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Chicken Feather Keratin Peptides for the Control of Keratinocyte Migration

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Featured Application: The work described in this paper has significant potential for medical applications. As an example, these peptides can be incorporated into wound dressings to treat chronic wounds, increasing their re-epithelization, or to treat other skin diseases, such as psoriasis.

Abstract: FAO estimates that in 2030 the poultry meat production could reach 120 million tons, which is a challenge in terms of waste management. Feathers are mainly composed of keratin, an important biomaterial. Using feathers as a source of keratin will minimize the waste generated, while contributing to supply an important material for several industries, such as pharmaceutical and biomedical. The peptides were extracted from the feathers by microbial degradation. In this study, we evaluated the peptides effect on keratinocyte metabolic activity and migration. The influence of these peptides on non-activated and activated macrophages was also assessed. It was demonstrated that depending on the keratin peptide fraction in contact with keratinocytes, it is possible to modulate the migration rate of the keratinocytes. Peptide fraction with low molecular weight increases migration, while peptides with a high range of molecular sizes decreases it. Some peptide fractions induce the secretion of TNF-α in non-activated macrophages and not on activated macrophages, demonstrating that these peptides should only be placed in contact with cells, in the context of an ongoing inflammatory process. This work is a step forward on the understanding of keratin peptides influence on keratinocytes and immune cells system cells, macrophages.

Keywords: chicken feathers; microbial hydrolysis; keratin peptides; keratinocytes; cellular migration; inflammation

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1. Introduction

The Food and Agriculture Organization estimates that in 2030 the poultry meat production could reach 120 million tons [1,2]. This fact poses a huge challenge in terms of waste management. Worldwide, it is calculated that the poultry-processing industry generates over 5 million tons of feather biomass [3]. This represents a serious waste hazard since only a small amount is processed into valuable products, such as feather meal or fertilisers [4]. Due to economic and environmental pressures, the industry is forced to find better ways to deal with feathers waste and generate economic value. This is particularly important due to the EU Directive 1999/31/EC on the landfill of waste [5], which restricts the disposal of waste with a significant concentration of biodegradable materials and with

high burning values, such as feathers. Finding an eco-friendly and effective way to reuse chicken feathers waste is of the utmost importance for this industry. The importance is not only due to the necessity to comply with the law and contribute to the 2030 United Nations goals [6] for sustainable development, but it is an opportunity to reduce costs in waste management. Furthermore, it is also an opportunity to create high-value products that can be used, for example, in the pharmaceutical and dermo-cosmetic industry. In this way, chicken feathers can be recycled and provide high-value materials, decreasing economic and environmental pressures [4]. The poultry industry could receive a boost based on renewable and sustainable growth.

The possibility to use feather waste material from the poultry industry in dermo-cosmetics is related to its high content (90%) in a specific protein, keratin, particularly β-keratin. Keratin is a widely used material in the pharmaceutical, cosmetic, medical, biomedical, and biotechnological industries due to its unique characteristics of bioactivity, biodegradability, and biocompatibility [3,7].

Several techniques can be used to extract keratin from feathers, such as acid and alkali hydrolysis, hydrothermal methods, high-density steam flash explosion, and ionic liquids. These techniques raise even more environmental concerns and have a low keratin yield [8]. The use of enzymatic and microbial keratinases can overcome all these problems [9]. The use of keratinases involves a few steps, namely keratinases production by bacteria or fungi strains, followed by its isolation and purification. Then, keratinases can be incubated with a rich keratin-containing substrate that can be hydrolysed to soluble peptides without the loss of essential amino acids [10,11]. Enzymatic keratinolysis is an effective, economic, and environmentally friendly method to extract keratin-based peptides from chicken feathers.

For the past years', keratin-based biomaterials have gained interest due to keratin biodegradability, biocompatibility, and mechanical durability [12]. Keratin is particularly interesting for skin applications, as it is the most abundant structural protein in epithelial cells after collagen [13]. Psoriasis, chronic cutaneous wound, and skin cancer are common skin diseases associated with the dysregulation of keratinocyte proliferation [14]. This event is often linked with a pathological pathway of regenerative maturation characterized by a higher rate of proliferation, aberrant response to growth factors, faulty differentiation, and increased migratory capability [14]. Keratins are typical intermediate filament proteins, having an important role in the mechanical stability and integrity of epithelial cells and tissues [15]. These proteins are also involved in regulatory functions and intracellular signalling pathways, such as wound healing [15].

