

Original Article

Screening sensibility and antifungal activity after topical application of a synthetic lactoferrin-derived antimicrobial peptide

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Received November 30, 2023; Accepted February 15, 2024; Epub February 15, 2024; Published February 28, 2024

Abstract: Objective: Onychomycosis is the most common disease of the nails and constitutes about half of all nail abnormalities. Onychomycosis is usually caused by dermatophytes and incomparably less frequently by yeast-like fungi and non-dermatophyte molds. Current treatment options for onychomycosis are ineffective. Methods: This study evaluated the performance of a commercial and CE-registered product containing antimicrobial peptide hLF1-11 in vitro for treating toenail onychomycosis. In a case-control setting, nail samples from 59 volunteers were obtained before and after treatment by a pedicurist and investigated for the presence of fungi by culturing, barcode sequencing, and MALDI-TOF-MS. Results: Of 89 samples, *T. rubrum* (19%) and *C. parapsilosis* (17%) were cultured. In total, 47 samples (53%) were positive for culture. MALDI-TOF-MS could identify 28, but 19 remained unidentified; those species were not included in the commercial MALDI-TOF reference database library. A positive effect of treatment by the hLF1-11 product on 41 volunteers (1 placebo, 18 low doses, 22 high doses) was observed. No adverse effects of the peptide were observed or reported by the pedicurist or any of the participants. Conclusions: This study showed a positive therapeutic effect of a commercial product containing hLF1-11 in the case of 88.9% of the patients with onychomycosis. The present formulation of hLF1-11 into PBS is stable enough to permit storage at room temperature for at least two years.

Keywords: Onychomycosis, lactoferrin peptide, hLF1-11, topical treatment, antifungal activity

Introduction

Fungal infections of the nail are collectively called onychomycosis. A recent review of population-based studies in Europe and the US reported a mean prevalence of 4.3-8.9% [1, 2]. Onychomycosis affects up to 10-12% of the global population, and its treatment represents a significant worldwide market [3, 4]. Onychomycosis is the most common disease of the nails, constituting about half of all nail abnormalities [5, 6]. The infections are commonly caused by dermatophytes that actively degrade the keratin of the nail, in particular species of the genera *Microsporum*, *Epidermophyton*, and *Trichophyton*, and about 90% of cases are caused by *Trichophyton rubrum* [7]. The disease is generally considered a problem of a cosmetic nature but can significantly impact

patients' quality of life [8]. Onychomycosis can be transmitted mainly through direct contact or contamination when walking barefoot over surfaces contaminated with dermatophyte propagules. A clinical hallmark of onychomycosis is that the nail becomes friable, and often characteristic visible spikes occur. Typically, topical treatment is recommended when up to 50% of the distal nail plate is involved, no more than three or four nails are affected, and for early distal and lateral subungual onychomycosis treatment and superficial white onychomycosis [9].

In many countries, topical formulations based on ciclopirox, azoles, or terbinafine can be obtained prescription-free over the counter at the generic drugstore without consulting a clinician/dermatologist. Unfortunately, the overall

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effectivity of these drugs is relatively low (<10%) [10], and the treatment must be applied for at least one year [11-13]. There is a need for effective antifungal treatment of toenail infections, ideally with a highly effective and safe drug that could be used without a prescription. Antimicrobial peptides (AMP) may provide an opportunity to support the impact of using peptides in a therapeutic setting [14-17]. Human lactoferrin (hLF), an AMP present as a component of human breast milk, showed high antimicrobial properties against a broad diversity of bacteria and fungi with an excellent safety record in humans [18-20]. A synthetic fragment of the first 11 amino acids of hLF, hLF1-11, is effective against a broad range of microorganisms, including fungi, either directly by binding to the cell wall, induction of cytolysis, or indirectly through the activation of the immune system [21]. The peptide's safety against pathogens in single and repetitive doses has been established in pre-clinical and clinical settings [22]. Large-scale production can be done in different ways, such as 1) extraction from natural resources (microalgae), 2) recombinant strategies, and 3) chemical synthesis from products [14, 22] (see [Supplementary Materials](#) for more details).

In this study, we determined the antifungal efficacy of a registered commercially available product based on hLF1-11 on onychomycosis. After clinical screening, fungi were collected from the toenail scrapings of volunteers suffering from onychomycosis. Toenail scrapings were cultured, and fungi were isolated, identified, and characterized *in vitro* by culture, barcode sequencing, and MALDI-TOF MS. After that, *in vitro* antifungal susceptibility testing was determined for the commercial product that contains the hLF1-11 peptide. The medical pedicurist determined the antifungal efficacy of topical application for 3-7 months of the hLF1-11 product on toenail infections in volunteers suffering from onychomycosis. We described the synthesis and monitored the (shelf-life) stability of hLF1-11 containing the peptide by varying the temperature and storage time, as commonly determined for clinically graded solutions. The antibacterial and antifungal activity of the peptide was followed over 24 months.

Materials and methods

General

All chemicals were obtained from commercial sources and were used without further purification.

Peptide hLF1-11

The synthetic peptide corresponds to residues 1-11 [GRRRRSVQWCA; C₅₆H₉₅N₂₅₀14_S, Mw. 1415.8 Da; purity of 98.54%] derived from human lactoferrin and will be further referred to as hLF1-11. The peptide's synthesis and quality control (QC) analysis is extensively described in [Supplementary Materials](#). For placebo control experiments, a control peptide without antimicrobial action *in vitro* comprising alanines at positions 2, 3, 6, and 10 [GAARRAVQWAA; Mw. 1156.4 Da] was purchased from Pepscan, Lelystad, The Netherlands [23]. For more details, see [Supplementary Materials](#) for quality analysis and stability testing.

Microorganisms

The following microbes were used for susceptibility testing of hLF1-11. The fluconazole-resistant *C. albicans* strain Y01-19 was obtained from the Department of Infectious Diseases, Leiden University Medical Center (LUMC), Leiden, The Netherlands, and identified using barcode sequencing and MALDI-TOF MS [23]. This yeast was further identified using Candiselect (Sanofi Pasteur, Paris, France) and confirmed by the pattern of sugar utilization (API-ID32C, bioMerieux, Marcy l'Etoile, France) as described by Pincus et al. [24]. The fluconazole resistance of this isolate was evaluated, with a Minimum Inhibitory Concentration (MIC) >256 µg/mL using Etest (AB Biodisk, Solna, Sweden). Two well-characterized *Candida spp.* strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD): *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 (CBS 604). The *C. neoformans* strain was provided by the Department of Infectious Diseases, LUMC, The Netherlands. Six strains for six dermatophyte species (*viz.*, *E. floccosum*, *M. canis*, *N. gypsea*, *T. mentagrophytes*, *T. rubrum*, and *T. tonsurans*) were obtained from the Department of Infectious Diseases, LUMC. The various fungal strains were used as a refer-

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ence to compare the susceptibility and efficacy of the peptide [23].

Antifungal efficacy assays for yeast

An *in vitro* assay was used for sensitivity/selectivity testing of hLF1-11 for the most common fungi isolated from toenails with diagnosed onychomycosis. As with mammalian cells, monitoring of cell viability and growth, the internal environment of fungal pathogens becomes more reduced as the cells proliferate [25], and this process was monitored spectrophotometrically or spectrofluorometrically. The efficacy of the peptides against the various strains was quantitated using an *in vitro* microdilution procedure according to the National Committee for Clinical Laboratory Standards (NCCLS) with some minor amendments [24]. Dilutions of each modified hLF1-11 peptide were prepared with $\frac{1}{4}$ strength of medium RPMI 1640 medium R8758 (Sigma Chemical Co., St. Louis, MO) without buffering supplement [26]. The increasing strength of the medium negatively affects the peptide activity because of its high binding capacity, even to plastic surfaces. Peptide activity was tested against these fungi in peptide-friendly one-quarter strength RPMI 1640 broth. The peptide dilutions were dispensed into 96-well round-bottom polypropylene low-binding microtiter plates (Greiner Bio-one, No.: 3474 Ultra-low Attachment, Germany), sealed, and stored at room temperature until needed. The yeast cells were adjusted to a concentration of $0.5\text{--}2.5 \times 10^5$ colony forming units (CFU)/mL in RPMI 1640 medium, and an aliquot of 100 μL of this solution was added to each well of the microdilution plate (CLSI, M27-A4). AlamarBlue™ has been used to monitor the susceptibility of several yeast pathogens, e.g., *C. albicans*, *C. glabrata*, and *C. neoformans*, to amphotericin B, fluconazole, and flucytosine [27, 28] and was added at a volume of 2 μL /well. The total volume in each well was 200 μL . The cells were incubated at 35°C, and AlamarBlue™ reduction was assessed after 3 and 24 hours. Yeast cell growth (endpoints) was determined by visual color reading. Growth was monitored using a spectrophotometer (SPECTRO star Nano Absorbance Reader, BMG Labtech, Germany) at 570 nm and 600 nm. The MIC was defined as the lowest concentration of drugs that produced a prominent decrease in turbidity compared to the drug-free control (score <2). AlamarBlue™ was added to show

cell viability through the measurement of oxidation. When growth was present, the well turned to red/pink color, and when no growth was present, the well remained dark blue/purple. OD at 570 nm and 600 nm was also measured to confirm those outcomes. Each experiment consists of at least three independent replications. Values were presented as the average of three individual experiments to compare peptide efficacy.

Modified antifungal susceptibility assays for filamentous fungi

Susceptibility to hLF1-11 for filamentous fungi was determined using microdilution according to protocol CLSI-M38-A2 with some modifications. Cultures of fungi isolated from nail samples and reference strains were sub-cultured on potato dextrose agar (PDA) and incubated at 30°C for five to seven days. After this period, the plates were flooded with 5 mL sterile physiological saline (0.85%), and the conidia were gently removed from the culture surface using a Drigalski spatula. The conidia were transferred with a sterile swab to a sterile plastic conical tube, and the final volume was adjusted to 5 mL with saline. The mixtures of conidia and hyphal fragments were mixed with a vortex for 30 s, and large particles were allowed to settle for five min. The suspensions were counted with a Bürker-Türk counting chamber to $1\text{--}5 \times 10^6$ conidia/mL. This suspension was diluted 100 \times with $\frac{1}{4}$ strength of RPMI 1640 medium to final concentrations of $1\text{--}5 \times 10^4$ conidia/mL. Aliquots of 100 μL of this solution were added to 100 μL of different peptide solutions and incubated using 96-well non-binding microdilution plates (Greiner bio-one, type 650901). Finally, the agent AlamarBlue™ was added at 2 μL /well. The cells were incubated at 35°C and examined daily for up to five days. Growth was monitored using a spectrophotometer as described above. Experiments were performed for at least three independent replications. MIC values are presented as single data for clarity and ease of comparison of peptide efficacy. Values were presented as the average of three individual experiments of peptide efficacy.

