diluted to concentrations of 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 20 ng/ml by volume with DMEM medium.

The cells were collected and total RNA was extracted using the High Pure RNA Isolation kit (Roche Diagnostics). Total RNA (1 µg) was retrotranscribed using the iScriptTM cDNA Synthesis Kit (Bio-Rad; Hercules, CA, USA), and the cDNA generated was analyzed by real-time PCR, using the iQTM SYBR Green Supermix (Bio-Rad; Hercules, CA, USA). Real-time PCR was performed using a CFX96 Real-time PCR Detection System (Bio-Rad; Hercules, CA, USA). The HPRT gene was used to standardize mRNA expression in each sample and gene expression was quantified using the 2-ΔΔCt method. A Marker for M1 polarization was tumor necrosis factor alpha (TNF-α) mRNA production.

Figure 14e shows the inhibition of LPS-induced TNFalpha mRNA production in human THP-1 cells.

As can be seen from Figure 14e AUP-12 culture medium inhibit LPS-induced TNFalpha mRNA production in human THP-1 cells similar to rhIL4. The effective dose of rhIL4 is between 1-20 ng/ml.

An * in Figure 14e means a p-value $P<0.05$ compared to LPS alone. Error bars represent the standard deviation.

Example 4d: Induction of M2-polarization in Thp1-derived macrophages

In order to induce M2 polarization, unpolarizied THP-1 derived macrophages obtained in Example 4c were incubated with dilutions of the AUP-12 stock solution obtained in Example 4a or with dilutions of a stock solution of recombinant human IL4 (rhIL4) having a final concentration of 20 ng/ml for 18 hours.

The AUP-12 stock solution, having a total protein concentration of 1 mg/ml, was diluted to concentrations of 0.1 % and 1 % by volume with DMEM medium.

Cells were analysed as described above. After polarization cells were collected and total RNA was extracted. Total RNA was retrotranscribed and the cDNA generated was analyzed by real-time PCR. Real-time PCR was performed using a CFX96 Real-time PCR Detection System. The HPRT gene was used to standardize mRNA expression in each sample and gene expression was quantified using the 2-ΔΔCt method. Markers for M2 polarization were macrophage mannose receptor 1 (Mrc1) and Kruppel-like factor 4 (KLF4).

Figure 14f shows the induction of RNA expression of M2 genes for the macrophage mannose receptor 1 (Mrc1) and Kruppel-like factor 4 (KLF4) in THP1-derived macrophages.

As can be seen from Figure 14f AUP-12 culture medium induces RNA expression of M2 genes for the macrophage mannose receptor 1 (Mrc1) and Kruppel-like factor 4 (KLF4) in THP1-derived macrophages similar to rhIL4. The effective dose of rhIL4 is between 2-20 ng/ml.

An * in Figure 14f means a p-value $P<0.05$ compared to M0 (M0 = PMA induced THP1) and Mn = Non-induced THP1. An ** in Figure 14f means a p-value $P<0.05$ compared to Mn = Non-induced THP1. Error bars represent the standard deviation.

Example 4e: STAT-6 transcriptional Activity of AUP-12 in various cell lines.

To determine the biological activity of recombinant IL-4 and AUP-12 on STAT6 transcriptional activity MO3.13 (human) and HEK293T (human) cells transiently co-transfected with the STAT-6 and STAT6-Luc plasmids (Addgene Inc, Cambridge, MA, USA) were used.

Human embryonic kidney 293T cells HEK293T (ATCC® CRL-3216™) were obtained from LGC Standards GmbH. The cells were seeded in 96-well microplates and incubated for 24 hours under the following conditions:

- Culture conditions: 37°C, 5% CO_2.
- Culture medium: DMEM medium, penicillin 50 U/ml, streptomycin 50 µg/ml, and fetal calve serum to a final concentration of 10% by volume.

Human Glial (Oligodendrocytic) Hybrid Cell Line MO3.13 was obtained from tebu-bio GmbH (Offenbach, Germany). The cells were seeded in 96-well microplates and incubated for 24 hours under the following conditions:

- Culture conditions: 37°C.
- Culture medium: DMEM medium, penicillin 50 U/ml, streptomycin 50 µg/ml, and fetal calve serum to a final concentration of 10% by volume.

