A yeast-derived peptide promotes skin wound healing by stimulating effects on fibroblast and immunomodulatory activities

Abbreviated title:
"Wound healing potential of a short yeast-derived peptide"

Mahta Mirzaei[a], Amin Shavandi[a*], Gianina Dodi[b], Ioannis Gardikiotis[b], Sorin-Aurelian Pasca[c], Saeed Mirdamadi[d], Nazila Soleymanzadeh[d], Houman Alimoradi[e], Muriel Moser[f*], Stanislas Goriely[f*]

a) Université libre de Bruxelles (ULB), École polytechnique de Bruxelles - BioMatter unit, Avenue F.D. Roosevelt, 50 - CP 165/61, 1050 Brussels, Belgium
b) Advanced Research and Development Center for Experimental Medicine, Grigore T. Popa University of Medicine and Pharmacy of Iasi, Romania
c) Pathology Department, Faculty of Veterinary Medicine, Ion Ionescu de la Brad Iasi University of Life Sciences
d) Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran
e) School of Biomedical Sciences, University of Otago, Dunedin, New Zealand
f) ULB Center for Research in Immunology (U-CRI), Laboratory of Immunobiology, Université Libre de Bruxelles, Gosselies, Belgium

*Corresponding author 1:
Amin Shavandi
Postal address: Université Libre de Bruxelles, Ecole polytechnique de Bruxelles, Service BioControl, BioInfo and BioMatter (3BIO), Campus du Solbosch - CP 165/61, Avenue F.D. Roosevelt, 50, 1050 Bruxelles.
Email address : Amin.shavandi@ulb.be

*Corresponding author 2:
Stanislas Goriely
Postal address : Faculty of Sciences, Charleroi Campus - Gosselies (Biopark) - CP 300, Rue Prof. Jeener & Brachet, 12, 6041 Gosselies.
Email address : **:** Stanislas.Goriely@ulb.be

*Corresponding author 3:
Muriel Moser
Postal address : Faculty of Sciences, Charleroi Campus - Gosselies (Biopark) - CP 300, Rue Prof. Jeener & Brachet, 12, 6041 Gosselies.
Email address: muriel.moser@ulb.be

Abstract
Functionalizing biomaterials with peptides that stimulate cell growth or modulate immune responses have been recently studied for tissue engineering applications. Recently we reported the antioxidant and angiotensin converting enzyme (ACE)-inhibitory activity of the short peptides derived from yeast containing hydrophobic and aromatic amino acids. Herein, we investigated yeast-derived peptides and their structural analogues for wound healing applications *in vitro* and *in vivo*. The immunomodulatory activities of the peptides were evaluated by measuring the levels of pro-and anti-inflammatory cytokines, including TNF-α and IL-6, in macrophages pretreated with the peptides. We also evaluated the peptides' influence on macrophages and fibroblasts proliferation, fibroblasts' migration and collagen production, as well as antimicrobial efficacy against skin pathogens. The peptide with the amino acid sequence of VLSTSFPPW (VW-9) was the most effective at reducing the levels of inflammatory cytokines TNF-α and IL-6 by 34.82% ± 5.88 and 44.75% ± 6.18, respectively and the level of CD64 marker by 17.50% ± 8.06. The peptide was also effective for stimulating fibroblast proliferation (125.48% ± 31.184), migration and collagen production (44.94% ± 6.24). Furthermore, treating full-thickness excisional wounds in rat skin with VW-9 solution resulted in a significant increase in the healing process as compared with controls ($p<0.05$).

Keywords: Yeast-derived peptides, Immunomodulatory peptides, Wound healing, Human macrophages, Fibroblasts.

Highlight points:

- Short yeast-derived peptides were investigated for wound healing potential.
- The short peptide, VLSTSFPPW (VW-9) showed anti-inflammatory activity.
- Skin fibroblast cells were stimulated by the peptides VW-9.
- Trp in the VW-9 C-terminus was shown to contribute to anti-inflammatory and stimulating action.
- Peptide VLSTSFPPW improved the wound healing process in rat skin.

1. Introduction

One of the challenging tasks in biomaterials engineering is to mimic native extra cellular matrix (ECM). Bioactive peptides, have recently emerged as promising and cost-effective biomolecules for creating functional biomaterials for tissue healing and regeneration [1]. Peptides from 2to 20 amino acids can be obtained from protein sources such as milk, egg, fish, soybean, spirulina, and yeast through protein hydrolysis [2]. Antioxidant, anti-inflammatory, antimicrobial activity, as well as their ability to improve cell adhesion, and modulate immune responses, are among the properties that make peptides suitable for functionalization of biomaterials and wound healing applications [3, 4].

Although it is well-established that the biological activities of peptides depend on their molecular weight and amino acid sequences, the link between the structure and function is not fully understood [5]. Small peptides below 1 KDa derived from different sources such as IPP and VPP from fermented milk [5], IRW and IQW from egg protein [6, 7], PLV and VPY from soy protein [8, 9], PTGADY from Alaska pollock protein [10], and PAY from salmon protein [11] can be absorbed by immune and skin cells and modulate cell functions [12]. In these peptides, hydrophobic amino acids are the key players in their anti-inflammatory activity and proliferative properties on fibroblasts. In addition, the majority of reported antimicrobial peptides are hydrophobic, cationic, or amphipathic in nature, with an average length of 8 to 22 amino acids [13-15].

Understanding the correlation between the amino acid sequence and the potential anti-inflammatory property of such peptides can promote the discovery of novel and efficient anti-inflammatory peptide-based therapeutics. To this end, we have previously deviated VLSTSFPPK (VL-9) and YGKPVAVPAR (YR-10) from yeast and developed their structural analogues, including VLSTSFPPW (VW-9), VLSTSFPPF (VF-9), VLSTSFCPK(VCK-9), VLSTSFYPK(VYK-9), VLSTSFHPH(VHK-9), STSFPPK(SK-7), YGKPVAVPAR(YR-10), YGKHVAVHAR(YHR-10), GKPVAVPA(GA-8), GKHVAVHA(GHA-8), and PAR(PAR-3). We showed that these peptides have antioxidant, cytoprotective and angiotensin-converting enzyme (ACE)-inhibitory effects [16-18].

Given our findings and other reports showing that short peptides containing hydrophobic amino acids have potent antioxidant and antibacterial properties, it appears that these peptides could benefit the wound healing process. These findings prompted us to investigate the ability of these peptides to influence microbial growth, cytokine production by immune cells, fibroblast proliferation, collagen synthesis and cell migration. In addition, the effect of one of the selected peptides (VW9) on wound healing process was studied in vivo.

2. Materials and methods

2.1. Peptides

Thirteen different peptides with >95% purity were synthesized chemically by SynthBio Engineering (Hefei, China) company using solid-phase approach. The synthesized peptides were purified using HPLC and the peptides sequences were confirmed by analytical HPLC-MS/MS.

2.2. Cell culture and cell viability assay

U937, a pro-monocytic human myeloid leukaemia cell line (ATCC, CRL-1593.2) and fibroblasts (ATCC, CCL-186), were obtained from American Type Culture Collection. The cells were cultured in RPMI 1640 and DMEM medium respectively, supplemented with 2 mM L-Glutamine, 10% fetal bovine serum (FBS), and 100 unit/mL penicillin/streptomycin (Lonza), according to ATCC guidelines. Cells were incubated at 37 °C in a humidified atmosphere (5% CO_2). U937 was differentiated to macrophages after being treated with PMA

(300 nM) for 24 h and then resting for another 24 hours.We performed a real-time Glo MT assay (Promega, USA) to assess the viability of cells following treatment of cells with different concentrations (0, 0.25, 0.5, 1mM) of peptides. At zero, 24 h, 30 h and 48 h time intervals, luminescence intensity (RLU) were monitored continuously using a microplate-reading luminometer (Promega, GloMaX) [19].