Study design

Study objectives: An investigator-initiated *in vitro* study aimed to identify and quantify the fungi before treating onychomycosis in infected

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toenails donated by volunteers. A medically registered pedicurist (affiliated with the Dutch branch association, Provoet, The Netherlands) saw all volunteers who were users/purchasers of the product and conducted the treatment with a commercially available product containing the antimicrobial peptide hLF1-11. This product is sold commercially on the open market in the Netherlands, has a CE certification (Class 1-NL-CA002-2019-45945), and is commercially registered as medical devices class 1 according to art.9, paragraph 3, European directive 93/42/EEC. Manufacturer: CBMR Scientific Nanoscience BV. This study performed *in vitro* analyses to determine the fungi's presence and susceptibility to hLF1-11. Only material from volunteers was involved in this study, and this donation involved just the nail clippings collected before and after treatment. The volunteers were provided extensive information and signed a statement to take photographs and donate nail clippings. Volunteers were given a number so that anonymity and privacy were guaranteed. We designed this study to analyze the effectiveness of this commercial product. We opted for concentrations (MIC values) that were effective in previous hLF1-11 studies. The objectives of the study are: 1) to analyze the presence and identity of fungi in toenail clippings before and after therapy, 2) to assess an effective dose and time for eradicating fungi in infected toenails, and 3) to follow up with volunteers on the recurrence of toenail infections.

Study setup: Volunteers were enrolled in the study after screening by a professional certified medical pedicurist. Those with clinically suspected toenail infection(s) were included for topical treatment of infected toenails. Only affected toenails were visually monitored for each subject, but only one big toenail (digitus primus pedis) was included per subject. Before admission, a photographic record of the condition of the toenail was made, and nail clipping samples were taken to analyze the presence of dermatophytes and other fungi. Nail clippings were collected and analyzed at the Westerdijk Fungal Biodiversity Institute, The Netherlands. Samples of nail scrapings were examined by culture, and subsequently, isolates were identified by ITS barcode sequencing or MALDI-TOF-MS. Clinical responses to treatment were monitored by culturing, visual inspection by three

independent observers and the subjects' personal experiences. Also, the affected nails were categorized into their disorders by (i) low to mild fungal nail problems (low-dose group) or (ii) mild to severely infected onychomycosis (high-dose group); this due to the two concentrations of peptides with which the therapy was initiated. At each visit to the pedicurist, eventual adverse effects were evaluated using a standardized questionnaire of four questions addressing sensitivity, pain, adverse effects noticed during therapy, and thickness/disintegration or hardening of the treated nails. A pedicurist chose treatment concentration in the first study setup. Volunteers were categorized for their skin and nail disorders into three groups: (1) persons without fungal nail problems, (2) persons with mild complaints, and (3) persons with serious complaints. Treatment using different concentrations was given for low to mild fungal nail problems (hLF1-11, 3 µg/mL group), mild to severe fungal nail problems (combination group hLF1-11, 3 µg/mL or 33 µg/mL), and the high infected onychomycosis (hLF1-11, 33 µg/mL) group. After obtaining the informed consent, the volunteers were randomly assigned to treatment with either low or high peptide concentrations or a placebo (33 µg/mL of scrambled hLF1-11), according to a computer-generated randomization schedule. Test and placebo solutions were administered twice daily for at least 3-4 months at a low dose of 3 µg/mL or a high dose of 33 µg/mL as one drop application.

Endpoints and sample collection: Volunteers receiving the placebo solution (scrambled hLF1-11, 33 µg/mL) were monitored for 3-7 months. One or two samples of affected toenails were collected per volunteer during treatment to follow the effectiveness of the peptide.

Culturing of the fungi from nail clippings

Nail samples were washed in sodium hypochlorite 4 g/100 mL H₂O for 15 to 30 s to prevent bacterial growth by washing off externally present microorganisms at the clipped nails. After washing with sodium hypochlorite, the sample was neutralized with milli-Q purified water for 15-30 s, except for tiny nail samples. Small samples (<1 mm) were directly plated on malt extract agar (MEA) plates containing penicillin and streptomycin (MEA p/s, streptomycin sul-

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fate, 0.4 mg/ml; penicillin G, 0.4 mg/mL) (Difco B112, Difco Laboratories Michigan, USA). Nail flakes obtained by scraping flakes of dis-eased nail parts with a scalpel were stuck into MEA p/s agar. A maximum of eight pieces of nail flakes were incubated per agar plate at 28°C for 4 weeks. Fungal colonies growing from each flake were isolated and transferred to a new MEA slant. To prevent possible infestations with mites, the tubes were sealed with a benzyl benzoate solution of 25% and incubated at 28°C until fungal growth was observed.

Identification of fungi using MALDI-TOF MS

For the identification of molds and yeasts, MALDI-TOF MS used a Bruker Microflex and standard procedures [29, 30, 34-36]. Isolates were incubated on a glucose-yeast extract-peptone-agar (GYPA) plate at 28°C until enough growth was observed, permitting identification. The MALDI-TOF MS procedure followed the full extraction protocol [31, 32]. A suspension was made with 100 µL demi-water, one colony of the GYPA plate, and 900 µL ethanol (96%) was added. The mixture was vortexed for 5 s and centrifuged for 3 m at 14,000 rpm, and after removing the supernatant and replenishing the solvent, it was centrifuged for 60 s at 14,000 rpm. The supernatant was removed, and 10 µL 70% formic acid (FA) was added to the sample. The mixture was vortexed for 5 s, and 10 µL acetonitrile was added and vortexed for another 2 s. Mixtures were centrifuged again for 1 m at 14,000 rpm. One µL of the prepared sample was added to a spot on the MALDI-TOF MS plate and left to dry completely. To this spot, 1 µL of the matrix α -cyano-4-hydroxycinnamic acid (α -CHCA) was added, kept in the dark, and thoroughly dried before the sample was identified by MALDI-TOF MS [33].

DNA extraction

At first, glass beads (1.5-2 mm) were added to 2 mL screw-cap tubes, after which 490 µL cetyltrimethylammonium bromide (Sigma-Aldrich, USA), CTAB-buffer 2x (CTAB 2%, NaCl 1.4 M, EDTA 20 mM, Tris 100 mM) was added. Parts of the fungal colonies obtained were added to the tubes. Ten µL proteinase K solution (ThermoFisher, Grand Island, NY, USA) was added, after which the suspension was thoroughly vortexed for 10 m on a vortex Mixer (Greiner, Bio-One, Germany). The samples were then incu-

bated for one hour at 60°C and cooled on ice for a few minutes. Hereafter, 0.1 mL SEVAG (chloroform diluted 24:1 in iso-amyl alcohol) was added and mixed to form an emulsion. The samples were centrifuged for 10 m at 14,000 rpm, and the clear supernatant was harvested. To this sample, 220 µL ice-cold isopropanol (=0.55× the DNA sample size) was added, and the emulsion was remixed and centrifuged for 10 m at 14,000 rpm. The isopropanol was poured off, and 1 mL of ice-cold ethanol 70% was added. Next, the samples were centrifuged at 14,000 rpm for 2 min, and the ethanol supernatant was removed. The samples were dried in a SpeedVac (Savant Instruments Inc., Farmingdale, NY, USA), and 100 µL Tris-EDTA (TE) (Sigma-Aldrich) buffer was added. The DNA quality was tested as follows. Three µL of the sample was run for 30 m at 50 V on a 0.8% agarose gel, and the concentration was determined with a Qubit 4 Fluorometer (Qubit Fluorometric Quantification, ThermoFisher). The samples were stored at -20°C.

PCR and sequencing

The PCR mix for 1 reaction was made by adding 2.5 µL NH_4^+ buffer (10×) (Sigma-Aldrich), 15 µL sterile water, 2.5 µL dNTP mix, 1 mM (Sigma-Aldrich), 1.0 µL Mg^{2+} , 50 mM (Sigma-Aldrich), 1.0 µL ITS1 primer ((TCCGTAGGTGAACCTGCGG) [41], 10 pmol), 1.0 µL ITS4 primer ((TCCTCCGCTTATTGATATGC), 10 pmol) and 1.0 µL Taq polymerase (Bio-line, 0.5 U, Germany) to a low-binding Eppendorf tube. One µL of the DNA sample was added to the tubes of the PCR strips, after which 24 µL of the PCR mix was added. Twenty-five µL of the PCR mix without DNA was added to a tube as a negative control with the following settings: 95°C pre-denaturation for 5 min, denaturation at 95°C for 45 s, annealing at 48°C for 30 s, elongation at 72°C for 60 s and post-elongation at 72°C for 6 m. The denaturation, annealing, and elongation were done for 35 cycles. The samples were transferred to a 1.0% agarose gel slot as a quality control and run at 135 V for 20 min. The sequence mix was made by adding 3 µL buffer (Tris/Borate/EDTA), 0.7 µL Big Dye™ sequencing buffer (ThermoFisher), 4.3 µL water, 1.0 µL primer (ITS1) or (ITS4) [34] and 1 µL of the PCR amplicon. The primers were firstly diluted to a concentration of 3-4 pmol, and amplicons were diluted in 0.1 M TE buffer to 3-10 ng/µL. A PCR

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Table 1. Occurrence of fungal species sampled from 53 subjects

Isolate	Number	Isolate	Number
<i>Aspergillus carbonarius</i>	1	<i>Hypocreales spp.</i>	2
<i>Aspergillus niger</i>	1	<i>Meyerozyma guilliermondii</i>	1
<i>Aspergillus sydowii</i>	1	<i>Naganishia albidosimilis</i>	1
<i>Aspergillus tamarii</i>	6	<i>Naganishia diffluens</i>	2
<i>Aspergillus terreus</i>	1	<i>Penicillium albocorenium</i>	1
<i>Aspergillus unguis</i>	2	<i>Penicillium canescens</i>	1
<i>Aspergillus versicolor</i>	1	<i>Penicillium chrysogenum</i>	2
<i>Candida haemulonii</i>	1	<i>Tintelnotia destructans</i>	1
<i>Candida metapsilosis</i>	5	<i>Trichophyton interdigitale</i>	2
<i>Candida parapsilosis</i>	19	<i>Trichophyton mentagrophytes</i>	1
<i>Cladosporium parahalotolerans</i>	1	<i>Trichophyton rubrum</i>	10
<i>Cystobasidium calyptogenae</i>	1	<i>Trichosporon mucoides</i>	4
<i>Cystobasidium lysinophilum</i>	1	<i>Westerdykella spp.</i>	1
<i>Debaryomyces hansenii</i>	1	<i>Unidentified</i>	91
<i>Fusarium solani</i>	1		

The samples were screened for fungi with MALDI-TOF-MS, and samples with a score below 1.700 were identified with ITS1 and ITS4 sequencing. *C. parapsilosis* (32%) and *T. rubrum* (22%) were most frequently retrieved from cultured nail samples.

was performed (for ITS1 and ITS4) with the following settings: 95°C for 1 minute and 30 cycles of the following steps: 95°C for 10 s, 5 s at 50°C, and 2-4 m at 60°C. The samples were submitted, purified, and sequenced with Sanger sequencing. Strains were sequenced bi-directionally, but only once. The isolates were identified using BLAST of the National Center for Biotechnology Information (NCBI) [35].