The transfections with the STAT-6 and STAT6-Luc plasmids were performed using RotiFect™ reagent (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) according to the manufacturer's recommendations. The cells were treated with 0.1 ng/ml, 1 ng/ml,10 ng/ml, 20 ng/ml and 100 ng/ml of recombinant human IL4 (rhIL-4) and dilutions of the AUP-12 stock solution for 6 hours. The AUP-12 stock solution was diluted to concentrations of 0.1%, 1%, 10% and 20% by volume with DMEM medium.

Then the cells were lysed in 25 mM Tris-phosphate pH 7.8, 8 mM MgCl2, 1 mM DTT, 1% Triton X-100, and 7% glycerol.

Luciferase activity was measured using an Autolumat LB 953 (EG&G Berthold, USA) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA). The results of the specific transactivation are expressed as a fold induction over untreated cells

Figure 14g shows the STAT-6 transcriptional Activity of AUP-12 and rhIL-4 in Human Glial Hybrid Cell Line (MO3.13).

Figure 14h shows the STAT-6 Transcriptional Activity of AUP-12 and rhIL-4 in Human Embryonic Kidney 293 Cells.

As can be seen from Figures 14g and 14h AUP-12 culture medium induces STAT-6 transcriptional Activity in a Human Glial Hybrid Cell Line (MO3.13) as well as 4 in Human Embryonic Kidney 293 Cells similar to rhIL4. The effective dose of rhIL4 is between 0,1 and 20 ng/ml.

Example 5: Wound closure experiments

The consequence of application of probiotic bacteria expressing human FGF-2, CSF-1 and IL-4 alone or in combination to full-thickness excisional wounds on db/db diabetic mice was examined in a combined pharmacokinetic (PK) / pharmacodynamic (PD) - efficacy study. Therein, bacteria expressing FGF2 (AUP-10), IL4 (AUP-12) and CSF1 (AUP-14) was evaluated alone or combined as three part combination (i.e. AUP-10 + AUP-12 + AUP14) and compared in respect of efficacy to a positive control (i.e. a combination of recombinant protein TGF-alpha + PDGF-BB). Furthermore, bacteria expressing FGF2, IL4 and CSF1 in a single bacterial cell (AUP-16) was evaluated and compared to a vehicle treatment (i.e. saline + film dressing or IM1 medium + film dressing) and the positive control (TGF-alpha + PDGF-BB).

Patients with diabetes are prone to impaired wound healing, with foot ulceration being particularly prevalent. This delay in wound healing also extends to diabetic animals, including the spontaneously diabetic (db/db) mouse – which was commercially obtained from The Jackson Laboratory (Bar Harbor, ME, USA).

99 diabetic mice (strain name BKS.Cg-Dock^{7m} +/+ Lepr^{db}/J - Stock Code 00642), all male and aged approximately 10 weeks were used in a first study and a subsequent second study.

Recombinant probiotic bacteria expressing human FGF-2, CSF-1 or IL-4 alone or as a three part combination as well as recombinant probiotic bacteria expressing FGF2, IL4 and CSF1 in a single bacterial cell were applied to wounds on the day of wounding (day 0) and their survival was examined 6 hours after application. In order to facilitate determination of the production of FGF-2, CSF-1 and/or IL-4 by bacteria applied to wounds, wound fluid samples were taken from wounds after 6 hours, and 1, 2 & 7 days. Systemic blood and key organs were harvested after 6 & 24 hours and 7 days to facilitate PK/PD analysis. Wounds in receipt of IM1 medium alone were used as controls for the PK/PD component of this study.

In order to examine the impact of these recombinant probiotic bacteria on the process of wound healing, bacteria expressing human FGF-2, CSF-1 and IL-4, as well as bacteria expressing human FGF-2, CSF-1 or IL-4 alone were applied to wounds on the day of wounding (day 0) and daily thereafter until post-wounding day 6. The healing of wounds in receipt of these recombinant probiotic bacteria was compared to that of similar wounds exposed to fresh IM1 medium only.