The interactions between keratins and skin cells have been studied for many years. At the beginning of the 1950s, Giroud and Leblond, and later in the 1970s, Sun et al. studied the intermolecular disulphide bond of keratins on keratinocytes and their influence on the final stage on cell differentiation [16,17]. This fact demonstrates the importance of keratin interactions. Moreover, it is known that actin cytoskeleton is crucial for motility of adult keratinocytes; there is evidence that keratins, which are normally basally restricted, appear suprabasally in keratinocytes at the wound margin [18]. It is also described that short filament keratins 6, keratin 16 and 17 are induced and appear to help retract other cellular keratins into juxtanuclear aggregates within the actively crawling cells [18,19]. These studies demonstrated the importance of keratin on wound healing, particularly migration.

The peptide molecular weight has a significant influence on the cellular metabolism, so on this work, keratin peptides obtained from microbial chicken-feather degradation were fractioned, according to their molecular weight, and their effect on keratinocyte migration and metabolic activity as well as on macrophage release of TNF- α was evaluated.

2. Materials and Methods

2.1. Microorganisms

The Bacillus subtilis (S188D) used in this study belong to the collection of Bacillus of the University of Azores (Portugal). This strain was chosen for this work due to its high capability to hydrolase chicken feathers (results obtained in previous tests, data not

shown). A single fresh colony of strain S188D was grown in 5 mL of lysogeny broth (LB) (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) at 28 °C for 18 h, for later use in the fermentation process.

2.2. Fermentation

Chicken feathers were thoroughly washed in current water, dried at 60 °C for 24 h and shredded in a blade mill before fermentation. For microbial fermentation, 2 g of shredded chicken feathers were incubated with 100 mL of minimum medium (1 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.1 g/L $MgSO_4.7H_2O$, 7 g K_2HPO_4 and 0.2 g yeast extract per litre, pH 7) [20], and 2 mL of LB S188D culture (0.5 MacFarland standard at 600 nm) transferred to the medium with feathers. The fermentation was performed at 28 °C and 120 rpm for 48 h. These conditions of solid/liquid ratio, temperature, agitation, and time were previously optimized in our group (data not shown).

2.3. Isolation, Purification and Fractionation

At the end of the fermentation, bacteria and contaminant products were separated by centrifugation at 8000 g for 10 min, using a fixed angle rotor (Megafuge, Heraeus). The supernatant was then filtered through a 0.22 µm cellulose acetate membrane (Millipore) and concentrated by tangential flow filtration (TFF). The TFF was performed using a polyethersulfone cassette (Millipore) with 10 KDa cut-off membranes to obtain the peptides (eluted fractions). The peptides were then subjected to different purification procedures. First, they were desalted using a C18 column and eluted with graded series of acetonitrile, 20%, 40% and 80%. Each eluted fraction was fractionated using a Superdex peptide column to obtain different molecular weight chromatographic peaks, P1, P2, P3, P4, and P5. The peptide was designated as P188D in accordance with the bacterial isolate used.

2.4. Cells

Human keratinocytes (NCTC 2544) were grown in Dulbecco's modified Eagle medium (DMEM; Lonza Group, Ltd., Braine l'Alleud, Belgium) with 10% foetal bovine serum (FBS; Biosera, Ringmer, UK) and 1% penicillin-streptomycin (complete medium). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Human dermal fibroblasts (Zen-Bio DF-F) were used in the preliminary viability assay (outsourced—Tebu-Bio). The cells were grown in Dulbecco's modified Eagle medium (DMEM; Lonza Group, Ltd., Braine l'Alleud, Belgium) with 10% foetal bovine serum (FBS; Biosera, Ringmer, UK) and 1% penicillin-streptomycin (complete medium). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Macrophage cell line Raw 264.7 was also used to evaluate secretion of TNF-α, pro-inflammatory cytokine, after incubation with the peptides. This cell line was routinely cultured with DMEM high glucose, supplemented with 10% heat-inactivated FBS and 10 mM 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid (HEPES) buffer in tissue culture flasks 5% (v/v) CO_2 at 37 °C.

2.4.1. Proliferation Assay

Keratinocytes were maintained with complete medium and seeded into 96-well plates (2×10^4 cells/mL). The cells were incubated overnight at 37 °C in 5% CO_2 atmosphere. After 24 h, the culture medium was replaced by complete medium with 45 µg/mL of each peptide solution. Resazurin sodium salt was diluted in PBS to a concentration of 0.15 g/L and filter (0.2 µm pore diameter). The resazurin working solution was prepared by diluting resazurin stock solutions 1:5 in complete medium. After 24 h of contact, the cells were washed with PBS and incubated with resazurin working solution for 2 h. At this time point, the supernatant was removed and placed into black, opaque 96-well plates to measure the conversion of rezaruin to resorfin. For that, a fluorimeter with an excitation wavelength at 540 nm and emission at 590 nm was used.

