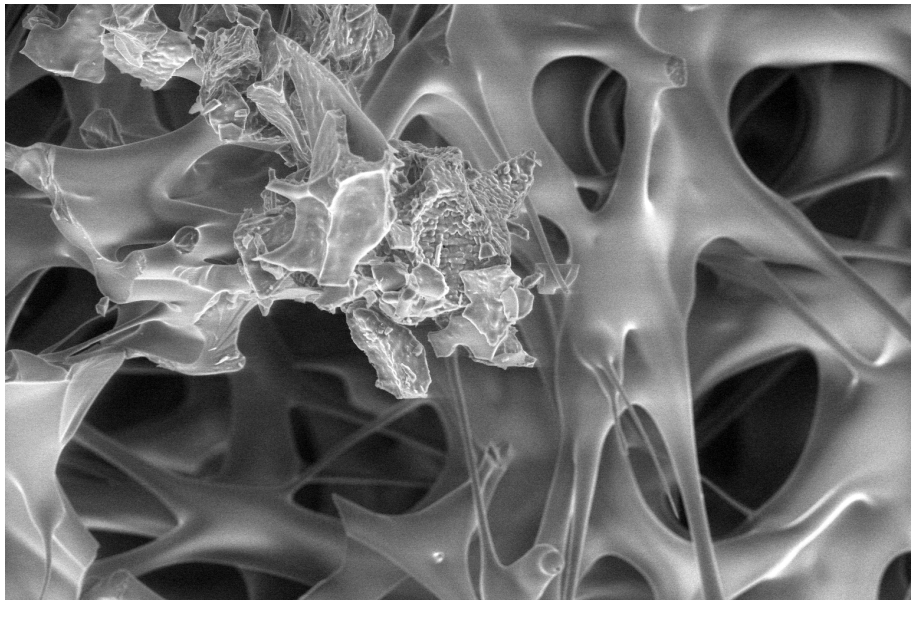
Live Biotherapeutics

Importance of formulation and lyophilization parameters
and an example of a clinical application

Nisha Tyagi



Licentiate Thesis
Swedish University of Agricultural Sciences
Uppsala

Live Biotherapeutics

Importance of formulation and lyophilization parameters and an example of a clinical application

Nisha Tyagi
Faculty of Natural Resources and Agricultural Sciences
Department of Molecular Sciences
Uppsala

SLU
SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

LICENTIATE THESIS
Uppsala 2023

Cover: A scanning electron microscopy image of a freeze-dried *Limosilactobacillus reuteri* R2LC in sucrose without annealing step at low (10^9 CFU/mL) bacterial concentration. (photo: Zandra Gidlöf)

ISSN 1652-6880
ISBN (print version) 978-91-8046-852-7
ISBN (electronic version) 978-91-8046-853-4
https://doi.org/10.54612/a.4g1r1pvjb4

Swedish University of Agricultural Sciences, Department of Molecular Sciences, Uppsala, Sweden

Print: SLU Grafisk Service, Uppsala 2023

Live Biotherapeutics – Importance of formulation and lyophilization parameters and an example of a clinical application

Abstract

In recent years, probiotics have expanded from their traditional classification as "health promoting food" to the development of live biotherapeutic products (LBP). Traditional probiotics are marketed as food/dietary supplements while LBPs are drug products intended for treatment or prevention of diseases. This type of products offers several advantages over traditional drugs, but also entail potential challenges with development, manufacturing, and demonstration of clinical safety. To obtain a sufficient quality, LBPs are typically produced by cultivation in a bioreactor, followed by formulation and lyophilization.

In the first part of the project, the impact of lyophilization parameters on physicochemical and biological properties of *Limosilactobacillus reuteri* R2LC was evaluated. Using sucrose as a lyoprotectant gave a better freeze-drying survival, vitality and storage stability than using trehalose. A high concentration (20%) of sucrose sometimes resulted in a collapsed structure and 15% gave the overall best properties of the lyophilized bacteria. Interestingly, vitality was positively affected by using a higher concentration (10^{10} cfu/ml) of bacteria. Another observation was that introducing an annealing step in the process was positive when using sucrose as lyoprotectant, but no effect was seen when using trehalose.

The second part of the project describes evaluation of the genetically modified *L. reuteri* R2LC expressing the human chemokine CXCL12 (ILP100-Topical) in a phase 1 trial on wound healing. The product was safe and well-tolerated. In addition, it gave a larger proportion of healed wounds (76 %) on Day 32 when compared to saline/placebo (59 %) (p=0.020) and the time of wound healing was reduced by 6 days on average and by 10 days at highest dose. Also, ILP100-Topical increased the density of $CXCL12^{+}$ cells in the wounds and local wound blood perfusion.

Keywords: Probiotics, Live Biotherapeutics, *Limosilactobacillus reuteri* R2LC, ILP100-Topical, freeze-drying, CXCL12, vitality, viability, safety, wound healing.

Levande bioterapeutika – Betydelsen av formulerings- och frystorkningsparametrar och ett exempel på en klinisk tillämpning

Sammanfattning

Under de senaste åren har probiotika expanderat från sin traditionella klassificering som "hälsofrämjande mat" till utveckling av levande bioterapeutiska produkter (LBP). Traditionella probiotika marknadsförs som kosttillskott medan LBP är läkemedelsprodukter avsedda för behandling och förebyggande av sjukdomar. Denna typ av produkter erbjuder flera fördelar gentemot traditionella läkemedel, men innebär också potentiella utmaningar med utveckling, tillverkning och bevisning av klinisk säkerhet. För att erhålla en tillräcklig kvalitet produceras LBP vanligtvis genom odling i en bioreaktor, följt av formulering och frystorkning.

I den första delen av projektet utvärderades effekten av frystorkningsparametrar på fysikalkemiska och biologiska egenskaper hos *Limosilactobacillus reuteri* R2LC. Att använda sackaros som torkskydd gav en bättre frystorkningsöverlevnad, vitalitet och lagringsstabilitet än att använda trehalos. En hög koncentration (20 %) av sackaros resulterade ibland i en kollapsad struktur, men 15 % gav de överlag bästa egenskaperna hos de frystorkade bakterierna. Intressant nog påverkades vitaliteten positivt genom att använda en hög koncentration (10^{10} cfu/ml) av bakterier. En annan observation var att införandet av ett annealingssteg i processen var positivt när man använde sackaros som lyoprotectant, men för trehalos hade annealing ingen effekt.

Den andra delen av projektet beskriver utvärderingen av en genetiskt modifierad *L. reuteri* R2LC som uttrycker humant kemokin CXCL12 (ILP100-Topical) i en fas 1-studie på sårläkning. Produkten var säker och tolererades väl. Dessutom gav det en större andel läkta sår (76 %) på dag 32 jämfört med saltlösning/placebo (59 %) (p=0.020) och tiden för sårläkning minskade med 6 dagar i genomsnitt och med 10 dagar för den högsta dosen. Dessutom ökade ILP100-Topical densiteten av CXCL12+-celler i såren och lokalt blodflöde.

Nyckelord: Probiotika, Levande bioterapeutika, Limosilactobacillus reuteri R2LC, ILP100-Topical, frystorkning, vitalitet, viabilitet, säkerhet, sårläkning.

Dedication

To my parents,

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Nisha Tyagi, Zandra Gidlöf, Daniel Tristan Osanlóo, Elizabeth S. Collier, Sandeep Kadekar, Lovisa Ringstad, Anna Milliqvist Fureby and Stefan Roos (2023). Impact of formulation and freeze-drying on the properties and performance of freeze-dried probiotic *Limosilactobacillus reuteri* R2LC. *Manuscript*.

II. Öhnstedt, E., Vågesjö, E., Fasth, A., Lofton Tomenius, H., Dahg, P., Jönsson, S., Tyagi, N., Åström, M., Myktybekova, Z., Ringstad, L., Jorvid, M., Frank, P., Heden, P., Roos, S. and Phillipson, M (2023). Engineered bacteria to accelerate wound healing: an adaptive, randomised, double-blind, placebo-controlled, first-in-human phase 1 trial. eClinicalMedicine, 45, 102014.

Papers II is reprinted with the permission of the publishers.

The contribution of Nisha Tyagi to the papers I and II included in this thesis was as follows:

I. Planning and designing of experimental work together with supervisors, performed most of experimental work, data analysis and manuscript writing.

II. Methodology designed together with coauthors. Method development, quality control and validation of Laser Speckle Contract Analysis Image analysis of perfusion on skin and in wounds and analysis of microcirculation of the treated wounds and surrounding skin.

List of tables

List of figures

Abbreviations

CFU	Colony forming unit
CXCL12	Chemokine also known as Stromal Derived Factor 1a
DFU	Diabetic foot ulcer
DOE	Design of experiment
DSC	Differential scanning calorimetry
ECM	Extra cellular matrix
FMT	Fecal microbiota transplantation
ILP100	Genetically modified *Limosilactobacillus reuteri* R2LC producing human chemokine CXCL12
KFT	Karl-Fischer titration
LBPs	Live Biotherapeutic Products
L. reuteri R2LC	*Limosilactobacillus reuteri* R2LC
MRS	De Man, Rogosa and Sharpe
SEM	Scanning electron microscope
Tg	Glass transition temperature

1. Introduction

1.1 Microbiota

The importance of the microbiota in health and disease have been described in many studies (Hou et al., 2022; Tungland, 2018). Human microbiotas are classified based on the sites they colonize, such as oral cavity, skin, gut etc. (Hou et al., 2022). The bacteria assists in metabolic processes, degradation of food components, production of vitamins, immune development, as well as protection against pathogens (Hou et al., 2022). Probiotics are defined as live microorganisms that when administered in adequate amount confer a health benefit on the host (Hill et al., 2014). Traditionally, strains used as probiotics have been isolated from the gastrointestinal tract (incl. faeces), breast milk or fermented food products (for example yogurt and kefir) and have been marketed as food, food/ dietary supplements (Fenster et al., 2019). Recent advancement in technologies such as DNA sequencing, bioinformatics and metabolomics have been strong drivers for the expansion of knowledge of the human microbiota at the molecular and strain levels. Projects such as Human Microbiome Project (https://hmpdacc.org/) and MetaHIT (https://sanger.ac.uk/resources/downloads/bacteria/metahit/) have played important roles in connecting knowledge about the gut microbiota to the broader medical research. The increased research within the field has also been a vital help in the discovery of new bacterial strains that can be used for developing potential new probiotics/ Live biotherapeutics.

First generation (also called traditional) probiotics are primarily used as food or dietary supplements, while next-generation probiotics or live biotherapeutic products (LBP) are intended to use as medicinal drug products to prevent or cure human diseases (O'Toole et al., 2017). LBPs also include

genetically modified microorganisms (GMM) (O'Toole et al., 2017). Live Biotherapeutic Products are defined as ‘a biological product that 1) contains live microorganisms, such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or conditions of human beings; and 3) is not a vaccine’ (U.S. Department of Health and Human Services, 2016). .

1.2 Legislation for LBPs

Globally, probiotics and LBPs are divided into different categories as per different country’s regulations (Arora & Baldi, 2015; Chieffi et al., 2022) as described in table 1. Generally, they are categorised as food/food supplement/dietary supplements or as functional food/drugs (Arora et al., 2013). FDA and European Commission had set the legislative and regulatory measures to address the use of live biotherapeutics in clinical settings (Regulation, 2007; U.S. Department of Health and Human Services, 2016). These measures aim to guarantee the safety and efficacy of live biotherapeutics by monitoring their development, testing and administration.

Table 1. Globally categorization of probiotics an LBPs

Country	Category	Regulatory group	References
Australia and New Zealand	Functional food and Therapeutic goods	FSANZ and TGA	(FSANZ, 2021; Kumar, 2021)
Brazil	Functional food and biological products	ANVISA	(Castanheira et al., 2011; Library, 2014)
Canada	Natural health products	Natural Health Products Directorate	(Canada, 2022)
China	Functional foods and drug	SFDA and CDE	(Administration, 2007)
Europe	Functional foods and drugs	EFSA and EDQM	(Cordaillat-Simmons et al., 2020; Regulation, 2007; Saxelin, 2008)
India	Functional food, and drugs	FSSA, PFA and FDA	(Arora & Baldi, 2015; Sharma et al., 2013; WHO/FAO, 2001)

Country	Category	Regulatory group	References
Japan	Functional foods, drugs, and nutraceuticals	MHLW, and FOSHU	(H. Amagase, 2008; Harunobu Amagase, 2008; MHLW, 2007)
Malaysia	Functional foods	FSQD, The Drug Control Authority, NPCB, and The Committee for the Classification of Food -Drug Interface Products Natural Health Products Directorate	(Lau, 2019)
USA	Dietary supplements	DSHEA	(Administration, 2022)
	Biological products	BLA	(FDA, 2021; Wu, 2019)
	Drugs, medicinal food, and LBPs	FDA	(Administration, 2018; Degnan, 2008, 2012; U.S. Department of Health and Human Services, 2016)

ANVISA: National Health Surveillance Agency Brazil, BLA: Biologic License Application, DSHEA: Dietary Supplement Health and Education Act, FAO/WHO: Foos and Agricultural Organization/World Health Organization, FOSHU: Food for Specified Health Use, FSANZ: Food Standards Australia and New Zealand, FSQD: Food Safety and Quality Division, FUFOSE: Functional Food Science in Europe, MHLW: Ministry of Health and Welfare, NPCB: National Pharmaceutical Control Bureau, PFA: Prevention of Food Adulteration Act, SFDA: State Food and Drug Administration

1.3 LBPs in treatment and prevention of human diseases

Probiotic products have been developed and distributed over the past century, while the first live biotherapeutic drugs just recently have been approved by US-FDA: Rebyota (Nov. 2022) and VOWEST (April 2023). Both products are based on a complete faecal microbiota, and thus have big similarities to the concept “faecal microbiota transplantation” (FMT; (Ooijevaar et al., 2019). Development of next-generation probiotic is an

exciting and promising advance of the healthcare sector. Another FMT product from BiomeBank was approved only in Australia to treat recurrent *Clostridioides difficile* infection. Whilst there is plethora ongoing research only a few LBP drug candidates have reached to clinical stage being evaluated in randomized controlled trials following the pharmaceutical regulatory frameworks (described in table 2). The market of live biotherapeutic products grow quickly and globally it is anticipated to reach $2.60 billion by 2030 (Analytic, 2022).

LBPs could potentially be helpful in treatment and prevention of a wide range of diseases such as metabolic disorders, cardiovascular diseases, infectious diseases, cancer, and inflammation (Lim & Song, 2019; Meng et al., 2023). Most LBPs under development are based on naturally occurring bacterial strains (60%) but leveraging the area of synthetic biology and metagenomics are now used for the development of genetically engineered LBP's (40%) (Table 2). Examples are Aurealis therapeutics that have developed AUP-16, a genetically engineering *Lactococcus lactis* expressing human fibroblast growth factor 2, IL-4 and CSF-1 to treat diabetic foot ulcers (Kurkipuro et al., 2022), and Ilya Pharma that have developed a strain of *Limosilactobacillus reuteri* that expresses the human chemokine CXCL12 for accelerated wound healing (paper II).

Table 2. List of LBPs approved and under clinical development.

Organization	**Product**	**Description**	**Targeted disease**	**Approved / Clinical phase status**	**References/ Clinical ID***
Ferring	Rebyota	Fecal microbiota transplantation (FMT) product	Recurrent *C. difficile* infection	Approved (US-FDA)	(Orenstein, 2023)
BiomeBank	Biomictra Fecal Microbiota	FMT-product	Restoration of gut microbiota and rCDI	Approved (Australia's Therapeutic Goods Administratio n	(Tucker et al., 2023)
Seres Therapeutics	SER-109	Purified Firmicutes spores	rCDI	Approved (US-FDA)	(Sims et al., 2023) https://www.fda. gov/vaccines-

Organization	Product	Description	Targeted disease	Approved / Clinical phase status	References/ Clinical ID*
					blood-biologics/vowst
Finch Therapeutics	CP101	Full-spectrum microbiota (FSM)	rCDI	Phase 2 (completed)	(Allegretti et al., 2022)
Vedanta Biosciences	VE303	Fecal microbiome	rCDI	Phase 2 (completed)	(Dsouza et al., 2022; Louie et al., 2023)
University of Michigan	VE303+ Vanco-mycin	LBPs (consisting of 8 non-pathogenic commensal strains of clostridia) + vancomycin	Hepatic Encephalop athy	Phase 2 (active, not recruiting)	NCT04899115
Genome & Company	Bavencio (GEN-001+ Avelumab)	*L. lactis* strain with immunomodulat ory activity in partnership with immune checkpoint inhibitors	Cancer (advanced solid tumours)	Phase 2 (recruiting)	NCT05419362
Kibow Therapeutics	KT-301	Natural probiotics formulation that metabolizes nitrogenous waste	Chronic kidney disease (CKD) phase IV	Phase 2 (recruiting)	NCT04913272
Regions-hospitalet Viborg, Skive	LACTIN-V + Clinda-mycin	*L. crispatus* + Clindamycin	Bacterial vaginosis (BV)	Phase 2 (recruiting)	(Haahr et al., 2020) NCT05166746
Osel, Inc.	LACTIN-V	Human *L. crispatus* CTV-05 strain	HIV	Phase 2 (recruiting)	NCT05022212
Ilya Pharma AB	ILP100	Genetically modified *L. reuteri* expressing human chemokine	Topically wound healing by activating immune response	Phase 2 (recruiting)	(Öhnstedt et al., 2023) NCT05608187

Organization	Product	Description	Targeted disease	Approved / Clinical phase status	References/ Clinical ID*
Vedanta Biosciences	VE202	Human derived 16 bacterial strains	Ulcerative colitis	Phase 1 (completed)	(Oka et al., 2020)
Aurealis Therapeutics AG	AUP-16	Genetically modified *L. lactis* expressing therapeutic human proteins	Diabetic foot ulcer (chronic wounds)	Phase 1 (completed)	(Kurkipuro et al., 2022)
SNIPR Biome Aps	SNIPR001	Genetically modified bacteriophages specifically targeting *E. coli*	*E. coli* infections and bloodstream infections	Phase 1 (completed)	NCT05277350
YSOPIA Bioscience	Xia1/Yso1	*Christensenella minuta*	Obesity and metabolic disease	Phase 1 (completed)	(Mazier et al., 2021), NCT04663139
Naked Biome, Inc.	NB01	Strain of *P. acnes*	Acne vulgaris	Phase 1 (completed)	NCT03450369
Bloom Science	BL-001	Two rationally selected gut microbes	Dravet syndrome (paediatric epilepsy)	Phase 1a (completed)	NCT05818306
Microbiotica	MB097	Nine bacterial strains that enhance the efficacy of immune checkpoints inhibitors (ICIs)	Melanoma	Phase 1b (completed)	(Robinson et al., 2022)
Precigen ActoBiotics	AG013	*L. lactis* expressing human trefoil factor 1	Oral mucositis	Phase 1b (completed)	(Caluwaerts et al., 2010; Limaye et al., 2013)
Leadiant Biosciences, Inc.	STP206		Necrotizing enterocolitis	Phase 1b (completed)	NCT01954017
Vedanta Biosciences	VE800	LBPs (11 non-pathogenic, non-toxicogenic,	Cancer	Phase 1 (Active, not recruiting)	NCT04208958

Organization	Product	Description	Targeted disease	Approved / Clinical phase status	References/ Clinical ID*
		commensal bacterial strains) +Nivolumab (Opdivo)			
Imperial College London	MRx0518	Proprietary strain of bacterium (*Enterococcus* species)	Cancer	Phase I (active, not recruiting)	NCT03934827
Federation Bio Inc.	FB-001	Consortium of 148 defined bacterial strains	Enteric hyperoxaluria	Phase 1 (recruiting)	NCT05650112
Synlogic	SYNB8802 -CP-002	Genetically engineered *E. coli Nissle* 1917 reduce oxalate levels by converting oxalate to formate and carbon dioxide	Enteric Hyperoxaluria	Early phase 1	NCT05377112
Azitra	ATR-12	Genetically modified *S. Epidermidis* strain expressing LEKTI	Netherton Syndrome	Phase 1 (FDA-cleared for first-in-human trial in 2023)	https://azitrainc.com/

* https://clinicaltrials.gov/study/

1.4 Considerations

Live microorganisms or 'bugs as drugs' holds immense potential (Lamouse-Smith et al., 2021; Wang et al., 2023), however, these live microorganisms should be carefully selected, and developed to provide the desired quality. Development of LBP's entails several challenges in terms of demonstration of safety, delivering efficacy, and ensuring a consistent production resulting in products with high viability and stability. The major safety concerns of LBPs include translocation of the microorganisms, colonization of the microorganism on mucosal surfaces or the skin of the host (depending on the

product), and gene transfer. Therefore, FDA has defined certain regulations that needs to be fulfilled (non-clinical and clinical study) before product approval (Dreher-Lesnick et al., 2017; FDA, 2021; Regulation, 2007; U.S. Department of Health and Human Services, 2016). The regulatory framework in the US is evolving and adapting quickly to the pipeline whilst this is not seen in Europe, rather the opposite with the new centralised procedure.

1.5 Lactic acid bacteria (LAB)

The mankind's use of lactic acid bacteria (LAB) goes long back to ancient times. LAB have played important roles in preservation of food, maintaining texture and incorporation of flavours in the food and as additive to improve gut health (Piccioni et al., 2021; Stiles, 1996). The importance of lactic acid bacteria for maintaining the gut flora and healing digestive problems was first suggested in 1907 (Metchnikoff, 1907), but the interest in the link to health has increased significantly during the last decades.

Limosilactobacillus reuteri, which was firstly described as a species in 1980 (Kandler et al., 1980), is a Gram-positive, rod shaped bacteria, which is aerotolerant and strictly fermentative (Vandenberghe et al., 2010). *L. reuteri* is found in the gastrointestinal tract and other mucosal surfaces of birds and mammals (Martinez et al., 2015; Valeur et al., 2004; Walter et al., 2011), including humans (Jiang et al., 2023). Strains of *Limosilactobacillus reuteri* produces a wide range of metabolic end-products, such as organic acids (like lactic, acetic, propionic, and phenyl lactic acid), low molecular weight antimicrobial molecules (like reuterin, hydrogen peroxide, ethanol, diacetyl, and acetaldehyde) (Mu et al., 2018) and other substances (like bacteriocins, histamine, and reutericyclin which all contributes to an antimicrobial activity (Abuqwider et al., 2022; Vandenberghe et al., 2010).