2.3. Cell stimulation and treatment with peptides

Immunomodulatory activity of the peptides was assessed by seeding macrophages at 400,000 cells in 2 mL per well in 24-well plates and pretreating with each of the peptides (0, 0.25, 0.5, 1, and 2 mM) for 22 h, followed by stimulation of cells with Lipopolysaccharide (LPS) (*E. coli* O55: B5, Sigma) (500 ng/mL) for 6 h or 19h to evaluate the levels of TNF-α and IL-6, respectively. When indicated, cells were treated by recombinant Interferon-gamma (IFN-γ) (20 ng/mL) for 24h.

2.3.1. Flow cytometry analysis

The treated U937 cells (1000,000 per tube) were subjected to staining using anti TNF-α antibody (Clone Mab11, conjugated with Alexa fluor 700) and anti-CD64 monoclonal antibody (clone 10.1, conjugated with FITC) (Invitrogen). To determine the amount of TNF-α, cells were treated with Brefeldin A (Invitrogen, USA) at a final concentration of 3 µg/mL for 6 h before staining. Live/Dead fixable Near-IR dead cell stain kit (Thermofisher, USA) was utilized in all experiments to assess the cell viability. Before intracellular staining, fixation, and permeabilization/wash buffer (Invitrogen, USA) were used. After 30 min staining at 4°C, cells were washed with PBS and suspended in PBS for analysis by FACS Calibur Flow cytometer (Becton Dickinson). Fluorescence intensity analysis was performed using a FlowJo (Version 10.7.1, Treestar Software, USA). After separating the possible noises, by selecting the main population in the side scatter area vs forward scatter area [FSC-A], single cells were selected using forward scatter height vs FSC-A plot for further analysis.

2.3.2. Enzyme-linked immunosorbent assay (ELISA).

Supernatants were collected to determine TNF-α and IL-6 with specific ELISA kits according to manufacturer`s instruction (DuoSet ELISA Development System, Biotechne). Optical density was read immediately using a microplate reader at 450 nm and cytokine concentrations were evaluated using a standard curve.

2.4. Quantification of IL-6, TNF-α, IL-1β, CCL2, and β-Actin mRNA levels by Real-Time PCR

Macrophages at the density of 400,000 cells per well (2 mL) were treated with peptides at a final concentration of 0.5 mM for 22 h. LPS (500 ng/mL) was added to each well and incubated for 0.5, 1, 3, and 6 h at 37°C, 5% CO_2. The treated cells were used to measure expression levels of IL6, IL10, IL1β, TNF, and CC-chemokine ligand 2 (CCL2) genes in the presence and absence of peptide. Total RNA was extracted from collected cells using NucleoSpin RNA Plus RNA isolation Kit according to the manufacture instruction (MACHEREY-NAGEL). cDNA was synthesized with total RNA (1µg) using Thermo Scientific RevertAid RT kit by oligo (dt) primer. Diluted cDNA (1:3) was subjected to quantitative real-time PCR amplification using LightCycler 480 probes master Roche kit in a Roche Real time PCR system. Data were expressed as relative gene expression to β -Actin using the 2^-ΔΔT method.

Table 1. Primer sequences for RT-PCR assay

Gene	Primer	Sequence
ACTβ	Actine F976 Actine R1065 Actine S997	GGA-TGC-AGA-AGG-AGA-TCA-CTG CGA-TCC-ACA-CGG-AGT-ACT-TG CCC-TGG-CAC-CCA-GCA-CAA-TG
CCL2	hCCL2 F95 hCCL2 R244 hCCL2 S127	GTG-CCT-GCT-GCT-CAT-AGC TT-GCT-GCT-GGT-GAT-TCT-TCT CCC-AAG-GGC-TCG-TCT-AGC-C
IL1ß	hIL-1 β F176 hIL-1 β R248 hIL-1 β S207	ACA-GAT-GAA-GTG-CTC-CTT-CCA GTC-GGA-GAT-TCG-TAG-CTG-GAT CTC-TGC-CCT-CTG-GAT-GGC-GG
TNF	h TNF-α F275 h TNF-α R358 h TNF-α S303 BHQ	CCC-AGG-GAC-CTC-TCT-CTA-ATC ATG-GGC-TAC-AGG-CTT-GTC-ACT TGG-CCC-AGG-CAG-TCA-GAT-CAT-C
IL6	h IL-6 F193 h IL-6 R316 h IL-6 S242	GA-CAG-CCA-CTC-ACC-TCT-TCA AG-TGC-CTC-TTT-GCT-GCT-TTC CCT-CGA-CGG-CAT-CTC-AGC-CC

2.5. Analyzing p38 mitogen-activated protein Kinase (MAPk) and NF-κB (nuclear factor kappa-light-chain-enhancer) signaling pathways

The effect of peptide VW-9 on NF-κB and p38 MAPK signaling pathways in the presence of LPS was investigated by Phospho-flow cytometry assay. In brief, macrophages were grown overnight in full media with FBS (10%) and starved for 3 h and then treated with peptide (0.5 mM) in starving media for 22 h and then with LPS (500 nM) for 5, 15, 30, 45, 60 min. The cells were immediately fixed by adding pre-warm Phos-flow fix buffer I (BD Biosciences) and incubated for 10 min at 37°C. Then cells were permeabilized with Phospho-flow perm/wash buffer I (BD Biosciences) and stored on ice for 30 min. Cells were spun down, washed twice with staining buffer (1X PBS, 1% FBS and 0.09% sodium azide) and resuspended in the staining buffer at a cell density of 10 million cells per well. 100 µL samples of cell suspension were then used for staining with anti-p65 conjugated with Alexafluor 647 and anti-p38 conjugated with PE. Finally, after 30 min staining at 4°C, the cells were washed with PBS and suspended in PBS for analysis by the FACS Calibur flow cytometry method [20].

2.6. Wound healing scratch assay

The peptides were tested for *in vitro* wound healing activity using wound scratch assay following the method (REF). Fibroblast cells were seeded (100,000 cells/well) in 2 mL in 48-well plates and allowed to grow for 24 h. When the cells reached 100% confluence, the

monolayer of cells was scratched with a yellow pipette tip to create a scratch and washed twice with PBS to remove floating cells. Each well was filled with 1 mL DMEM (with 2% FBS) containing peptides at a final concentration of 0.5 mM. Throughout the 24 h period, images of wounded cell monolayer were taken every six hours using a Zeiss AxioObserver Z1 (inverted wide-field microscope) with a 10x/0.3 EC Plan Neofluar Dry Ph1 objective. Fiji software was used for image analysis [21]. The migrated cells were imaged, and the covered areas were measured using the WimScratch software program (Wimasis, Munich, Germany).

2.7. Effect of peptides on fibroblasts collagen production

In 24-well plates, fibroblasts were seeded at a density of 50,000 cells per 2 mL. 24 h later, the cells were treated with various concentrations of the peptide for 24 h. The collagen concentration in 20 µL of two-times diluted supernatant samples was measured using a collagen assay kit (MAK322, Sigma- Aldrich, USA) and a fluorescent microplate reader (BioTeck, USA) at λex = 375/λem = 465 nm.