Stability and shelf-life

Samples containing peptides were evaluated at different temperatures. Prediction of shelf life can be defined as the period during which the peptide retains its original quality. The manufacturing, formation process, and processing steps of lactoferrin from human origin must be visualized and optimized to minimize its denaturation. Procedures and results are described in [Supplementary Materials](#).

Statistical analysis

All data are presented as mean values or a percentage of the total number of patients. The Student two-tailed independent sample t-test was used to analyze differences between the treatment groups. All analyses and calculations were performed using Microsoft Office Excel 2019.

Results

Effect of peptides on the treatment of onychomycosis

The study included 59 volunteers with different clinical signs related to the severity of onychomycosis of the toenails (range 1 to 4, according to the score for the proximity of disease to the nail matrix) [43]. Six volunteers halted for personal reasons after 2-4 weeks, and those were excluded from the study. Most infected toenails were diagnosed as severe onychomycosis (high score group), and >30% of the toenail surface was affected for at least one year. From the nail samples, 89 samples were incubated for fungal growth. Forty-seven nail samples showed growth of microorganisms (52.8%), corresponding to earlier results [6, 36, 37]. Other infected toenail clippings that were culture-negative were likely infected with fungi that are difficult to culture (**Table 1**). Fifteen percent showed growth of fungal species, of which *Trichophyton spp.* and *Candida spp.* were most frequently retrieved with 22% and 32% of cultured nail samples, respectively. We found other species in 20% of volunteers, such as *Aspergillus spp.*, *Cladosporium spp.*, *Fusarium spp.*, and *Penicillium spp.* (**Table 2**). Furthermore, 28 fungal strains collected from the nail clippings were harvested and, when identified using MALDI-TOF, gave score values ≤ 1.7 (**Table 1**),

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Table 2. *In vitro* MIC 90 values of hLF1-11 in case of fungal strains isolated from the toenail samples taken from 53 volunteers

Isolate	MIC 90 (microgram/mL)	Isolate	MIC 90 (microgram/mL)
<i>Aspergillus carbonarius</i>	12.5	<i>Penicillium albocorenium</i>	50
<i>Aspergillus niger</i>	25	<i>Penicillium canescens</i>	50
<i>Aspergillus terreus</i>	25	<i>Penicillium chrysogenum</i>	25
<i>Candida haemulonii</i>	25	<i>Trichophyton interdigitale</i>	12.5
<i>Candida metapsilosis</i>	12.5	<i>Trichophyton mentagrophytes</i>	6.25
<i>Candida parapsilosis</i>	12.5	<i>Trichophyton rubrum</i>	12.5
<i>Cladosporium parahalotolerans</i>	6.25	<i>Trichosporon mucoides</i>	6.25
<i>Fusarium solani</i>	6.25		

After being cultured and isolated from the nails, the samples were identified with barcode sequencing or MALDI-TOF-MS. Minimal Inhibitory Concentration (MIC) MIC 90 values were determined in RPMI 1640 one-fourth of broth strength as assay medium. The assays were repeated in triplicate. Representative single assay data are given for clarity and ease of comparison of peptide efficacy.

Table 3. Observed visual effect of peptide antifungal activity after 12 months

hLF treatment	Patients (n)	Cured (% of total)	No effect (% of total)
Low (3 microgram/mL)	21	18 (85.7%)	3 (14.3%)
High (33 microgram/mL)	24	22 (91.7%)	2 (8.3%)
Control (placebo)	8	1 (12.5%)	7 (87.5%)

Fifty-three volunteers were treated with either placebo, or low or high dose peptide for 12 months with 2 drops daily on the infected nail. Treatment positively affected 40 out of 45 volunteers (88.9%).

and these were not further determined in this study.

Observations by a medical pedicurist about the toenail status after the treatment of volunteers at the end of the period were included in evaluating the final results (**Table 3; Figure 1A, 1B**). A positive outcome was established for 41 volunteers, of whom one received the placebo, 18 received a low dose (34%), and 22 received a high dose (42%) of hLF1-11 (**Table 3**). No effects were observed with 12 volunteers, of which 7 received the placebo. Three of them received a low dose, with one sample showing a positive culture of *C. parapsilosis*, and the other two could not be tested because they were culture-negative. The two volunteers with the highest dose during 12 months of fungal nail treatment yielded *T. interdigitale* and *Hypocreales spp.* isolates. Concentrations of both treatments of low (3 mg/L) and high (33 mg/L) doses were below the MIC value of the fungi isolated from those samples. MIC values of those strains were compared for susceptibility with MIC values from reference strains [38] (**Table 4**).

A microbicidal effect was recorded in most cases. The lowest dose of hLF1-11 was equal to

3 mg/L and yielded a positive effect in 18 volunteers, and for 22 volunteers, the dose of hLF1-11 used was equivalent to 33 mg/L. Of the 12 non-responders, seven received the placebo, three subjects received a low dose, and two received a high dose for 12 months of treatment of the toenail.

The medical-certified pedicurist's visible inspection revealed that the nail quality of volunteers with low-dose treatment improved after 4-7 months, whereas those treated with the highest dose treatment showed an improvement after 3-6 months. After 12 months, visual improvements of the nail were observed by almost 89% of the volunteers. 75.5% responded positively to the treatment, of which 9.4% had an effect after treatment with the higher peptide dose. Furthermore, 13.2% did not respond to treatment, and 1.9% (n=1) had a positive effect when treated with the placebo.

Comments from subjects obtained during the 12-month treatment with both doses of peptide revealed that sensitive and painful nails became less sensitive, and no more painful skin problems were encountered after two weeks. None of the participants reported any

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Figure 1. A. Examples of volunteers' nails. Visual effect of the peptide after 4-7 months of treatment with daily 1-2 drops (33 mg/L dose of hLF1-11 peptide). Images before and after treatment of the product and no treatment (placebo) are shown. B. Images of the feet of two volunteers at the start, after 3 months, and after 7 months of treatment.

adverse side effects, and visual inspection confirmed that thick nails became thinner and shinier (Table 5).

Discussion

This study showed that various fungal isolates obtained from onychomycosis could be identified by MALDI-TOF-MS, and their susceptibility to antimicrobial peptide hLF1-11 could be determined. Moreover, a commercially available product containing hLF1-11 reduced onychomycosis caused by these fungi. Of the 53 volunteers involved in the research, 88.9% achieved an infection-reducing effect. During the 12 months of treatment, no adverse side effects were reported. Based on this, together with the previously reported safety of systemi-

cally applied hLF1-11 [22], the treatment can be considered safe. We followed the visual outcome from the treatment with both peptide concentrations wherein the peptide is applied to the eponychium and/or proximal nail fold. The growth of nails starts in the nail root, hidden under the cuticle. When new cells at the nail's root grow, the new nail cells push out the old nail cells. These old cells flatten and harden thanks to keratin, a protein these cells make [39]. The newly formed nail then slides along the nail bed, the flat surface that protects nails. Without being bound by theory, it is thought that the antimicrobial peptide hLF1-11, when applied to the eponychium and/or the proximal nail fold, is incorporated in the newly formed nail and protects it from infection. Cell Penetrating Peptides (CPPs), such as hLF1-11,

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Table 4. Minimum Inhibitory Concentration values of the antimicrobial compound hLF1-11

Isolate	MIC 90 (microgram/mL)	Isolate	MIC 90 (microgram/mL)
Candida albicans YO1-19	12.5	Microsporum canis	25
Candida albicans ATTC 90028	12.5	Nannizzia gypsea	25
Candida parapsilosis ATTC 22019	12.5	Trichophyton mentagrophytes	25
Cryptococcus neoformans WT	12.5	Trichophyton rubrum	50
Hypocreales spp.	50	Trichophyton tonsurans	25

MIC 90 values of antifungal compound hLF1-11 peptide were determined in RPMI 1640, one-fourth strength of the broth as assay medium. Experiments: 1×10^5 CFU of various strains/mL were incubated for 24-48 h at 37 °C into one-fourth medium of RPMI 1640 with the previous concentrations of hLF(1-11), respectively. As controls, reference strains were incubated for 24-48 h at 37 °C with no agent, and then growth was monitored using a spectrophotometer at 600 nm. Results are means of at least three independent experiments. Representative single assay data are given for clarity and ease of comparison of peptide efficacy. MIC = Minimum Inhibitory Concentration.

Table 5. Questionnaire of volunteers at the end of the trial period on sensitivity/pain relief, healthier skin, thickness reduction, and color change of the nails

Observation	Improvement (% of total)
Sensitivity/pain relief	29%
Healthier skin	72%
Thickness reduction of the nail	85%
Color change of the nail	29%

can penetrate through barriers like skin, cornea, and the blood-brain barrier (BBB). However, it is difficult to rank the efficacy of CPPs because transport efficiency and tissue selectivity depend not only on the CPP itself but also on the tissue itself. The antifungal effect of hLF1-11 also relates to direct interaction with the fungal surface and allows the killing of yeast cells [23, 40].