Furthermore, recombinant human platelet-derived growth factor-BB (rh-PDGF-BB) in combination with recombinant human Transforming Growth Factor-alpha (rh-TGF-α) was used as the “positive control” in this study. The positive control was prepared in a 0.25% Hydroxypropyl methylcellulose (HPMC) vehicle, which was prepared by dissolving 0.5g of Hydroxypropyl methylcellulose, obtained from Sigma Aldrich (St. Louis, MO, USA) in 100 ml distilled water and adding sodium hydroxide to bring the pH up to 7.0.

The combination of PDGF-BB and TGF is based on a study done by Brown RL et al. (Brown RL, Breeden MP, Greenhalgh DG.: “PDGF and TGF-alpha act synergistically to improve wound healing in the genetically diabetic mouse. J. Surg. Res. 56(6), 1994, pages 562 to 570). Brown et al. describe that improvements in wound closure were observed with a combination of PDGF-BB and TGF-alpha when compared to treatment with the individual growth factors.

Wound healing was studied at both the macroscopic and histological levels. Wound healing was studied at the macroscopic level in terms of (i) initiation of neo-dermal repair responses, and (ii) wound closure.

Wound closure, and its components wound contraction and wound re-epithelialisation, were determined from digital photographs taken on post-wounding days 0, 4 & 7 post-wounding. Histological assessments of granulation tissue formation (depth) and wound width (cranio-caudal contraction) were undertaken on routine (H&E) stained sections. These histological assessments were undertaken on tissues harvested on post-wounding day 7.

The development of adverse effects were monitored and fully documented.

Creation of full-thickness experimental wounds and application of treatments

After experimental wounding, animals were housed in individual cages (cage size 500 cm^2 with sawdust bedding, changed three times per week), in an environment maintained at an ambient temperature of 23°C with 12-hour light/dark cycles. They were provided with food (CRM-P, product code 801722, SDS diets, UK) and water ad libitum. To acclimatise the animals to their surroundings, prior to experimentation, they were housed for a period of 5 - 7 days without disturbance, other than to refresh their bedding and to replenish their food and water provisions. Following all anaesthetic events, animals were placed in a warm environment and were monitored until they have fully recovered from the procedure. All animals received appropriate analgesia (buprenorphine) after surgery and received additional analgesics as required.

All animal procedures were carried out in a UK Home Office licensed establishment under UK Home Office Licences (PCD: 50/2505; PPL: 40/3614; PIL: IBCEFDF55; PIL: I34817249). The health of animals was monitored on a daily basis throughout the study.

Animals were randomized to one of the 18 treatment regimes according to Tables 2a and 2b.

On day 0, animals were anaesthetised (isofluorane & air) and the dorsum was shaved and cleaned with saline-soaked gauze. The skin was then be swabbed with 70% EtOH and wiped dry with sterile gauze. A single standardised full-thickness wound (10.0mm x 10.0mm) was created in the left dorsal flank skin of each experimental animal.

Wounds on animals in all groups were then be dressed with a circumferential band of the transparent film dressing Bioclusive (Systagenix Wound Management, Gatwick, UK); after which they received one of the treatments described below (see Table 2a and 2b) which was applied by injection through the Bioclusive film using a 27-gauge needle.

For each treatment the respective un-induced recombinant probiotic bacteria were provided in 10 ml IM1 medium having the following cell density (colony forming units (CFU) per ml medium):

Designation	Sample	CFU/ml
AUP-10	Un-induced NZ3900(pFGF2) obtained in Example 2a	3×10^8
AUP-14	Un-induced NZ3900(pCSF1) obtained in Example 2c	3×10^8
AUP-12	Un-induced NZ3900(pIL4) obtained in Example 2b	3×10^8
Combination	Un-induced NZ3900(pFGF2) obtained in Example 2a	1×10^8
	Un-induced NZ3900(pCSF1) obtained in Example 2c	1×10^8
	Un-induced NZ3900(pIL4) obtained in Example 2b	1×10^8
AUP-16	Un-induced NZ3900(pC-F-I) cells obtained in Example 2d	3×10^8

The treatment scheme “Combination” represents a three part combination resulting from a mixture of un-induced NZ3900(pFGF2) obtained in Example 2a, un-induced NZ3900(pCSF1) obtained in Example 2c, and un-induced NZ3900(pIL4) obtained in Example 2b. The mixture has a total cell density of 3×10^8 CFU/ml.