Limosilactobacillus reuteri R2LC is a strain of *L. reuteri* isolated from rat colon (Fabia et al., 1993; Wang et al., 1995), which in preclinical studies has been described to reduce acetic-acid/DSS induced colitis in rats (Holma et al., 2001) and mice (Ahl et al., 2016; Liu et al., 2021). R2LC has also been shown to possess an antimicrobial activity mediated by a polyketide (Ozcam et al., 2019), protect the mucosal barrier from enterotoxigenic *E. coli* (Karimi et al., 2018). In addition, it has recently been demonstrated (Liu et al., 2021) that R2LC without colonizing, protects the intestine from inflammation by

conveying probiotic signals in Peyer's patches by regulating B-cells subsets and thereby mediate increased IgA production.

1.5.1 Production of LAB/probiotics based on *lactobacilli*

The first step in the production of lactic acid bacteria/microorganisms to be used in LBPs is cultivation followed by formulation and drying. During growth, lactic acid bacteria ferment carbohydrates into end products such as lactic and acetic acids or ethanol (Stanbury et al., 2017). Based on the pathway used for sugar fermentation LAB are divided into two groups: homofermentative (lactic acid as the main end product) and heterofermentative (lactic acid, carbon dioxide, and ethanol or acetic acid as main end products). *L. reuteri* R2LC belongs to the latter group (Yu et al., 2018). It has previously been demonstrated that the biological activities of probiotics are directly influenced by cultivation parameters such as time of harvest, growth media, and environmental parameters (e.g. temperature and pH) (Meng et al., 2008). Freeze-drying (also known as lyophilization) is a widely used method for drying probiotics (including LBP's) to increase the shelf life of the product. The process is divided into three steps: a) freezing, b) primary drying, and c) secondary drying (Fonseca et al., 2015) (Figure 1). During the freezing step, samples are cooled by decreasing shelf temperature resulting in ice crystal formation. Usually, the temperature is between -40°C and -20°C. During this step bacteria is exposed to osmotic shock, mechanical and oxidative stress, which can later affect the biological activity of the probiotics/LBPs. To partly overcome these stressful conditions during freezing, fast cooling and including an annealing step could be utilized, which facilitates water vapour transport and improves the drying (Merivaara et al., 2021). Annealing is a process to optimize the freezing and facilitate the primary drying i.e., lower than glass transition temperature (Tg) for certain period in order to increase the ice crystal growth and promote the rate of primary drying (Badal Tejedor et al., 2020). Previously, it has been shown that annealing improved the viability and stability of probiotics (Ekdawi-Sever et al., 2003). During the primary drying, the pressure is decreased which leads to ice sublimation, and it's important to have an optimal combination of shelf temperature and chamber pressure to achieve an efficient sublimation (Fonseca et al., 2015). In the last step, the secondary drying, unfrozen water is removed via desorption. In this step the

temperature is slowly increased to around 20°C while maintaining a low pressure.

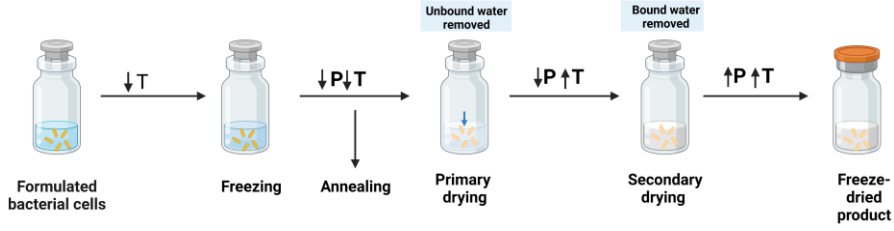


Figure 1. Freeze-drying process

The effectiveness of the drying process depends on several parameters like time, pressure, temperature, and properties of the lyoprotectant (Tang & Pikal, 2004). The lyoprotectant plays a vital role in protecting cells from damage and helps in maintaining the stability, viability during the freeze-drying process. Sugars as lyoprotectants are efficient by replacing the water molecules (Santivarangkna et al., 2008; Wessman et al., 2011), and e.g. sucrose and trehalose are well known lyoprotectants in the production of probiotics and biological drugs. It has been shown that non-reducing disaccharides protect biological drugs/probiotics by forming a glassy matrix structure that prevent damage due to ice crystal formation. These sugars also have a high glass transition temperature that stabilizes the product and prolong shelf life at higher temperature (Bodzen et al., 2021a; Bogdanova et al., 2022; Crowe et al., 1996; Montel Mendoza et al., 2014; Onwe et al., 2022; Wessman et al., 2013). It has also been shown that lactose and trehalose increases the bioavailability and efficiency of some drugs/biologics (Zhang et al., 2020).

2. Aims

Development of live biotherapeutic products (LBP's) entails challenges like designing an efficient product concept, development of a production methods that ensures a high and consistent quality, and demonstration of both clinical safety and efficacy. This thesis is a continuation on previous publications that describe intriguing interactions and effects of *Limosilactobacillus reuteri* R2LC in preclinical models (Ahl et al., 2016; Liu et al., 2021); and efficient wound healing by using R2LC that has been genetically engineered to express the chemokine CXCL12 (ILP100-Topical). The thesis consists of the following two parts:

- The effect of formulation and lyophilization parameters on biological as well as physicochemical properties of freeze-dried *L. reuteri* R2LC. The following parameters were investigated: type of lyoprotectant, concentration of lyoprotectant, bacterial concentration and using a freeze-drying process with or without an annealing step. The following biological characteristics were monitored: process survival, vitality, and shelf life. In addition, the correlation between physicochemical properties of the lyophilized products and the biological characteristics were investigated. (**Paper I**).

- In a first-in-human clinical phase-I trial on wound healing, the safety, tolerability, and biologic effect of genetically modified *L. reuteri* R2LC (ILP100-Topical) was evaluated after topical single and multiple dose administration to experimentally induced skin wounds in healthy subjects/volunteers. Furthermore, influence of ILP100-Topical on the microcirculation/blood perfusion in the

wound or in the edge surrounding wound was investigated. (**Paper II**)

3. Methods

In **paper I**, *Limosilactobacillus reuteri* R2LC was used as a model microorganism for studying the impact of freeze-drying and formulation on its performance while in **paper II** a genetically modified *Limosilactobacillus reuteri* R2LC expressing the chemokine CXCL12 (ILP100-Topical; (Vagesjo et al., 2018) was evaluated in a first-in-human phase-1 trial.

3.1 Impact of production parameters on physicochemical and biological properties of freeze-dried *L. reuteri* R2LC **(Paper I)**

3.1.1 Experimental design

To understand the impact of formulation and freeze-drying on physicochemical and biological properties of freeze-dried *L. reuteri* R2LC, four experimental factors were combined to in total 24 different variants using a Design-of-Experiment approach and a full factorial study design (using the software Modde (Eriksson et al., 2008)). Type of lyoprotectant (sucrose or trehalose); Concentration of lyoprotectant (10, 15 or 20%); Bacterial concentration (10^9 or 10^{10} cfu/ml); and introducing an annealing step or not in the freeze-drying process. (Figure 2).

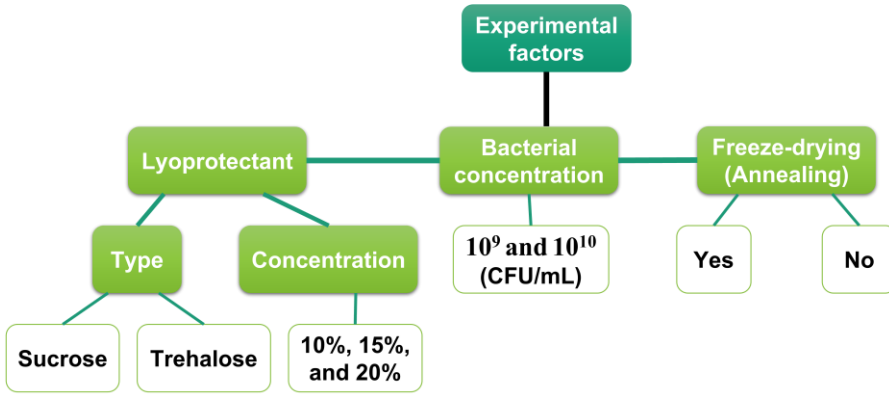


Figure 2. Different experimental factors used in the study to understand the effect of formulations and freeze-drying parameters.

3.1.2 Cultivation, formulation and freeze-drying of *L. reuteri* R2LC

L. reuteri R2LC was cultivated in a pilot 5-L scale bioreactor (also called fermenter) containing deMan, Rogosa, Sharpe broth (MRS, Merck) as shown in figure 3. The stirring speed, pH, and temperature of bioreactor were set to 200 rpm, 5.7 and 37°C respectively. During fermentation the growth was monitored by measuring optical density as well as by plating the sample on MRS agar plates via serial dilution at different timepoints (1, 2, 3 hr and so on). The detailed materials and methods description is provided in paper I.

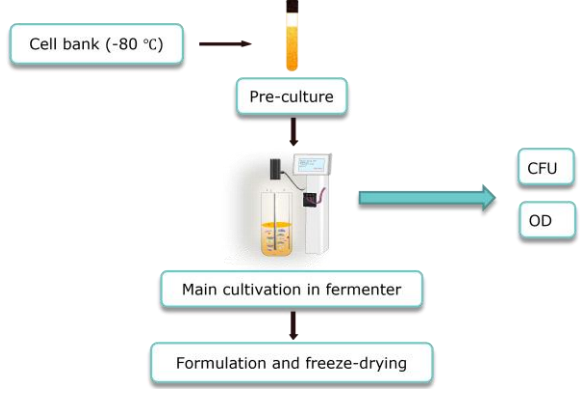


Figure 3. Production of *L. reuteri* R2LC

After fermentation the cell suspension was concentrated using a diafiltration column (750 kDa cut off) and then mixed with different lyoprotectants at different concentrations as shown in figure 4. The

formulated samples were firstly divided into sets (1) samples freeze-dried with annealing and (2) samples freeze-dried without annealing step. Two Christ, Epsilon 2-6D, LSC plus, (Martin Christ GmbH, Germany) freeze dryers were used. Detailed description of freeze-drying process can be found in **paper I**.

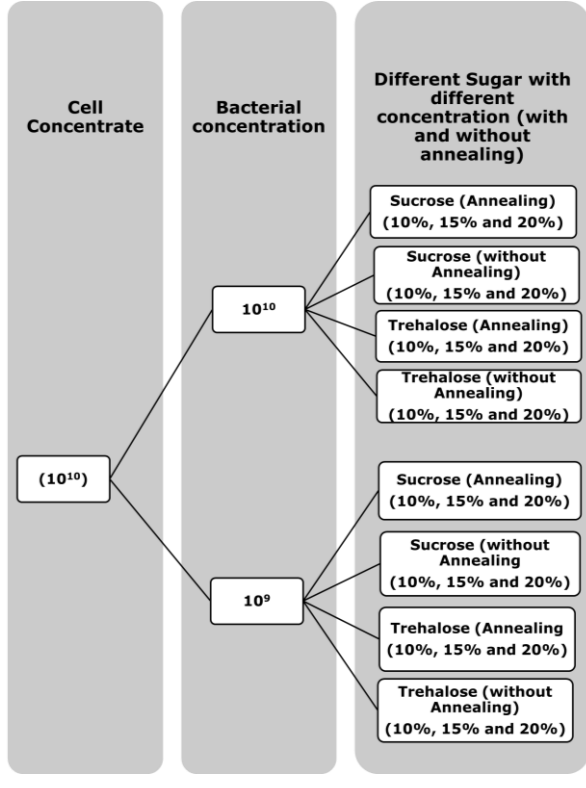


Figure 4. Formulation of different bacterial concentrations in lyoprotectants with different concentrations. The bacterial suspensions were lyophilized with and without annealing. In total 24 variants were produced.

3.1.3 Biological Characterization

To ensure the functionality of freeze-dried probiotics/LBP's, certain criteria need to be fulfilled. It is important that the bacteria (i) are alive and have the expected concentration, (ii) have a high metabolic activity, and (iii) have a sufficient long-term stability. Therefore, the effects of the different production parameters on biological activities of freeze-dried R2LC was analysed using the different methods described below.

Bacterial viability

Monitoring of the bacterial survival after freeze-drying was done by plate count analysis of samples taken before and after the drying. The samples were plated on MRS agar plates after serial dilution (as per different bacterial concentration).

Cell vitality

The vitality of the bacterial cells was measured by a pH drop method. During cultivation R2LC produces lactic acid which reduce the pH of the media. Metabolically active bacterial cells (having high vitality) therefore give a larger drop in pH than cells with low vitality. After reconstitution of freeze-dried bacteria in growth media, pH drop was measured at 3 timepoints 0, 1, and 2 hours and delta pH were calculated.

Accelerated storage stability

The stability of the freeze-dried product is related to the storage temperature (Meng et al., 2008). A probiotics/LBP is normally stored at 4-25°C, but an accelerated stability study was performed at 37°C. We investigated the viability, vitality, and water content of freeze-dried *L. reuteri* R2LC before and after storage at 37°C for 2 and 4 weeks.

3.1.4 Physiochemical characterisation

The freeze-dried samples were visually analysed, and the appearance of the cakes was scored according to the following scale: Score 1) intact and homogenous cake; 2) intact but non-homogenous cake, colour change of the bottom part of the cake; 3) shrinkage of the cake around the edges; 4) partially collapsed cake (20-40%), and 5) collapsed cake. (Figure 5).

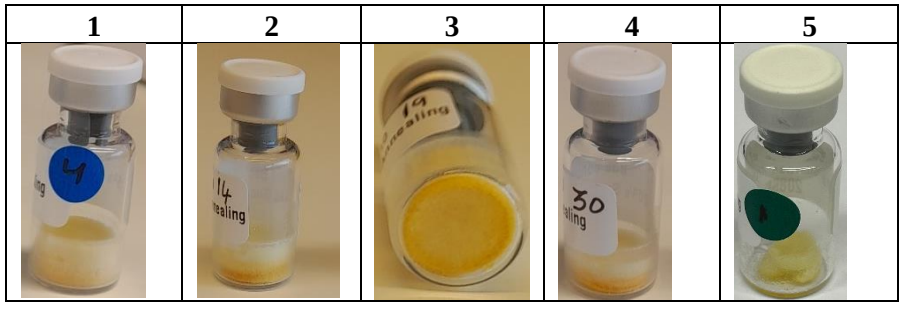


Figure 5. Cake appearance and their corresponding scoring numbers

Glass transition temperature (Tg) of all *L. reuteri* formulations were measured by differential scanning calorimetry (DSC) as described in **paper I.** Apart from DSC, we also studied the distribution of freeze-dried bacteria in the lyophilized cake and porosity of different formulations, after investigating the material with scanning electron microscopy (SEM; the same samples that were analysed by DSC). Sections from top and bottom were visualized at 100 x, 500 x, 1000 x and 2500 x magnifications. Porosity of the cake was measured using the Image J software according to the procedure presented by Saraf et al. (Saraf et al., 2019).

Bacterial aggregation in the lyophilized samples was measured using flow cytometry, where clumps larger than >6 µm were defined as aggregates. Prior to analysis samples were diluted 1:100 and 1:200 with saline solution. 100,000 events were recorded for all formulations. FlowJo software was used for calculating the aggregation (%). The aggregation (%) was calculated as (no. of events counted in bead region x no. of events in bacterial region)/100.

Water content of all formulations was analysed by using a Karl Fischer coulometric method. All samples were reconstituted in dry methanol and incubated at room temperature for 1 hour to extract all water. Supernatants was analysed by Metrohm 831 Karl Fischer Coulometry to determine water content.

3.1.5 Statistical analysis

DOE experimental setup was designed by use of MODDE 13, Umetrics. Statistical analyses were performed by using JASP 0.17.2.1 software.

3.2 Evaluation of ILP100 in a first-in-human phase-1 trial on wound healing (Paper II)

Paper II presents an adaptive, randomized, double-blind, placebo-controlled first-in-human study designed to evaluate safety, tolerability, clinical and biologic effects on wound healing of single and multiple ascending doses of ILP100-Topical (*L. reuteri* expressing CXCL12 administered topically to experimentally induced skin wounds in healthy subjects). The study comprises of a treatment and assessment phase up to 6 weeks after last treatment and a 5-year long-term follow-up.

In the study, 240 wounds were induced in 36 healthy volunteers. Single (SAD) and multiple ascending dose (MAD) of ILP100-Topical in 3 sequential cohorts (Figure 6). The dose levels of ILP100-Topical in SAD part were 5×10^4, 5×10^7, and 1×10^9 CFU/cm^2 and in MAD part 5×10^5, 5×10^7, and 1×10^9 CFU/cm^2 in wound area for cohort 1, 2, and 3 respectively. In the SAD (2 wounds/arm) part, 4 subjects were assigned to each cohort; while for the MAD (4 wounds/arm) part, 8 subjects were assigned to each cohort.

3.2.1 Microcirculation/blood perfusion in wound

Microcirculation/blood perfusion around wound edges was measured using PeriCam PSI NR system from perimed, which is based on laser speckle contrast analysis (LASCA) imaging techniques that assess blood perfusion in real time. Region of interest (ROI) was defined as areas within a perfusion image where the analysis was done. The following three different ROIs were made a) the wound area, b) the area 5 mm, and c) 10 mm outside the wound area (Figure 7). The PIMSoft software was used to assess the blood perfusion images of the wound and the surrounding. The detailed procedure is described in paper II (Öhnstedt et al., 2023).

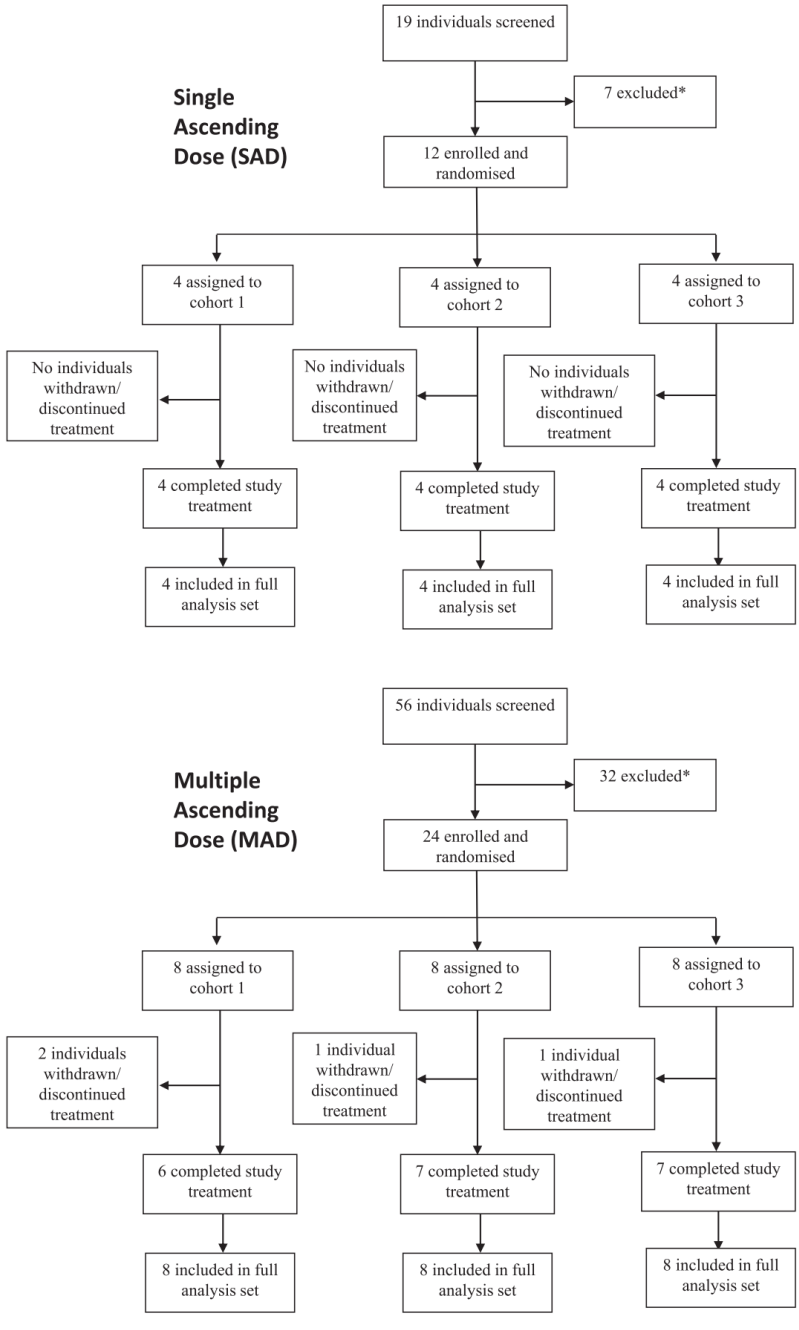


Figure 6. Trial profile for SAD and MAD. *Excluded = ineligible, reserves, or other (Öhnstedt et al., 2023).

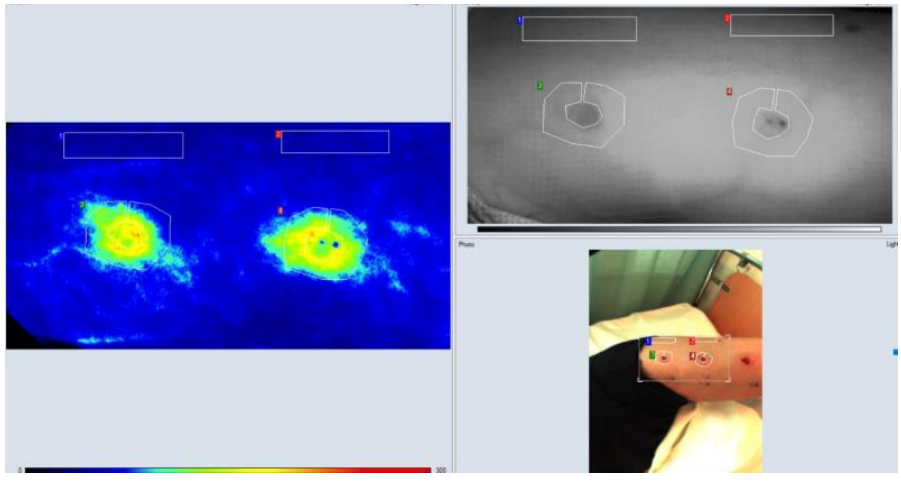


Figure 7. Blood perfusion analysis. Blood perfusion of wound edges was measured using LASCA (Laser Speckle Contrast Analysis) at MAD visits and analysed off-site as the difference in blood perfusion between the wound edge (drawn areas surrounding wounds marked green and dark red) and reference areas (top boxes, blue and red region) in each individual.