2.8. Antimicrobial activity

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were acquired from the Persian Type Culture Collection (PTCC). Strains were cultivated on nutrient agar (Lab-Lemco powder 1.0 g/L, yeast extract 2.0 g/L, peptone 5.0 g/L, NaCl 5 g/L, agar15 g/L) and antimicrobial activity testing were conducted using Mueller Hinton broth (Scharlu, Spain). The antimicrobial activity of the synthesized peptides was determined using the CLSI-recommended microdilution method (M7-M100, 2018). The bacterial inoculum was prepared by direct colony suspension method in normal saline (OD_{600nm} = 0.1- 0.12). The suspension was then prepared in Mueller Hinton broth at a concentration of 10,000000 CFU/mL. In 96-well round-bottom microplates, 50 µL of a 1000,000 CFU/mL suspension of bacterial was added to peptide solution at a gradient concentration range of 0.02 -10 mM. The microplates were incubated at 37 °C for 16-20 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which there was no bacterial growth. To estimate the minimal bactericidal concentration (MBC), we used the spread plate method to culture the whole content of the wells with no growth on a nutrient agar medium.

2.9. Wound healing assay

Adult male Wistar rats (270-400 g, 14 months of age) were housed in temperature- and humidity-controlled individually ventilated cages (12:12-h light-dark cycle) with free access to water and a standard pellet diet. The animal experiments were performed according to international guidelines and were approved by the Ethical Committee of the Grigore T. Popa University of Medicine and Pharmacy of Iasi, Romania. The rats were divided into three groups (5/group) as follows: group 0 (blood donors, sacrificed to prepare platelet-rich plasma -PRP-solution), the PRP group (positive control group), and the peptide group (test group containing VW-9, 0.5 mM).

Rats in Group 0 were anesthetized by inhalation with isoflurane gas mixed with normal air (5% for induction and 2% maintenance), and 10-14 mL of blood was drawn via the central abdominal artery and introduced into clear tubes. The rats were euthanized after blood harvest through exsanguination and during 5% isoflurane hyperdose. Blood cell elements were separated using a laboratory centrifuge (Universal 32R, Hettich, Germany) at 3500 rpm (1317 RCF) for 5 minutes at 20°C, resulting in two basic components: red blood cells at the bottom and platelet plasma on top, yielding 3-4.5 mL. The platelet-rich layer was separated by further centrifugation, in different tubes, at 6300 rpm for 20 min, and the PRP solution (1 mL) was isolated from the supernatant and stored at 4°C until use. The PRP isolation process was freshly prepared for each experimental day during the entire study.

The back of the rats was shaved, and the skin was cleaned with chlorhexidine di-gluconate (20%, 10.65 mg) and benzalkonium chloride (50%, 9.85 mg) solution. Two circular full-thickness cutaneous wounds with a diameter of 1.5 cm were marked and were then carefully excised by using sterile sharp scissors. The wounds were made on the dorsal surface with one on each side and left undressed to the open environment. The right for the PRP or VW-9 treatment and on the left for negative control (sterile pure water). The day of wound creation was defined as day 0 of the study. 50 µL solution of PRP or VW-9 were applied using a pipette to cover the wounded area twice per day up to day 7. For the animals' pain management, an intra-peritoneal injection of 40 mg/kg tramadol was used on the day of surgery (day 0). Photographs of the wounds were taken using a digital camera (Sony, model no. DSC-W570 with 16.1 megapixels) at days 0, 3, 5 and 7 during the healing process.

The wound sizes were measured on the 3rd and 7th day using a paper ruler. The wound-healing ratio (HR) was calculated by the equation as follows:

$$HR(\%) = \frac{S_0 - S_n}{S_0} \times 100\ ,$$

where S_0 and S_n are the wound areas at day 0 and the predetermined experimental day, respectively.

On day 7, the animals were euthanized by anesthesia hyperdose and the full thickness wound tissue with a minimum of 0.5 cm normal tissue margins was collected for histological studies.

2.10. Histolopathological examination

The dissected skin specimens were fixed in 10% neutral formalin, processed, embedded in paraffin, cross-sectioned and stained with hematoxylin-eosin to study the wound closure properties. Histological photographs were performed in 5 µm tissue sections using a light microscope 211 Leica DM750 (Leica Microsystems GmbH, Germany) with an attached digital camera 212 Leica ICC50 HD (Leica Microsystems GmbH, Germany). A systematic examination of 40 random fields under 40X magnification was performed in order to semi-quantitatively score the following histological parameters: necrosis, inflammation, granulation tissue formation, angiogenesis, re-epithelization and collagen tissue deposit.

2.11. Statistical Analysis

All experiments were replicated three times; results are expressed as the standard error of the mean (SEM). Statistical analysis were performed using GraphPad Prism software (Version 9) by one-way ANOVA analysis of variance followed by Dunnett's or Šidák's multiple comparisons test. Two-way ANOVA test followed by Tukey's multiple comparisons was chosen to compare groups in the cell viability tests. $p<0.05$ was considered statistically significant.

3. Results and discussion

In order to investigate the wound healing potential of yeast-derived peptides such as VLSTSFPPK (VL-9) and YGKPVAVPAR (YR-10) and their structural analogues (listed in Table 1), we evaluated several key properties, including their immunomodulatory, growth promoting and antimicrobial activities. Table 1 contains the amino acid sequence and theoretical features of the peptides. The molecular weight, isoelectric point (pI), and net charge at pH 7 were predicted using the Pepdraw tool (http://www.tulane.edu/biochem/WW/PepDraw/), and the GRAVY indexes were predicted using the Protparam tool (http://web.expasy.org/protparam). The positive and negative values of the GRAVY index correspond to peptides' hydrophobic and hydrophilic properties, respectively. The molecular weight of the peptides ranged from 342.40 to 1137.31 Da. The isoelectric point ranged from 5.53 to 11.29.

Table 1. The physicochemical properties of the peptides investigated in this study

Peptides	Sequence	Molecular weight (Da)	Net charge at pH 7	Theoretical isoelectric pH	GRAVY index
VL-9	VLSTSFPPK	974.54	+1	9.80	0.156
VY-9	VLSTSFPPY	1009.51	0	5.53	0.444
VW-9	VLSTSFPPW	1032.52	0	5.58	0.489
VF-9	VLSTSFPPF	993.51	0	5.56	0.900
VCK-9	VLSTSFCPK	980.49	+1	8.67	0.611
VYK-9	VLSTSFYPK	893.48	+1	9.48	0.189
VHK-9	VLSTSFHPK	1014.54	+1	9.80	-0.022
SK-7	STSFPPK	768.32	+1	9.8	-0.943
YR-10	YGKPVAVPAR	1057.26	+2	10.41	-0.130
YHR-10	YGKHVAVHAR	1137.31	+2	10.41	-0.450
GA-8	GKPVAVPA	737.90	+1	10.2	0.562
GHA-8	GKHVAVHA	817.45	+1	10.12	0.163
PAR-3	PAR	342.40	+1	11.29	-

The molecular weight, isoelectric point (pI), and net charge at pH 7 were predicted using the Pepdraw tool (http://www.tulane.edu/biochem/WW/PepDraw/), and the Gravy indexes were predicted using the Protparam tool (http://web.expasy.org/protparam).

3.1. Macrophage viability

Using the CellTiter-Glo Luminescent cell viability test, we investigated the effect of different peptides concentrations (0.25, 0.5, and 1mM) on U937 macrophage viability after 24, 30, and 48 h of incubation at 37 °C. After 48 h, both treated and control samples showed a significant decrease in cell viability. So data are only presented for 24 and 30 h of treatment (Fig.1), and as shown, cell proliferation mainly happened after 24 h.

In agreement with these results, Wang and co-workers also observed a higher cell growth rate after 24 h compared to 48 and 72 h by studying the proliferation effects of peptides from bovine gelatin hydrolysate on MC3T3-E1 cell line, indicating that peptides can stimulate cell growth in a short time [22].

None of the peptides examined showed significant cytotoxicity throughout the study, and the peptides VL-9, VY-9, VW-9, VF-9, VCK-9, VHK-9, VYK-9, SK-7, and YR-10 significantly increased cell growth.