Before application of treatment, the respective un-induced recombinant probiotic bacteria provided in 10 ml IM1 medium were incubated at 30°C without shaking and the optical density was determined spectrophotometrically at a wavelength of 600 nm (OD600).

At an OD600 of 0.5 nisin was added to a final concentration of 5 ng/ml. After additional incubation for 30 min. at 30°C the induced culture was transferred to 10°C. Subsequently, the respective induced culture was applied by injection through the Bioclusive film using a 27-gauge needle.

Treatments were applied according to the scheme described in Table 2a and 2b. The animals of treatment groups 1 to 9 received a single application of 50 µl at day 0. The animals of treatment groups 10 to 18 received seven consecutive applications divided as a single application of 50 µl per day at days 0, 1, 2, 3, 4, 5, and 6.

Six hours after the first application of treatment, wound fluid was harvested from all animals in groups 1 through 4. The wound fluid harvested from the combination group (grp 1) was processed in such a manner to permit CFU counting of the recombinant probiotic bacteria. The fluid taken from wounds in the remaining groups was held on wet ice prior to transfer to -80ºC for longer term storage. After wound fluid collection, all animals in these groups were terminated (by a UK Home Office approved method). Prior to termination, systemic blood was taken by cardiac puncture into EDTA treated tubes. One half of the blood sample was centrifuged to derive plasma; the remaining half was held at 4°C overnight prior to shipment to Langford Veterinary Services Ldt. (Bristol, UK), where full and differential blood counts were undertaken. Post-mortem samples of liver, kidney, spleen, heart, lung tissue and draining lymph nodes were harvested from all animals (plasma and tissues was be placed in cryovials which were snap-frozen in liquid nitrogen – after which they were stored at -80ºC).

24 hours after the first application of treatment, wound fluid were harvested from all animals in groups 5 through 9 and were stored as described above. Blood was collected and stored and animals were then be terminated. Wound fluid, whole blood, plasma and tissue samples were harvested and stored as described above.

One day after the first application and at daily intervals thereafter until day 6, treatments were re-applied to all wounds in groups 10 through 15.

2 days after the first application of treatment (1 day after second application), wound fluid was harvested from all animals in groups 10 through 15 and was stored as described above.

7 days after the first application of treatment (one day after the final application of treatment on day 6), wound fluid was harvested from all animals in groups 10 through 15 and was stored as described above. Blood was collected and stored and animals were then be terminated. Following termination, wound and marginal tissue was harvested and processed for histological investigation (see section 3.7). Wound fluid, whole blood, plasma and tissue samples were harvested and stored as described above.

On post-wounding days 4 and 7, animals in groups 10 to 15 were re-anaesthetised and their wounds were assessed and digitally photographed together with a calibration/identity plate. On day 4 this was undertaken with the Bioclusive film dressing in place. On day 7, dressings and any free debris was removed and wounds were then be cleaned using saline-soaked sterile gauze and mopped dry with sterile gauze. Wounds were assessed and digitally photographed together with a calibration/identity plate.

Table 2a: Experimental groups of study 1

Tx Group	Treatment (BFD = Bioclusive Film Dressing)	Application of treatment	Time of termination	Fluid sample	Group name	"n"
PK/PD STUDY						
1	combination (FGF2, IL4, CSF1) OD 0.5 CFU + BFD (1 x 50 µl) (for CFU count)	Day 0	+6h	-	COMBO-6h	5
2	single (FGF2) OD0.5 + BFD (1 x 50 µl)	Day 0	+6h	50µl	FGF-6h	5
3	single (IL4) OD 0.5 + BFD (1 x 50 µl)	Day 0	+6h	50µl	IL4-6h	5
4	Vehicle (IM1 medium) + BFD (1 x 50 µl)	Day 0	+6h	50µl	VEH-6h	5
5	combination (FGF2, IL4, CSF1) OD 0.5 + BFD (1 x 50 µl)	Day 0	+1	50µl	COMBO-24h	5
6	single (FGF2) OD 0.5 + BFD (1 x 50 µl)	Day 0	+1	50µl	FGF-24h	5
7	single (IL4) OD 0.5 + BFD (1 x 50 µl)	Day 0	+1	50µl	IL4-24h	5
8	single (CSF1) OD0.5 + BFD (1 x 50 µl)	Day 0	+1	50µl	CSF1-24h	5
9	Vehicle (IM1 medium) + BFD (1 x 50 µl)	Day 0	+1	50µl	VEH-24h	5