4. Results and discussion

4.1 Paper I

In paper I, impact of different factors (type of lyoprotectant, lyoprotectant concentration, bacterial concentration and annealing) on biological and physicochemical characteristics of freeze-dried R2LC was investigated. Previous studies have mostly focused on investigating the impact of different factors (such as type of lyoprotectant/combination of lyoprotectants and concentration of lyoprotectant, and freeze-drying process) on biological activity or on physicochemical properties of freeze-dried probiotics (Bodzen et al., 2021b; Oluwatosin et al., 2022; Saarela et al., 2005; Savedboworn et al., 2019; Wessman et al., 2011), but none of them include a broad characterization of biological activity.

Sucrose and trehalose are widely used lyoprotectants, and previous studies have demonstrated that trehalose generally give a better protection than sucrose (Celik & O'Sullivan, 2013; Crowe et al., 1996; Lestari et al., 2018; Onwe et al., 2022). We observed positive effects of sucrose over trehalose but could also show that the type of lyoprotectant along with concentration of lyoprotectant and bacterial concentration affected the biological outcomes. The medium (15%) concentration of sucrose at high (10^{10}) bacterial concentration gave the best freeze-drying survival (Figure 8), vitality (Figure 8), and storage stability (Figure 9). In addition, we also observed that aggregation was higher when using high (10^{10} CFU/mL) bacterial concentration and a positive correlation between aggregation and vitality was also seen. (Figure 10; Table 3).

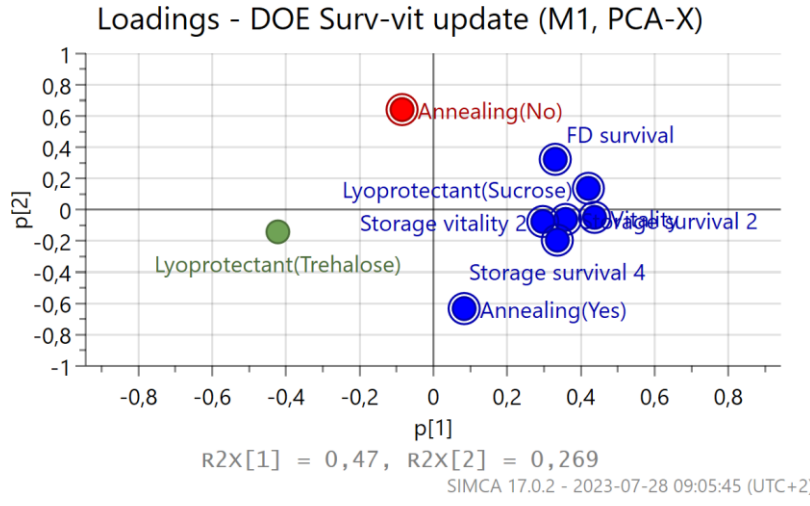


Figure 8. PCA plot to describe the overall effect of factors on performance of freeze-dried R2LC. BC: Bacterial concentration; LC: Lyoprotectant concentration, LT: type of lyoprotectant, and Ann: Annealing. Blue: shows statistically significantly correlated factors while text in red and green color shows not statistically significant factors

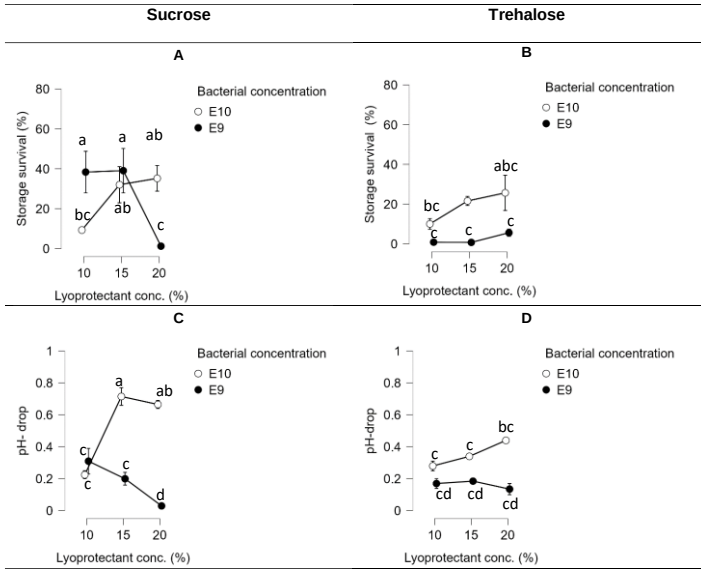


Figure 9. Evaluation of accelerated stability after 2 weeks. Effects of different factors and their interactions on survival (A, B) and vitality (C, D) of freeze-dried R2LC. Datasets with different letters are significantly different (p<0.01; data presented in A & B are compared and C & D are compared)

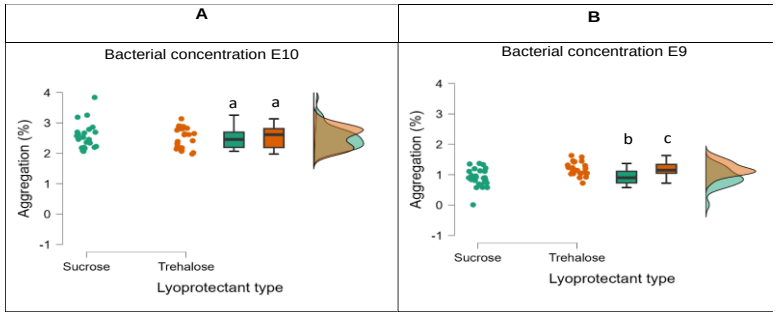


Figure 10. Effect of low (A) and high (B) bacterial concentration and type of lyoprotectant (sucrose and trehalose) on aggregation of freeze-dried R2LC. Datasets with different letters are significantly different (p<0.01; data presented in A and B are compared)

Table 3. Pearson's correlation between biological and physicochemical outcomes.

Pearson's Correlations

		Pearson's r	*p*
vitality	- FD survival	0.463	< **.001**
vitality	- Aggregation	0.503	< **.001**
vitality	- Porosity	0.282	0.181
vitality	- Water content	0.063	0.770
vitality	- Glass transition temperature	-0.551	**0.005**
vitality	- Cake appearance	-0.118	0.426
FD survival	- Aggregation	0.056	0.707
FD survival	- Porosity	0.352	0.091
FD survival	- Water content	0.203	0.341
FD survival	- Glass transition temperature	-0.602	**0.002**
FD survival	- Cake appearance	0.045	0.759
Aggregation	- Porosity	0.233	0.273
Aggregation	- Water content	-0.432	**0.035**
Aggregation	- Glass transition temperature	0.254	0.232
Aggregation	- Cake appearance	-0.306	**0.035**
Porosity	- Water content	-0.214	0.316
Porosity	- Glass transition temperature	-0.142	0.508
Porosity	- Cake appearance	-0.088	0.682
Water content	- Glass transition temperature	-0.537	**0.007**
Water content	- Cake appearance	0.613	**0.001**
Glass transition temperature	- Cake appearance	-0.124	0.564

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.2 Paper II

ILP100-Topical is a genetically modified *L. reuteri* R2LC expressing human chemokine CXCL12-1a (designated ILP100-Topical) that has been designed to accelerate wound healing (Vagesjo et al., 2018). The chemokine CXCL12 binds CXCR4 expressed by immune cells and keratinocytes. Macrophages and neutrophils are major immune cells at the wound site, where they are important for controlling invading microorganisms and for facilitating the healing process by secreting additional chemokines, growth factors, and matrix digesting enzymes. During this process, macrophages shift phenotype and become anti-inflammatory, and subsequently promote closing of the wound. This is induced by macrophage phagocytosis of cell debris and by microenvironmental signals such as CXCL12. It has also been shown that on-site delivery of ILP100-Topical reduces the pH of the wound environment which inactivates the protease CD26 and helps in increasing the bioavailability of CXCL12. The chemokine expressing *L. reuteri* R2LC have previously been shown to accelerate wound healing in healthy mice, ischemic, and hyperglycaemic murine models (Vagesjo et al., 2018).

The aim of this study was to determine the safety and tolerability as well as clinical and biologic effects of ILP100-Topical after topical single and multiple dose administration to experimentally induced skin wounds in healthy subjects. Results showed neither adverse events (AEs) nor colonization of the genetically modified *L. reuteri* R2LC in blood and faeces samples of the patients. Also, no increase of concentration of circulating CXCL12 was observed in individuals treated with single or multi-dose of ILP100-Topical. Furthermore, there was no increase in wound rupture in connection to treatment with ILP100-Topical, while two cases of wound scars were reported in the placebo group. All dataset, single as well as multi-dosage were considered safe and well-tolerated over 3 weeks of administered ILP100. The microcirculation was measured in the wounds and the skin surrounding the wound areas. A wound was defined as healed when the wound area was completely re-epithelialized. In multi-dosing ILP100, significant difference in treatment-related wound healing in cohort 1 at day 32 and day 19 and 22 (Day 32, p=0.058) was observed. Pooled analyses of all cohorts showed that compared to control ILP100 significantly improved wound healing (p=0.020) as shown in Figure 6 and Table 4.

Recently, the focus to accelerate wound healing by changing the wound environment by topical application of growth factors or cell therapies have been increased (Kosaric et al., 2019; Mahdipour & Sahebkar, 2020). In the previous preclinical study by Vågesjö et al. (Vagesjo et al., 2018) it was demonstrated that the blood flow in hyperglycaemic mice was normalized in wounds treated with ILP100. In another study, genetically modified *L. lactis* expressing FGF2, CSF1, and IL-4 resulted in accelerated wound healing in a mouse model (Kurkipuro et al., 2022).

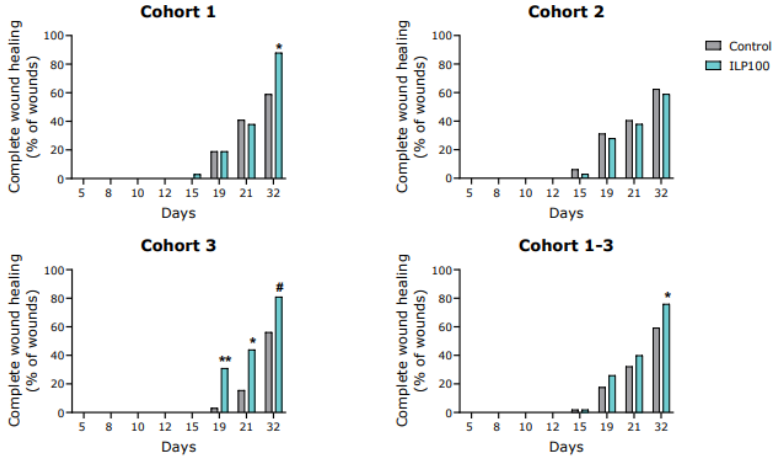


Figure 11. Proportion of wounds healed for control and wounds treated with ILP100-topical (MAD) during different visit. Wounds assessed as healed (yes/no) by three blinded IEs. p (#) =0.058, p (*) ≤0.05, and p (**) ≤0.001. A) p-values were calculated by Fisher's exact test

Table 4. Wound edge blood perfusion analysed using non-invasive imaging Laser Speckle Contrast Analysis (LASCA) at Days 2, 8 and 15 in control and ILP100-Topical treated groups (MAD)

LASCA perfusion imaging	n, m	Day 2		Day 8		Day 15	
		Control	ILP100	Control	ILP100	Control	ILP100
Cohort 1	7–8, 26–32	43.8 ± 5.5	53.2 ± 5.6	34.8 ± 12.9	46.3 ± 12.1	30.4 ± 4.8	46.5 ± 10.4
Cohort 2	6–8, 24–32	56.1 ± 5.5	76.6 ± 6.0**	31.6 ± 3.1	33.6 ± 3.0	10.9 ± 2.0	13.3 ± 1.8
Cohort 3	7–8, 28–32	37.1 ± 3.4	89.3 ± 5.4****	32.2 ± 4.8	34.8 ± 4.4	5.4 ± 2.9	8.7 ± 2.7
Cohort 1-3	21–24, 84–96	45.7 ± 2.9	74.1 ± 3.6****	32.7 ± 4.3	38.2 ± 4.3	17.1 ± 2.5	24.4 ± 4.5

The MAD cohorts treated with 5×10^5 CFU/cm^2 wound area (cohort 1), 5×10^7 CFU/cm^2 (cohort 2) and 1×10^9 CFU/cm^2 (cohort 3), respectively, were assessed for wound edge perfusion at visits Day 2, 8 and 15. Values are delta perfusion units (dPFU) and represent perfusion of the wound edge (skin area within 5 mm from the wound border) minus the reference non-wounded skin perfusion at the same image presented as Mean ± SEM. Controls represent saline–or placebo-treated wounds. n, number of individuals; m, number of wound frames analysed per group. Mann–Whitney test **p = 0.010, ****≤0.0001.

***Table 2*: Wound edge blood perfusion analysed using non-invasive imaging Laser Speckle Contrast Analysis (LASCA) at Days 2, 8, and 15 in control- and ILP100-Topical-treated groups (MAD).**

5. Conclusions

Paper I: We have observed that the factors: type of lyoprotectant, lyoprotectant concentration, bacterial concentration and annealing affect the properties and performance of freeze-dried R2LC. Sucrose as a lyoprotectant gave better freeze-drying survival, vitality, and storage stability of R2LC than trehalose as a lyoprotectant. Overall, sucrose at 15% with an annealing step showed the best results in the analyses of freeze-drying survival, vitality, and storage stability of R2LC. The high concentration of sucrose (20%) at low bacterial concentration (10^9 CFU/mL) resulted in elevated water content and resulted in partial and collapsed cake formation. Finally, the high (10^{10} CFU/mL) R2LC concentration resulted in the best vitality but also resulted in more aggregates.

Paper II: *L. reuteri* R2LC expressing CXCL12 (ILP100-Topical) has previously shown promising effects in accelerating wound healing by onsite delivery of CXCL12, enhancing the activity of macrophages. In a human clinical phase-1 trial **(paper II)** we showed that ILP100-Topical is safe, well tolerable, and have an effective biologic effect in accelerating wound healing. Time to first healing was shortened by 6 days on average, and by 10 days in highest dose.

6. Future perspective

- Study I - we have investigated the impact of different process parameters on the properties of freeze-dried *L. reuteri* R2LC. A future aim could be to investigate the biological properties of freeze-dried LBP in different model such as an animal model or an *in vitro* artificial small intestine (SHIME). This system provides the possibility to perform a realistic assessment of probiotic/LBP properties in an environment with extensive similarities to the gastrointestinal tract. SHIME could be operated to simulate different intestinal environments such as adult, infants, elderly, and specific conditions (e.g., pathogen infection).
- Study II - The first-in-human study have demonstrated the product to be safe and effective in accelerating wound healing. There are one ongoing phase 2a trial investigating the ILP100-Topical in diabetic patients with diabetes and non-healing wounds and an IND cleared for a pivotal trial evaluating the ILP100-Topical in post-surgical wounds in prediabetic, diabetic and obese patients.

References

Surname, First name, initial. (Year of Publication). *Title*. Diss. University. Place of publication: Publisher. Constant link.

Article author (Year). Title. *Title of the journal*. Volume (Number), Page Number/Article Number. Constant link.

Abuqwider, J., Altamimi, M., & Mauriello, G. (2022). Limosilactobacillus reuteri in Health and Disease. *Microorganisms*, *10*(3). https://doi.org/10.3390/microorganisms10030522

Federal food, drug and cosmetic act (FD&C act), (2018). https://www.fda.gov/regulatory-information/laws-enforced-fda/federal-food-drug-and-cosmetic-act-fdc-act

Administration, F. a. D. (2022). *FDA 101: Dietary supplements* https://www.fda.gov/consumers/consumer-updates/fda-101-dietary-supplements

Administration, S. F. a. D. (2007). *The guideline for registration of functional food*. Retrieved from http://sfda.com/

Ahl, D., Liu, H., Schreiber, O., Roos, S., Phillipson, M., & Holm, L. (2016). Lactobacillus reuteri increases mucus thickness and ameliorates dextran sulphate sodium-induced colitis in mice. *Acta Physiol (Oxf)*, *217*(4), 300-310. https://doi.org/10.1111/apha.12695

Allegretti, J., Kelly, C., Fischer, M., Cohen, T., Budree, S., & Khanna, S. (2022). Tu1519: CP101, AN INVESTIGATIONAL MICROBIOME THERAPEUTIC FOR THE PREVENTION OF RECURRENT C. DIFFICILE INFECTION: A COMBINED ANALYSIS OF THE PRISM3 (RANDOMIZED PLACEBO-CONTROLLED) AND PRISM-EXT (OPEN-LABEL) TRIALS. *Gastroenterology*, *162*, S-995. https://doi.org/10.1016/S0016-5085(22)62357-6

Amagase, H. (2008). Current marketplace for probiotics: a Japanese perspective. *Clinical Infectious Diseases*, *46 Suppl 2*, S73-75; discussion S144-151. https://doi.org/10.1086/523338

Amagase, H. (2008). Current Marketplace for Probiotics: A Japanese Perspective. *Clinical Infectious Diseases*, *46*(Supplement_2), S73-S75. https://doi.org/10.1086/523338

Analytic, I. (2022). *Live Biotherapeutic Market worth $ 2.60 billion by 2030* https://www.prnewswire.com/news-releases/live-biotherapeutic-product-lbp-market-worth--2-60-billion-by-2030---exclusive-report-by-insightace-analytic-301582129.html

Arora, M., & Baldi, A. (2015). Regulatory categories of probiotics across the globe: A review representing existing and recommended categorization. *Indian*

Journal of Medical Microbiology, *33*, S2-S10. https://doi.org/https://doi.org/10.4103/0255-0857.150868

Arora, M., Sharma, S., & Baldi, A. (2013). Comparative Insight of Regulatory Guidelines for Probiotics in USA, India and Malaysia: A Critical Review. *International Journal of Biotechnology for Wellness Industries*, *2*, 51-64.

Badal Tejedor, M., Fransson, J., & Millqvist-Fureby, A. (2020). Freeze-dried cake structural and physical heterogeneity in relation to freeze-drying cycle parameters. *Int J Pharm*, *590*, 119891. https://doi.org/10.1016/j.ijpharm.2020.119891

Bodzen, A., Jossier, A., Dupont, S., Mousset, P. Y., Beney, L., Lafay, S., & Gervais, P. (2021a). Design of a new lyoprotectant increasing freeze-dried Lactobacillus strain survival to long-term storage. *BMC Biotechnol*, *21*(1), 66. https://doi.org/10.1186/s12896-021-00726-2

Bodzen, A., Jossier, A., Dupont, S., Mousset, P. Y., Beney, L., Lafay, S., & Gervais, P. (2021b). Increased Survival of Lactococcus lactis Strains Subjected to Freeze-Drying after Cultivation in an Acid Medium: Involvement of Membrane Fluidity Cultivation in Acid Medium to Improve Bacterial Survival of Freeze-Drying. *Food Technol Biotechnol*, *59*(4), 443-453. https://doi.org/10.17113/ftb.59.04.21.7076

Bogdanova, E., Fureby, A. M., & Kocherbitov, V. (2022). Influence of Cooling Rate on Ice Crystallization and Melting in Sucrose-Water System. *J Pharm Sci*, *111*(7), 2030-2037. https://doi.org/10.1016/j.xphs.2022.01.027

Caluwaerts, S., Vandenbroucke, K., Steidler, L., Neirynck, S., Vanhoenacker, P., Corveleyn, S., Watkins, B., Sonis, S., Coulie, B., & Rottiers, P. (2010). AG013, a mouth rinse formulation of Lactococcus lactis secreting human Trefoil Factor 1, provides a safe and efficacious therapeutic tool for treating oral mucositis. *Oral Oncol*, *46*(7), 564-570. https://doi.org/10.1016/j.oraloncology.2010.04.008

Canada, G. o. (2022). *Natural and Non-prescription Health Products Directorate*. Retrieved from https://www.canada.ca/en/health-canada/corporate/about-health-canada/branches-agencies/health-products-food-branch/natural-non-prescription-health-products-directorate.html

Castanheira, L. G., Barbano, D. B. A., & Rech, N. (2011). Current development in regulation of similar biotherapeutic products in Brazil. *Biologicals*, *39*(5), 308-311. https://doi.org/https://doi.org/10.1016/j.biologicals.2011.06.021

Celik, O. F., & O'Sullivan, D. J. (2013). Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria. *Journal of Dairy Science*, *96*(6), 3506-3516. https://doi.org/https://doi.org/10.3168/jds.2012-6327

Chieffi, D., Fanelli, F., & Fusco, V. (2022). Chapter 2 - Legislation of probiotic foods and supplements. In E. Leite de Souza, J. L. de Brito Alves, & V. Fusco (Eds.), *Probiotics for Human Nutrition in Health and Disease* (pp. 25-44). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-323-89908-6.00013-3

Cordaillat-Simmons, M., Rouanet, A., & Pot, B. (2020). Live biotherapeutic products: the importance of a defined regulatory framework. *Exp Mol Med*, *52*(9), 1397-1406. https://doi.org/10.1038/s12276-020-0437-6

Crowe, L. M., Reid, D. S., & Crowe, J. H. (1996). Is trehalose special for preserving dry biomaterials? *Biophys J*, *71*(4), 2087-2093. https://doi.org/10.1016/S0006-3495(96)79407-9

Degnan, F. H. (2008). The US Food and Drug Administration and probiotics: regulatory categorization. *Clinical Infectious Diseases*, *46 Suppl 2*, S133-136; discussion S144-151. https://doi.org/10.1086/523324

Degnan, F. H. (2012). Clinical studies involving probiotics: when FDA's investigational new drug rubric applies-and when it may not. *Gut Microbes*, *3*(6), 485-489. https://doi.org/10.4161/gmic.22158

Dreher-Lesnick, S. M., Stibitz, S., & Carlson, P. E. (2017). U.S. Regulatory Considerations for Development of Live Biotherapeutic Products as Drugs. *Microbiology Spectrum*, *5*(5). https://doi.org/ARTN BAD-0017-2017
10.1128/microbiolspec.BAD-0017-2017

Dsouza, M., Menon, R., Crossette, E., Bhattarai, S. K., Schneider, J., Kim, Y. G., Reddy, S., Caballero, S., Felix, C., Cornacchione, L., Hendrickson, J., Watson, A. R., Minot, S. S., Greenfield, N., Schopf, L., Szabady, R., Patarroyo, J., Smith, W., Harrison, P., . . . Norman, J. M. (2022). Colonization of the live biotherapeutic product VE303 and modulation of the microbiota and metabolites in healthy volunteers. *Cell Host Microbe*, *30*(4), 583-598.e588. https://doi.org/10.1016/j.chom.2022.03.016

Ekdawi-Sever, N., Goentoro, L. A., & de Pablo, J. J. (2003). Effects of annealing on freeze-dried Lactobacillus acidophilus. *Journal of Food Science*, *68*(8), 2504-2511. https://doi.org/DOI 10.1111/j.1365-2621.2003.tb07052.x

Eriksson, L., Johansson, E., Kettaneh-Wold, N., WikstrÄom, C., & Wold, S. (2008). *Design of Experiments: Principles and Applications*.