After 24 h of treatment with the peptides at a concentration of 0.25 mM, cell viability increased by 142% ± 18 (VL-9), 107% ± 21 (VY-9), 164% ± 22 (VW-9), 182% ± 34.92 (VF-9), 121% ± 22 (VCK-9), 82% ± 17 (VYK-9), 105% ± 28.5 (VHK-9), 86% ± 16 (SK-7), and 18.25 ± 4.45 (YR-10).

By comparing the cell viability effect of the peptides at a concentration of 0.25 mM (Fig. 1B), we discovered that replacing Lys in the C-terminus of VL-9 with Trp and Phe increased the percent of cell proliferation from 142 % ± 21 to 182.46 % ± 23 and 164.56 ± 16, respectively, indicating the positive role of Trp and Phe in the C-terminus of peptides VF-9 and VW-9.

Pro has been previously identified as an important amino acid residue that contributes to the proliferation and differentiation of MC3T3-E1 cells [20]. Our findings confirmed this result and revealed that Cys,Tyr and His are also important because peptides containing Pro (VL-9), Cys (VCK-9), Tyr (VYK-9), and His (VHK-9) in the antepenultimate position have the same effect on macrophage proliferation, with no preference for Pro over Cys, Tyr, and His at the same position (Fig. 1B).

Finally, VF-9 and VW-9 were the most effective peptides for cell proliferation. The low molecular weight (<1 KDa) of VF-9 and VW-9 is a key characteristic in peptide cell penetration ability, and numerous studies have shown that small-size peptides have improved proliferative activity on different cell lines [22, 23]. It can also be due to the hydrophobic nature of peptides and the presence of aromatic amino acids, Phe and Trp, at C-terminus, because aromatic amino acids have previously reported as key criteria in cell proliferation [24].

3.4. Immunomodulatory activity of peptides

In this study, we employed macrophages as an *in vitro* model for investigating inflammatory markers and macrophage activation by LPS and IFN-γ. LPS is detected by macrophages via specific receptors, primary Toll-like receptor complex, resulting in activation of signaling pathways mostly including NF-κB and MAPKs that leads to overexpression of genes and increased production of inflammatory cytokines such as TNF-α, IL-6 and IL-1β. IFN-γ is also a powerful macrophage activator that increases the cell's ability to perform an immune response. The main pathway elicited by IFN-γ is the JAK-mediated phosphorylation of STAT1 (REF). IFN-γ induces the expression of the high-affinity receptor for IgG and FcγR1(CD64) [25]. Modulation of the production of anti-inflammatory cytokines (TNF-α, IL-6, and IL-1β in LPS-treated macrophages and the surface expression of CD64 in IFN-γ-stimulated cells are two useful read-outs to evaluate the anti-inflammatory potential effects of these components.

As expected, LPS treatment promoted cytokine production by U937 cells. The population of cells with positive TNF-α increased from 0.38% ± 0.01 to 24.3% ± 0.98 (Fig 2.A), MFI increased from 253 ± 3.8 to 424 ± 6.90 and TNF-α concentration in cell-free supernatant increased from under detectable level to 6196 ± 134.17 pg/mL (Fig. 2C). Furthermore, LPS treatment increased the concentrations of IL-6 in cell free supernatant from under detectable level to 596 ± 14.00 (Fig. 2D). MFI value for CD64 of IFN-γ-stimulated cells increased from 21521 ± 3421 to 50216 ± 3170 (Fig. 2E).

TNF-α, IL-6, and CD64 levels in cells pretreated with the peptides and stimulated with LPS and IFN-γ were evaluated to assess their immunomodulatory activity. Flow cytometry and

ELISA tests revealed that pretreatment with VW-9 significantly modulated the capacity of cells to produce TNF-α. TNF-α positive cells reduced from 24.3 % ± 0.98 to 16.8% ± 1.039 and MFI values from 424 ± 6.9 to 351 ± 2.6 (Fig. 2A, and B). The levels of IL-6 also decreased by 44.75% ± 6.18 in cells pretreated with VW-9. VW-9 was also effective in reducing the MFI value of CD64 positive cells by 17.50% ± 8.06 (Fig. 2E). The anti-inflammatory effect of VW-9, as shown in Fig. 2F lacked dose-dependency at the examined concentrations.

The structural and physicochemical properties of anti-inflammatory peptides have been poorly studied. Most anti-inflammatory peptides that have been reported in various research are small-sized peptides. For example anti-inflammatory peptides VPP and IPP resulting from bacterial fermentation of milk, IRW and IQW obtained from egg casein, and PAY isolated from salmon byproduct protein, and PTGADY from Alaska pollock protein [10] have molecular weight less than 1 kDa [26]. The exact relationship between peptide size and its anti-inflammatory activity is not well-established, but some reports attributed the higher anti-inflammatory of small-sized peptides to the easier absorbance by immune cells [12]. Peptides that penetrate cells usually have a sequence length of 5 to 42 amino acids [27]. The size of VW-9 may also have influenced its anti-inflammatory activity.

Acidic nature (pI= 5.58), the presence of hydrophobic amino acids, Val, Leu, and Pro, as well as aromatic amino acids Trp and Phe have been reported for several anti-inflammatory peptides [2, 28-30] and may also justify the higher anti-inflammatory effects of VW-9 in comparison to other peptides. In addition, our findings revealed that Trp is a key amino acid residue in C-terminus of anti-inflammatory peptides. It was discovered by comparing the activity of VL-9, VW-9, VF-9, and VY-9, all of which had similar sequences but a distinct C-terminus, including Lys, Trp, Phe, and Tyr, respectively. Trp was also previously found in the same place in the sequence of two anti-inflammatory peptides derived from egg protein, IRW and IQW [6, 7].

3.6. Anti-inflammatory mechanisms

VW-9 peptide showed the highest inhibitory effect on TNF-α and IL-6 cytokines in LPS-stimulated cells and CD64 levels in IFN-γ-stimulated cells (Fig. 2). Therefore to test for potential anti-inflammatory mechanisms, we selected VW-9 peptide.

3.6.1. Modulation of signaling pathways: NF-κB and p38 MAPk

When macrophages are activated with LPS, TNF-α is one of the first major proinflammatory mediators produced and NF-κB, a heterodimer of p65 and p50 proteins, plays a critical role in regulating TNF-α gene transcription. The expression of a large number of other genes involved in inflammation is also controlled by NF-κB such as COX-2, VEGF, pro-inflammatory cytokines (IL-1, IL-2, IL-6, and TNF-α) [31]. The mitogen-activated protein kinases (MAPK) are also a highly conserved family of serine-threonine protein kinases that regulate gene transcription and transcription factor activities involved in inflammation [31].

As demonstrated in Fig.3A, LPS treatment increased the levels of phosphorylated p38 and p65, which peaked at 30 and 45 min, respectively. We found that VW-9`s anti-inflammatory activity in cells is not attributed to blocking the NF-κB or MAPK pathways by looking at the effect of VW-9 on phosphorylation of p38 and p65 in the cells stimulated with LPS for different time intervals (5, 15, 30, and 45 min).

3.6.2. Effect of VW-9 on the expression of pro and anti-inflammatory genes

To further investigate the mechanism by which VW-9 attenuated the LPS-induced inflammation, gene expression of TNF-α, IL-6, IL-1β, and CCL-2 was measured in the cells pretreated with peptide for 22 h and stimulated with LPS for 30 min, 60 min, 3 h, and 6 h. The effect of the peptide on gene expression was also investigated in non-stimulated cells.