10	combination (FGF2, IL4, CSF1) OD0.5 + BFD (7 x 50 μl)	Days 0,1,2,3,4,5,6	+7	50μl on days 2 and 7	COMBO-7d	5
11	single (FGF2) OD0.5 + BFD (7 x 50 μl)	Days 0,1,2,3,4,5,6	+7	50μl on days 2 and 7	FGF-7d	5
12	single (IL4) OD0.5 + BFD (7 x 50 μl)	Days 0,1,2,3,4,5,6	+7	50μl on days 2 and 7	IL4-7d	5
13	single (CSF1) OD0.5 + BFD (7 x 50 μl)	Days 0,1,2,3,4,5,6	+7	50μl on days 2 and 7	CSF1-7d	5
14	Vehicle (IM1 medium) + BFD (7 x 50 μl)	Days 0,1,2,3,4,5,6	+7	50μl on days 2 and 7	VEH-7d	5
15	rh-PDGF-BB [10μg] + rh-TGF-α [1ug] – (7 x 50μl) in 0.5% HMPC + BFD	Days 0,1,2,3,4,5,6	+7	50μl on days 2 and 7	**+ve**-7d	5

Table 2b: Experimental groups of study 2

16	Saline + BFD (7 x 50 μl)	Days 0,1,2,3,4,5,6	+12		VEH-12d	8
17	rh-PDGF-BB [10μg] + rh-TGF-α [1ug] – (7 x 50μl) in 0.5% HMPC + BFD	Days 0,1,2,3,4,5,6	+12		**+ve**-12d	8
18	AUP-16 (FGF2, IL4, CSF1) OD0.5 + BFD (7 x 50 μl)	Days 0,1,2,3,4,5,6	+12		COMBO-12d	8

Image analysis of wound closure

Image Pro image analysis software (version 4.1.0.0, Media Cybernetics, USA) was used to measure wound closure from images of wounds in groups 10 through 15 and 16 through 18 over time. For each wound at each time point (days 4 & 7 for groups 10 through 15 and days 4, 7, 8, and 12 for groups 16 through 18), open wound area was measured and expressed in terms of % wound area relative to day 0.

Survival of recombinant probiotic bacteria after 6 hours after application.

Six hours after introducing the combination of FGF2, IL4 and CSF1 expressing bacteria into the wound, wound fluid was extracted and a bacterial colony forming unit (CFU) counting was performed. The results of the CFU counting from 5 wounds are given in Table 3.

The result confirms that the bacteria are viable in the wound and were still capable of proliferating, after being in the wound for 6 hours, highlighting the fact that the above

mentioned recombinant probiotic bacteria can be used to deliver therapeutic protein combinations to the wounds.

Table 3: Results of the CFU counting

Wound number	Colony count	Dilution	Cfu/ml	Average count
1	342	10^{-4}	3.42×10^{7}	3.76×10^{7}
	41	10^{-5}	4.10×10^{7}	
2	141	10^{-4}	1.41×10^{7}	-
3	346	10^{-4}	3.46×10^{7}	3.13×10^{7}
	28	10^{-5}	2.80×10^{7}	
4	78	10^{-2}	7.80×10^{4}	-
5	287	10^{-1}	2.87×10^{4}	2.93×10^{4}

Wound healing response in the different treatment groups

The wound healing response in the different treatment groups on post-wounding was evaluated by visual inspection 4 and 7 days after starting treatment.

Visual inspection by naked eye as well as quantitative analysis by Image Pro image analysis software of groups 10 through 15 reveals that wound healing occurs even as early as four days after starting treatment. Moreover, on day seven the mice treated with the three part combination of recombinant probiotic bacteria expressing FGF2, IL4, and CSF1 (treatment groups 1, 5, and10) demonstrate a superior healing response (demonstrating pink/red granulation tissue deposition as well as epithelialization of the wound) compared to the individual treatment groups of FGF2 expressing probiotic bacteria (AUP-10), IL4 expressing probiotic bacteria (AUP-12) and CSF1 expressing probiotic bacteria (AUP-14), as well as compared to the positive control (i.e. TGF-alpha and PDGF-BB). The results are depicted in Figure 15a.