Fabia, R., Ar'Rajab, A., Johansson, M. L., Willen, R., Andersson, R., Molin, G., & Bengmark, S. (1993). The effect of exogenous administration of Lactobacillus reuteri R2LC and oat fiber on acetic acid-induced colitis in the rat. *Scand J Gastroenterol*, *28*(2), 155-162. https://doi.org/10.3109/00365529309096063

Importing Biologics and CBER Regulated Products, (2021). https://www.fda.gov/industry/importing-fda-regulated-products/importing-biologics-and-cber-regulated-products#regulate

Fenster, K., Freeburg, B., Hollard, C., Wong, C., Ronhave Laursen, R., & Ouwehand, A. C. (2019). The Production and Delivery of Probiotics: A Review of a Practical Approach. *Microorganisms*, *7*(3). https://doi.org/10.3390/microorganisms7030083

Fonseca, F., Cenard, S., & Passot, S. (2015). Freeze-drying of lactic acid bacteria. *Methods Mol Biol*, *1257*, 477-488. https://doi.org/10.1007/978-1-4939-2193-5_24

Food Standards Australia New Zealand: Health and related claims, (2021). https://www.foodstandards.gov.au/Pages/default.aspx

Haahr, T., Freiesleben, N. C., Pinborg, A., Nielsen, H. S., Hartvig, V., Mikkelsen, A. L., Parks, T., Uldbjerg, N., Jensen, J. S., & Humaidan, P. (2020). Effect of clindamycin and a live biotherapeutic on the reproductive outcomes of IVF patients with abnormal vaginal microbiota: protocol for a double-blind, placebo-controlled multicentre trial. *BMJ Open*, *10*(10), e035866. https://doi.org/10.1136/bmjopen-2019-035866

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol*, *11*(8), 506-514. https://doi.org/10.1038/nrgastro.2014.66

Holma, R., Salmenpera, P., Lohi, J., Vapaatalo, H., & Korpela, R. (2001). Effects of Lactobacillus rhamnosus GG and Lactobacillus reuteri R2LC on acetic acid-induced colitis in rats. *Scand J Gastroenterol*, *36*(6), 630-635. https://doi.org/10.1080/003655201750163114

Hou, K., Wu, Z.-X., Chen, X.-Y., Wang, J.-Q., Zhang, D., Xiao, C., Zhu, D., Koya, J. B., Wei, L., Li, J., & Chen, Z.-S. (2022). Microbiota in health and diseases. *Signal Transduction and Targeted Therapy*, *7*(1), 135. https://doi.org/10.1038/s41392-022-00974-4

Jiang, J. Y., Li, K., Xiao, Y., Zhong, A. A., Tang, J. J., Duan, Y. F., & Li, Z. J. (2023). Limosilactobacillus reuteri Regulating Intestinal Function: A Review. *Fermentation-Basel*, *9*(1). https://doi.org/ARTN 19
10.3390/fermentation9010019

Kandler, O., Stetter, K. O., & Kohl, R. (1980). Lactobacillus-Reuteri Sp-Nov, a New Species of Heterofermentative Lactobacilli. *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene I Abteilung Originale C-Allgemeine Angewandte Und Okologische Mikrobiologie*, *1*(3), 264-269. https://doi.org/Doi 10.1016/S0172-5564(80)80007-8

Karimi, S., Jonsson, H., Lundh, T., & Roos, S. (2018). Lactobacillus reuteri strains protect epithelial barrier integrity of IPEC-J2 monolayers from the detrimental effect of enterotoxigenic Escherichia coli. *Physiol Rep*, *6*(2). https://doi.org/10.14814/phy2.13514

Kosaric, N., Kiwanuka, H., & Gurtner, G. C. (2019). Stem cell therapies for wound healing. *Expert Opin Biol Ther*, *19*(6), 575-585. https://doi.org/10.1080/14712598.2019.1596257

Kumar, A. (2021). The Indications for Probiotics in Australia and Their Regulation. *Journal of Alternative, Complementary & Integrative Medicine*, *7*, 1-2. https://doi.org/10.24966/ACIM-7562/100206

Kurkipuro, J., Mierau, I., Wirth, T., Samaranayake, H., Smith, W., Kärkkäinen, H. R., Tikkanen, M., & Yrjänheikki, J. (2022). Four in one-Combination therapy using live Lactococcus lactis expressing three therapeutic proteins

for the treatment of chronic non-healing wounds. *PLoS One*, *17*(2), e0264775. https://doi.org/10.1371/journal.pone.0264775

Lamouse-Smith, E., Kelly, D., & De Cremoux, I. (2021). Designing bugs as drugs: exploiting the gut microbiome. *Am J Physiol Gastrointest Liver Physiol*, *320*(3), G295-G303. https://doi.org/10.1152/ajpgi.00381.2019

Lau, T.-C. (2019). Chapter 34 - Regulations, opportunities, and key trends of functional foods in Malaysia. In D. Bagchi (Ed.), *Nutraceutical and Functional Food Regulations in the United States and around the World (Third Edition)* (pp. 561-573). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-816467-9.00034-4

Lestari, S. W., Ilato, K. F., Pratama, M. I. A., Fitriyah, N. N., Pangestu, M., Pratama, G., & Margiana, R. (2018). Sucrose 'Versus' Trehalose Cryoprotectant Modification in Oocyte Vitrification : A Study of Embryo Development. *Biomedical and Pharmacology Journal*, *11*(1), 97-104. https://doi.org/10.13005/bpj/1351

Library, T. F. (2014). *Nutraceuticals in Latin America: Brazil and Beyond Latin America: nutraceutical boom or bust?* https://www.thefreelibrary.com/Latin+America%3a+nutraceutical+boom+or+bust%3f-a0189971350

Lim, D., & Song, M. (2019). Development of bacteria as diagnostics and therapeutics by genetic engineering. *J Microbiol*, *57*(8), 637-643. https://doi.org/10.1007/s12275-019-9105-8

Limaye, S. A., Haddad, R. I., Cilli, F., Sonis, S. T., Colevas, A. D., Brennan, M. T., Hu, K. S., & Murphy, B. A. (2013). Phase 1b, multicenter, single blinded, placebo-controlled, sequential dose escalation study to assess the safety and tolerability of topically applied AG013 in subjects with locally advanced head and neck cancer receiving induction chemotherapy. *Cancer*, *119*(24), 4268-4276. https://doi.org/10.1002/cncr.28365

Liu, H. Y., Giraud, A., Seignez, C., Ahl, D., Guo, F., Sedin, J., Walden, T., Oh, J. H., van Pijkeren, J. P., Holm, L., Roos, S., Bertilsson, S., & Phillipson, M. (2021). Distinct B cell subsets in Peyer's patches convey probiotic effects by Limosilactobacillus reuteri. *Microbiome*, *9*(1), 198. https://doi.org/10.1186/s40168-021-01128-4

Louie, T., Golan, Y., Khanna, S., Bobilev, D., Erpelding, N., Fratazzi, C., Carini, M., Menon, R., Ruisi, M., Norman, J. M., Faith, J. J., Olle, B., Li, M., Silber, J. L., & Pardi, D. S. (2023). VE303, a Defined Bacterial Consortium, for Prevention of Recurrent Clostridioides difficile Infection: A Randomized Clinical Trial. *JAMA*, *329*(16), 1356-1366. https://doi.org/10.1001/jama.2023.4314

Mahdipour, E., & Sahebkar, A. (2020). The Role of Recombinant Proteins and Growth Factors in the Management of Diabetic Foot Ulcers: A Systematic Review of Randomized Controlled Trials. *J Diabetes Res*, *2020*, 6320514. https://doi.org/10.1155/2020/6320514

Martinez, I., Stegen, J. C., Maldonado-Gomez, M. X., Eren, A. M., Siba, P. M., Greenhill, A. R., & Walter, J. (2015). The gut microbiota of rural papua new guineans: composition, diversity patterns, and ecological processes. *Cell Rep*, *11*(4), 527-538. https://doi.org/10.1016/j.celrep.2015.03.049

Mazier, W., Le Corf, K., Martinez, C., Tudela, H., Kissi, D., Kropp, C., Coubard, C., Soto, M., Elustondo, F., Rawadi, G., & Claus, S. P. (2021). A New Strain of Christensenella minuta as a Potential Biotherapy for Obesity and Associated Metabolic Diseases. *Cells*, *10*(4), 823. https://www.mdpi.com/2073-4409/10/4/823

Meng, J., Liu, S., & Wu, X. (2023). Engineered probiotics as live biotherapeutics for diagnosis and treatment of human diseases. *Critical reviews in microbiology*, 1-15. https://doi.org/10.1080/1040841X.2023.2190392

Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., & Ross, R. P. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry*, *106*(4), 1406-1416. https://doi.org/https://doi.org/10.1016/j.foodchem.2007.04.076

Merivaara, A., Zini, J., Koivunotko, E., Valkonen, S., Korhonen, O., Fernandes, F. M., & Yliperttula, M. (2021). Preservation of biomaterials and cells by freeze-drying: Change of paradigm. *J Control Release*, *336*, 480-498. https://doi.org/10.1016/j.jconrel.2021.06.042

MHLW. (2007). *Ministry of Health, Labour and Welfare: Food for Specified Health Uses (FOSHU)*. Retrieved from https://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html

Montel Mendoza, G., Pasteris, S. E., Otero, M. C., & Fatima Nader-Macias, M. E. (2014). Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. *J Appl Microbiol*, *116*(1), 157-166. https://doi.org/10.1111/jam.12359

Mu, Q., Tavella, V. J., & Luo, X. M. (2018). Role of Lactobacillus reuteri in Human Health and Diseases. *Front Microbiol*, *9*, 757. https://doi.org/10.3389/fmicb.2018.00757

O'Toole, P. W., Marchesi, J. R., & Hill, C. (2017). Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nat Microbiol*, *2*, 17057. https://doi.org/10.1038/nmicrobiol.2017.57

Öhnstedt, E., Vågesjö, E., Fasth, A., Lofton Tomenius, H., Dahg, P., Jönsson, S., Tyagi, N., Åström, M., Myktybekova, Z., Ringstad, L., Jorvid, M., Frank, P., Hedén, P., Roos, S., & Phillipson, M. (2023). Engineered bacteria to accelerate wound healing: an adaptive, randomised, double-blind, placebo-controlled, first-in-human phase 1 trial. *EClinicalMedicine*, *60*, 102014. https://doi.org/10.1016/j.eclinm.2023.102014

Oka, A., Mishima, Y., Bongers, G., Baltus, A., Liu, B., Herzog, J., Ohkusa, T., Atarashi, K., Honda, K., San Mateo, L., Plevy, S., & Sartor, R. B. (2020). P074 HUMAN-DERIVED CLOSTRIDIUM VE202 STRAINS REDUCE ENTEROBACTERIACEAE AND FUSOBACTERIA AND REVERSE EXPERIMENTAL COLITIS INDUCED BY HUMAN GUT

MICROBIOTA. *Inflammatory Bowel Diseases*, *26*(Supplement_1), S36-S37. https://doi.org/10.1093/ibd/zaa010.094

Oluwatosin, S. O., Tai, S. L., & Fagan-Endres, M. A. (2022). Sucrose, maltodextrin and inulin efficacy as cryoprotectant, preservative and prebiotic – towards a freeze dried Lactobacillus plantarum topical probiotic. *Biotechnology Reports*, *33*, e00696. https://doi.org/https://doi.org/10.1016/j.btre.2021.e00696

Onwe, R. O., Onwosi, C. O., Ezugworie, F. N., Ekwealor, C. C., & Okonkwo, C. C. (2022). Microbial trehalose boosts the ecological fitness of biocontrol agents, the viability of probiotics during long-term storage and plants tolerance to environmental-driven abiotic stress. *Sci Total Environ*, *806*(Pt 1), 150432. https://doi.org/10.1016/j.scitotenv.2021.150432

Ooijevaar, R. E., Terveer, E. M., Verspaget, H. W., Kuijper, E. J., & Keller, J. J. (2019). Clinical Application and Potential of Fecal Microbiota Transplantation. *Annu Rev Med*, *70*, 335-351. https://doi.org/10.1146/annurev-med-111717-122956

Orenstein, R. (2023). The Role of Microbiome-Based Therapeutics in Clostridioides difficile Infection: Durable, Long-Term Results of RBX2660. *Infect Dis Ther*, *12*(1), 1-7. https://doi.org/10.1007/s40121-022-00714-9

Ozcam, M., Tocmo, R., Oh, J. H., Afrazi, A., Mezrich, J. D., Roos, S., Claesen, J., & van Pijkeren, J. P. (2019). Gut Symbionts Lactobacillus reuteri R2lc and 2010 Encode a Polyketide Synthase Cluster That Activates the Mammalian Aryl Hydrocarbon Receptor. *Appl Environ Microbiol*, *85*(10). https://doi.org/10.1128/AEM.01661-18

Regulation, A. (2007). *ATMP REGULATION (EC) No 1394/2007 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCILof 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004*. Official Journal of the European Union Retrieved from https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:en:PDF

Robinson, M., Vervier, K., Harris, S., Popple, A., Klisko, D., Hudson, R., Bakdash, G., Castan, L., Villemin, C., Adams, D., Milne, D., Booth, C., Parkinson, C., Rabbie, R., Welsh, S., Barker, E., Dalchau, K., Corrie, P., & Lawley, T. (2022). *Abstract P074: MB097: A therapeutic consortium of bacteria clinically-defined by precision microbiome profiling of immune checkpoint inhibitor patients with potent anti-tumor efficacy in vitro and in vivo* (Vol. 10). https://doi.org/10.1158/2326-6074.TUMIMM21-P074

Saarela, M., Virkajarvi, I., Alakomi, H. L., Mattila-Sandholm, T., Vaari, A., Suomalainen, T., & Matto, J. (2005). Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of Bifidobacterium animalis ssp. lactis cells produced without milk-based ingredients. *J Appl*

Microbiol, *99*(6), 1330-1339. https://doi.org/10.1111/j.1365-2672.2005.02742.x

Santivarangkna, C., Higl, B., & Foerst, P. (2008). Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiol*, *25*(3), 429-441. https://doi.org/10.1016/j.fm.2007.12.004

Saraf, S., Singh, A., & Desai, B. G. (2019). Estimation of Porosity and Pore size distribution from Scanning Electron Microscope image data of Shale samples: A case study on Jhuran formation of Kachchh Basin, India. *ASEG Extended Abstracts*, *2019*(1), 1-3. https://doi.org/10.1080/22020586.2019.12073197

Savedboworn, W., Teawsomboonkit, K., Surichay, S., Riansa-Ngawong, W., Rittisak, S., Charoen, R., & Phattayakorn, K. (2019). Impact of protectants on the storage stability of freeze-dried probiotic Lactobacillus plantarum. *Food Sci Biotechnol*, *28*(3), 795-805. https://doi.org/10.1007/s10068-018-0523-x

Saxelin, M. (2008). Probiotic Formulations and Applications, the Current Probiotics Market, and Changes in the Marketplace: A European Perspective. *Clinical Infectious Diseases*, *46*(Supplement_2), S76-S79. https://doi.org/10.1086/523337

Sharma, S., Arora, M., & Baldi, A. (2013). Probiotics in India: Current status and future prospects. *PharmAspire*, *4*, 68.

Stanbury, P. F., Whitaker, A., & Hall, S. J. (2017). Chapter 1 - An introduction to fermentation processes. In P. F. Stanbury, A. Whitaker, & S. J. Hall (Eds.), *Principles of Fermentation Technology (Third Edition)* (pp. 1-20). Butterworth-Heinemann. https://doi.org/https://doi.org/10.1016/B978-0-08-099953-1.00001-6

Tucker, E. C., Haylock-Jacobs, S., Rapaic, M., Dann, L. M., Bryant, R. V., & Costello, S. P. (2023). Stool donor screening within a Therapeutic Goods Administration compliant donor screening program for fecal microbiota transplantation. *JGH Open*, *7*(3), 172-177. https://doi.org/10.1002/jgh3.12874

Tungland, B. (2018). *Human microbiota in health and disease : from pathogenesis to therapy*. Elsevier/Academic Press.

U.S. Department of Health and Human Services, F. a. D. A. G. f. I. (2016). *Early Clinical Trials with Live Biotherapeutic Products: Chemistry, Manufacturing, and Control Information: Guidance for Industry*. Center for Biologics Evaluation and Research Retrieved from https://www.fda.gov/regulatory-information/search-fda-guidance-documents/early-clinical-trials-live-biotherapeutic-products-chemistry-manufacturing-and-control-information

Vagesjo, E., Ohnstedt, E., Mortier, A., Lofton, H., Huss, F., Proost, P., Roos, S., & Phillipson, M. (2018). Accelerated wound healing in mice by on-site production and delivery of CXCL12 by transformed lactic acid bacteria.

Proc Natl Acad Sci U S A, *115*(8), 1895-1900. https://doi.org/10.1073/pnas.1716580115

Valeur, N., Engel, P., Carbajal, N., Connolly, E., & Ladefoged, K. (2004). Colonization and immunomodulation by Lactobacillus reuteri ATCC 55730 in the human gastrointestinal tract. *Appl Environ Microbiol*, *70*(2), 1176-1181. https://doi.org/10.1128/AEM.70.2.1176-1181.2004

Vandenberghe, L., Spier, M., Medeiros, A., Yamaguishi, C., De Dea Lindner, J., Pandey, A., & Thomaz-Soccol, V. (2010). The Potential of Probiotics: A Review. *Food Technology and Biotechnology (imacan@pbf.hr); Vol.48 No.4*, *48*.

Walter, J., Britton, R. A., & Roos, S. (2011). Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm. *Proc Natl Acad Sci U S A*, *108 Suppl 1*(Suppl 1), 4645-4652. https://doi.org/10.1073/pnas.1000099107

Wang, J., Ghosh, D., & Maniruzzaman, M. (2023). Using bugs as drugs: Administration of bacteria-related microbes to fight cancer. *Adv Drug Deliv Rev*, *197*, 114825. https://doi.org/10.1016/j.addr.2023.114825

Wang, X. D., Soltesz, V., Molin, G., & Andersson, R. (1995). The role of oral administration of oatmeal fermented by Lactobacillus reuteri R2LC on bacterial translocation after acute liver failure induced by subtotal liver resection in the rat. *Scand J Gastroenterol*, *30*(2), 180-185. https://doi.org/10.3109/00365529509093259

Wessman, P., Hakansson, S., Leifer, K., & Rubino, S. (2013). Formulations for freeze-drying of bacteria and their influence on cell survival. *J Vis Exp*(78). https://doi.org/10.3791/4058

Wessman, P., Mahlin, D., Akhtar, S., Rubino, S., Leifer, K., Kessler, V., & Hakansson, S. (2011). Impact of matrix properties on the survival of freeze-dried bacteria. *J Sci Food Agric*, *91*(14), 2518-2528. https://doi.org/10.1002/jsfa.4343

WHO/FAO. (2001). Report of a joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. *Food and Nutriton Paper*. https://www.fao.org/3/a0512e/a0512e.pdf

Wu, J. G. a. R. (2019). Labeling for Biological Products. CDER Perscription Drug Labelling Conferance,

Yu, J., Zhao, J., Song, Y., Zhang, J., Yu, Z., Zhang, H., & Sun, Z. (2018). Comparative Genomics of the Herbivore Gut Symbiont Lactobacillus reuteri Reveals Genetic Diversity and Lifestyle Adaptation. *Front Microbiol*, *9*, 1151. https://doi.org/10.3389/fmicb.2018.01151

Zhang, Y., Zhang, H., & Ghosh, D. (2020). The Stabilizing Excipients in Dry State Therapeutic Phage Formulations. *AAPS PharmSciTech*, *21*(4), 133. https://doi.org/10.1208/s12249-020-01673-5

Acknowledgements

This work was carried out at the Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), Research Institutes of Sweden (RISE) and at Ilya Pharma AB. Uppsala, Sweden. The work in this thesis was funded by Ilya Pharma AB, H2020 SME Instrument Phase II under Grant Agreement No 804438, Competence Centre NextBioForm, Vinnova Swedish Governmental Agency for Innovation, Swedish Research Council under grant number 2018-04730 and Knut and Alice Wallenberg Foundation grant to Mia Phillipson.