Visible effects were already observed 3-4 weeks after the onset of treatment. When applied regularly, e.g., daily, the antimicrobial peptide will be continuously added to the new nail, protecting the growing nail from infection by fungi and/or bacteria. This evidence for an effective treatment is because there was a visual improvement in the nail appearance of an onychomycotic toenail, and thick nails became thinner and shinier as a response to the treatment as determined by a certified pedicurist.

Each volunteer visited the pedicurist once a month during the treatment period. Four outcome measures were followed during treatment: pain, infection, function/color of the toe (nail and skin), and the volunteer's perception.

29% of volunteers reported relief of pain and discomfort and improved well-being as a considerable improvement. At the end of the period, 72% of the volunteers reported skin improvement and an 85% reduction in the thickness of the nails (**Table 5**). All fungal isolates obtained from the clipped toenails responded *in vitro* well to the antimicrobial activity of hLF1-11. The two volunteers that showed no antifungal effects

with the peptide were proposed to use a concentration of 100 µg/mL per drop twice a day for the next set of months. For both these volunteers, a positive effect resulting in improved toenails was established within three months after the onset of this higher treatment regime. For three volunteers who received a low dose, only one yielded a culture of *C. parapsilosis*, and the other two subjects likely contained toenail infections with non-culturable microorganisms. From the nail samples in this study, 52.8% yielded a culture in concordance with the literature [6, 36, 41]. Ghannoum et al. discussed that reassessing the definition of an onychomycosis cure is critical [42]. In most investigations, many toenail samples collected from subjects contained visible fungal hyphae that subsequently failed to grow upon culture, and it was difficult to differentiate between "live" and "dead" fungi. It has been proposed that if there is no response to topical treatment for infected toenails, the length of treatment should be extended up to 18 months.

Many people suffering from onychomycosis do not have the discipline to complete a longitudinal treatment. With an average nail growth of about 1 mm per month (nail growth rate varies

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per person from 0.9-1.3 mm), treating onychomycosis should last at least 9 to 12 months. In volunteers receiving a low dose, the treatment showed an improvement after 4-7 months, whereas volunteers treated with the highest dose showed an improvement after 3-6 months. After 12 months, the big toe (hallux; digitus primus pedis) visual improvements were achieved in approximately 89% of the subjects. In addition, our study showed that hLF1-11 is highly effective *in vitro* against all fungi tested.

This study's limitations were the arbitrary endpoint chosen based on the cure of the toenail infection, but we did not study re-infections in the long term. The cure outcome cannot be defined because multiple factors contribute to many people dealing with fungal re-infections of toenails. In addition to the medical background (e.g., genetic predisposition to fungal nails) [12], diabetes, or immune status, many of these patients were not evaluated separately [43]. Visual inspection by a pedicurist will be beneficial in determining treatment effectiveness. Moreover, regular visits to a pedicurist will improve foot hygiene, and toenail filing may improve therapeutical outcomes and reduce cross-contamination. Cross-infection can occur from materials (shoes and socks) directly contacting microorganisms growing on feet, including nails [4].

The stability of peptides might be a problem in making products to treat onychomycosis. In general, pharmacological conditions must be applied for convenient storage. Especially with a low concentration of peptides, they often lose activity during storage at room temperature (RT). The shelf life of a peptide formulation could be limited, especially when peptides contain amino acids like cysteine, tryptophan, and glutamine. The various heat treatments did not affect modified hLF1-11, as indicated by HPLC, mass spectrometry, and antibacterial and antifungal activity. The present formulation of hLF1-11 into PBS is stable enough to permit storage at room temperature for at least two years.

Conclusions

This study showed that a commercially available product containing the antimicrobial peptide hLF1-11 could be applied to treat onychomycosis effectively. The results showed the high efficacy of the hLF1-11 peptide in reducing

fungal growth in the nails of volunteers with diagnosed onychomycosis within a few weeks of the treatment. No adverse effects were reported by any of the study participants, which suggests a promising application of this innovative formula. Fortunately, the formulation is stable enough to permit storage for at least two years.

Acknowledgements

We thank Peter Nibbering from Leiden University Medical Centre for providing a fluconazole-resistant *Candida albicans* isolate and other strains. TB thanks the Distinguished Scientists Fellowship Program from King Saud University for support. The authors thank Ferry Hagen and Bert Gerrits van den Ende for their valuable comments on the manuscript. We thank Youp van der Linden and Tony Smits for their technical support.

Disclosure of conflict of interest

CB and MR are co-founders of CBMR Scientific Inc.

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Supplementary Materials

Synthesis and stability testing of a synthetic lactoferrin-derived antimicrobial peptide

Manufacturing

Objectives: In this section, we describe the synthesis and monitored the (shelf-life) stability of hLF1-11 containing the peptide by varying the temperature and storage time, as commonly determined for clinically graded solutions. The antibacterial and antifungal activity of the peptide was followed over 24 months.

Materials used for manufacturing human lactoferrin (hLF) peptide derived from the first 11 amino acids of hLF (hLF1-11) [1-4] are the resin and the amino acid derivatives, were tested and released against approved specifications and test methods manuals [5, 6]. The structure of hLF1-11 consists of the following formula: Gly-Arg-Arg-Arg-Arg-Ser-Val-Gln-Trp-Cys-Ala. The essential raw materials used for the synthesis of hLF1-11 peptides were Fmoc-Ala-Wang resin, Boc-Gly, Fmoc-Ala, Fmoc-Cys(X), Fmoc-Trp(X), Fmoc-Gln(X), Fmoc-Val, Fmoc-Ser(X), Fmoc-Arg(X). Where Boc is tert.-butyloxycarbonyl, Fmoc is 9-fluorenylmethyloxycarbonyl, and X represents the various side-chain protecting groups.

Synthesis of hLF1-11 peptides: The process of manufacturing hLF1-11 peptides is depicted in [Figure S1](#). Here, we describe the method of every step of a solid phase synthesis of a basic peptide. The purity and length of the peptide chain measure the peptide synthesis. For GMP and GCP, synthesized peptides must undergo a quality check procedure such as HPLC and mass spectrometry. Medical products also require amino acid analysis and sequencing.

Resin-bound and fully protected hLF1-11 peptide was prepared by solid phase peptide synthesis (SPPS). SPPS immobilizes the growing peptide on an insoluble polymer (in this case, Fmoc-Alanine-wang resin). The synthesis of hLF1-11 was carried out following the general solid-phase procedure first described by Merrifield [7]. The alpha-amino group of each amino acid was protected with a 9-Fluorenylmethyloxycarbonyl group except for the N-terminal amino acid in the sequence, glycine, which was protected with tert.-butyloxycarbonyl ([Figure S2](#)).

The peptide chain was assembled by first deprotecting the Fmoc group from Fmoc-Alanine-wang resin and coupling the following amino acid-protected derivative in the sequence [Fmoc-Cysteine(X)] to Alanine [8]. The Fmoc-group was then removed from Fmoc-Cysteine(X), and the following protected amino acid derivative in the sequence [Fmoc-Trp(X)] was coupled. Repeating the cycle with the appropriate amino acid derivatives resulted in the desired sequence in a fully protected peptide resin. All amino acids in the sequence were of the naturally occurring L-configuration. Progress of the coupling reaction was monitored using the ninhydrin test, which should be negative, indicating the absence of unreacted amine. When the ninhydrin test was positive, indicating the presence of unreacted amine, the coupling was repeated using the amino acid derivative and coupling reagents, or the resin was acetylated using acetic anhydride in the presence of methylene chloride (DCM) before continuing with the synthesis.

The amino acid sequence of resin-bound hLF1-11 was as follows: Boc-Gly-Arg(X)-Arg(X)-Arg(X)-Arg(X)-Ser(X)-Val-Gln(X)-Trp(X)-Cys(X)-Ala-Wang resin.

After coupling the N-terminal amino acid (Boc-Gly), the fully protected peptide resin is washed with methanol, filtered, and dried under vacuum to a constant weight. At this stage, the peptide resin was ready for further processing.

Cleavage and deprotection stage: The protected peptide-resin precursor for hLF1-11 was cleaved from the resin and simultaneously deprotected (functional side chain groups) by acidolysis using trifluoroacetic acid (TFA, Sigma-Aldrich No. 1.08262). To prevent side reactions, scavengers are added to react with any intermediates that the cleavage reaction might generate. After cleaving the crude hLF1-11, the resin

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was filtered and washed with the TFA solution 3 times. The filtrate from the washes was evaporated under a vacuum, and the crude hLF1-11 was precipitated with ether, washed with ether, dried to a constant weight, and then purified as follows (Figure S3).

Purification stage: The crude hLF1-11 material was purified by reverse-phase high-performance liquid chromatography (RP-HPLC). This technique incorporates the difference in peptide hydrophobicities to separate peptides of closely related structures. Silica-based support containing octadecyl (C18) functional groups is the material for column packing. The interactions of peptides with this support can often separate moderate-sized peptides that differ by a single amino acid. Various peptides can be eluted by adjusting the concentration of the organic solvent. After desorption, the peptide no longer interacts with the column packing material (Figure S4).

The first purification step employed an appropriate buffer system for acetonitrile linear gradient [9]. An additional buffer system to the acetonitrile linear gradient was used for the second separation. An additional buffer system to the acetonitrile linear gradient was used for the second separation if necessary. Fractions that have the required purity, as assessed by analytical HPLC determination, were pooled, and the acetonitrile content was reduced by either evaporation or dilution with deionized water. These solutions were reloaded onto the column, and the product was converted to the hydrochloride salt form. Fractions that did not meet the required purity were discarded, pooled, and recycled through the first and/or second HPLC buffer purification processes. The product was then lyophilized to powder form using a Speedvac (Savant Instruments Inc., Farmingdale, NY, USA). The final product was packaged in glass bottles, fitted with Teflon[®]-lined polypropylene caps, and stored at -20°C.

Impurities: The hLF1-11 peptide purity was higher than or equal to 95.0%, with total unknowns determined at ≤5.0% as assessed by analytical HPLC. The most likely contaminant is the dimer formed during purification, which, because of the small difference in retention time, was extremely difficult to isolate and characterize quantitatively.

The product specifications included tests for electrospray mass spectral analysis for molecular mass determination, Edman degradation for peptide sequencing, and amino acid analysis for determination of the amino acid ratios, all used to establish the product's identity. An analytical reverse-phase HPLC technique determined the purity of the product and related substances, and nitrogen estimation was used to determine the content of the active substance.