Figure 15a illustrates the wound healing response in different treatment groups.

The individual treatments groups show a comparable healing response, which is superior to the IM1 medium treated group (vehicle treated group). The results showed the synergistic effect of combining FGF2, IL4 and CSF1 in wound healing.

FGF2 (AUP-10) treated mice further showed granulation tissue formation, as well as vascularization, but not epithelialization. It can also be seen that there is leakiness from the blood vessels when FGF2 expressing probiotic bacteria was used alone. IL4 expressing probiotic bacteria (AUP-12) and CSF1 expressing probiotic bacteria (AUP-14) showed mainly epithelialization of the wound, but not granulation, whereas wounds treated with the three part combination of recombinant probiotic bacteria (AUP-10 + AUP-12 + AUP-14) demonstrate granulation tissue formation as well as epithelialization of the wound. No leakiness of blood vessels can be seen.

It is important to know that in diabetic foot ulcers (in humans) epithelialization is dependent on granulation tissue formation. Without granulation tissue formation, epithelialization can not occur and the wound does not close.

In the groups 16 through 18 AUP-16 (recombinant bacteria expressing FGF2, IL4 and CSF1 in a single bacterial cell) was compared to vehicle treatment (saline + dressing) and positive control (i.e. TGF-alpha + PDGF-BB). Throughout the days 4, 8 and 12 AUP-16 demonstrated superiority in wound healing compared to the positive control group (TGF-alpha + PDGF-BB). The results are depicted in Figure 15b.

A comparison of the wound-healing status of control (saline treated group 16), positive control (treatment group 17) and AUP-16 treated tissue (treatment group 18) after post-wounding and after 4, 8 and 12 days is shown in Figure 15b.

Day 0 represents the original wound immediately post-wounding. The same wound is also shown at days 4, 8 and 12. The respective photographs were approximately equally scaled to visualise wound closure. The black bar in each photograph represents 1 cm.

A comparison of the remaining wound area after day 8 and day 12 of the positive control (treatment group 17) and AUP-16 treated tissue (treatment group 18) is depicted in Figure 15c.

The remaining wound area was measured with Image Pro image analysis software in images of wounds and expressed in terms of % wound area relative to day 0.

At day 12 almost full closure of the wound was observed in wounds treated with the combination (AUP-16). This is reflected by a remaining wound area of 1,37 % relative to the wound area at day 0 immediately post-wounding.

Furthermore, it can be seen from Figures 15a and 15b that in wounds treated with the three part combination as well as AUP-16 a significant better wound closure was observed compared to the wounds treated with recombinant human platelet-derived growth factor-BB in combination with recombinant human transforming growth factor-alpha (positive control), especially at day 12.

All bacterial treatment regimes investigated in this experiment, AUP-10, AUP-12 and AUP-14 - applied alone or as a three part combination as well as AUP-16, were found to promote wound repair in the diabetic db/db mouse, which is a widely accepted and one of the best validated animal model of delayed wound healing in humans.

Excisional wounds in db/db mice show a statistically significant delay in wound closure, decreased granulation tissue formation, decreased wound bed vascularity, and markedly diminished proliferation, as for example described in Michaels et al. (2007) (“db/db mice exhibit severe wound-healing impairments compared with other murine diabetic strains in a silicone-splinted excisional wound model”, Wound Rep. Reg. 15, pages 665 to 670).

The experimental results clearly show that application of recombinant probiotic bacteria of the present invention provides a significant improvement in the treatment of wounds in humans, especially of chronic wounds.

Application of recombinant probiotic bacteria of the present invention induces granulation tissue formation, increased wound bed vascularity and proliferation, thus leading to an improved and accelerated wound closure even in a model exhibiting severe wound-healing impairments.

These results, thus, show that inflammatory, preferably chronic inflammatory, skin dysfunctions such as frostbite, eczema, psoriasis, dermatitis, ulcer, wound, lupus eritematosus, neurodermitis, and combinations thereof, preferably dermatitis, ulcer, wound, and combinations thereof, further preferably ulcer, benefit from the application of recombinant probiotic bacteria of the present invention.