First of I would like to thank my supervisor **Stefan Roos** for providing me an excellent opportunity to work as a PhD candidate in your group. I am also grateful for your constant support, guidance, and mentoring throughout this journey.

I would also like to extend my thank to my manager **Lovisa Ringstad** for all your guidance and support throughout this work.

I would like to thank my co-supervisors **Hava Lofton Tomenius, Mia Phillipson, Hans Jonsson,** and **Evelina Vågesjö**, for providing me an opportunity to work on this excellent project and their constant support and supervision throughout this research.

I would like to thank my colleagues at Ilya Pharma, **Kerstin**, **Fredrik**, **Sofia**, **Emelie**, and **Margareth**. **Sandeep**, **Yanhong**, and **Zhanar** for helping me during the fermentation and stability studies. Further, I would also like to thank **Daniel** and **Zandra** from RISE for their help with the SEM and DSC analysis of freeze dried R2LC samples. **Elizabeth** for suggestions on statistical analysis of data. **Anton**, and **Ludwig** for scientific and friendly conversations. **Punya** for being a nice friend and officemate. To all the long list of my friends and colleagues, at SLU and Ilya Pharma Uppsala.

I would like to thank my parents for your encouragement, guidance and support throughout the life, my sister **Shikha** and brother **Bhanu** for all your love and support. To my husband **Mohit** thanks for motivating me, being the best friend and a constant support. During this journey I also had a new addition to our family, **Ira**, who despite of making my everyday life busy, provides me a great source of selfless love. Thanks for bringing joy to our life, Ira.

II

Engineered bacteria to accelerate wound healing: an adaptive, randomised, double-blind, placebo-controlled, first-in-human phase 1 trial

Emelie Öhnstedt,[a,b] Evelina Vågesjö,[a,b] Andreas Fasth,[a] Hava Lofton Tomenius,[a] Pia Dahg,[a] Sofia Jönsson,[a] Nisha Tyagi,[a,d] Mikael Åström,[a] Zhanar Myktybekova,[a] Lovisa Ringstad,[a] Margareth Jorvid,[a] Peter Frank,[a] Per Hedén,[c] Stefan Roos,[d] and Mia Phillipson[b,e,*]

[a]Ilya Pharma AB, Dag Hammarskjölds Väg 30, 752 37 Uppsala, Sweden
[b]Uppsala University, Department of Medical Cell Biology, 751 23 Uppsala, Sweden
[c]Sofiahemmet, Valhallavägen 91, 114 86 Stockholm, Sweden
[d]Swedish University of Agricultural Sciences, Department of Molecular Sciences, Uppsala BioCenter, 750 07 Uppsala, Sweden
[e]Science for Life Laboratory, Uppsala University, Sweden

eClinicalMedicine 2023;60: 102014
Published Online 25 May 2023
https://doi.org/10.1016/j.eclinm.2023.102014

Summary

Background Impaired wound healing is a growing medical problem and very few approved drugs with documented clinical efficacy are available. CXCL12-expressing lactic acid bacteria, *Limosilactobacillus reuteri* (ILP100-Topical), has been demonstrated to accelerate wound healing in controlled preclinical models. In this first-in-human study, the primary objective was to determine safety and tolerability of the drug candidate ILP100-Topical, while secondary objectives included assessments of clinical and biologic effects on wound healing by traditionally accepted methods and explorative and traceable assessments.

Methods SITU-SAFE is an adaptive, randomised, double-blind, placebo-controlled, first-in-human phase 1 trial (EudraCT 2019-000680-24) consisting of a single (SAD) and a multiple ascending dose (MAD) part of three dose cohorts each. The study was performed at the Phase 1 Unit, Uppsala University Hospital, Uppsala, Sweden. Data in this article were collected between Sep 20th, 2019 and Oct 20th 2021. In total 240 wounds were induced on the upper arms in 36 healthy volunteers. SAD: 12 participants, 4 wounds (2/arm), MAD: 24 participants, 8 wounds (4/arm). Wounds in each participant were randomised to treatment with placebo/saline or ILP100-Topical.

Findings In all individuals and doses, ILP100-Topical was safe and well-tolerated with no systemic exposure. A combined cohort analysis showed a significantly larger proportion of healed wounds ($p = 0.020$) on Day 32 by multi-dosing of ILP100-Topical when compared to saline/placebo (76% (73/96) and 59% (57/96) healed wounds, respectively). In addition, time to first registered healing was shortened by 6 days on average, and by 10 days at highest dose. ILP100-Topical increased the density of $CXCL12^+$ cells in the wounds and local wound blood perfusion.

Interpretation The favourable safety profile and observed effects on wound healing support continued clinical development of ILP100-Topical for the treatment of complicated wounds in patients.

Funding Ilya Pharma AB (Sponsor), H2020 SME Instrument Phase II (#804438), Knut and Alice Wallenberg foundation.

Keywords: CXCL12; ILP100; Limosilactobacillus; Phase 1 clinical trial; Immunotherapy; SITUSAFE

Introduction

Complicated or non-healing wounds, encompassing wounds that do not heal for 4 or more weeks with standard of care, are a growing medical problem associated with metabolic diseases and aging.[1–4] These problematic wounds negatively impact life quality and reduce life expectancy, and they often become infected and increase the risk for sepsis. There is a high unmet need for effective therapies, as there are very few available therapeutics with proven efficacy of accelerated wound healing. Instead, antibiotics are being overused in these patients, and up to 75% receive systemic

*Corresponding author. Department of Medical Cell Biology, Science for Life Laboratory, Uppsala University, Box 571, 75123 Uppsala, Sweden.
E-mail address: mia.phillipson@mcb.uu.se.
Translation: For the Swedish translation of the abstract see Supplementary Materials section.

Research in context

Evidence before this study

We searched PubMed for original articles, meta-analyses, and systematic reviews published until April 25th, 2023, describing the role of CXCL12-α in regeneration search terms included but were not limited to: SDF-1, CXCL12, regeneration, wound. At the start of the study in 2019, therapeutic functions to promote wound healing had been successfully addressed in preclinical models using genetically modified cells or bacteria that delivered CXCL12 locally. There are to our knowledge no reports of CXCL12 being tested in human wounds prior to this study.

Added value of this study

To our knowledge, this study is the first to provide support for safety and effects on wound healing by the novel first-in-class drug candidate with therapeutic CXCL12-α (ILP100-Topical) in a blinded, randomised, and placebo-controlled clinical trial setting. In addition, we demonstrate that this newly designed biotechnological platform enables delivery of proteins with short half-life, e.g. chemokines such as CXCL12-α, in a clinical use, and offers a novel approach for immunotherapies with local effects.

Implications of all the available evidence

No safety or tolerability issues were identified following treatment with ILP100-Topical to induced wounds. Clinical effect of accelerated wound healing was observed for the highest dose and when pooling data from all three multidose cohorts. The favourable safety profile and observed effect together support continued clinical development of ILP100-Topical for the treatment of difficult skin wounds in patients.

antibiotics despite often lacking documented clinical infection.[5,6]

Wound healing is driven by cells of the immune system regulated by signals from the wound micro-environment.[7–9] Immunomodulatory drugs are currently transforming oncology and autoimmune diseases, while therapeutic targeting of immune cells within wounds has not yet been successful. This is at least in part due to that topical administration is hampered by the proteolytic wound environment, which limits the bioavailability of candidate therapeutic molecules.[10] Therefore, genetically modified bacteria producing, delivering, and stabilising immunomodulatory proteins within the wounds could be disruptive in the field of immunotherapy, as they enable the use of proteins with short half-lives as scalable therapeutics.

A first-in-class drug candidate, ILP100-Topical (emilimogene sigulactibac), was designed by engineering *Limosilactobacillus reuteri* R2LC (*L. reuteri* R2LC), a strain of non-human origin, to produce and release the human chemokine CXCL12-α on-site to the wound bed.[11] Accelerated healing after topical delivery has been well-documented in multiple non-clinical studies, depends on increased numbers of wound macrophages of a restorative phenotype expressing TGF-β, and a favourable safety profile was demonstrated.[5,12] Here, we present results from the randomised, blinded, and placebo-controlled first-in-human study designed to primarily assess safety and tolerability of ILP100-Topical, whereas the secondary and exploratory objectives aimed to evaluate clinical and biologic effects on wound healing. To complement and validate the conventional assessments performed by Investigators during visits, blinded and high-resolution wound imaging techniques were used, which provided objective analyses of healing in fully traceable and reproducible data sets. This pioneering study demonstrates a favourable safety profile together with proven clinical and biologic effects on accelerated wound healing, which supports the continued clinical development of ILP100-Topical, a new modality and local immunotherapeutic.

Methods

Study design

This single-centre adaptive, randomised, double-blind, placebo-controlled, first-in-human phase 1 trial (SITU-SAFE) was conducted at the Phase 1 Unit, Uppsala University Hospital, Sweden, in 240 induced skin wounds in 36 healthy volunteers. The study included a treatment phase followed by an assessment phase running up to 6 weeks after last dose and an ongoing 5-year long-term follow up. The results presented in this paper were collected between September 20th, 2019 and October 20th, 2021 include results up to 13 months, i.e. 12 months follow up after last dose in the MAD part. The primary objective was to determine the safety and tolerability profile, whereas other objectives included assessments of clinical and biologic effects on wound healing, as well as presence and biodistribution of ILP100-Topical. ILP100-Topical consists of *L. reuteri* R2LC genetically modified with the pSIP_CXCL12-α plasmid to express CXCL12-α, hereunder referred to as CXCL12, following induction by the activation peptide SppIP.[11,13,14] The ready-to-use drug product consists of *L. reuteri* R2LC carrying the pSIP_CXCL12 plasmid reconstituted with SppIP-containing buffer. As a control within each participant, placebo (SppIP-containing buffer), or saline (NaCl 0.9%) was used. The study was designed to comprise a single ascending dose (SAD) part of three cohorts, and a multiple ascending dose (MAD) part of another three cohorts, where safety confirmation of the SAD part preceded MAD initiation (Supplementary Fig. S1).

The studies were undertaken in accordance with Good Clinical Practice and the Declaration of Helsinki, and with approval of the Swedish Ethical Review Authority (Approval no. 2019-02802) and the Medical Product Agency in Uppsala, Sweden. Informed consent was obtained from the study individuals. The trial is registered in EudraCT (2019-000680-24).

Participants

Healthy male and female individuals aged 25–45 years who were willing to comply with the study procedures (experimental incision of 4 or 8 wounds in the SAD and MAD, respectively, equally distributed at the upper inner arms) and who have given written informed consent were considered eligible to participate in the study. Prior to consent, all individuals were given extensive information about the procedures and the potential risks with the study, such as punch biopsy procedure and risk of scarring. All individuals included had to understand and be willing to comply with study procedures. Individuals with a history of any bleeding disorder, including prolonged or habitual bleeding, individuals on blood-thinning medication or individuals with e.g. a tattoo or apparent skin abnormality on the upper inner arms were not included in the study, neither were pregnant or lactating women.

Randomisation and masking

The Investigational medicinal products (IMPs) were prepared by unblinded pharmacists, masked in order to maintain the blind, and administered topically in volumes of 50 µl per wound to blindfolded individuals. A computer-generated randomisation list (SAS Proc Plan, SAS Version 9.4, Institute, Inc., Cary, NC, USA) was kept by the randomiser in a sealed envelope until database lock.

Procedures

Enrolled individuals were admitted to the clinic on Day 1 for pre-dose safety assessments and full thickness wound punching (biopsy punch, 6 mm in diameter) on the ventral aspect of the upper arms (SAD: 2 wounds/arm; MAD: 4 wounds/arm) following treatment of local anaesthesia (injected Xylocaine 10 mg/mL) and cleaning of the area (70% ethanol) (Supplementary Fig. S1). The wounds were photographed in a standardised setting before treatment on Day 1, and at all subsequent visits. For assessment of wound healing, the non-epithelialised wound area was measured by the IEs using ImageJ Software (U. S. National Institutes of Health, USA). To address exploratory objectives, wounds of the MAD part were scanned using a 3D spectroscopic scanner to evaluate scar area, scar volume and scar redness (Cherry Imaging, Yokneam, Israel, Supplementary methods) and blood perfusion of the wound bed and adjacent skin was measured (Laser Speckle Contract Analysis, LASCA; Perimed AB, Järfälla, Sweden, Supplementary Fig. S2 and Supplementary methods).[15,16] Wound biopsies were taken 48 h post-dosing in the SAD part for assessment of local mechanisms of action (Supplementary methods).

The SAD part of the study comprised of 3 sequential cohorts, each including 4 individuals with 2 experimentally induced wounds on each arm, in total 12 individuals and 48 wounds. For each individual, a single dose of ILP100-Topical (5×10^4, 5×10^7 or 1×10^9 CFU/cm^2 wound area in cohort 1, cohort 2 and cohort 3, respectively) and placebo were randomised to 2 wounds on the left arm and 2 wounds on the right arm, in a 1:1 ratio.

The MAD part comprised of 3 sequential cohorts, each including 8 individuals with 4 experimentally induced wounds on each arm, in total 24 individuals and 192 wounds. The IMP was randomised in a 4:2:2 ratio, with ILP100-Topical (cohort 1: 5×10^5 CFU/cm^2, cohort 2: 5×10^7 CFU/cm^2 and cohort 3: 1×10^9 CFU/cm^2) to 4 wounds on left or right arm, and placebo or saline to 2 wounds each on the arm on which wounds did not receive ILP100-Topical. Saline was used as a control to assess the potential effect on wound healing by the SppIP-containing buffer in placebo. Each wound was administered with repeated doses of IMP on Day 1, 2 and 3, followed by 3 times a week over the course of 3 weeks (10 doses in total).

Outcomes

Clinical safety assessments were performed at visits and included adverse events (AEs), clinical laboratory parameters, vital signs, ECG, physical examination, local tolerability reactions, formation of anti-CXCL12 antibodies (ADA, supplementary methods), systemic exposure of CXCL12 in plasma (Supplementary methods), as well as presence of *L. reuteri* R2LC containing the pSIP_CXCL12 on the skin surrounding the wound, blood, and faeces (Supplementary methods).

Tolerability, clinical and biologic effects were assessed at each visit (SAD: Day 1, 2, 3, 7, 14, and at 6 weeks, 3 months and 12 months from start of treatment at Day 1; MAD: Day 1, 2, 3, 5, 8, 10, 12, 15, 17, 19, 21, 32, and at 6 weeks, 3 months and 12 months after last dose at Day 19). All assessments were blinded and occurred by on-site visual inspections of the wounds by the Principal Investigator (or co-investigator), as well as off-site by traceable evaluation and detailed wound area measurements from 2D photographs of all wounds by 3 Independent Evaluators (IEs) with expertise in wound healing. Tolerability was graded 0–3 according to predefined criteria based on wound appearance (wound and wound edge inflammation, surrounding skin inflammation, haemorrhage, presence of exudate, slough or necrotic tissue, granulation tissue, or hypergranulation). For the clinical effect on wound healing, a wound was defined as healed when the wound area was completely re-epithelialised and there were no dressing

requirements, and if the assessments by one or more IEs deviated more than two steps on the 4-grade scale, the three IEs assembled to adjudicate the definitive grade. In addition, 3D scans were used to assess changes in scar size, while analyses of the mechanism of action included blood flow measurements (MAD part only) and molecular changes by histology and local CXCL12 levels by ELISA in the wound biopsies (SAD part only).

Statistical analysis

The sample size was considered sufficient to provide adequate information for the primary and related safety and tolerability objectives. For detailed description about statistical analysis, please see supplementary methods. In the post-hoc analyses of the biologic and clinical effects on wound healing, Fisher's exact test and the Mann–Whitney test were used for comparing the different treatment groups for proportion healed wounds and average time to first registered healing. All descriptive summaries and pre-defined statistical analyses were performed using SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA). Post-hoc analyses were performed using StatXact Version 11.1.0 (Cytel Inc., Waltham, MA, USA), SAS Version 9.4, and GraphPad Prism 9.1.1.225 (GraphPad Software, San Diego, CA, USA).

Role of the funding source

Ilya Pharma AB is the Sponsor of the study fulfilling all sponsor responsibilities. The trial was in part supported by a grant from the European Commission, H2020 SME Instrument Phase II (#804438) and by Knut and Alice Wallenberg foundation.

Study Sponsor was responsible for the study design, analysis of data from 3D scanning and LASCA measurements, and decision to publish the data. Study report and data interpretation (except for 3D scanning and LASCA measurements) was performed by CRO and reviewed by the Sponsor.

EÖ, EV, AF, HLT, PD, SJ, NT, MÅ, ZM, LR, MJ, PF, PH, SR, and MP all had access to the dataset and accept responsibility for the decision to submit for publication.

Results

Thirty-six healthy study individuals between 25 and 45 years old were enrolled at a Phase 1 Unit at Uppsala University Hospital, Uppsala, Sweden between 20th of September 2019 and 1st of October 2020 (Fig. 1). Baseline characteristics and demographics of the individuals are summarised in Supplementary Tables S1 and S2 for the SAD and MAD parts of the study, respectively.

The primary objective of the study was to determine the safety and tolerability profile. For all individuals, single- or multi-dosing of ILP100-Topical (1 and 10 administrations over 3 weeks, in the SAD and MAD, respectively) were considered safe and well-tolerated. No clinically significant changes from baseline of any parameters were detected during any visits. There were no serious adverse events or AEs leading to discontinuation from the study (Supplementary Tables S3 and S4). Overall, the AE profile of wounds treated with ILP100-Topical was comparable to that of wounds treated with placebo or saline (Table 1). For all cohorts, *L. reuteri* R2LC containing pSIP_CXCL12 was only identified on the skin surrounding wounds 1–2 days after treatment, no colonisation occurred, and *L. reuteri* R2LC containing pSIP_CXCL12 was not detected in blood or faeces at any time point. In addition, circulating levels of CXCL12 were not increased after single- or multi-dosing, and ADAs against CXCL12 could not be detected at any time point.

In both SAD and MAD, transient inflammation of the wound and surrounding skin was observed to a higher degree for the highest ILP100-Topical levels (Supplementary Tables S5 and S6), while the prevalence of wound infections was similar between saline, placebo, and ILP100-Topical treated wounds (Table 1). Treatment with ILP100-Topical was associated with increased exudation in the two lowest doses and in the highest dose to the amount of slough/necrotic tissue, as assessed by the IEs, but not according to the Investigators (Supplementary Tables S8 and S9 and data not included). There were no evident associations between the amount of, granulation tissue, haemorrhage or hypergranulation between the different treatments in either SAD or MAD (Supplementary Tables S8, S10, and S11). Irrespective of treatment in cohort 1 in the MAD part, the Investigators reported eczema and inflammation of the skin in contact with the dressing (Table 1), which resulted in discontinuation of treatment of in total 28 wounds (Supplementary Table S4). The dressing type was therefore changed for cohort 2 and 3. ILP100 treatment did not increase wound rupture, as this was only reported for scars of two placebo-treated wounds.

Secondary objectives included assessments of clinical and biologic effects on wound healing. No differences in wound healing were detected between the saline–or placebo-treated wounds by either Investigators or IEs, and saline- and placebo-treatment were therefore pooled. The Investigators' assessments did not show any difference in wound healing between treatment groups. In the MAD part, the IEs' assessments revealed treatment-related differences in wound healing at Days 32 in cohort 1, and at Days 19 and 21 (Day 32, $p = 0.058$) in cohort 3, where higher proportions of wounds treated with ILP100-Topical were assessed as healed by all three IEs compared to control-treated wounds (Fig. 2A). A pooled analysis of all cohorts showed that ILP100-Topical significantly improved wound healing compared to controls ($p = 0.020$), as 76% (73/96) of the ILP100-Topical treated wounds were healed at or prior to

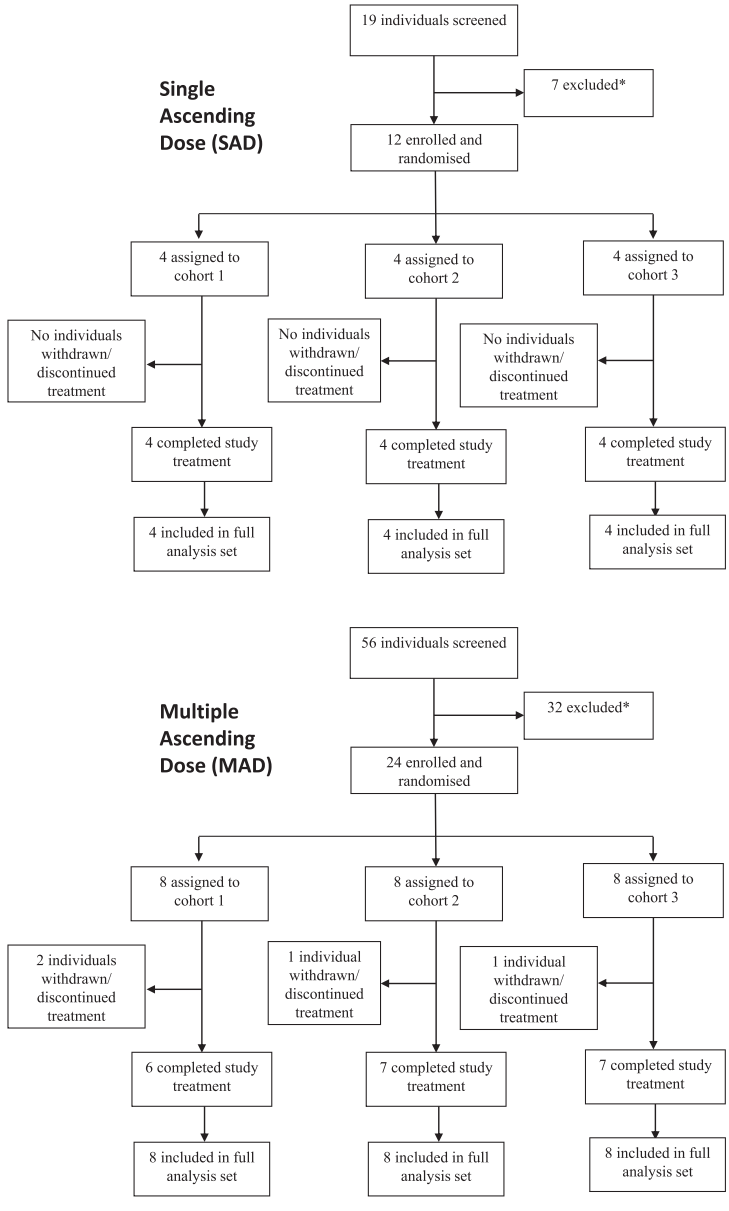


Fig. 1: **Trial profile for SAD and MAD.** *Excluded = ineligible, reserves, or other.