2.4.2. Migration Assay

Keratinocytes were seeded into a six-well plate (5×10^5 cells/mL) and incubated overnight at 37 °C in 5% CO2 atmosphere. A scratch on the confluent cell layer was performed with a pipette tip. The culture medium was then replaced by complete medium and 45 µg/mL of each keratin peptides solution. Cell migration was monitored microscopically, and images were acquired at 0 h, 6 h and 24 h, in the same region.

The area of the scratch was measured at different time points using Image J's plugin, MRI Wound Healing Tool.

2.4.3. Quantification of TNF-α

The macrophage cell line Raw 264.7 was routinely cultured in DMEM supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, and 25 mM HEPES buffer, in tissue culture flasks (Nagle Nunc, Int., Hereford, UK) in a humified atmosphere with 5% (v/v) CO_2 at 37 °C (Binder CB150; Tuttlingen, Germany).

After confluent growth, macrophage cells were washed with fresh medium and recovered by scrapping. Viable cells were counted by Trypan blue exclusion in the haemocytometer and resuspended in DMEM to a final concentration of 1×10^5 cells/mL. Then, 500 µL of the macrophage suspension was cultured in 24-well tissue culture plates. Cells were incubated overnight with increasing lipopolysaccharide (LPS) concentrations (0.08, 0.33, and 1 µg/mL). After incubation, supernatants were collected, and the cells were treated with different peptide fractions. Then, 24 h after treatment the cells' supernatants were stored at −20 °C for TNF-α quantification. The Mouse TNF alpha Elisa Ready-SET-Go® (Sensitivity: 8 pg/mL) was used according to the manufacturer's instructions (Affymetrix, eBioscience). Cells incubated only with DMEM were considered negative controls. Two other controls were also performed: cells treated only with peptides and cells treated with different concentrations of LPS.

2.5. Mass Spectrometry Analysis and Peptide/Protein Identification

Mass spectrometry was performed at the UniMS—Mass Spectrometry Unit, ITQB/IBET, Oeiras, Portugal. Briefly, 20 µL of the samples were and diluted 1:20 in buffer A (0.1% formic acid in water, Fisher Chemicals, Geel, Belgium).

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed on an ekspert™ NanoLC 425 cHiPLC® system coupled with a TripleTOF® 6600 with a DuoSpray Turbo V ion source (Sciex, Framingham, MA, USA). Peptides were separated through reversed-phase chromatography (RP-LC). Separation was performed at 1µL/min, on a HALO C18 column (Sciex 0.5 × 50 mm, 2.7 µm, 90 Å). The gradient was as follows: 0–1 min, 5% B (0.1% formic acid in acetonitrile, Fisher Chemicals, Geel, Belgium); 1–31 min, 5–30% B; 31–34 min, 30–95% B; 34–36 min, 95% B; 36–38 min, 95–5% B; 38–42 min, 5% B.

Peptides were sprayed into the MS through an ESI electrode (50 µm, Eksigent). The source parameters were set as follows: 20 GS1, 0 GS2, 30 CUR, 5.5 keV ISVF and 100 °C IHT. An information-dependent acquisition (IDA) method was set with a TOF-MS survey scan of 400–2000 *m/z*. The 40 most intense precursors were selected for subsequent fragmentation and the MS/MS were acquired in high-sensitivity mode. The obtained spectra were processed and analysed using ProteinPilot™ software, with the Paragon search engine (version 5.0, Sciex). A database containing the sequences of the proteins of interest was used (*Gallus gallus* feather keratin, from Uniprot 09-2017). The instrument selected was TripleTOF 6600. The ID focus was on biological modifications and amino acid substitutions. The search effort was set as thorough. Only the proteins with an unused protein score above 2.2 and 99% confidence were considered.

2.6. Statistical Analysis

All measurements were performed in triplicate and data presented as mean. For the selected evaluation tests, the means of all tested formulations were compared with

each other by means of one paired Student's *t*-test or one-way ANOVA. The statistical significance level (p) was set at <0.05.

3. Results and Discussion

3.1. Cell Metabolic Activity and Migration

Cellular migration is of the utmost importance for several mechanisms, such as immune response, wound repair and tissue homeostasis [21]. On the other hand, an aberrant cell migration leads to several pathologies such as the invasion of malignant cells into the surrounding tissue, with the formation of tumour metastasis, leading to a poorer prognostic for the patient [22].