For example, chronic wounds such as chronic venous ulcers, chronic arterial ulcers, chronic diabetic ulcers, and chronic pressure ulcers, can be treated by application of the recombinant probiotic bacteria of the present invention, since the wound-healing impairments observed in the respective chronic wounds are overcome after application of the recombinant probiotic bacteria of the present invention.

Claims

1. Recombinant probiotic bacteria, comprising
(a) nucleic acid sequence(s) encoding for a first heterologous factor, (a) nucleic acid sequence(s) encoding for a second heterologous factor, and preferably (a) nucleic acid sequence(s) encoding for a third heterologous factor, with the proviso that said first factor, said second factor, and said third factor are functionally different from each other,
wherein said first factor is a growth factor, wherein said second factor is selected from the group consisting of M2-polarizing factors, and wherein preferably said third factor is selected from the group consisting of M2-polarizing factors and growth factors,
wherein said M2-polarizing factor is selected from the group consisting of interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), colony stimulating factor-1 (CSF1), interleukin 34 (IL34), and mixtures thereof, and
wherein said growth factor is selected from the group consisting of fibroblast growth factors (FGF), vascular endothelial growth factors (VEGF), epidermal growth factors (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), insulin-like growth factors (IGF), platelet-derived growth factors (PDGF), transforming growth factor beta (TGF-ß), and mixtures thereof; and
wherein the nucleic acid sequence(s) encoding for the first heterologous factor, the nucleic acid sequences(s) encoding for the second heterologous factor, and the nucleic acid sequence(s) encoding for the third heterologous factor (when present) are located in different and/or the same sub-populations of the recombinant probiotic bacteria.

2. The recombinant probiotic bacteria according to claim 1, wherein the nucleic acid sequence(s) encoding for the heterologous factors are located in one population of the recombinant probiotic bacteria.

3. Recombinant probiotic bacteria according to claim 1 or 2, wherein said nucleic acid sequence(s) is/are located on at least one of a chromosome and a plasmid of said recombinant probiotic bacteria.

4. Recombinant probiotic bacteria according to any one of claims 1-3, wherein an expression of said nucleic acid sequence(s) is/are controlled by a constitutive promoter or an inducible promoter.

5. Recombinant probiotic bacteria according to claim 4, wherein the expression of said nucleic acid sequence(s) is/are controlled by an inducible promoter, said nucleic acid sequence(s) being expressible in the presence of at least one inducer.

6. Recombinant probiotic bacteria according to any one of claims 4 or 5, wherein said inducible promoter, which is inducible by an inducer, is a promoter for a microbial gene which encodes for a lantibiotic peptide a functional analogue thereof, wherein preferably said inducible promoter is PnisA, PnisZ, PnisQ, PnisF, PnisU, or combinations thereof of *Lactococcus lactis*.

7. Recombinant probiotic bacteria according to any one of claims 5 or 6, wherein the inducer is at least one lantibiotic peptide or a functional analogue thereof, preferably selected from the group consisting of nisin A, nisin Z, nisin Q, nisin F, nisin U, functional analogues thereof, and mixtures thereof.

8. Recombinant probiotic bacteria according to any one of claims 1 to 7, wherein said probiotic bacteria further comprises at least one inactivated gene encoding for an essential protein necessary for viability of said probiotic bacteria.

9. Recombinant probiotic bacteria according to claim 8, wherein said at least one inactivated gene necessary for viability of said probiotic bacteria is selected from the group consisting of alanine racemase (alaR), thymidylate synthase (thyA), asparagine synthase (asnH), CTP synthase (pyrG), tryptophan synthase (trpBA), and combinations thereof.

10. Recombinant probiotic bacteria according to any one of claims 8 or 9, wherein said gene is inactivated by deletion of said gene, mutation of said gene, epigenetic modification of said gene, RNA interference (RNAi) mediated gene silencing of said gene, translational inhibition of said gene, or combinations thereof.

11. Recombinant probiotic bacteria according to any one of claims 1 to 10, wherein at least one of said first factor, said second factor, and said third factor is releasable from said recombinant probiotic bacteria.