	System organ class	LP100 (n = 32/cohort)		Placebo (n = 16/cohort)		Saline (n = 16/cohort)		Placebo+Saline (n = 32/cohort)		Total (n = 64/cohort)	
	Preferred term	n (%)	m	n (%)	m	n (%)	m	n (%)	m	n (%)	m
Cohort 1	General disorders and administration site conditions	13 (40%)	13	5 (31%)	5	9 (56%)	9	14 (44%)	14	27 (42%)	27
	Administration site eczema	11 (34%)	11	4 (25%)	4	8 (50%)	8	12 (38%)	12	23 (36%)	23
	Administration site inflammation	2 (6.3%)	2	1 (6.3%)	1	1 (6.3%)	1	2 (6.3%)	2	4 (6.3%)	4
	Infections and infestations	5 (16%)	5	5 (31%)	5	4 (25%)	4	9 (28%)	9	14 (22%)	14
	Eczema infected	0	0	2 (13%)	2	2 (13%)	2	4 (13%)	4	4 (6.3%)	4
	Wound infection	5 (16%)	5	3 (19%)	3	2 (13%)	2	5 (16%)	5	10 (16%)	10
	Skin and subcutaneous tissue disorders	6 (19%)	6	2 (13%)	2	2 (13%)	2	4 (13%)	4	10 (16%)	10
	Pruritus	2 (6.3%)	2	0	0	0	0	0	0	2 (3.1%)	2
	Skin mass	4 (13%)	4	2 (13%)	2	2 (13%)	2	4 (13%)	4	8 (13%)	8
	Injury, poisoning and procedural complications	2 (6.3%)	2	4 (25%)	5	1 (6.3%)	1	5 (16%)	6	7 (11%)	8
	Wound complication	0	0	4 (25%)	5	1 (6.3%)	1	5 (16%)	6	5 (8.0%)	6
	Wound haemorrhage	2 (6.3%)	2	0	0	0	0	0	0	2 (3.1%)	2
Cohort 2	Injury, poisoning and procedural complications	26 (81%)	31	9 (56%)	11	12 (75%)	13	21 (66%)	24	47 (73%)	55
	Wound complication	1 (3.1%)	1	0	0	0	0	0	0	1 (1.5%)	1
	Wound haemorrhage	25 (78%)	30	9 (56%)	11	12 (75%)	13	21 (66%)	24	46 (72%)	54
	General disorders and administration site conditions	0	0	0	0	1 (6.3%)	1	1 (3.1%)	1	1 (1.6%)	1
	Application site pruritus	0	0	0	0	1 (6.3%)	1	1 (3.1%)	1	1 (1.6%)	1
Cohort 3	Injury, poisoning and procedural complications	7 (22%)	9	5 (31%)	7	5 (31%)	6	10 (31%)	13	17 (27%)	22
	Wound complication	4 (13%)	4	3 (18%)	3	2 (13%)	2	5 (16%)	5	9 (14%)	9
	Wound hematoma	0	0	1 (6.3%)	1	0	0	1 (3.1%)	1	1 (1.6%)	1
	Wound haemorrhage	5 (16%)	5	3 (19%)	3	3 (19%)	4	6 (19%)	7	11 (17%)	12
	Infections and infestations	1 (3.1%)	1	0	0	0	0	0	0	1 (1.6%)	1
	Wound infection	1 (3.1%)	1	0	0	0	0	0	0	1 (1.6%)	1

Percentages are based on the number of wounds in the study period included in the full analysis set, n, number of wounds; m, number of events. Pre-treatments are not included.

Table 1: **Adverse events in Cohort 1, 2, and 3 up to 13 months follow-up (MAD).**

Day 32, as assessed by all IEs, compared to 59% (57/96) of control wounds (Fig. 2A). Further, when all doses/cohorts were pooled, the time to first registration of healed by all three IEs was on average shortened by 6 days by ILP100-Topical ($p = 0.039$) compared to controls. For the highest ILP100-Topical dose group, the time to first registration of healed was 10 days faster compared to controls ($p = 0.0046$, Fig. 2B). Similar results for time to wound healing and the proportion of healed wounds were obtained with paired statistical methods (data not included).

Irrespective of treatment, blood perfusion of the wound bed peaked at Day 8 (Supplementary Table S12), whereas wound edge perfusion decreased over time as the wounds gradually healed (Table 2) with the exception for cohort 1 where dressing-induced eczema and skin inflammation were reported (Table 1). Treatment with ILP100-Topical was found to increase wound edge blood perfusion dose dependently at Day 2 when compared to the control-treated wounds of cohorts 2 and 3, but not at Day 8 or 15 (Table 2).

Immunohistochemistry of wound biopsies from the SAD wounds revealed increased numbers by 59% of $CXCL12^{+}$ cells in the wound edge dermis by the highest dose of ILP100-Topical (1018 ± 134 vs 1623 ± 315 for control and ILP100-treated wounds, respectively) (Table 3, Supplementary Fig. S3). No differences were detected for CXCL12 levels within tissue (Supplementary Table S13).

Scar formation was assessed as normal for all healed wounds at all visits. The 3D spectroscopic scanning revealed no difference in scar areas between cohorts or treatments (Supplementary Table S14), while the sensitivity of scar volume scans did not allow for comparisons between treatment groups (Supplementary Tables S15 and S16). Scar redness normalised to skin colour was also assessed, but no differences between treatments could be detected (Supplementary Tables S17 and S18).

Discussion

In this first-in-human trial, topical application of the first-in-class drug candidate ILP100-Topical was suggested to be safe and well-tolerated. In addition, multiple doses of ILP100-Topical supported clinical efficacy on wound healing, as demonstrated by a larger proportion of healed wounds from Day 19 and shortened time to first registered healing. Thus, genetically modified *L. reuteri* R2LC engineered to deliver and stabilise CXCL12 was suggested to be safe and effective in accelerating healing of induced wounds.

Therapeutic means to support healing has recently been focusing on altering the wound microenvironment

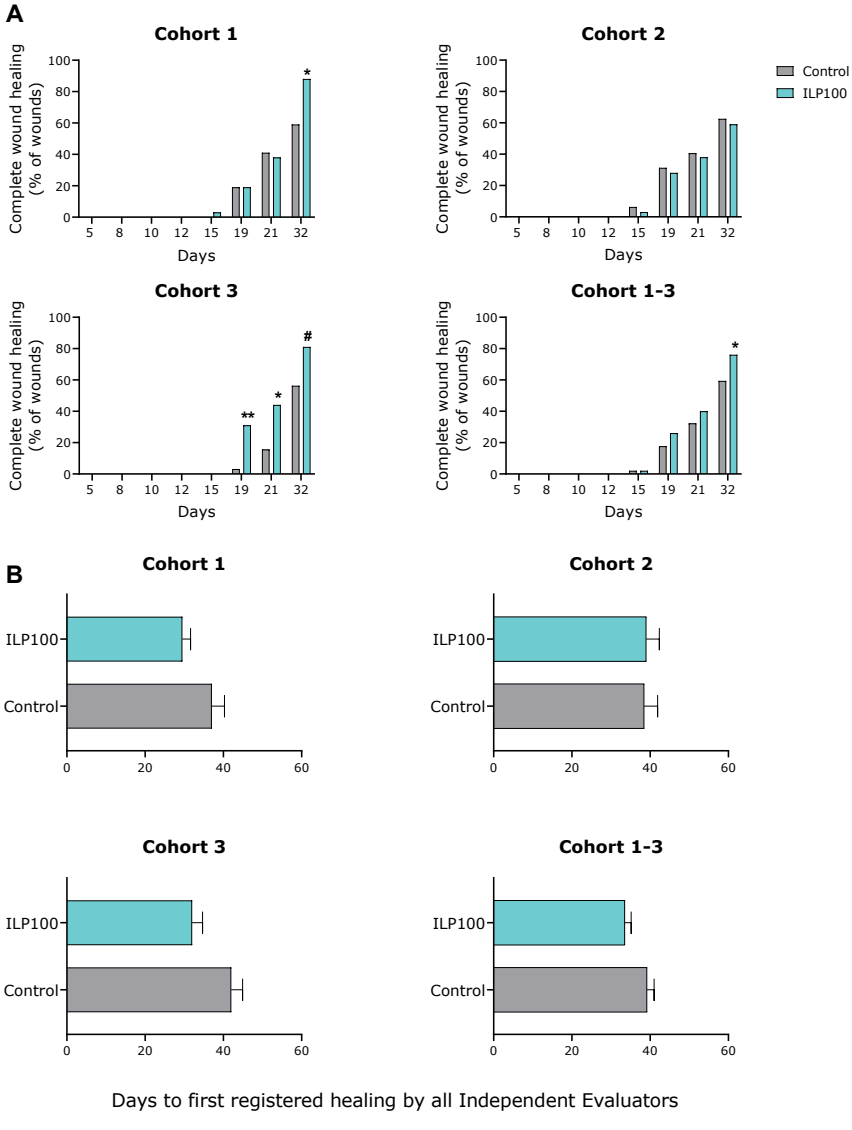


Fig. 2: **Proportion of healed wounds at visits, and time to first registration of healed (days) for control-treated wounds and wounds treated with ILP100-Topical (MAD)**. The MAD cohorts treated with 5×10^5 CFU/cm^2 wound area (cohort 1), 5×10^7 CFU/cm^2 (cohort 2) and 1×10^9 CFU/cm^2 (cohort 3), respectively, were assessed for complete wound healing at visits. A) Wounds were defined as healed when the wound area was completely re-epithelialised (yes/no) by three blinded IEs in the MAD cohorts. #p = 0.058, *p ≤ 0.05, **p ≤ 0.01, compared to control. p-values (two-sided) are calculated by Fisher's exact test. B) The time point where a wound was first registered as healed at visits for the

LASCA perfusion imaging	n, m	Day 2		Day 8		Day 15	
		Control	ILP100	Control	ILP100	Control	ILP100
Cohort 1	7–8, 26–32	43.8 ± 5.5	53.2 ± 5.6	34.8 ± 12.9	46.3 ± 12.1	30.4 ± 4.8	46.5 ± 10.4
Cohort 2	6–8, 24–32	56.1 ± 5.5	76.6 ± 6.0**	31.6 ± 3.1	33.6 ± 3.0	10.9 ± 2.0	13.3 ± 1.8
Cohort 3	7–8, 28–32	37.1 ± 3.4	89.3 ± 5.4****	32.2 ± 4.8	34.8 ± 4.4	5.4 ± 2.9	8.7 ± 2.7
Cohort 1-3	21–24, 84–96	45.7 ± 2.9	74.1 ± 3.6****	32.7 ± 4.3	38.2 ± 4.3	17.1 ± 2.5	24.4 ± 4.5

The MAD cohorts treated with 5×10^5 CFU/cm^2 wound area (cohort 1), 5×10^7 CFU/cm^2 (cohort 2) and 1×10^9 CFU/cm^2 (cohort 3), respectively, were assessed for wound edge perfusion at visits Day 2, 8 and 15. Values are delta perfusion units (dPFU) and represent perfusion of the wound edge (skin area within 5 mm from the wound border) minus the reference non-wounded skin perfusion at the same image presented as Mean ± SEM. Controls represent saline–or placebo-treated wounds. n, number of individuals; m, number of wound frames analysed per group. Mann–Whitney test **p = 0.010, ****≤0.0001.

Table 2: **Wound edge blood perfusion analysed using non-invasive imaging Laser Speckle Contrast Analysis (LASCA) at Days 2, 8, and 15 in control- and ILP100-Topical-treated groups (MAD).**

by topical application of growth factors, plasma-derived products or cell therapies.[17–19] These strategies are often hampered by restricted access of administered cells to wound tissue, and by limited bioavailability of therapeutic proteins due to high levels of proteases present in wounds. Another disruptive and recently recognised approach is to use genetically modified bacteria to deliver endogenous proteins to wounds.[20] So far, two attempts have been reported to accelerate healing in mouse models: *Lactococcus lactis* expressing FGF2, CSF1, and IL-4 (AUP-1602-C) currently tested in a first-human trial (NCT04281992), and the herein investigated ILP100-Topical, *L. reuteri* R2LC expressing CXCL12.[11,21] In addition to the onsite bacterial production, the lactic acid produced by *L. reuteri* R2LC was demonstrated to reduce CXCL12 degradation within the wound, and thereby further boosting the CXCL12-induced tissue restorative functions of macrophages.[11] Accelerated healing of wounds by ILP100-Topical was also confirmed in minipigs.[12]

For new modalities, trial design capturing drug-specific characteristics are vital for continued clinical development. The present trial was designed to allow independent evaluations of wounds, reduce the number of individuals and overcome interindividual variability by having wounds treated with ILP100-Topical, placebo and saline in the same participant. The individuals were closely monitored using an extensive set of safety and tolerability assessments, and all wounds were imaged for subsequent off-site, unbiased, high-resolution, and traceable analyses, in addition to the on-site assessments. Notably, no clinically significant deviations from baseline were detected when safety and tolerability were assessed, and no serious AEs were recorded. Treatment of acute wounds with ILP100-Topical was therefore demonstrated to be both safe and well-tolerated at all timepoints and doses tested.

Complete wound healing is the regulatory endpoint considered to be the most clinically meaningful by FDA. In this study, wound healing was assessed by blinded and fully traceable, off-site analyses of high-resolution wound images. All three IEs found that a higher proportion of wounds treated with the highest dose of ILP100-Topcial were healed from Day 19. Further, the time to first registration of complete healing was shortened by 10 days following repeated ILP100-Topical treatment with the highest dose. These results are indeed clinically very relevant given that 1–2 days of accelerated healing in acute wounds or healing of 10–15% more non-healing ulcers in patients with diabetes compared to standard of care is regarded as clinically meaningful and suffice the requirements for marketing authorisation by regulatory authorities.[22]

To increase the probability of capturing AEs and effects on wound healing in this first-in-human trial, we combined the clinical assessment of the wounds with objective, explorative techniques measuring local blood perfusion and scar formation. These different techniques together generated more than 100 000 data points analysed in a blinded manner. While the small size of the scars precluded comparisons between treatments, a transient and dose-dependent hyperaemia around the ILP100-Topical-treated wounds were detected at early time points. Together with the observed accelerated healing and limited number of transient inflammation-related AEs, this likely reflects biologic effects of the treatment, rather than inflammatory response to bacteria. Thus, the obtained results support continued clinical development of ILP100-Topical for the treatment of difficult-to-heal skin wounds in patients. In fact, two phase 2 trials investigating ILP100-Topical as treatment in different wound types is now approved by European and US health authorities.

three cohorts of the MAD part, as well as for pooled cohorts. Mann–Whitney test *p ≤ 0.05, **p ≤ 0.01. Average time, error bars indicate standard error of the mean (SEM). Wounds with missing timepoint of wound healing or not judged as healed by the end of the study has been imputed as healed after 61 days being the next timepoint of assessment. Control group includes saline–or placebo-treated wounds.

CXCL12⁺ cells in wound biopsies at 48 h	n, m	Higher density of CXCL12⁺ cells in ILP100 treated wounds	Placebo (m = 4/cohort)	ILP100 (m = 4/cohort)	Ratio
Cohort 1	4, 8	3 of 4 (75%)	479 ± 163	454 ± 126	0.95
Cohort 2	4, 8	1 of 4 (25.0%)	689 ± 52	485 ± 106	0.70
Cohort 3	4, 8	4 of 4 (100.0%)	1018 ± 134	1623 ± 315	1.59
Cohort 1–3	12, 24	8 of 12 (66.7%)	729 ± 94	846 ± 196	1.16

Data represents mean values (±SEM) of placebo-treated and ILP100-Topical-treated wounds compared within each subject in the cohort. N, number of individuals; m, number of wound biopsies analysed per treatment group.

Table 3: **CXCL12⁺ cells quantified following immunohistochemistry of wound edge biopsies 48 h following single dose administration of ILP100-Topical or placebo (SAD).**

Limitations of this study include the single-centre design, the rather small number of study individuals and that different investigators were involved in clinical assessments. Changes in the investigator team during MAD cohort 2 might have influenced the wounding procedures and thereby explain inconsistent results compared to other cohorts. In addition, the individuals included in this study were all healthy, non-obese, and under the age of 45, and is thereby not predisposed for these factors associated with impaired or complex wound healing. Hence, while ILP100-Topical in this study show results supporting an accelerated wound healing in otherwise healthy patients (eg in trauma-related wounds), the effect might not be directly translatable to a patient population exhibiting risk factors for delayed wound healing. As a natural next step in the clinical development efficacy is already being investigated in different well-defined patient populations with pathologies linked to impaired wound healing. Strengths include its design allowing large numbers of wounds, minimal bias as wounds treated with active and control treatment in the same individuals reduce the risk for factors influencing wound healing in different treatment groups, as well as high comparability between treatments for tolerability and effects on healing. This is especially important in a First-in-Human study with few study individuals and at the same time allows for a smaller samples size with fewer individuals exposed to an experimental investigational product in early clinical development. The well-being of the study individuals was thoroughly considered, and each study individual was informed about the study procedures and risks for scar formation before giving consent. Only individuals able to fully understand the study information and comply with the protocol procedures were considered for the study. Further, each study individual fulfilled the inclusion criteria and did not present any of the exclusion criteria including conditions associated with abnormal scar formation and other physical risks, but not mental illness risks. The latter was not considered necessary to evaluate specifically, given that the individuals were informed and accepted the study risk and were assessed for their overall eligibility for participation. Another strength is the wound assessments from high-resolution images, which allows blinded, detailed analyses of both tolerability and wound healing. In conclusion, the favourable safety profile together with the clinical and biologic effects on wound healing support continued clinical development of ILP100-Topical for the treatment of complicated, non-healing wounds in patients. In addition, the study demonstrates that genetically modified bacteria is a new modality enabling the use of short-lived proteins, such as CXCL12, as therapeutics.

Contributors

The authors Emelie Öhnstedt (EÖ), Evelina Vågesjö (EV), Andreas Fasth (AF), Hava Lofton Tomenius (HLT), Pia Dahg (PD), Sofia Jönsson (SJ), Nisha Tyagi (NT), Mikael Åström (MÅ), Zhanar Myktybekova (ZM), Lovisa Ringstad (LR), Margareth Jorvid (MJ), Peter Frank (PF), Per Hedén (PH), Stefan Roos (SR), and Mia Phillipson (MP) contributed as follows. EV, AF, PH, SR, MJ, and MP were responsible for the conceptualisation. Formal analysis and investigation were the responsibility of EÖ, EV, AF, SR, and MP. EÖ, SJ, ZM, NT, LR, PF, and HLT contributed to the methodology. Project administration was performed by PD and AF, and supervision by EV, AF, SR, and MP. Validation and verification of the data were performed by EÖ, AF, and MP. The first draft of the manuscript was written by MP and review and editing was performed by EÖ;, EV, AF, PH, MÅ, and MP.

EÖ, EV, AF, HLT, PD, SJ, NT, MÅ, ZM, LR, MJ, PF, PH, SR, and MP all had access to the dataset and accept responsibility for the decision to submit for publication.

Data sharing statement

Deidentified participant data will be made available on request for scientific purposes by contacting the corresponding author. Study protocol are available at: https://www.ilyapharma.se/media/1249/situ-safe-ip-ct-001-sap-summary-of-changes.pdf, https://www.ilyapharma.se/media/1248/situ-safe-ip-ct-001-csp-v30-24feb2020.pdf and https://www.ilyapharma.se/media/1247/situ-safe-ip-ct-001-csp-summary-of-changes.pdf.

Declaration of interests

MP, SR, PF, MJ, PH and EV are shareholders of Ilya Pharma. EV, AF, EÖ, HLT, LR, NT, PF, SJ, and ZM have stocks options in Ilya Pharma. AF, EÖ, EV, HLT, LR, NT, PF, SJ and ZM are employees, part time or full time, of Ilya Pharma AB. MJ, MÅ, and PD are consultants paid by Ilya Pharma AB for their services. MP and SR renumeration for work in the company Board of Directors.

Acknowledgements

The study was supported by Ilya Pharma AB (Sponsor), H2020 SME Instrument Phase II (#804438) to Sponsor, and Knut and Alice Wallenberg Foundation to Mia Phillipson.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.eclinm.2023.102014.

References

1 Öhnstedt E, Lofton Tomenius H, Vågesjö E, et al. The discovery and development of topical medicines for wound healing. *Expert Opin Drug Discov*. 2019;14(5):485–497. https://doi.org/10.1080/17460441.2019.1588879.

2 Vågesjö E, Grigoleit P, Fasth A, et al. How can we optimize the development of drugs for wound healing? *Expert Opin Drug Discov*. 2022;17(2):93–96. https://doi.org/10.1080/17460441.2022.1992381.

3 Wilkinson HN, Hardman MJ. Wound healing: cellular mechanisms and pathological outcomes. *Open Biol*. 2020;10(9):200223.

4 Anderson K, Hamm RL. Factors that impair wound healing. *J Am Coll Clin Wound Spec*. 2014;4(4):84–91.

5 Howell-Jones RS, Wilson MJ, Hill KE, et al. A review of the microbiology, antibiotic usage and resistance in chronic skin wounds. *J Antimicrob Chemoterapy*. 2005;55:143–149.

6 Öien R, Forsell HW. Ulcer healing time and antibiotic treatment before and after the introduction of the Registry of Ulcer Treatment: an improvement project in a national quality registry in Sweden. *BMJ Open*. 2013;3:e003091.

7 Rodrigues M, Kosaric N, Bonham CA, et al. Wound healing: a cellular perspective. *Physiol Rev*. 2019;99(1):665–706.

8 Krzyszczyk P, Schloss R, Palmer A, et al. The role of macrophages in acute and chronic wound healing and interventions to promote pro-wound healing phenotypes. *Front Physiol*. 2018 May 1;9:419.