Stability testing

Testing stability of hLF1-11: The following microbes were used for antimicrobial drugs and AMP hLF1-11 susceptibility testing. The fluconazole-resistant *C. albicans* strain Y01-19 was purchased from Leiden University Medical Center (LUMC), Leiden, The Netherlands and identified using barcode sequencing and MALDI-TOF MS. This yeast was further identified using Candiselect (Sanofi Pasteur, Paris, France) and confirmed by the pattern of sugar utilization (API-ID32C, bioMerieux, Marcy l'Etoile, France) as described [10]. Fluconazole resistance was evaluated as minimal inhibitory concentration (MIC >256 µg/mL) using the Etest (AB Biodisk, Solna, Sweden). *Staphylococcus aureus* 2141 (MRSA), a clinical isolate from the LUMC, The Netherlands, was highly resistant to a variety of antibiotics, including methicillin (MIC >256 mg/L) and vancomycin (MIC ≥32 mg/L). This strain was only used for stability testing of the effect on heat treatment and shelf-life.

Peptides were exposed for 20 min to 40°C, 60°C, 80°C, or 100°C and then immediately analyzed using HPLC and mass spectrometry and in antimicrobial assays as read-outs. hLF1-11 peptides were dissolved to 1 mg/mL H₂O and stored (75-100% humidity). Experiments were done at room temperature 25°C with 60-70% relative humidity. In a second stability experiment to determine the shelf-life, peptides were stored in the dark at various intervals (24 h, one week, four weeks, 13 weeks, 26 weeks, and 52 weeks) at 20°C or 37°C. After that, the peptides were analyzed as described above.

Antifungal efficacy assays for yeast: An *in vitro* assay was used to perform sensitivity/selectivity of hLF1-11 as described [11]. Antifungal effects of hLF1-11 also interact directly with the fungal surface and kill

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the yeast cells, as described by Mónica Viejo-Díaz [12] and Soukka [13]. The bactericidal effect was described by Arnold [14]. Antifungal activities of the peptides against the various strains were quantitated using an *in vitro* microdilution procedure as described [15].

As with mammalian cells, monitoring of cell viability and growth, the internal environment of fungal pathogens becomes more reduced as the cells proliferate [16]. This process can be monitored spectrophotometrically or spectrofluorometrically. The efficacy of the peptides against the various strains was quantitated using an *in vitro* microdilution procedure according to the CLSI (NCCLS - National Committee for Clinical Laboratory Standards) [17] with some minor amendments. Dilutions of each modified hLF1-11 peptide were prepared with $\frac{1}{4}$ strength of medium RPMI 1640 medium R8758 (Sigma Chemical Co., St. Louis, MO) without buffering supplement. The increasing strength of the medium negatively affects the peptide activity because of its high binding/sticking capacity, including plastic surfaces. The peptide activity was tested against these bacteria in peptide-friendly one-quarter strength RPMI 1640 broth. The peptide dilutions were dispensed into 96-well round-bottom polypropylene low-binding microtiter plates (Greiner Bio-one, No.: 3474 Ultra-low Attachment), sealed, and stored at room temperature until needed. The yeast cells were adjusted to a concentration of $0.5\text{-}2.5 \times 10^5$ colony forming units (CFU)/mL in RPMI 1640 medium, and an aliquot of 100 μL of this solution was added to each well of the microdilution plate (CLSI, M27-A4). Finally, AlamarBlue™ was added at a volume of 2 μL /well. AlamarBlue™ has been used as a read-out of the susceptibility of several fungal pathogens, e.g., *Candida* spp., *C. glabrata*, and *C. neoformans* to amphotericin B, fluconazole, and flucytosine. The total volume in each well was 200 μL . The cells were incubated at 35°C, and AlamarBlue™ reduction was assessed after 3 and 24 hours. Yeast cell growth (endpoints) was determined by observing visual color reading. Growth was monitored using a spectrophotometer (SPECTRO star Nano Absorbance Reader, BMG Labtech, Germany) at 570 nm and 600 nm. The MIC was defined as the lowest concentration of drug that produced a prominent decrease in turbidity compared to a drug-free control (score <2). AlamarBlue was added to show cell viability through the measurement of oxidation. When growth was present, the well turned to red/pink color, and when no growth was present, the well remained dark blue/purple. The OD600 nm was also measured to confirm outcomes. All experiments required at least three independent replications. Values were presented as the average of three individual experiments for clarity and ease of comparison of peptide efficacy.

Modified antifungal susceptibility assays for filamentous fungi: Susceptibility for fungi was determined by microdilution according to protocol CLSI-M38-A2 for filamentous strains with modification method as follows as described by Brouwer and Cudic [11, 15]. Cultures and reference strains were sub-cultured on potato dextrose agar (PDA) and incubated at 30°C for five to seven days. After this period, the plates were flooded with 5 mL of sterile physiological saline (0.85%), and the conidia were gently removed from the culture surface using a Drigalski spatula. The conidia were transferred to a sterile conical tube with a sterile swab, and the final volume was adjusted to 5 mL with saline. The conidia and hyphal fragments were mixed for 30 s and large particles were allowed to settle for 5 min. The suspension was counted in a Bürker-Türk counting chamber to $1\text{-}5 \times 10^6$ conidia/mL concentrations. This suspension was diluted 100 \times with $\frac{1}{4}$ strength of RPMI 1640 medium to final concentrations of $1\text{-}5 \times 10^4$ conidia/mL. Aliquots of 100 μL of this solution were added to 100 μL of different peptide solutions and incubated using a microdilution plate. Finally, the agent AlamarBlue™ was added with a volume of 2 μL /well. The cells were incubated at 35°C and examined daily for up to five days. Growth was monitored spectrophotometrically as described above. Experiments were performed for at least three independent replications. Values were presented as the average of three individual experiments for clarity and ease of comparison of peptide efficacy. MIC values were presented as single data for clarity and ease of comparison of peptide efficacy.

Antibacterial efficacy assays: The bactericidal activity of the peptide hLF1-11 against strains of *S. aureus* (MRSA) was determined using an *in vitro* killing assay as described [11]. Briefly, bacteria were grown on Muller Hinton agar plates for 18-24 h at 37°C. Cell concentrations were estimated by measuring the ultraviolet absorbance at 600 nm and applying the formula $\text{CFU/ml} = A_{600} \times 3.8 \times 10^8$, where CFU is the number of colony-forming units. The suspension was diluted in the same media used for the growth to reach 4×10^5 CFU/ml. The MIC was determined by broth dilution in a round-bottomed polypropylene

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Low-Binding 96-well microtiter plate (Greiner Bio-one, No.: 3474 Ultra-low Attachment). Twenty microliter aliquots of 1 mg/mL concentrated peptide in sterilized water were added to each well that contained 180 μ l one-fourth strength RPMI 1640 Medium + Glutamax (Gibco 61870). Serial dilutions were made by transfer of 100 μ l to the next well. Thereafter, 100 μ l of 10^5 CFU/ml of the test micro-organism was added to each well. Two controls were set on each plate: 1) The bacterial inoculum without any antimicrobial compound for determining the bacterial growth, and 2) Medium not inoculated for sterility control. The plate was overnight incubated at 37°C. AlamarBlue was added to show cell viability through measurement of oxidation: when growth was present, the well turned red/pink, and when no growth was present, the well remained dark blue/purple. To confirm those outcomes, the OD was measured at 600 nm on a microplate reader (SPECTRO star Nano Absorbance Reader, BMG Labtech, Germany). The MIC was calculated as the lowest antibiotic concentration at which growth was inhibited (inhibitory concentration IC₉₀). All experiments required at least three independent replications. Values were presented as the average of three individual experiments for clarity and ease of comparison of peptide efficacy.

Results

Effect of heat treatment on the stability of peptides and shelf-life: Notably, the various heat treatments (RT, 40°C, 60°C, 80°C and 100°C) did not significantly affect the fungicidal or bactericidal activity of hLF1-11 ([Table S1](#)).

MIC values for *S. aureus* 2141 (MRSA) (6.25-12.5 mg/mL), the strain used for stability testing was *C. albicans* Y01-19 (25-50 mg/mL) incubation at RT up to 100°C did not affect the peptide stability ([Table S1](#)). Mass spectrometry analysis showed that the various heat treatments did not affect the mass of hLF1-11 ([Figure S5](#)).

HPLC analysis showed that hLF1-11 was eluted at approximately 20.846 min (with 93.74% recovery). Dimerization after heat treatment was observed at 21.250 min. In addition to the highest peak, HPLC analysis showed five other tiny peaks at 20.126 min, 20.594 min, 21.250 min, 21.483 min, and 23.112 min, respectively (breakdown particles) ([Figure S6](#); [Table S2](#)).

HPLC analysis of the various peptides stored for the indicated periods on the shelf at RT showed no differences compared to a freshly dissolved peptide. These data were confirmed by mass spectrometry. Moreover, storage on the shelf (either at 20 or 37°C) over 24 months did not affect the antifungal or antibacterial activity of hLF1-11 ([Figure S7](#)).

MIC values for *C. albicans* Y01-19 (25-50 mg/mL) and *S. aureus* 2141 (MRSA) (6.25-12.5 mg/mL) were stable over the whole interval of testing (i.e., 24 h, one week, four weeks, 13, 26, 52, and 104 weeks samples at 20 and 37°C) ([Table S3](#)).

Evaluation

Our results showed that hLF1-11 peptides dissolved in a liquid formula, such as an isotonic buffer with a low pH (pH 4), remained stable for a long time.

A lack of stability of peptides could be a problem in making products to treat infections. In general, pharmacological conditions must be applied for convenient storage. Especially with a low concentration of peptides, they often lose activity during storage at RT. The shelf life of peptide formulation could be limited, especially when peptides contain amino acids like cysteine, tryptophan, and glutamine.

Once the peptide is dissolved, a buffer or salt solution may be added to obtain the desired concentration. Generally, peptides are more stable under acidic conditions, and a pH between 3.0 and 6.0 is recommended for cosmetic or medical products. If production cannot be placed under strictly ISO conditions, we recommend sterile filtration of the final stock solution.

It is usually impossible to recover a peptide that has aggregated in the presence of salt. If sufficient peptide is available, testing the solubility on a small sample must be done first. For example, DMSO can completely oxidize the free thiol groups of cysteine in short-chain peptides within minutes and should

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be avoided as a solvent for these peptides. Peptides that contain methionine, cysteine, or tryptophan may oxidize and generate impurities. For products, the use of oxygen-free solvents is recommended. Lyophilized peptides are generally extraordinarily stable at sub-zero temperatures, often showing little or no degradation even after years. We recommend storage at -18°C or lower.