When compared to the control cells, stimulation with LPS increased the expression levels of TNF, IL6, IL1β, and CCL-2 after 1-3 h. Treatment with peptide did not result in lower expression of the genes, but also increased the expression levels of TNF (after 1 and 3h) and IL6 (after 6 h), Whereas IL1β and CCL-2 mRNA levels did not change following treatment with the peptide (Fig. 3B). The findings suggested that VW-9 anti-inflammatory activity is unrelated to decreased gene expression levels of the tested cytokines.

We did not observe suppressing effects of the peptide on NF-κB and MAPK pathways based on phosphorylation and mRNA assays (Fig. 3). Inflammation is a complex immune response involving multi genes and cytokines [32]. Numerous processes would be studied to ascertain the precise mechanism underlying anti-inflammatory activity. For instance, the effect on pro-inflammatory c-Jun N-terminal kinase (JNK, a kind of MAPKinase) pathway has been reported for the anti-inflammatory activity of peptides VPP and IPP resulting from bacterial fermentation of milk [33]. Inhibition of NO/iNOS and PGE2/COX-2 pathways has been reported for peptide PAY obtained from salmon protein hydrolysate [26, 34]. Regulation of the STAT1/IRF3 signaling pathway has been reported for the anti-inflammatory activity of Bee venom [35] and regulation of PI3K/AKT was reported to be involved in anti-inflammatory activity of Xuanwei ham derived peptides in LPS-stimulated macrophages [5]. Additionally, destabilization of cytokine mRNA and action on post-transcriptional steps may be responsible for the final suppression of mRNA to protein translation [36]. More research is required to pinpoint the precise mechanism(s) of the anti-inflammatory activity of VW-9.

3.7. Effect of the peptides on fibroblasts

Fibroblasts play an important role in tissue repair, beginning with the late inflammatory phase and continuing to the complete epithelization. Fibroblast proliferation and migration initiate the proliferative phase of healing, and they also contribute to wound healing by secreting growth factors, cytokines, collagen, and other extracellular matrix (ECM) compounds [37]. The effect of synthetic peptides on fibroblast proliferation, migration, and collagen production was investigated in this study.

3.7.1. Fibroblast cell viability

The peptides investigated in this study were not toxic to fibroblasts at concentrations up to 1mM for 48 h. At doses ranging from 0.25 mM to 1 mM, the peptides VL-9, VY-9, VW-9, VF-9, VCK-9, VYK-9, VHK-9, and GHA-8 significantly promoted fibroblast cell proliferation ($p<0.05$ vs. control). After 30 h, the proliferative effects of VW-9, VY-9, and VYK-9 were most noticeable, and after 48 h, all of the peptides had the least proliferation effect. The greatest effective concentration was 0.5 mM for VW-9, 0.25 mM for VYK-9 and GHA-9, and 1mM for VF-9, 0.25 and 0.5 mM for VY-9 (Fig. 4A).

Our finding confirmed the importance of peptide hydrophobicity on their proliferative effects. Hydrophilic peptides, including SK-7, YR-10, and YHR-10 did not affect the cell proliferation, whereas hydrophobic peptides, including, VL-9, VY-9, VW-9, VF-9, VCK-9, VYK-9, VHK-9, and GHA-8 enhanced the cell proliferation. Previous researches also showed that the high content of hydrophobic amino acids promotes fibroblast proliferation due to interaction with cells and modulating cell growth [14, 15, 38].

We used the highest proliferation values of each peptide after 24 h to compare the activity of the peptides (Fig. 4B). On this basis, the highest proliferation effect was obtained for GHA-8 (146.11% ± 39.89), VW-9 (125.48% ± 31.184), and VF-9 (110.92% ± 18.90). Replacing Lys in the VL-9`s C-terminus, with aromatic amino acids Trp and Phe caused a significant increase in cell proliferation by 50.16 % ± 12.13 and 40.89% ± 8.38, respectively. The results reveal the important role of aromatic amino acid residues, Trp, and Phe at the C-terminus of peptides on

fibroblast growth. In addition, substituting Pro with Tyr, Cys, and His in the VL-9 sequence did not influence cell growth. These findings are consistent with the data obtained from the macrophages, that Pro, Cys, Tyr, and His in the antepenultimate position of peptides had the same effect on the cell proliferation.

3.7.2. In vitro wound scratch assay

The ability of the peptides on collagen production and fibroblast migration was investigated for a better understanding of their wound healing potential. We tested the collagen synthesis of the cells following incubation with 0.5 mM of the peptides for 24 h. Except for VYK-9, VHK-9, and SK-7, all other peptides significantly increased collagen production in fibroblasts (Fig. 4A). VF-9, GHA-8, PAR-3 were the most effective peptides, which induced 111.59% ± 17.75, 108.24% ± 3.57, and 108.24 ± 9.44 increases in collagen production. VW-9 also effectively stimulated cells to produce 44.94% ± 6.24 more collagen.

Cell migration was also observed after 16 h in cells treated with VF-9, VW-9, and VYK-9 compared to the control (Fig. 5B, C).

The presence of Val and Leu at the N-terminus of the peptide was observed as a common feature of VL-9, VY-9, VW-9, and VF-9 (under investigation in this research) and VLPVPQK described by Kumar et al. [39] for fibroblast`s stimulating effect. In addition, SK-7 which has the same sequence as VL-9 but lacks Val and Leu at N-terminus, did not affect fibroblasts, demonstrating the important role of Val and Leu on fibroblast`s stimulation.

Our findings show that the substitution of Lys with Phe in VL-9 resulted in a 40% increase in collagen formation, suggesting the more favorable impact of Phe as an aromatic hydrophobic amino acid compared to Lys in the C-terminus of peptides with positive effect on fibroblasts., VL-9 and VCK-9 with Pro and Cys in the antepenultimate position had a greater influence on collagen formation compared to VYK-9 and VHK-9 with Tyr and His at same position . These results indicate the beneficial role of Pro and Cys in the antepenultimate position of peptides for such effects. Pro was also reported in the antepenultimate position of VLPVPQK with the same effect on fibroblasts [39].

By comparing the sequence of VYK-9 (VLSTSFYPK) studied in this research and previously reported peptides (e.g. KKVVFWVKFK [21] and VLPVPQK [39]), it can be concluded that maybe Lys in the C-terminus contributes to the stimulating activity of peptides. However, VHK-9 (VLSTSFHPK), VCK-9 (VLSTSFCPK), and VL-9 (VLSTSFPPK), which all had Lys at the same place, had no significant positive effect. As the lone variation amongst this set of peptides, the presence of Tyr in the antepenultimate position of VYK-9 instead of His in VHK-9, Cys in VCK-9, and Pro in VL-9 may be a reason for VYK-9's increased activity. Furthermore, VHK-9's hydrophilic nature may be a factor in its decreased activity, as it makes it difficult for the peptide to connect with and penetrate cells [15].

VF-9 and VW-9 had the greatest effect on fibroblast migration and collagen production, demonstrating the beneficial role of aromatic amino acid in C-terminus of peptides with fibroblast's stimulating effect and also confirming the previous findings on the critical role of peptide hydrophobicity in fibroblast's stimulation, as it results in improved peptide-cell interaction and a more modulatory effects [14, 15].

3.8. Antimicrobial activity

All synthetic peptides were tested for antimicrobial activity against two common skin pathogens, *E.coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). Four of the 13 peptides tested, including GA-8, VHK-9, YR-10, and SK-7 demonstrated antimicrobial

activity. GA-8 was found to be antimicrobial against both *E. coli* and *S. aureus*. GA-8's MIC and MBC against *E. coli* and *S. aureus* were 10 mM and 20 mM, respectively. VHK-9 and YR-10 were found to have antimicrobial activity against *S. aureus*, with MICs of 5 mM for both. SK-7 showed antimicrobial activity against *S. aureus*, with a MIC of 10 mM. None of the peptides studied in this research showed multifunctional immunomodulatory and antimicrobial activities.