9 Lucas T, Waisman A, Ranjan R, et al. Differential roles of macrophages in diverse phases of skin repair. *J Immunol*. 2010 Apr 1;184(7):3964–3977.

10 Kim HS, Sun X, Lee J-H, et al. Advanced drug delivery systems and artificial skin grafts for skin wound healing. *Adv Drug Deliv Rev*. 2019;146:20–239.

11 Vågesjö E, Öhnstedt E, Mortier A, et al. Accelerated wound healing in mice by on-site production and delivery of CXCL12 by transformed lactic acid bacteria. *Proc Natl Acad Sci U S A*. 2018;115(8):1895–1900.

12 Öhnstedt E, Lofton Tomenius H, Frank P, et al. Accelerated wound healing in minipigs by on-site production and delivery of CXCL12 by transformed lactic acid bacteria. *Pharmaceutics*. 2022;14(2):229.

13 Sørvig E, Grönqvist S, Naterstad K, et al. Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum*. *FEMS Microbiol Lett*. 2003;229(1):119–126.

14 Sørvig E, Mathiesen G, Naterstad K, et al. High-level, inducible gene expression in *Lactobacillus sakei and Lactobacillus plantarum* using versatile expression vectors. *Microbiology (Read)*. 2005;151(Pt 7):2439–2449.

15 Salameh F, Shehadeh W, Sprecher E, et al. A stereoscopic optical system for objective quantification of the change in cumulative acne scar depth following various treatment interventions. *J Cosmet Dermatol*. 2022;21(5):2099–2105.

16 Briers JD. Laser Doppler, speckle and related techniques for blood perfusion mapping and imaging. *Physiol Meas*. 2001;22(4):R35–R66.

17 A phase I/II, multicentre, randomised, controlled, and open-label trial comparing the efficacy and safety of three dose regimens of BioChaperone PDGF-BB to becaplermin gel for the treatment of diabetic foot ulcer. Available from: https://clinicaltrials.gov/ct2/show/NCT01098357. Accessed September 27, 2022.

18 Mahdipour E, Sahebkar A. The role of recombinant proteins and growth factors in the management of diabetic foot ulcers: a systematic review of randomized controlled trials. *J Diabetes Res*. 2020;2020:6320514.

19 Kosaric N, Kiwanuka H, Gurtner GC. Stem cell therapies for wound healing. *Expet Opin Biol Ther*. 2019;19(6):575–585.

20 Davis FM, Gallager K. Time heals all wounds… but wounds heal faster with lactobacillus. *Cell Host Microbe*. 2018;23(4):432–434.

21 Kurkipuro J, Mierau I, Wirth T, et al. Four in one – combination therapy using live Lactococcus lactis expressing three therapeutic proteins for the treatment of chronic non-healing wounds. *PLoS One*. 2022;17:e0264775.

22 Guidance for industry chronic cutaneous ulcer and burn wounds — developing products for treatment. Available from: https://www.fda.gov/media/71278/download; 2006. Accessed September 27, 2022.

Supplementary appendix

Table of Contents

List of Figures

List of Tables

Supplementary methods

Study procedures

The SAD part of the study included 6 visits for treatment and initial follow-up (Day 1 to Day 14). In the MAD part, there were 12 visits for treatment and initial follow-up (Day 1 to Day 32). All individuals are part of a 5-year long-term follow-up. This report includes results from visits up to 6 weeks, and at 3, 6 and 12 months after the last dose was administered, referred to as 2, 4, 7 and 13 months indicating time post wound induction. After the initial screening visit, individuals fulfilling the study criteria were enrolled.

In SAD, separate adhesive transparent film dressings were used to isolate the wounds from each other. In cohort 1 in the MAD the same dressing was applied throughout the treatment period (Day 1 to Day 19). With repeated administrations of IMP occlusive film dressing caused eczema on the skin surrounding the wounds in the majority of study participants. In cohorts 2 and 3 of the MAD part, the dressing was therefore changed and covered with adhesive, transparent film during 48 hours after the first and second IMP application. From Day 3 and onwards, the wounds were treated with IMP and then covered with adhesive, transparent film for 1 hour only. Thereafter the film was removed, the wounds were allowed to air dry and were then be covered with non-occlusive dressing in accordance with standard wound care procedures. Each wound was dressed until healed.

3D spectroscopic scanner evaluation

In order to understand the precision and the limitations of the 3D spectroscopic scanner (Cherry Imaging, Yokneam, Israel), an evaluation of the scanner was performed using scars scanned in the SAD part. The scar volume and area evaluation were performed 2 months post wound induction, where four scars from the same subject were scanned consecutively for four times in order to assess inter-scan variability. To assess intra-scan variability, each scar was measured five times using the Cherry Imaging software. The scars used for this evaluation were very small with areas and volumes ranging from 23-26 mm^2 and 0.7-1.6 mm^3, respectively, and the results are presented in Supplementary table 15. Evaluation of the measurements of the redness of scars was performed in a similar manner where wounds or skin areas with a redness score of 0.1 to 0.9 were measured repeatedly. For the inter-scan variability 10 areas were used. In total, 20 areas per wound were included in the intra-scan variability assessment, and the defined area within the scan was repeatedly measured five times using the Cherry Imaging software. The results are presented in Supplementary table 17.

Study outcomes

CXCL12 levels in human plasma were analysed using ELISA according to the manufacturer's instructions (Human CXCL12/SDF-1a Quantikine ELISA kit and Quantikine Immunoassay Control Group 3, R&D Systems, Minneapolis, MN, USA). To determine the presence of ADAs, human plasma samples were analysed using a GLP-validated electrochemiluminescent immunoassay (ECLIA). Presence of *L. reuteri* R2LC containing the pSIP_CXCL12 plasmid was analysed in faeces, blood samples and swabs of the area surrounding the wounds by bacterial culturing. PCR and sequencing were used in the occurrence of bacterial culture colonies.

In the SAD part, one placebo-treated wound and one ILP100-Topcial-treated wound were biopsied again at 48 hrs post wounding with an 8 mm in diameter biopsy punch. The biopsy was split in two halves, one was used for histology and one for analysis of tissue CXCL12 by ELISA (Quantikine ELISA Human CXCL12 / SDF-1α Immunoassay, R&D Systems, Minneapolis, MN, USA). The tissue saved for histology was paraffin embedded and stained for CXCL12 (NSJ Bioreagents, RQ4559).

The 3D scans of scar area, volume and pigmentation were analysed (Trace software, Cherry Imaging), and validated (Supplementary Methods and Supplementary Tables 1-2). Blood flow was recorded in an area of 5x10 cm around 2 wounds at a time during at least 2 minutes. Reference perfusion was measured in an area remote from the wound (Supplementary Figure 2), whereas wound edge perfusion was measured in the surrounding skin within 5 mm from the wound border. Blood perfusion of the wound edge is reported as delta perfusion units (dPFU; wound edge perfusion subtracted by reference perfusion). In the SAD part of the study, local mechanism of action was assessed by histology of wound biopsies stained for CXCL12 (NSJ Bioreagents, RQ4559), as well as ELISA to measure total local CXCL12 levels in the wound and immediate surrounding tissue.

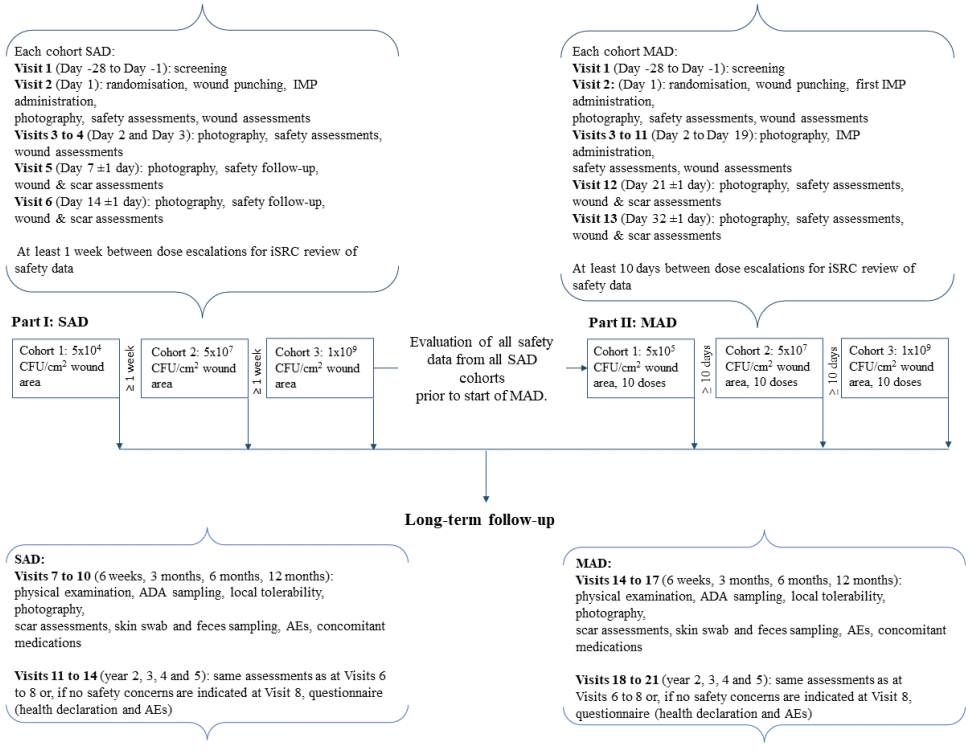
Statistical analysis

The statistical analyses for safety and clinical efficacy endpoints included all randomised individuals who received at least one dose of the IMP (Full analyses set; FAS).

No formal sample size calculations were performed for this first-in-human study with the primary objective to study safety and tolerability. The duration of the Treatment Period was selected as sufficiently long in order to assess the safety, PK/PD and preliminary efficacy of ILP100-Topical treatment. This is anticipated long enough to provide initial information about a clinical efficacy during treatment and sufficiently long to capture delayed AEs, a delayed onset of action and requirement of maintenance treatment versus a single dose.

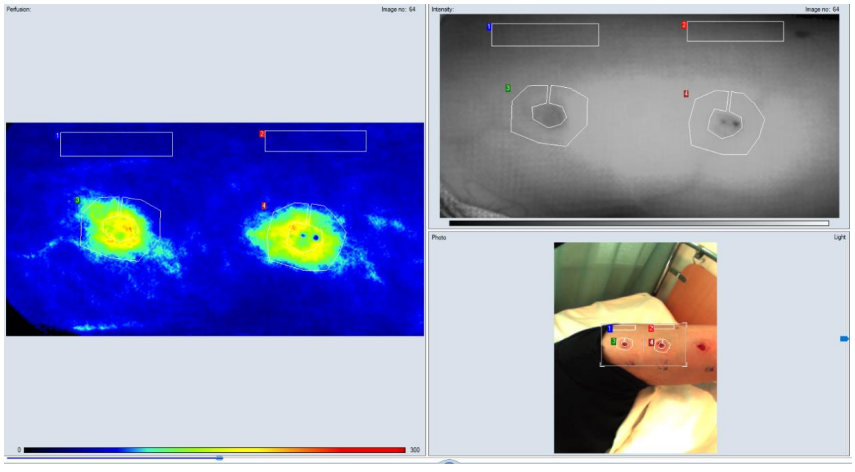
Predefined statistical analyses included a mixed linear regression model for analyses of pairwise (left and right arm as well as wound position on the arm) treatment comparison of time to first registered wound healing and McNemar's paired test for proportion of healed wounds at each timepoint. However, at study design and regulatory approval, as well as at database lock, the wounds in the different treatments groups were considered to be most appropriately analysed as independent based on the influence from biologic parameters related wound healing associated with different use of the dominant and non-dominant hands. These parameters include blood circulation, muscle mass, activity, metabolism and structures of underlying muscles and other tissues, as well as mechanical impact on underlying tissues and abrasion of the skin. In the post-hoc analyses, the biologic and clinical effects were analysed using Fisher´s exact test and the Mann-Whitney test for comparing the different treatment groups for proportion healed wounds and average time to first registered healing. Since the IEs did not perform assessments after Day 32, and the Investigators assessed all wounds as healed on Day 61 (2 months) after wound induction, 61 days was imputed as the timepoint for healing if no earlier timepoint was registered for healing or for wounds with missing data. A safety review committee reviewed all safety and tolerability data throughout the treatment phase. Given the primary objective to assess the safety and tolerability, and the hypothesis-testing nature of the biologic and clinical assessments of wound healing, no adjustments for multiplicity was made.

Supplementary figures

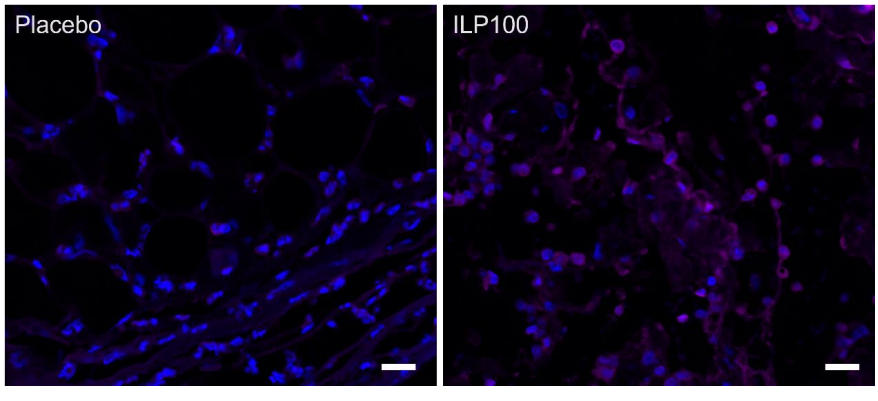


AE=Adverse Event, IE=Independent Evaluator, IMP=Investigational Medicinal Product, iSRC=Independent Safety Review Committee, LASCA=Laser Speckle Contrast Analysis, MAD=Multiple Ascending Dose, SAD=Single Ascending Dose. IMP refers to ILP100, Saline, or Placebo. Safety assessments included ECG, physical examination, vital signs, blood sampling for lab safety, local tolerability, AEs, and CXCL12 anti-drug antibody formation. Wound assessments included CXCL12 blood levels (ELISA), CXCL12 exposure in the wound (immunohistochemistry), microcirculation in the wound (LASCA), as well as evaluation of wound area, wound rupture, and wound healing by IEs (2D photographs). Scar assessments included 3D scanning measurements, as well as evaluation of scar formation by the Investigator and IEs.

Supplementary Figure 1. Overview of the study design



Supplementary Figure 2. Blood perfusion analysis. Blood perfusion of wound edges was measured using LASCA (Laser Speckle Contrast Analysis) at MAD visits and analysed off-site as the difference in blood perfusion between the wound edge (drawn areas surrounding wounds marked green and dark red) and reference areas (top boxes, blue and red region) in each individual.



Supplementary Figure 2. CXCL12 in wound biopsies in control- or ILP100-Topical-treated groups (SAD). Representative images of immunofluorescence staining of CXCL12 measured 48 hours post-wound induction in ILP100-Topical or placebo-treated wound biopsies. Blue: nuclei, Magenta: CXCL12, White bar 20 µm.

Supplementary tables

Supplementary Table 1. Baseline characteristics and demographics (SAD)

		Cohort 1 (N=4)	Cohort 2 (N=4)	Cohort 3 (N=4)	Total (N=12)
Age (years)	n	4	4	4	12
	Mean (SD)	35.8 (4.6)	33.8 (3.8)	35.8 (4.3)	35.1 (4.0)
	Median (Min, Max)	36.5 (30, 40)	34.0 (30, 37)	36.5 (30, 40)	36.0 (30, 40)
Body Mass Index (kg/m2)	n	4	4	4	12
	Mean (SD)	23.8 (3.6)	24.2 (2.9)	25.8 (3.6)	24.6 (3.2)
	Median (Min, Max)	23.6 (21, 28)	24.6 (21, 27)	26.0 (22, 29)	24.6 (21, 29)
Height (cm)	n	4	4	4	12
	Mean (SD)	173.5 (6.5)	166.5 (12.7)	187.8 (7.8)	175.9 (12.5)
	Median (Min, Max)	173.5 (167, 180)	166.0 (152, 182)	188.0 (178, 197)	178.0 (152, 197)
Weight (kg)	n	4	4	4	12
	Mean (SD)	71.8 (13.5)	67.0 (8.8)	90.5 (8.3)	76.4 (14.2)
	Median (Min, Max)	70.0 (58, 89)	65.0 (59, 79)	88.5 (83, 102)	77.0 (58, 102)
Sex	Female	2 (50%)	1 (25%)	0	3 (25%)
	Male	2 (50%)	3 (75%)	4 (100%)	9 (75%)
Race	American Indian Or Alaska Native	0	0	0	0
	Asian	1 (25%)	1 (25%)	0	2 (17%)
	Black Or African American	0	0	0	0
	Native Hawaiian Or Other Pacific Islander	0	0	0	0
	White	3 (75%)	3 (75%)	4 (100%)	10 (83%)

Supplementary Table 2. Baseline characteristics and demographics (MAD)

		Cohort 1 (N=8)	Cohort 2 (N=8)	Cohort 3 (N=8)	Total (N=24)
Age (years)	n	8	8	8	24
	Mean (SD)	31.9 (5.5)	30.5 (3.6)	36.0 (5.6)	32.8 (5.3)
	Median (Min, Max)	30.5 (26, 42)	31.0 (26, 36)	35.5 (26, 44)	33.0 (26, 44)
Body Mass Index (kg/m2)	n	8	8	8	24
	Mean (SD)	24.2 (2.0)	24.6 (2.0)	25.8 (2.1)	24.9 (2.1)
	Median (Min, Max)	24.0 (20, 27)	25.2 (21, 27)	24.9 (24, 29)	24.9 (20, 29)
Height (cm)	n	8	8	8	24
	Mean (SD)	175.8 (4.6)	178.9 (9.5)	176.5 (9.3)	177.0 (7.9)
	Median (Min, Max)	176.0 (169, 181)	179.0 (169, 191)	178.5 (158, 189)	178.0 (158, 191)
Weight (kg)	n	8	8	8	24
	Mean (SD)	74.8 (7.8)	79.3 (12.4)	80.3 (8.8)	78.1 (9.8)
	Median (Min, Max)	77.0 (58, 84)	81.5 (60, 92)	78.5 (69, 99)	77.5 (58, 99)
Sex	Female	5 (63%)	0	2 (25%)	7 (29%)
	Male	3 (38%)	8 (100%)	6 (75%)	17 (71%)
Race	Asian	0	1 (13%)	0	1 (4.2%)
	Black Or African American	0	1 (13%)	0	1 (4.2%)
	White	8 (100%)	6 (75%)	8 (100%)	22 (92%)

Supplementary Table 3. Overview of adverse events up to 13 months follow-up in the SAD part. Percentages are based on the number of individuals in the study period included in the full analysis set, n, number of individuals; m, number of events. Study treatment refer to ILP100-Topical, placebo or saline. Percentages are based on the number of individuals included in the full analysis set. Pre-treatment events are not included.

	Cohort 1 N=4		**Cohort 2 N=4**		**Cohort 3 N=4**		**Total N=12**	
	n (%)	**m**	**n (%)**	**m**	**n (%)**	**m**	**n (%)**	**m**
Any AE	4 (100%)	42	4 (100%)	36	4 (100%)	30	12 (100%)	108
Any SAE	0	0	0	0	0	0	0	0
Any AE leading to withdrawal of study drug	0	0	0	0	0	0	0	0
Any AE leading to death	0	0	0	0	0	0	0	0
Relationship with study treatment								
Unlikely Related	4 (100%)	19	4 (100%)	13	4 (100%)	9	12 (100%)	41
Possibly Related	4 (100%)	23	4 (100%)	22	4 (100%)	18	12 (100%)	63
Probably Related	0	0	1 (25%)	1	3 (75%)	3	4 (33%)	4
Severity								
Mild	4 (100%)	34	4 (100%)	36	4 (100%)	30	12 (100%)	100
Moderate	1 (25%)	8	0	0	0	0	1 (8.3%)	8
Severe	0	0	0	0	0	0	0	0
Life-Threatening	0	0	0	0	0	0	0	0
Death	0	0	0	0	0	0	0	0

Supplementary Table 4. Overview of adverse events up to 13 months follow-up in the MAD part. Study treatment refers to ILP100-Topical, placebo or Saline. n equals number of individuals, m, number of events, SAE, Serious Adverse Events, and AE, Adverse Events. Percentages are based on the number of individuals included in the full analysis set. Pre-treatment events are not included.

	Cohort 1 N=8		**Cohort 2 N=8**		**Cohort 3 N=8**		**Total N=24**	
	n (%)	**m**	**n (%)**	**m**	**n (%)**	**m**	**n (%)**	**m**
Any AE	8 (100%)	81	8 (100%)	88	8 (100%)	57	24 (100%)	226
Any SAE	0	0	0	0	0	0	0	0
Any AE leading to discontinuation of study treatment	2 (25%)[a]	28	1 (13%)[b]	1	1 (13%)[c]	1	4 (17%)	30
Any AE leading to death	0	0	0	0	0	0	0	0
Relationship of AE with study treatment								
Unlikely Related	7 (88%)	23	7 (88%)	30	7 (88%)	25	21 (88%)	78
Possibly Related	8 (100%)	58	8 (100%)	58	8 (100%)	32	24 (100%)	148
Probably Related	0	0	0	0	0	0	0	0
Severity								
Mild	8 (100%)	77	8 (100%)	87	8 (100%)	56	24 (100%)	220
Moderate	4 (50%)	4	1 (13%)	1	0	0	5 (21%)	5
Severe	0	0	0	0	1 (13%)	1	1 (4.2%)	1
Life-Threatening	0	0	0	0	0	0	0	0
Death	0	0	0	0	0	0	0	0

a. One study individual discontinued treatment due to wound infection after 4 doses of ILP100-Topical, 4 doses of placebo, and 6 doses of saline. Another study individual discontinued treatment on placebo and saline treated wounds due to wound site eczema after receiving 4 doses.

b. One study individual did not perform day 12 to 19 due to upper respiratory tract infection, received 6 doses of ILP100-Topical, placebo, and saline.

c. One study individual did not perform day 17 due to diarrhea. This study individual received 9 doses of ILP100-Topical, placebo and saline.