12. Recombinant probiotic bacteria according to any one of claims 1 to 11, wherein said fibroblast growth factors (FGF) is selected from the group consisting of fibroblast growth factor 1, fibroblast growth factor 2, fibroblast growth factor 7 (keratinocyte growth factor), fibroblast growth factor 10 (keratinocyte growth factor 2), and mixtures thereof.

13. Recombinant probiotic bacteria according to any one of claims 1 to 12, wherein said first, second, and third heterologous factor is a combination of

- fibroblast growth factor 2, colony stimulating factor-1 and interleukin 4,
- fibroblast growth factor 2, interleukin 34 and interleukin 4,
- fibroblast growth factor 2, colony stimulating factor-1 and interleukin 13,
- fibroblast growth factor 2, interleukin 34 and interleukin 13,
- fibroblast growth factor 2, colony stimulating factor-1 and interleukin 10,
- fibroblast growth factor 2, interleukin 34 and interleukin 10,
- fibroblast growth factor 7, colony stimulating factor-1 and interleukin 4,
- fibroblast growth factor 7, interleukin 34 and interleukin 4,
- fibroblast growth factor 7, colony stimulating factor-1 and interleukin 13,
- fibroblast growth factor 7, interleukin 34 and interleukin 13,
- fibroblast growth factor 7, colony stimulating factor-1 and interleukin 10,
- fibroblast growth factor 7, interleukin 34 and interleukin 10,
- transforming growth factor beta, colony stimulating factor-1 and interleukin 4,
- transforming growth factor beta, interleukin 34 and interleukin 4,
- transforming growth factor beta, colony stimulating factor-1 and interleukin 13,
- transforming growth factor beta, interleukin 34 and interleukin 13
- transforming growth factor beta, colony stimulating factor-1 and interleukin 10,
- transforming growth factor beta, interleukin 34 and interleukin 10,
- transforming growth factor alpha, colony stimulating factor-1 and interleukin 4,
- transforming growth factor alpha, interleukin 34 and interleukin 4,
- transforming growth factor alpha, colony stimulating factor-1 and interleukin 13,
- transforming growth factor alpha, interleukin 34 and interleukin 13
- transforming growth factor alpha, colony stimulating factor-1 and interleukin 10,
- transforming growth factor alpha, interleukin 34 and interleukin 10
- platelet derived growth factor BB, colony stimulating factor-1 and interleukin 4,
- platelet derived growth factor BB, interleukin 34 and interleukin 4,
- platelet derived growth factor BB, colony stimulating factor-1 and interleukin 13,

- platelet derived growth factor BB, interleukin 34 and interleukin 13
- platelet derived growth factor BB, colony stimulating factor-1 and interleukin 10, or
- platelet derived growth factor BB, interleukin 34 and interleukin 10.

14. Recombinant probiotic bacteria according to any one of claims 1 to 13, wherein said first, second, and third heterologous factor is is a combination of fibroblast growth factor 2, colony stimulating factor-1 and interleukin 4, functional analogs thereof, and biosimilars thereof.

15. Recombinant probiotic bacteria according to any one of claims 1 to 14, wherein said recombinant probiotic bacteria are lactic acid bacteria, preferably a *Lactobacillus* or a *Lactococcus* species.

16. Recombinant probiotic bacteria according to claim 15, wherein said *Lactococcus* species is *Lactococcus lactis*, preferably *Lactococcus lactis* subspecies *cremoris*.

17. Recombinant probiotic bacteria according to any one of claims 1 to 16, wherein said recombinant probiotic bacteria are in a solution, frozen or dried, preferably lyophilised or spray dried.

18. Recombinant probiotic bacteria according to any one of claims 1 to 17, wherein said recombinant probiotic bacteria are to be administered topicallly and/or by subcutaneous injection.

19. Recombinant probiotic bacteria for use in the treatment of a chronic wound, wherein said recombinant probiotic bacteria are recombinant probiotic bacteria according to any one of claims 1 to 17.

20. Recombinant probiotic bacteria according to claim 19, wherein said chronic wound is at least one of a chronic venous ulcer, a chronic arterial ulcer, a chronic diabetic ulcer, a chronic pressure ulcer and a chronic preulceration stage thereof.