Despite the fact that the structure-function relationship of antimicrobial peptides is poorly understood, it has been demonstrated that the mechanism of action is dependent on peptide type, microbial target and experimental conditions [40]. It has been shown that membrane interaction is the most important component in peptide antimicrobial activity, regardless of whether the target is a membrane or intercellular compounds [41].

When we compared the antimicrobial activity of YR-10, YHR-10, GA-8, GHA-8, and PAR-3 with mostly identical sequences and only one or two amino acids difference, GA-8, which has one positive charge and most hydrophobic property (7 of 8 amino acids are hydrophobic) has bactericidal and bacteriostatic activity against both Gram-positive and Gram-negative bacteria. YR-10 which had two positive charges, and seven hydrophobic amino acids out of ten had only bacteriostatic activity against *S. aureus*. Peptides with cationic property can interact electrostatically with anionic components of Gram-positive and Gram-negative bacterial cell membrane, causing membrane integrity to be disrupted and/or translocate across the membrane to the cytoplasm to act on intracellular targets [42]. Having both hydrophilic and hydrophobic domains is also a key element for interacting antimicrobial peptides with the target cytoplasmic membrane. The charged domain of peptide interacts with hydrophilic groups of phospholipids, while the hydrophobic domain interacts with the hydrophobic core of the lipid bilayer, forcing the peptide further into the membrane [41, 43, 44].

Table 2. MIC and MBC values for the peptides with antimicrobial activity.

Peptide	**Sequences**	**MIC (mM)**		**MBC (mM)**	
		E. coli ATCC 25922	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*S. aureus* ATCC 25923
GA-8	GKPVAVPA	10	10	20	20
VHK-9	VLSTSFHPK	-	5	-	-
YR-10	YGKPVAVPAR	-	5	-	-
SK-7	STSFPPK	-	10	-	-

Aside from electric charges and peptide hydrophobicity, the type and sequence of amino acids play a role in antimicrobial action. The presence of hydrophobic amino acids, Gly, two Val, two Ala, and two Pro may contribute to the antimicrobial activity of both YR-10 and GA-8 as it has been reported previously [45, 46]. YR-10 and GA-8 differ in two amino acids, Tyr and Arg in the N and C terminals of YR-10, which are missing in the GA-8 sequence. Tyr is an aromatic amino acid and Arg is a polar, positive charge amino acid. The omission of one or both of the Tyr and Arg is thought to improve the peptide`s interaction with *E. coli* cell membrane.

When we compared the antimicrobial activity of peptides with the same sequence but one or two different amino acids, including VL-9, VY-9, VW-9, VF-9, VCK-9, VYK-9, VHK-9, and SK-7, we found that only VHK-9 which contains 4 of 9 hydrophobic amino acids, and SK-7 which contains 3 of 7 hydrophobic amino acid, and both with one positive charge, exhibited bacteriostatic activity against *S. aureus* (Table 2*)*. Apart from the amino acid type and sequence, positive charge and the hydrophobic/hydrophilic properties may facilitate the interaction of VHK-9 and SK-7 with the cell membrane, forming an amphipathic structure and penetrate the cells to exert antimicrobial activity [47].

3.9. *In vivo* wound healing potential

Next, an in vivo preclinical model was carried out to investigate the effect of peptide VW-9 with the most anti-inflammatory activity and fibroblast stimulating effect on wound healing using full-thickness excisional wounds created on rat skin.

The *in vivo* study of wound healing profiles for the two treatments, namely PRP (Platelet-Rich Plasma, as the positive control) and peptide solution, and their controls were illustrated in Fig. 6A. The results of wound healing ratio during the wound healing process were exemplified in Fig. 6B. PRP was considered positive control, since it is recognized to directly enhance healing by local release of growth factors, cytokines, as well as inflammatory mediators, essential for complete regeneration [48]. No sign of edema, erythema or suppuration in the wound area was observed on the treated groups over the 7 experimental days, suggesting that a regular course of wound healing was promoted. The results showed that both PRP and VW-9 solutions significantly reduced the wound area compared to the control wound region ($p<0.05$).

On day 3 of the study, the wounds showed similar characteristics for the two experimental groups, as there was no significant difference in the wound size between the treated groups and control, 26.81±9.99% and 20.44±3.84% for the PRP and the VW-9 treated groups respectively. On day 5, the PRP group seemed to be better (47.56± 5.02%) in promoting wound healing than the VW-9 (44±3.77%), as seen in the visual comparisons from Fig. 6A. As observed, the wound area of the control groups have significantly (28 ±5.02%) decreased at day 5.

On the 7th day, the percentage of wounds healing in the PRP and VW-9 groups reached nearly 70% and a significant difference ($p<0.05$) was observed for both PRP and VW-9 treated wounds when compared with their respective controls (Fig. 6B).

3.10. The histopathological observations

Histological evaluation with H&E staining shown in Fig. 6C was exemplified for wound sections at days 3 and 7. Results exhibited that the healing conditions of the tested groups were better than that of the control groups in the specimen on day 7.

On day 3, the wounds of the control groups present significant areas of necrosis and leukocyte exudation processes that replace the epidermis and partially destroyed dermis. In the PRP group, the wound is covered by a meshwork of fibrin, necrotic epithelial cells and neutrophils, and the dermis is invaded by severe congestion and a leukocyte-rich fibrin infiltrate. Although some inflammatory cells were noticeable in the dermis, the necrotic and fibrinous material rich in mononuclear cells could also be observed at the superficial level.

At the VW-9 wound surface, an important fibrino-necrotic material, infiltrated with neutrophils, substitutes the damaged epidermis. At the dermis level, the inflammatory process is present, but more faded than in the PRP and control groups. Also, the cellular and fibrinous exudations are much lower, with less pronounced congestion, reduced edema, significant fibrinous exudation and reduced leukocyte influx. The condensed inflammatory cells (rare macrophages and fibroblasts) identified in the tissue matrix were resumed to microcirculation congestion and diffuse edema.

The wound sections on day 7 showed more pronounced differences between batches. As expected, the control groups are still in the inflammatory phase of healing. The intense inflammatory process is characterized by edema with significant leukocyte exudation. However, regarding re-epithelialization of the lesions treated with only ultrapure water present, it is clear that the keratinocytes layer is absent on the surface. In the wounds treated with PRP solution, a minor necrosis area could be observed, along with connective tissue neogenesis (fibroblasts and collagen fibers) and neoformation blood vessels. The newly formed scar tissue

is loose and immature, suggesting reduced proliferation of keratinized stratified squamous epithelium (reduced re-epithelialization). On the contrary, in the lesions treated with the peptide solution, the re-epithelialization is mature with semi-oriented, dense young connective tissue, new covering epithelium resulting from the proliferation of keratinocytes at the edge of the wounds extending over the unformed one, demonstrating that the healing process is accelerated. More, a high number of blood vessels can be identified in the wounds treated with VW-9, indicating that peptide solutions strongly promote angiogenesis in vivo.
The level of wound closure was indicated also, by using scoring of histological parameters from minimal-to-very intense or complete appearance. The wounds treated with aqueous peptide fraction showed better histologic scores than PRP and control wounds, as shown in Table 3.
Table 3. Semi-quantitative evaluation of histological parameters for the assessment of wound healing. Notes: + mild; ++ moderate; +++ extensive; ++++ very intense; − absence.