Proteases of microbial origin may be excluded using freshly distilled water and sterile filtration. The recommended method to store reconstituted peptides is at -18°C or below after re-lyophilizing appropriate aliquot material. Each peptide's stability is unique and depends on its amino acid sequence. Most peptides, in powder form, are stable for more than two years under -20°C conditions. If this is impossible, store the stock solution in aliquots at -18°C or below and avoid repeated freeze-thaw cycles. Most peptides are stable and dried at ambient temperatures, and it is not necessary to refrigerate them for short periods during transportation.

Extended exposure to warm temperatures should be avoided. Peptides containing one or more free cysteine residues are unstable. Considering the use of biologically active peptides for cosmetic and medical production, it is necessary to evaluate the stability of those peptides under different manufacturing and storage conditions. Lactoferrin is mainly extracted from bovine milk and added to commercial products such as food (nutritional supplements), cosmetics, and toothpaste. Lactoferrin (bovine) is sensitive to denaturation by temperature [18]. When the temperature increases up to $50-70^{\circ}\text{C}$, the rate of degradation increases. Cosmetic and/or medical products containing peptides must be evaluated at different temperatures. Prediction of shelf life is defined as the period during which the peptide retains its original quality. The manufacturing, formation process, and processing steps of lactoferrin from human origin were visualized and optimized to minimize its denaturation.

The various heat treatments did not affect modified hLF1-11, as indicated by HPLC, mass spectrometry, and antibacterial and antifungal activity. MIC values for *C. albicans* Y01-19 (25-50 microgram/mL) and *S. aureus* 2141 (MRSA) (6.25-12.5 microgram/mL) were stable over the whole interval of testing (i.e., 104 weeks at 20 and 37°C). The present formulation of hLF1-11 into PBS was stable enough to permit storage at room temperature for at least two years.

Study setup and ethical statement

Study setup

The investigator-initiated intervention study aimed to identify the fungi before the treatment of onychomycosis in infected toenails donated by volunteers. A certified medical pedicurist (affiliated with the Dutch branch association Provoet, The Netherlands) conducted the treatment with a commercially available product containing the antimicrobial peptide hLF1-11. This product is sold commercially on the open market in the Netherlands, has a CE certification (Class 1-NL-CA002-2019-45945), and is commercially registered as medical devices class 1 according to art.9, paragraph 3, European directive 93/42/EEC. Manufacturer: CBMR Scientific Nanoscience BV. Only material from volunteers was involved in this in vitro study, and this donation involved just the nail clippings collected before and after treatment by the medical pedicurist. The volunteers were provided extensive information and signed a statement to donate nail clippings. Volunteers were given a number so that anonymity and privacy were guaranteed. A pedicurist chose treatment concentration in the first study setup. Volunteers were categorized for their skin and nail disorders into three groups: (1) persons without fungal nail problems, (2) persons with mild complaints, and (3) persons with serious complaints. Treatment using different concentrations was given for low to mild fungal nail problems (hLF1-11, $3\ \mu\text{g}/\text{mL}$ group), mild to severe fungal nail problems (combination group hLF1-11, $3\ \mu\text{g}/\text{mL}$ or $33\ \mu\text{g}/\text{mL}$), and the high infected onychomycosis (hLF1-11, $33\ \mu\text{g}/\text{mL}$ group). After obtaining the informed consent, the volunteers were randomly assigned to treatment with either low or high peptide concentrations or a placebo ($33\ \mu\text{g}/\text{mL}$ of scrambled hLF1-11), according to a computer-generated randomization schedule. Test and placebo solutions were administered twice daily for at least 3-4 months at a low dose of $3\ \mu\text{g}/\text{mL}$ or a high dose of $33\ \mu\text{g}/\text{mL}$ as one drop application. Volunteers receiving the placebo solution (scrambled hLF1-11, $33\ \mu\text{g}/\text{mL}$) were monitored for 3-7 months. One or two samples of affected toenails were collected per volunteer during treatment to follow the effectiveness of the peptide.

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Ethical statement

The Central Committee on Research Involving Human Subjects (CCMO, registration of clinical trials in the Netherlands) indicates that studies qualifying as in vitro drug research (IVDR) performance studies without risk to the subject are considered consumer research (see [Figure S8](#)). These studies do not need to be reviewed by an accredited CCMO (<https://de-mdr-ivdr.tuvsud.com/Medical-Device-Regulation-MDR.html>), (https://health.ec.europa.eu/document/download/12f9756a-1e0d-4aed-9783-d948553f1705_en). Thus, the study does not need to be reviewed by an accredited CCMO (IVDR article 57, 58-2; <https://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=CELEX%3A32017R0746&from=NL>).

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Topical use of lactoferrin peptide against toenail fungus

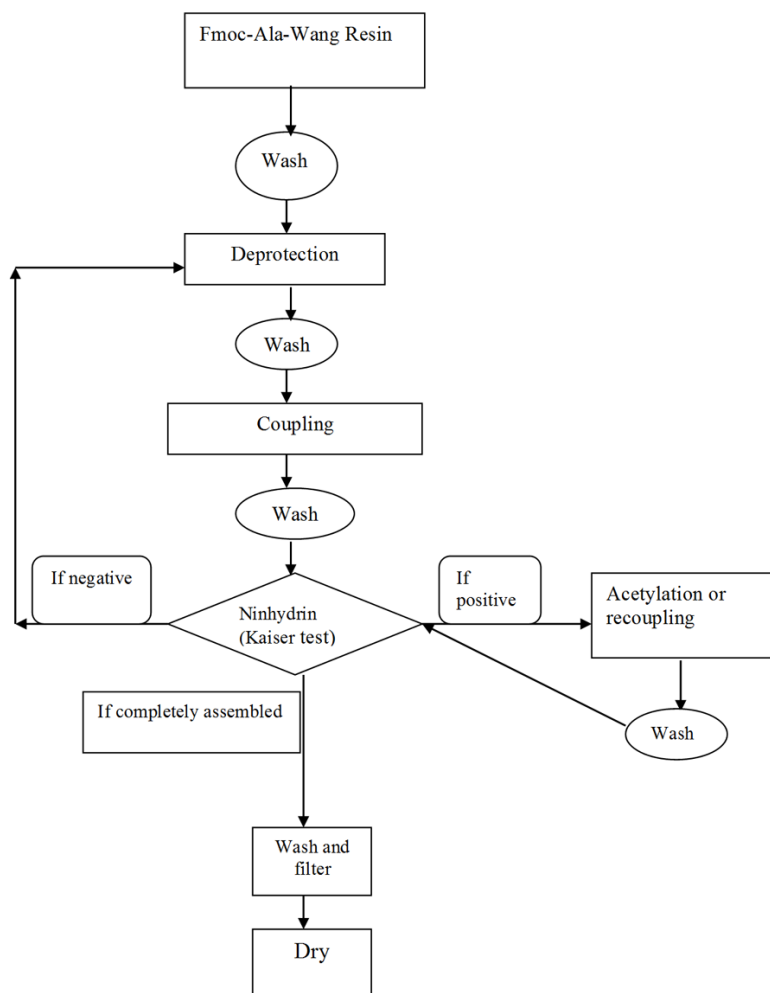


Figure S1. hLF1-11 synthesis flow chart.

Topical use of lactoferrin peptide against toenail fungus

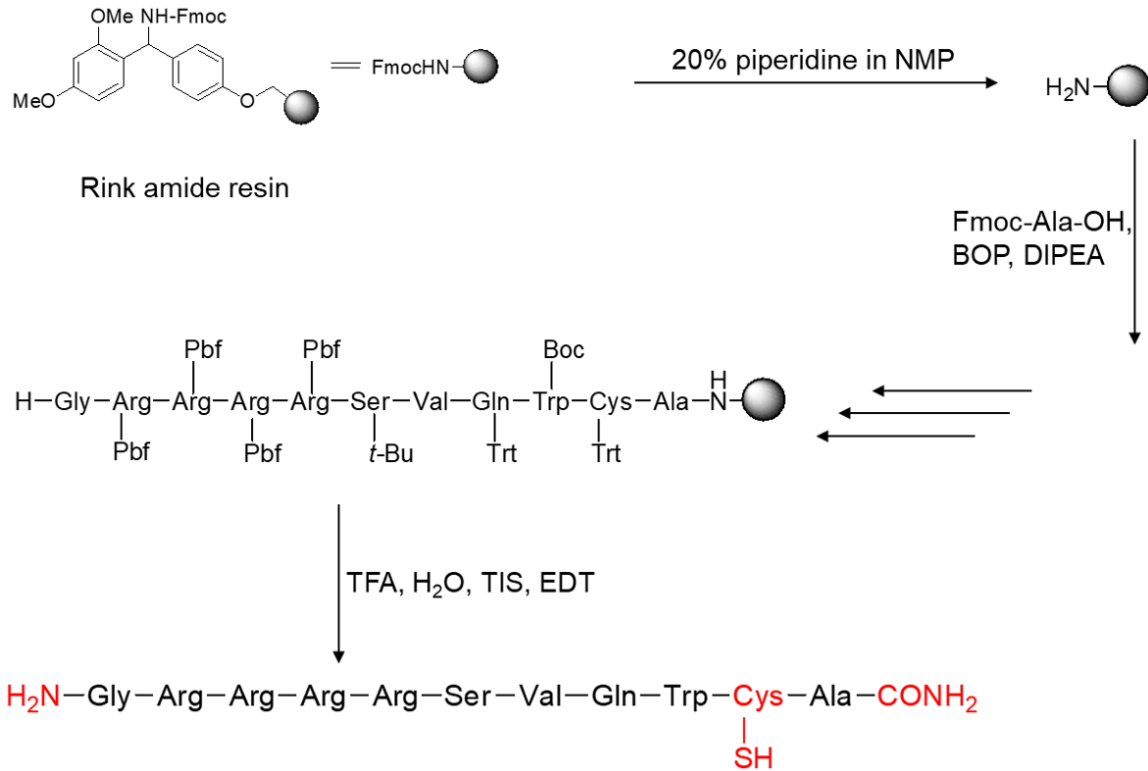


Figure S2. hLF1-11 peptide chain flow chart.