Supplementary Table 5. Inflammation of skin surrounding the wound score by Independent Evaluators. Mean tolerability score for inflammation of skin surrounding the wound assessed by the three IEs. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group. Control group includes saline- or placebo-treated wounds pooled. Mean score comparisons were made for each time point by Mann-Whitney test, * indicates p-value <0.05.

			Inflammation of skin surrounding the wound											
			Pre	**Post**	**2**	**3**	**5**	**8**	**10**	**12**	**15**	**19**	**21**	**32**
Cohort 1	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.43*	0.66*	0.74	0.66	0.43	0.48	0.55	0.56	0.68	0.42	0.41	0.062
		SEM	0.038	0.072	0.095	0.069	0.046	0.091	0.11	0.10	0.13	0.083	0.13	0.023
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.56	0.92	0.73	0.51	0.48	0.61	0.40	0.48	0.35	0.64	0.59	0.093
		SEM	0.051	0.078	0.096	0.074	0.045	0.098	0.078	0.075	0.062	0.12	0.13	0.040
Cohort 2	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.41	0.68	0.90	0.42	0.48	0.29	0.20	0.10	0.073	0.031	0.062	0.0
		SEM	0.047	0.094	0.12	0.079	0.086	0.047	0.033	0.031	0.029	0.017	0.028	0.0
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.40	0.67	0.65	0.31	0.31	0.22	0.18	0.18	0.062	0.052	0.062	0.010
		SEM	0.058	0.074	0.052	0.033	0.069	0.032	0.042	0.033	0.023	0.022	0.028	0.010
Cohort 3	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.43	0.51	0.91*	0.87	0.40	0.42	0.31	0.26	0.13	0.093	0.073	0.010
		SEM	0.038	0.048	0.085	0.084	0.053	0.060	0.047	0.049	0.042	0.031	0.036	0.010
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.41	0.57	0.69	0.67	0.40	0.27	0.26	0.20	0.14	0.073	0.073	0.052
		SEM	0.033	0.058	0.080	0.084	0.044	0.046	0.047	0.047	0.033	0.029	0.053	0.052

Supplementary Table 6. Inflammation of wound and wound edge score by Independent Evaluators. Mean tolerability score for inflammation of wound and wound edge assessed by the three IEs. Mean ±SEM. N, number of individuals; m, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled. Mean score comparisons were made for each time point by Mann-Whitney test, * indicates p-value <0.05.

			Inflammation of wound and wound edge											
			Pre	**Post**	**2**	**3**	**5**	**8**	**10**	**12**	**15**	**19**	**21**	**32**
Cohort 1	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	1.4	1.4	1.5	1.5	1.0	1.2	1.2	1.5	1.3	0.90	0.73	0.54
		SEM	0.095	0.096	0.10	0.11	0.12	0.14	0.14	0.12	0.14	0.12	0.11	0.065
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	1.5	1.6	1.5	1.3	1.1	1.2	1.3	1.4	1.1	0.98	0.94	0.48
		SEM	0.071	0.083	0.11	0.13	0.13	0.16	0.14	0.12	0.12	0.12	0.12	0.070
Cohort 2	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.97	1.2	1.4	1.6	2.1*	1.8	1.4	1.1	0.53	0.36	0.40	0.14
		SEM	0.073	0.093	0.12	0.10	0.056	0.069	0.10	0.11	0.10	0.084	0.085	0.075
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	1.1	1.3	1.3	1.4	1.9	1.9	1.6	1.2	0.83	0.41	0.35	0.26
		SEM	0.074	0.11	0.11	0.11	0.058	0.065	0.084	0.12	0.11	0.082	0.092	0.088
Cohort 3	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.73	1.2	1.3	1.9*	2.1*	1.9	1.6*	1.1	0.68	0.51	0.37	0.052*
		SEM	0.072	0.11	0.11	0.073	0.044	0.074	0.080	0.090	0.081	0.087	0.079	0.043
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.68	1.1	1.3	1.2	1.8	1.9	1.4	1.1	0.80	0.70	0.54	0.17
		SEM	0.073	0.098	0.11	0.11	0.082	0.078	0.086	0.069	0.086	0.085	0.080	0.076

Supplementary Table 7. **Haemorrhage score by Independent Evaluators**. Mean tolerability score for haemorrhage assessed by the three IEs. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled. Mean score comparisons were made for each time point by Mann-Whitney test, * indicates p-value <0.05.

			Haemorrhage											
			Pre	**Post**	**2**	**3**	**5**	**8**	**10**	**12**	**15**	**19**	**21**	**32**
Cohort 1	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	1.5	1.2	1.1	0.56	0.33	0.20	0.15	0.11	0.073	0.042	0.031	0.0
		SEM	0.10	0.11	0.13	0.084	0.060	0.047	0.062	0.049	0.029	0.020	0.017	0.0
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	1.4	1.4	1.1	0.59	0.45	0.20	0.16	0.18	0.15	0.062	0.042	0.010
		SEM	0.12	0.13	0.13	0.048	0.055	0.042	0.042	0.056	0.047	0.023	0.020	0.010
Cohort 2	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	1.2	1.1	0.38*	0.40	0.39	0.28	0.26	0.16	0.16	0.30	0.18	0.031
		SEM	0.099	0.15	0.10	0.081	0.098	0.065	0.080	0.050	0.067	0.11	0.091	0.017
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	1.1	0.91	0.76	0.40	0.51	0.40	0.35	0.35	0.23	0.22	0.17	0.063
		SEM	0.12	0.094	0.13	0.10	0.12	0.13	0.10	0.11	0.075	0.10	0.091	0.032
Cohort 3	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	2.0	0.89	0.48	0.26	0.47	0.30	0.39	0.39	0.32	0.18	0.21	0.021
		SEM	0.13	0.12	0.088	0.053	0.099	0.078	0.11	0.072	0.064	0.058	0.070	0.014
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	2.0	1.1	0.93	0.53	0.46	0.47	0.42	0.32	0.23	0.17	0.10	0.0
		SEM	0.13	0.13	0.17	0.15	0.10	0.098	0.085	0.063	0.048	0.087	0.053	0.0

Supplementary Table 8. Exudate score by Independent Evaluators. Mean tolerability score for exudate assessed by the three IEs. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled. Mean score comparisons were made for each time point by Mann-Whitney test, * indicates p-value <0.05.

			Exudate											
			Pre	**Post**	**2**	**3**	**5**	**8**	**10**	**12**	**15**	**19**	**21**	**32**
Cohort 1	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.25*	0.41	1.9	1.9*	2.1*	1.9*	1.4*	1.1*	1.1*	0.46	0.30	0.0*
		SEM	0.030	0.051	0.089	0.11	0.089	0.072	0.14	0.14	0.13	0.12	0.097	0.0
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.43	0.48	1.7	1.4	0.90	0.96	0.81	0.56	0.39	0.35	0.30	0.083
		SEM	0.050	0.052	0.12	0.12	0.10	0.096	0.083	0.090	0.060	0.058	0.055	0.030
Cohort 2	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.27	0.57*	0.76*	1.6	2.0*	1.8*	1.5*	1.2*	0.91*	0.48	0.21	0.0*
		SEM	0.043	0.11	0.11	0.092	0.12	0.095	0.12	0.13	0.12	0.097	0.068	0.0
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.28	0.81	1.7	1.5	1.2	0.72	0.72	0.54	0.50	0.44	0.29	0.094
		SEM	0.034	0.10	0.11	0.10	0.11	0.045	0.076	0.049	0.064	0.075	0.067	0.031
Cohort 3	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.39*	0.72	1.4	1.7	0.68*	0.77	0.81*	0.82*	0.52	0.33	0.26	0.15*
		SEM	0.034	0.094	0.13	0.13	0.068	0.077	0.054	0.10	0.052	0.056	0.055	0.042
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.28	0.84	1.6	1.5	1.2	0.97	0.66	0.53	0.49	0.34	0.21	0.031
		SEM	0.045	0.060	0.13	0.087	0.096	0.081	0.077	0.045	0.050	0.055	0.044	0.023

Supplementary Table 9. Slough score by Independent Evaluators. Mean tolerability score for slough assessed by the three IEs. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled. Mean score comparisons were made for each time point by Mann-Whitney test, * indicates p-value <0.05.

			Slough											
			Pre	**Post**	**2**	**3**	**5**	**8**	**10**	**12**	**15**	**19**	**21**	**32**
Cohort 1	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.0	0.016	0.073	0.28	0.31	0.44	0.45	0.33	0.34	0.27	0.12	0.0*
		SEM	0.0	0.016	0.029	0.050	0.042	0.089	0.081	0.045	0.044	0.041	0.033	0.0
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.0	0.031	0.062	0.19	0.39	0.48	0.51	0.37	0.36	0.27	0.21	0.083
		SEM	0.0	0.017	0.023	0.033	0.054	0.065	0.065	0.044	0.053	0.041	0.042	0.033
Cohort 2	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.010	0.094*	0.20	0.24	0.21	0.22	0.31	0.34	0.17	0.042	0.16	0.031
		SEM	0.010	0.034	0.043	0.067	0.068	0.055	0.063	0.069	0.033	0.025	0.040	0.017
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.0	0.031	0.073	0.16	0.11	0.22	0.20	0.18	0.15	0.11	0.083	0.052
		SEM	0.0	0.023	0.029	0.033	0.032	0.046	0.039	0.040	0.042	0.041	0.026	0.022
Cohort 3	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.042	0.073	0.31*	0.40*	0.35	0.52*	0.32	0.27	0.20	0.21	0.073	0.042
		SEM	0.020	0.029	0.072	0.088	0.098	0.11	0.083	0.041	0.029	0.042	0.025	0.020
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.031	0.010	0.073	0.078	0.22	0.25	0.23	0.18	0.21	0.20	0.14	0.094
		SEM	0.017	0.010	0.029	0.027	0.045	0.067	0.038	0.040	0.033	0.036	0.029	0.027

Supplementary Table 10. Granulation score by Independent Evaluators. Mean tolerability score for granulation assessed by the three IEs. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled. Mean score comparisons were made for each time point by Mann-Whitney test, * indicates p-value <0.05.

			Granulation											
			Pre	**Post**	**2**	**3**	**5**	**8**	**10**	**12**	**15**	**19**	**21**	**32**
Cohort 1	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,28
		Mean	0.36	0.33	0.39	0.41	0.51	0.51	0.80	1.2	0.98	0.81	0.50	0.11
		SEM	0.035	0.030	0.040	0.051	0.074	0.083	0.10	0.12	0.14	0.16	0.13	0.074
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,28
		Mean	0.29	0.40	0.37	0.44	0.43	0.56	0.84	1.1	1.1	0.75	0.62	0.30
		SEM	0.033	0.028	0.038	0.042	0.056	0.086	0.098	0.12	0.14	0.15	0.15	0.12
Cohort 2	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,31	8,32
		Mean	0.30	0.27	0.44	0.47	0.47	0.62	0.77	0.85	0.58	0.36	0.29	0.078
		SEM	0.023	0.035	0.064	0.057	0.065	0.090	0.10	0.10	0.13	0.12	0.12	0.046
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,28	8,32
		Mean	0.27	0.35	0.37	0.35	0.49	0.67	0.94	0.87	0.49	0.45	0.26	0.11
		SEM	0.028	0.033	0.046	0.057	0.046	0.084	0.11	0.13	0.12	0.13	0.11	0.058
Cohort 3	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,31	8,32
		Mean	0.16	0.14	0.34	0.30	0.64	0.53	0.73	0.48*	0.23	0.078	0.20	0.094
		SEM	0.030	0.029	0.043	0.054	0.061	0.085	0.091	0.11	0.075	0.064	0.081	0.052
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,31	8,32
		Mean	0.12	0.19	0.34	0.41	0.54	0.65	0.82	0.82	0.35	0.22	0.17	0.0
		SEM	0.029	0.030	0.040	0.054	0.064	0.072	0.10	0.13	0.11	0.092	0.062	0.0

Supplementary Table 11. Hypergranulation score by Independent Evaluators. Mean tolerability score for hypergranulation assessed by the three IEs. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled. Mean score comparisons were made for each time point by Mann-Whitney test, * indicates p-value <0.05.

			Hypergranulation											
			Pre	**Post**	**2**	**3**	**5**	**8**	**10**	**12**	**15**	**19**	**21**	**32**
Cohort 1	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,28
		Mean	0.0	0.021	0.0	0.0	0.0	0.0	0.010	0.021	0.031	0.052	0.073	0.012
		SEM	0.0	0.014	0.0	0.0	0.0	0.0	0.010	0.014	0.023	0.026	0.029	0.012
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,28
		Mean	0.010	0.0	0.0	0.010	0.0	0.0	0.010	0.031	0.062	0.18	0.11	0.060
		SEM	0.010	0.0	0.0	0.010	0.0	0.0	0.010	0.017	0.028	0.083	0.038	0.025
Cohort 2	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,31	8,32
		Mean	0.0	0.0	0.010	0.031	0.021	0.010	0.010	0.010	0.010	0.021	0.086	0.0
		SEM	0.0	0.0	0.010	0.017	0.014	0.010	0.010	0.010	0.010	0.014	0.046	0.0
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,28	8,32
		Mean	0.0	0.0	0.010	0.0	0.031	0.010	0.0	0.021	0.010	0.031	0.095	0.0
		SEM	0.0	0.0	0.010	0.0	0.017	0.010	0.0	0.014	0.010	0.017	0.054	0.0
Cohort 3	**ILP100**	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.0	0.0	0.010	0.010	0.0	0.010	0.031	0.021	0.073	0.083	0.073	0.0
		SEM	0.0	0.0	0.010	0.010	0.0	0.010	0.017	0.014	0.051	0.083	0.073	0.0
	Control	**n,N**	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32	8,32
		Mean	0.0	0.0	0.0	0.0	0.0	0.010	0.11	0.052	0.083	0.016	0.042	0.0
		SEM	0.0	0.0	0.0	0.0	0.0	0.010	0.044	0.026	0.064	0.016	0.042	0.0

Supplementary Table 12. Wound bed perfusion analysed using non-invasive imaging Laser Speckle Contrast Analysis (LASCA) at Days 2, 8, and 15 in ILP100-Topical and control wounds (MAD). Delta perfusion units (dPFU) are given and represent perfusion of the wound bed minus the reference perfusion within the same image. Mean ±SEM. n, number of individuals; N, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled. p= p-value by Mann-Whitney test.

LASCA perfusion imaging		**Day 2**			**Day 8**			**Day 15**		
	n, N	**ILP100**	**Control**	**p**	**ILP100**	**Control**	**p**	**ILP100**	**Control**	**p**
Cohort 1	7-8, 26-32	99.7±6.2	99.2±6.5	0.99	127.3±15.0	112.3±14.5	0.39	115.6±15.6	125.7±13.2	0.36
Cohort 2	6-8, 24-32	109.3±6.8	101.5±6.0	0.12	171.4±8.5	163.7±11.9	0.54	80.1±7.4	85.5±9.3	0.48
Cohort 3	7-8, 28-32	108.4±5.9	92.7±7.4	0.13	163.4±10.6	155.3±7.8	0.30	62.8±6.6	67.0±7.5	0.70
Cohort 1-3	21-24,84-96	106.1±3.7	97.6±3.9	0.06	154.8±6.8	144.7±7.1	0.18	89.6±7.6	96.6±7.1	0.23

Supplementary Table 13. Biopsies analysed for total CXCL12 levels at 48 hours following single dose administration of ILP100-Topical or placebo in the SAD part. Values are presented as Mean ±SEM, comparing placebo-treated and ILP100-Topical-treated wounds for each subject in the cohort. n, number of individuals; m, number of wound biopsies analysed.

CXCL12 in wound biopsies at 48 hours normalised to total protein (pg/mg total protein)	n, m	ILP100 (m=4/cohort)	Placebo (m=4/cohort)	Ratio
Cohort 1	4, 8	113.7±50.6	109.5±36.5	1.0
Cohort 2	4, 8	64.7±9.1	95.8±13.3	0.7
Cohort 3	4, 8	85.0±24.1	95.9±26.1	1.0
Cohort 1-3	12, 24	87.8±3.5	100.4±3.5	0.9

Supplementary Table 14. Scar area measured using 3D spectroscopic scanning 2, 4, 7, and 13 months post wound induction in ILP100 and control group (MAD). Measurements were made using 3D spectroscopic scanning. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group, Control group include saline- or placebo-treated wounds pooled. p= p-value by Mann-Whitney's exact test.

Scar area	n, N	2 months			4 months			7 months			13 months		
		ILP100	Control	p	ILP100	Control	p	ILP100	Control	p	ILP100	Control	p
Cohort 1	7-8,28-32	32.2±9.2	32.1±9.4	0.92	34.9±7.1	36.7±6.5	0.21	44.4±10.8	45.0±10.9	0.90	51.4±12.8	55.1±12.4	0.14
Cohort 2	7-8, 28-31	37.3±8.1	40.8±8.9	0.30	46.8±13.1	45.0±11.9	0.90	48.0±10.2	48.3±8.5	0.66	49.7±13.8	49.1±12.0	0.93
Cohort 3	8,31-32	36.6±10.5	34.1±5.8	0.75	39.2±7.3	38.5±9.2	0.79	45.3±8.8	43.7±10.9	0.28	47.6±8.8	44.6±8.9	0.21
Cohort 1-3	23-24,88-95	35.4±9.5	35.6±8.9	0.76	40.0±10.5	39.9±9.9	0.62	45.9±10.0	45.6±10.3	0.77	49.5±12.4	49.5±11.9	0.99

Supplementary Table 15. Intra-scan variability and inter-scan variability of 3D spectroscopic scanning of scars. Standard deviation (SD) range and coefficient of variance (CV) range for 5 repeated measurements performed on the scar in the same 3D scan (intra-scan variability) and in 5 different scans taken consecutively of the same scar (inter-scan variability). Mean SD refers to the mean value of all SD measurements in the evaluation.

		Intra-scan variability			Inter-scan variability		
	# scars	SD range	SD mean	CV (%) range	SD range	SD mean	CV (%) range
Area (mm^2)	4	0.664-2.574	1.720	3-9	0.562-1.868	1.283	2-7
Volume (mm^3)	4	0.037-0.482	0.087	7-61	0.106-0.483	0.252	8-44

Supplementary Table 16. Scar volume measured using 3D spectroscopic scanning 2, 4, 7, and 13 months post wound induction in ILP100 and control group (MAD). Measurements were made using 3D spectroscopic scanning. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group. Control group include saline- or placebo-treated wounds pooled.

Scar volume	n, N	2 months		4 months		7 months		13 months	
		Control	ILP100	Control	ILP100	Control	ILP100	Control	ILP100
Cohort 1	7-8,28-32	1.28±1.23	1.05±0.81	1.98±1.68	1.71±1.40	2.77±2.98	2.88±2.82	1.49±4.62	0.73±1.14
Cohort 2	7-8, 28-31	2.92±2.64	1.57±1.79	3.50±3.49	3.80±4.34	2.71±2.70	3.49±3.63	2.30±3.02	3.00±4.86
Cohort 3	8,31-32	1.84±1.62	2.47±2.47	2.32±1.93	2.71±1.85	2.11±2.28	2.30±1.50	1.93±2.64	2.06±2.42
Cohort 1-3	23-24,88-95	2.00±2.01	1.72±1.92	2.57±2.51	2.70±2.85	2.52±2.64	2.88±2.79	1.91±3.49	1.91±3.25

Supplementary Table 17. **Intra-scan variability and inter-scan variability of 3D spectroscopic scanning of the colouring of wound areas.** Standard deviation (SD) range and coefficient of variance (CV) range for 5 repeated measurements performed on skin areas or wounds in the same 3D scan (intra-scan variability) and in 5 different scans taken consecutively of the same skin area or wound (inter-scan variability). Mean SD refers to the mean value of all SD measurements in the evaluation.

	Intra-scan variability				Inter-scan variability			
	# areas	**SD range**	**SD mean**	**CV (%) range**	**# areas**	**SD range**	**SD mean**	**CV (%) range**
Redness (all)	20	0.00-0.109	0.049	0-50	10	0.011-0.054	0.034	0-25
Redness score 0.7-0.9	14	0.00-0.109	0.059	6-13	8	0.011-0.054	0.036	1-6
Redness score 0.4-0.6	3	0.00	0.000	0	-	-	-	-
Redness score 0.1-0.3	3	0.000-0.044	0.018	0-25	2	0.011, 0.175	0.028	6, 25%

Supplementary Table 18. Scar redness measured using 3D spectroscopic scanning 2, 4, 7, and 13 months post wound induction in ILP100 and control group (MAD). Measurements were made using 3D spectroscopic scanning. Mean ±SEM. n, number of individuals; m, number of wound frames analysed per group, Control group include saline- or placebo-treated wounds pooled. p= p-value by Mann-Whitney test.

Scar redness	**n, N**	**2 months**			**4 months**			**7 months**			**13 months**		
		Control	**ILP100**	**p**	**Control**	**ILP100**	**p**	**Control**	**ILP100**	**p**	**Control**	**ILP100**	**p**
Cohort 1	7-8, 28-32	0.71±0.019	0.77±0.024	0.075	0.72±0.019	0.70±0.026	0.86	0.54±0.017	0.52±0.027	0.32	0.46±0.041	0.47±0.047	0.49
Cohort 2	7-8, 28-31	0.62±0.017	0.64±0.020	0.66	0.62±0.024	0.60±0.023	0.46	0.47±0.036	0.50±0.026	0.98	0.26±0.037	0.23±0.038	0.59
Cohort 3	8, 31-32	0.63±0.022	0.65±0.030	0.43	0.61±0.028	0.61±0.024	0.99	0.40±0.033	0.37±0.047	0.63	0.25±0.039	0.17±0.049	0.24
Cohort 1-3	23-24, 87-95	0.66±0.012	0.68±0.016	0.16	0.65±0.015	0.64±0.015	0.56	0.47±0.018	0.46±0.021	0.53	0.32±0.024	0.28±0.029	0.53

ISBN (print version) 978-91-8046-852-7
ISBN (electronic version) 978-91-8046-853-4