Histological parameters	Day 3			Day 7		
	Control	PRP	VW-9	Control	PRP	VW-9
Necrosis	+++	+++	+++	+++	+	-
Congestion	+++++	+++++	+++++	+++	+	+
Inflammatory edema	+	+	+++	++++	-	-
Fibrinous exudation	+++++	+++++	+++	++++	-	-
Leukocyte infiltrate (neutrophils, macrophages, lymphocytes, histiocytes)	+++++	+++++	+++	++++	++	+
Resorption of the fibrinous matrix	-	-	-	+	++++	+++++
Cell differentiation in the wound (endothelial cells, fibroblasts)	-	-	-		++++	+++++
Fibrillar neogenesis (collagen fibers) and neoformation of blood vessels	-			++	++++	+++++
Re-epithelialization	-	-	-	+	++	+++

3.10. Conclusion

We studied the effects of 13 distinct peptides on macrophages and fibroblasts, as well as their antimicrobial activity, to assess their wound healing potential. On macrophages and fibroblasts, VW-9 showed the highest anti-inflammatory activity and stimulating effect. Our findings also confirm the importance of hydrophobicity on the immunomodulatory and proliferative effects of peptides. The two most abundant amino acid residues in the C-terminus of peptides, Trp and Phe, contributed to the anti-inflammatory and stimulatory action of peptides. VW-9's wound

healing ability was confirmed *in vivo*, and it may have the potential to functionalize wound healing biomaterials.

Acknowledgment
The first author (Mahta Mirzaei) acknowledges the fellowship provided by the European Program in IF@ULB-MARIE SKLODOWSKA-CURIE cofound action (European Horizon 2020). We gratefully acknowledge the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST) for antimicrobial assay. Graphical abstract is created by Biorender.com. Scratch assay analysis was done with the WimScratch software program (Wimasis, Munich, Germany).

Conflict of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
The data that supports this study is available from the authors upon reasonable request.

Credit author statement
Mahta Mirzaei: Conceptualization, Methodology, Data curation, Writing-review & editing, Funding acquisition, **Amin Shavandi**: Project administration, Investigation, Visualization. **Gianina Dodi, Ioannis Gardikiotis, Sorin-Aurelian Pasca**: In vivo data curation, Writing-review&editing, **Saeed Mirdamadi:** Investigation, Visualization, **Nazila Soleymanzadeh**: Data curation. **Houman Alimoradi**: Review & editing, **Muriel Moser, Stanislas Goriely:** Project administration, investigation, visualization.

References:

[1] J. Martin, A. Desfoux, J. Martinez, M. Amblard, A. Mehdi, L. Vezenkov, G. Subra, Bottom-up strategies for the synthesis of peptide-based polymers, Progress in Polymer Science 115 (2021) 101377.
[2] M. Chalamaiah, W. Yu, J. Wu, Immunomodulatory and anticancer protein hydrolysates (peptides) from food proteins: A review, Food Chemistry 245 (2018) 205-222.
[3] M.L. Mangoni, A.M. McDermott, M. Zasloff, Antimicrobial peptides and wound healing: biological and therapeutic considerations, Experimental Dermatology 25(3) (2016) 167-73.
[4] M. Dadar, Y. Shahali, S. Chakraborty, M. Prasad, F. Tahoori, R. Tiwari, K. Dhama, Anti-inflammatory peptides: current knowledge and promising prospects, Inflammation Research 68 (2019) 125-145.
[5] L. Fu, L. Xing, Y. Hao, Z. Yang, S. Teng, L. Wei, W. Zhang, The anti-inflammatory effects of dry-cured ham derived peptides in RAW264.7 macrophage cells, Journal of Functional Foods 87 (2021) 104827.
[6] K. Majumder, S. Chakrabarti, S.T. Davidge, J. Wu, Structure and Activity Study of Egg Protein Ovotransferrin Derived Peptides (IRW and IQW) on Endothelial Inflammatory Response and Oxidative Stress, Journal of Agricultural and Food Chemistry 61(9) (2013) 2120-2129.

[7] Q.Z. Huanli Jiao, Yuanbang Lin, Ying Gao, Peng Zhang, The Ovotransferrin-Derived Peptide IRW Attenuates Lipopolysaccharide-Induced Inflammatory Responses, Biomed Research International, Biomedical research International, 2019, doi: 10.1155/2019/8676410.
[8] J. Kovacs-Nolan, H. Zhang, M. Ibuki, T. Nakamori, K. Yoshiura, P.V. Turner, T. Matsui, Y. Mine, The PepT1-transportable soy tripeptide VPY reduces intestinal inflammation, Biochimica et Biophysica Acta (BBA) - General Subjects 1820(11) (2012) 1753-1763.
[9] S.-J. Kwak, C.-S. Kim, M.-S. Choi, T. Park, M.-K. Sung, J.W. Yun, H. Yoo, Y. Mine, R. Yu, The Soy Peptide Phe–Leu–Val Reduces TNFα-Induced Inflammatory Response and Insulin Resistance in Adipocytes, Journal of Medicinal Food 19(7) (2016) 678-685.
[10] H. Hou, Y. Fan, S. Wang, L. Si, B. Li, Immunomodulatory activity of Alaska pollock hydrolysates obtained by glutamic acid biosensor – Artificial neural network and the identification of its active central fragment, Journal of Functional Foods 24 (2016) 37-47.
[11] C.-B. Ahn, Y.-S. Cho, J.-Y. Je, Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate, Food Chemistry 168 (2015) 151-156.
[12] Z. Shi, B. Dun, Z. Wei, C. Liu, J. Tian, G. Ren, Y. Yao, Peptides Released from Extruded Adzuki Bean Protein through Simulated Gastrointestinal Digestion Exhibit Anti-inflammatory Activity, Journal of Agricultural and Food Chemistry 69(25) (2021) 7028-7036.
[13] T.A.E. Ahmed, R. Hammami, Recent insights into structure–function relationships of antimicrobial peptides, Journal of Food Biochemistry 43(1) (2019) e12546.
[14] C. Ozaki, S. Somamoto, S. Kawabata, Y. Tabata, Effect of an artificial silk elastin-like protein on the migration and collagen production of mouse fibroblasts, Journal of Biomaterials Science, Polymer Edition 25(12) (2014) 1266-1277.
[15] S. Benjakul, S. Karnjanapratum, W. Visessanguan, Hydrolysed collagen from Lates calcarifer skin: its acute toxicity and impact on cell proliferation and collagen production of fibroblasts, International Journal of Food Science & Technology 53(8) (2018) 1871-1879.
[16] M. Mirzaei, S. Mirdamadi, M.R. Ehsani, M. Aminlari, Production of antioxidant and ACE-inhibitory peptides from Kluyveromyces marxianus protein hydrolysates: Purification and molecular docking, Journal of Food and Drug Analysis 26(2) (2018) 696-705.
[17] M. Mirzaei, S. Mirdamadi, M.R. Ehsani, M. Aminlari, E. Hosseini, Purification and identification of antioxidant and ACE-inhibitory peptide from Saccharomyces cerevisiae protein hydrolysate, Journal of Functional Foods 19 (2015) 259-268.
[18] M. Mirzaei, S. Mirdamadi, M. Safavi, Antioxidant activity and protective effects of Saccharomyces cerevisiae peptide fractions against H2O2-induced oxidative stress in Caco-2 cells, Journal of Food Measurement and Characterization 13(4) (2019) 2654-2662.
[19] I. Alimov, S. Menon, N. Cochran, R. Maher, Q. Wang, J. Alford, J.B. Concannon, Z. Yang, E. Harrington, L. Llamas, A. Lindeman, G. Hoffman, T. Schuhmann, C. Russ, J. Reece-Hoyes, S.M. Canham, X. Cai, Bile acid analogues are activators of pyrin inflammasome, Journal of Biological Chemistry, 294(10) (2019) 3359-3366.
[20] P. Krutzik, J. Irish, G. Nolan, O. Perez, Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications, Clinical Immunology (Orlando, Fla.) 110 (2004) 206-21.
[21] H.-J. Park, M. Salem, A. Semlali, K.P. Leung, M. Rouabhia, Antimicrobial peptide KSL-W promotes gingival fibroblast healing properties in vitro, Peptides 93 (2017) 33-43.
[22] J. Wang, B. Zhang, W. Lu, J. Liu, W. Zhang, Y. Wang, M. Ma, X. Cao, Y. Guo, Cell Proliferation Stimulation Ability and Osteogenic Activity of Low Molecular Weight Peptides Derived from Bovine Gelatin Hydrolysates, Journal of Agricultural and Food Chemistry 68(29) (2020) 7630-7640.
[23] M. Lu, X.-H. Zhao, The growth proliferation, apoptotic prevention, and differentiation induction of the gelatin hydrolysates from three sources to human fetal osteoblasts (hFOB 1.19 Cells), Molecules 23(6) (2018) 1287.