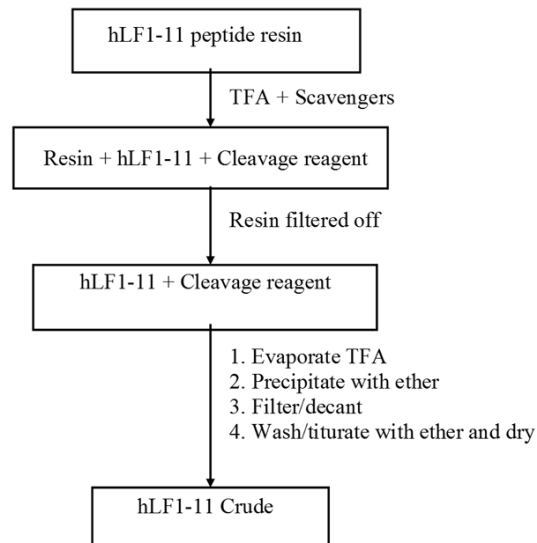


Figure S3. hLF1-11 cleavage/deprotection flow chart.

Topical use of lactoferrin peptide against toenail fungus

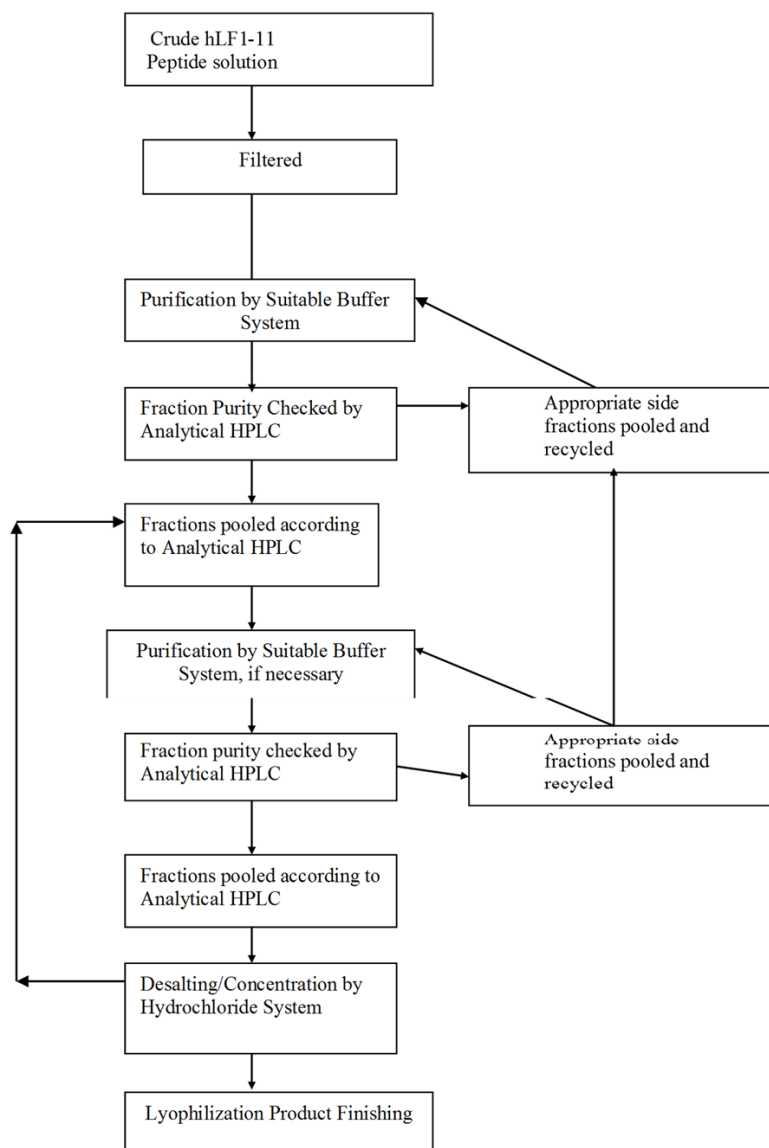


Figure S4. hLF1-11 purification flow chart.

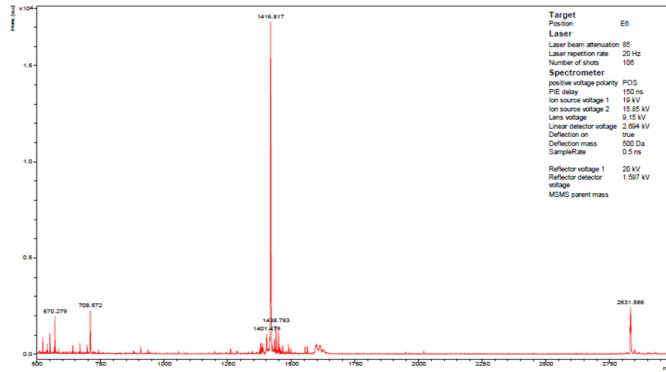
Table S1. Effect of heat treatment on the chemistry and biological activity of hLF1-11 peptide

Temperature	Mass spectrometry		MIC ($\mu\text{g}/\text{mL}$)	
	Main peak	% area	<i>MRSA</i>	<i>Candida</i>
RT	1416.817	98.23	6.25	25-50
40 °C	1415.780	98.48	6.25-12.5	25-50
60 °C	1416.921	98.35	6.25	25-50
80 °C	1416.882	98.73	6.25-12.5	50
100 °C	1416.962	98.54	12.5	25-50

20 min incubation of hLF1-11 at RT, 40 °C, 60 °C, 80 °C, and 100 °C did not affect the stability of this peptide, confirmed by HPLC, mass spectrometry analysis, and the maximum inhibition concentration (MIC Values). MIC values were determined in RPMI 1640 one-fourth broth strength as assay medium [26, 28, 29]. The assays were repeated four times; here, representative assay data are given for clarity and ease of comparison of peptide efficacy (range).

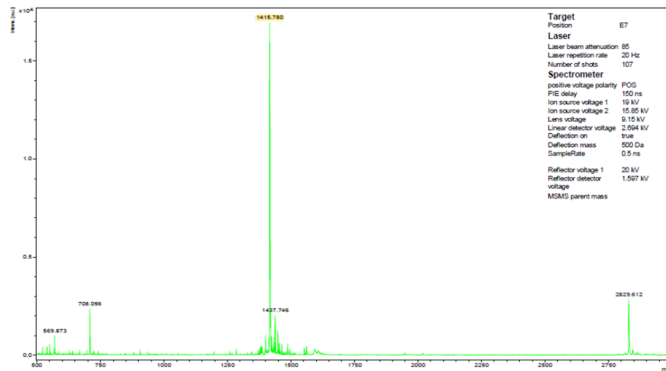
Topical use of lactoferrin peptide against toenail fungus

RT

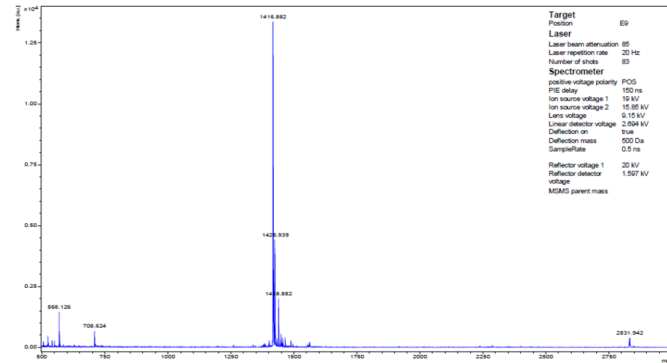


Temperature	Mass spectrometry			
	Main peak	% Area	Dimer	% Area
RT	1416.817	98.23	1438.783	0.7688
40 °C	1415.780	98.48	1437.746	0.3013
60 °C	1416.921	98.35	1438.912	0.7344
80 °C	1416.882	98.73	1438.882	0.8372
100 °C	1416.962	98.54	1438.960	0.6797

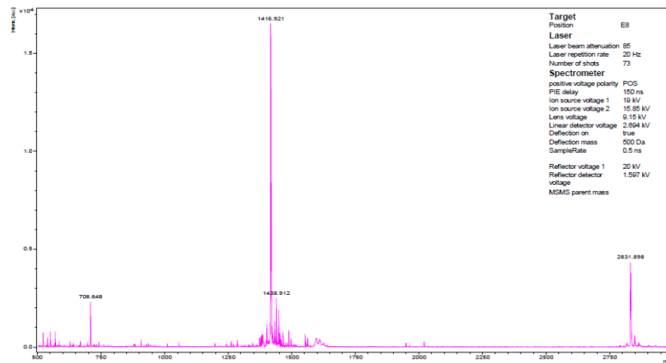
40°C



80°C



60°C



100°C

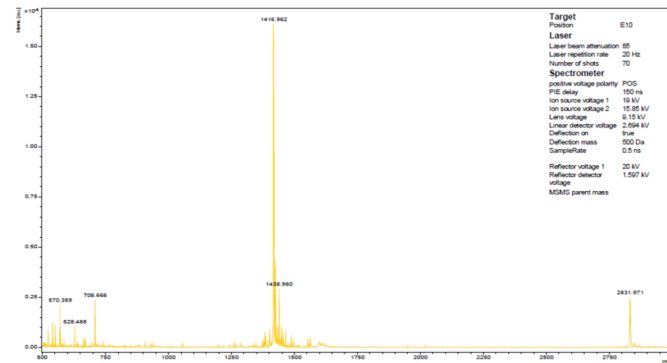


Figure S5. Mass spectrometry: analysis of hLF1-11 peptide exposed for 20 min in MQ water to different temperatures: RT, 40 °C, 60 °C, 80 °C, and 100 °C.

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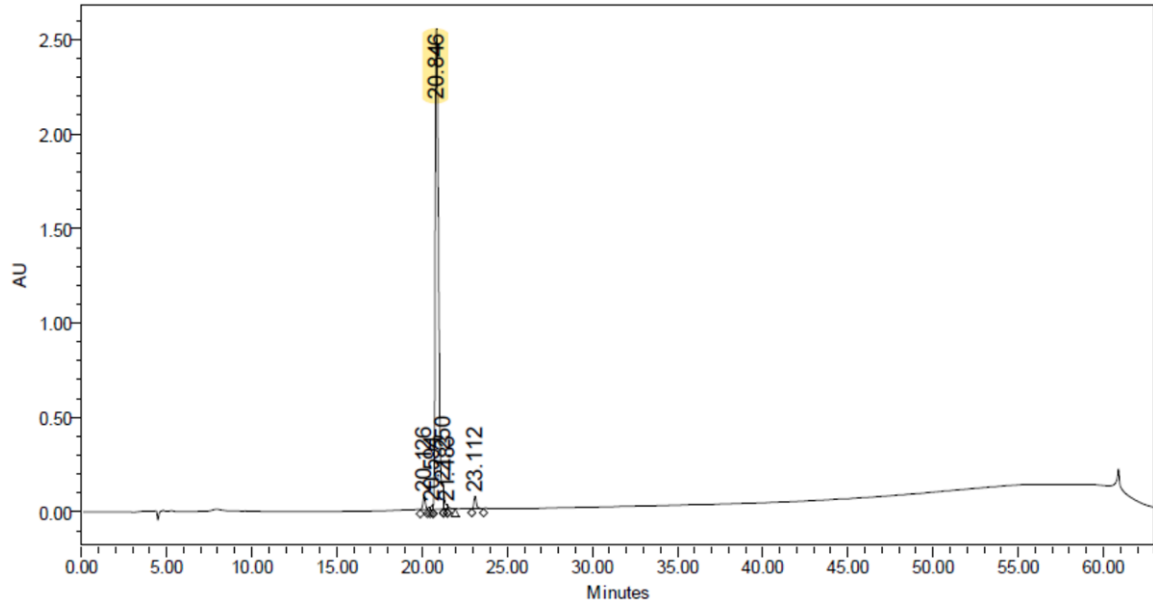


Figure S6. RP-HPLC: evaluation of the typical HPLC profile of hLF1-11. The sample size is 25 μ L of hLF1-11 (1.5 mg/mL MQ water).

Table S2. RP-HPLC

Peak	RT (min)	Area (units)	% area	Height (AU)
1	20.126	708,332	1.90	73,207
2	20.594	179,905	0.48	22,194
3	20.846	34,873,280	93.74	2,545,983
4	21.250	654,171	1.76	125,531
5	21.483	123,655	0.33	21,057
6	23.112	664,096	1.79	66,629

Analysis of hLF1-11 eluted at 20.846 min with 93.74% recovery. Elution of the dimer was observed at 21.250 min. AU = arbitrary units.

Topical use of lactoferrin peptide against toenail fungus

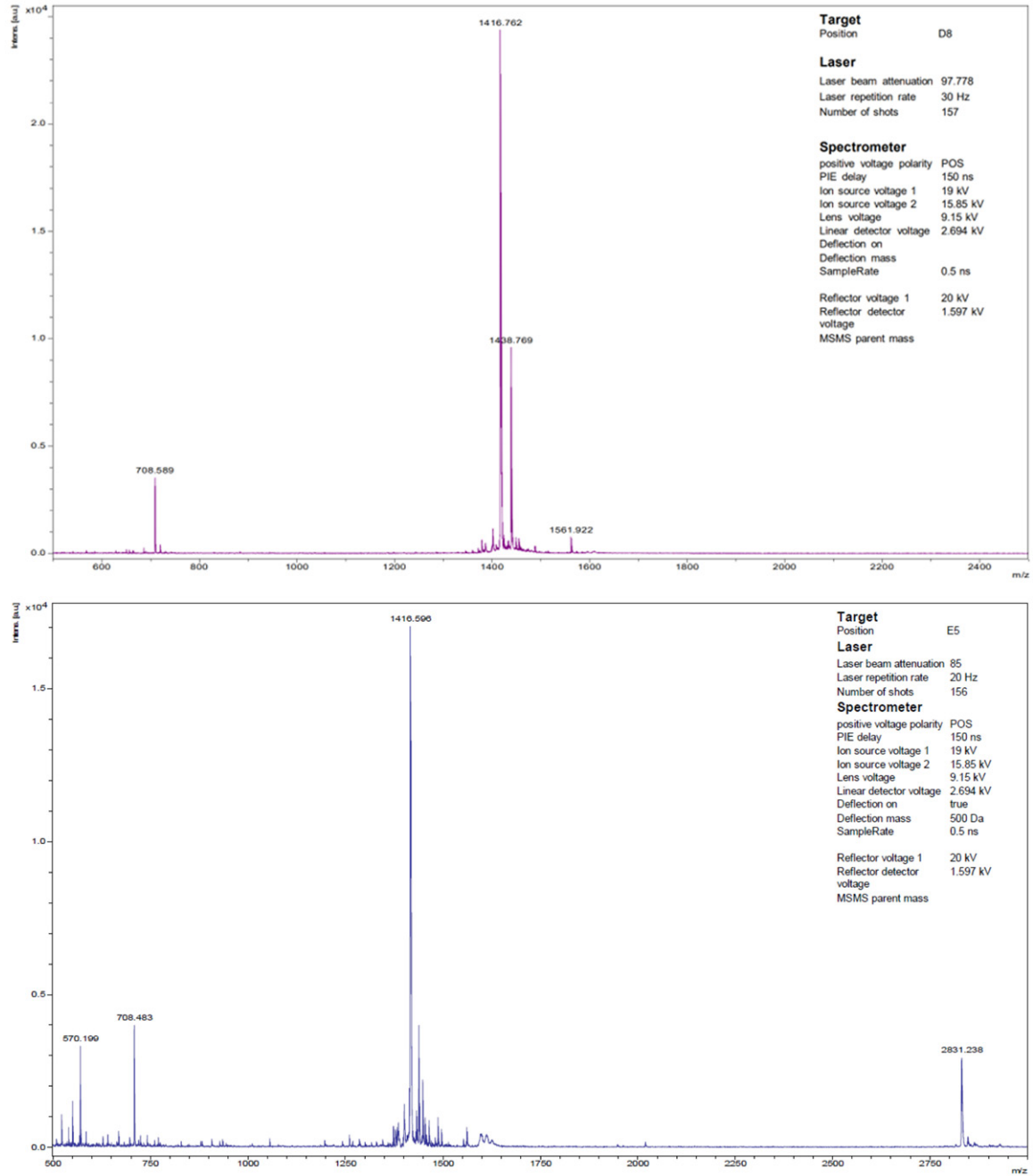


Figure S7. Mass spectrometry: analysis of hLF1-11 before (Top) and after (Bottom) 24 months on the shelf. The sample size was 1 microliter of a peptide solution (concentration and buffer). Performed on A Bruker Microflex MALDI-TOF (in 49.9% v/v acetonitrile, 4).

Topical use of lactoferrin peptide against toenail fungus

Table S3. Stability testing of hLF1-11 from 24 h up to >104 weeks after storing on the shelf at RT and 37 °C by measuring MIC values towards bacteria and fungi

Storage/time	Temp °C	Strain	MIC values (microgram/mL)				
24 h	20 °C	MRSA	6.25	6.25	12.5	6.25	12.5
	37 °C		12.5	6.25	12.5	6.25	6.25
1 week	20 °C		12.5	6.25	6.25	6.25	12.5
	37 °C		6.25	6.25	6.25	6.25	6.25
4 weeks	20 °C		12.5	6.25	12.5	6.25	6.25
	37 °C		6.25	12.5	12.5	12.5	6.25
13 weeks	20 °C		12.5	6.25	6.25	6.25	12.5
	37 °C		6.25	6.25	12.5	6.25	12.5
26 weeks	20 °C		6.25	6.25	6.25	12.5	12.5
	37 °C		12.5	12.5	12.5	6.25	6.25
52 weeks	20 °C		12.5	6.25	6.25	6.25	6.25
	37 °C		12.5	6.25	12.5	6.25	6.25
104 weeks	20 °C		12.5	6.25	12.5	6.25	12.5
	37 °C		12.5	6.25	12.5	6.25	6.25
24 h	20 °C	<i>C. albicans</i>	25	25	50	25	50
	37 °C		25	50	25	25	25
1 week	20 °C		25	50	25	25	50
	37 °C		50	25	25	25	25
4 weeks	20 °C		25	25	50	50	25
	37 °C		25	50	25	50	50
13 weeks	20 °C		50	25	25	25	25
	37 °C		50	50	50	25	25
26 weeks	20 °C		50	25	25	25	25
	37 °C		25	50	25	50	50
52 weeks	20 °C		50	25	25	25	25
	37 °C		25	50	25	25	25
104 weeks	20 °C		50	50	25	50	25
	37 °C		25	50	25	25	50

MIC values (mg/mL) for multi-drug resistant *Staphylococcus aureus* (MRSA isolate type) and drug-sensitive type isolate *Candida albicans* were determined in RPMI 1640 (R8758) [26, 28, 29]. Experiments: 1×10^5 CFU of various strains/mL were incubated at 37 °C into RPMI 1640 with the previous concentrations of hLF1-11, respectively. The results are of five independent experiments.

Medical Devices

Annex II: Flowchart to help determine whether an IVD is a CDx

This flowchart should be followed for each intended purpose of the device.

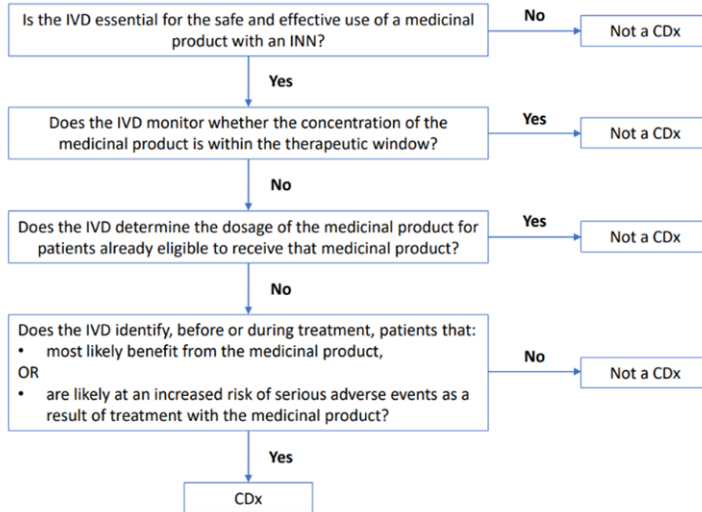


Figure S8. Decision tree to determine the status of the research is classified as *in vitro* Diagnostic Medical Devices (IVD) or a Companion diagnostic device (CDx), which is essential for the safe and effective use of a corresponding medicinal product. This document has been endorsed by the Medical Device Coordination Group (MDCG), established by Article 103 of Regulation (EU) 2017/745.