[24] A. Jacquot, S.F. Gauthier, R. Drouin, Y. Boutin, Proliferative effects of synthetic peptides from β-lactoglobulin and α-lactalbumin on murine splenocytes, International Dairy Journal 20(8) (2010) 514-521.
[25] K. Kårehed, A. Dimberg, S. Dahl, K. Nilsson, F. Öberg, IFN-γ-induced upregulation of Fcγ-receptor-I during activation of monocytic cells requires the PKR and NFκB pathways, Molecular Immunology 44(4) (2007) 615-624.
[26] C.B. Ahn, Y.S. Cho, J.Y. Je, Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate, Food Chemistry 168 (2015) 151-6.
[27] E.C.L. de Oliveira, K. Santana, L. Josino, A.H. Lima e Lima, C. de Souza de Sales Júnior, Predicting cell-penetrating peptides using machine learning algorithms and navigating in their chemical space, Scientific Reports 11(1) (2021) 7628.
[28] S. Guha, K. Majumder, Structural-features of food-derived bioactive peptides with anti-inflammatory activity: A brief review, Journal of Food Biochemistry 43(1) (2019) e12531.
[29] H.J. Vogel, D.J. Schibli, W. Jing, E.M. Lohmeier-Vogel, R.F. Epand, R.M. Epand, Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides, Biochemistry and Cell Biology 80(1) (2002) 49-63.
[30] L. Zhao, X. Wang, X.-L. Zhang, Q.-F. Xie, Purification and identification of anti-inflammatory peptides derived from simulated gastrointestinal digests of velvet antler protein (Cervus elaphus Linnaeus), Journal of Food and Drug Analysis 24(2) (2016) 376-384.
[31] N. Yahfoufi, N. Alsadi, M. Jambi, C. Matar, The Immunomodulatory and Anti-Inflammatory Role of Polyphenols, Nutrients 10(11) (2018).
[32] S. Chen, S. Bonifati, Z. Qin, C. St. Gelais, K.M. Kodigepalli, B.S. Barrett, S.H. Kim, J.M. Antonucci, K.J. Ladner, O. Buzovetsky, K.M. Knecht, Y. Xiong, J.S. Yount, D.C. Guttridge, M.L. Santiago, L. Wu, Samhdi suppresses innate immune responses to viral infections and inflammatory stimuli by inhibiting the NF-κB and interferon pathways, Proceedings of the National Academy of Sciences 115(16) (2018) E3798-E3807.
[33] K. Aihara, H. Ishii, M. Yoshida, Casein-derived tripeptide, Val-Pro-Pro (VPP), modulates monocyte adhesion to vascular endothelium, Journal of Atherosclerosis and Thrombosis 16(5) (2009) 594-603.
[34] C.-B. Ahn, J.-Y. Je, Y.-S. Cho, Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis, Food Research International 49(1) (2012) 92-98.
[35] H.J. Chung, J. Lee, J.S. Shin, M.R. Kim, W. Koh, M.J. Kim, J.W. Lee, E.J. Kim, I.H. Lee, W.K. Kim, Y.J. Lee, S.K. Lee, I.H. Ha, In Vitro and In Vivo Anti-Allergic and Anti-Inflammatory Effects of eBV, a Newly Developed Derivative of Bee Venom, through Modulation of IRF3 Signaling Pathway in a Carrageenan-Induced Edema Model, PLoS One 11(12) (2016) e0168120.
[36] J. Han, R.J. Ulevitch, Emerging targets for anti-inflammatory therapy, Nature Cell Biology 1(2) (1999) E39-E40.
[37] R. Addis, S. Cruciani, S. Santaniello, E. Bellu, G. Sarais, C. Ventura, M. Maioli, G. Pintore, Fibroblast Proliferation and Migration in Wound Healing by Phytochemicals: Evidence for a Novel Synergic Outcome, International Journal of Medical Science 17(8) (2020) 1030-1042.
[38] H. Ohara, S. Ichikawa, H. Matsumoto, M. Akiyama, N. Fujimoto, T. Kobayashi, S. Tajima, Collagen-derived dipeptide, proline-hydroxyproline, stimulates cell proliferation and hyaluronic acid synthesis in cultured human dermal fibroblasts, The Journal of Dermatology 37(4) (2010) 330-338.
[39] N. Kumar, S. Reddi, S. Devi, S.B. Mada, R. Kapila, S. Kapila, Nrf2 dependent antiaging effect of milk-derived bioactive peptide in old fibroblasts, Journal of Cellular Biochemistry 120(6) (2019) 9677-9691.

[40] K.D. Saint Jean, K.D. Henderson, C.L. Chrom, L.E. Abiuso, L.M. Renn, G.A. Caputo, Effects of Hydrophobic Amino Acid Substitutions on Antimicrobial Peptide Behavior, Probiotics Antimicrob Proteins 10(3) (2018) 408-419.
[41] M. Mahlapuu, J. Håkansson, L. Ringstad, C. Björn, Antimicrobial Peptides: An Emerging Category of Therapeutic Agents, Frontiers in Cellular and Infection Microbiology 6(194) (2016).
[42] D. Mohanty, R. Jena, P.K. Choudhury, R. Pattnaik, S. Mohapatra, M.R. Saini, Milk Derived Antimicrobial Bioactive Peptides: A Review, International Journal of Food Properties 19(4) (2016) 837-846.
[43] T.A.E. Ahmed, R. Hammami, Recent insights into structure-function relationships of antimicrobial peptides, Journal of Food Biochemistry 43(1) (2019) e12546.
[44] P.A.B. Branco, Purification and biochemical/molecular characterisation of antimicrobial peptides produced by Saccharomyces cerevisiae and evaluation of their mode of action, Lisboa:ISA (2018) 246 P.
[45] Q.-Y. Zhang, Z.-B. Yan, Y.-M. Meng, X.-Y. Hong, G. Shao, J.-J. Ma, X.-R. Cheng, J. Liu, J. Kang, C.-Y. Fu, Antimicrobial peptides: mechanism of action, activity and clinical potential, Military Medical Research 8(1) (2021) 48.
[46] C. Imjongjirak, P. Amphaiphan, W. Charoensapsri, P. Amparyup, Characterization and antimicrobial evaluation of SpPR-AMP1, a proline-rich antimicrobial peptide from the mud crab Scylla paramamosain, Developmental & Comparative Immunology 74 (2017) 209-216.
[47] G. Moscoso-Mujica, A.I. Zavaleta, Á. Mujica, I. Arnao, C. Moscoso-Neira, M. Santos, J. Sánchez, Antimicrobial peptides purified from hydrolysates of kanihua (Chenopodium pallidicaule Aellen) seed protein fractions, Food Chemistry 360 (2021) 129951.
[48] P. Everts, K. Onishi, P. Jayaram, J.F. Lana, K. Mautner, Platelet-Rich Plasma: New Performance Understandings and Therapeutic Considerations in 2020, International Journal of Molecular Science 21(20) (2020) 7794.