A patient with a chronic wound has a compromised quality of life due to excruciating pain and the constant need for medical care, resulting in a significant burden to the health system worldwide. Briefly, the physiological process of wound healing has several steps, such as haemostasis, inflammation, proliferation, and remodelling. The haemostasis starts as soon as the injury occurs and is characterized by vasoconstriction and blood clotting. This stage is of the utmost importance as it prevents excessive blood loss and provides the required matrix for cell migration. Upon the inflammation stage in which phagocytic cells are recruited, the proliferation stage starts. At this stage, several growth factors and cytokines are recruited by different cell types, such as keratinocytes. The final stage is characterized by the formation and organization of collagen bundles [21]. If an arrest or delay occurs in any of these stages, particularly in the inflammation stage, it can lead to the formation of a chronic wound. It is known that keratinocytes have an important role in wound healing, particularly in re-epithelization [23]. The non-migration of these cells to the injury site is associated with the clinical phenotype of chronic wounds.

Even though keratinocytes are not motile in normal steady-state condition, in the presence of signalling molecules, they can migrate to re-epithelize the wound site [24]. Consequently, the enhancement of the keratinocyte migration rate is of the utmost importance for wound re-epithelization, particularly in the case of chronic wounds [25].

On the other hand, cancer metastization or invasion into the surrounding tissue leads to a poorer prognostic for the patient, as in the case of invasive squamous cell carcinoma [22]. Squamous cell carcinoma and basal cell carcinoma are keratinocyte carcinomas, particularly diagnosed in fair-skinned populations [26,27]. The decrease in cell migration might result in a less invasive tumour.

The ability of keratinocytes to migrate from one edge of the scratch, performed on a cell confluent monolayer, to its opposite edge was evaluated for a period of 24 h, in the presence and absence of the peptides designated by P188D. First, a preliminary cell viability study using fibroblasts demonstrated that the presence of 25 and 50 μg/mL of peptides resulted in 94.2 ± 0.77 and 92.4 ± 1.55% of viable cells, respectively. Therefore, 45 μg/mL was the chosen concentration for the following tests.

The effect of P188D peptide on keratinocyte migration was evaluated up to 24 h hours of contact. The presence of the peptide seems to decrease the keratinocyte migration rate in comparison to the control, this effect being clear at 24 h of contact (Figure 1). Thus, it seems that the presence of the peptides negatively affects keratinocyte migration, which could be important to reduce the invasiveness of skin cancer cells.

The microbial degradation of a protein results in several peptides with different sizes, amino acid composition and sequence. It is known that these characteristics have a striking effect on several physiologic mechanisms [28]. Therefore, to better understand the effect observed on keratinocyte migration, peptides were fractioned into smaller fractions using different percentages of acetonitrile 20%, 40% and 80% (20%, 40% and 80% fraction peptide). The final result was a solution with a narrowed peptides distribution. The influence of these fractions on the keratinocyte metabolic activity and migration was once again evaluated.

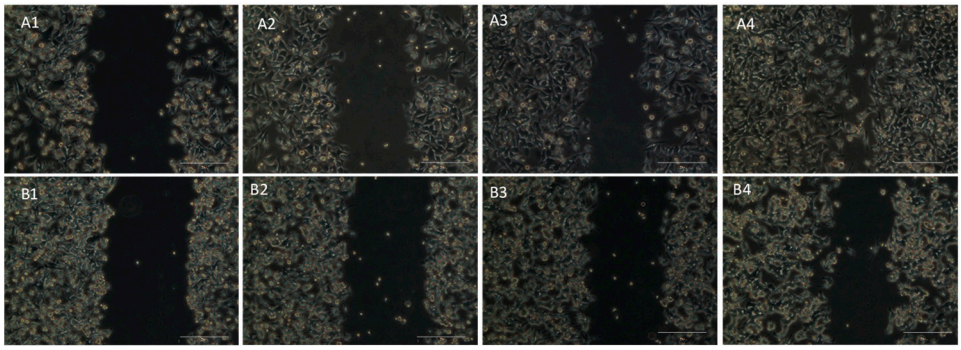

Figure 1. Keratinocyte migration in absence (**A**) and presence (**B**) of P188D peptide over time. 1—before contact, 2—2 h, 3—6 h, 4—24 h, respectively. Scale bar—200 mm.

As can be seen in Figure 2A,B, the presence of the 20% peptide fraction decreased the metabolic activity and migration rate of the keratinocytes, although it did not reach statistical significance. On the other hand, fractions 40 and 80% do not seem to have any effect on either mechanism. As these fractions did not induce significant changes in the keratinocyte proliferation and migration rate, an additional fractionation was performed, where peptides were separated according to their molecular weight, ranging from 9090 to 401 Da for the 20% fraction (P188D 20%) and from 8082 to 382 Da for the 40% fraction (P188D 40%) (Table 1). Once again, the keratinocyte proliferation and migration rate were determined in contact with these new peptide fractions.

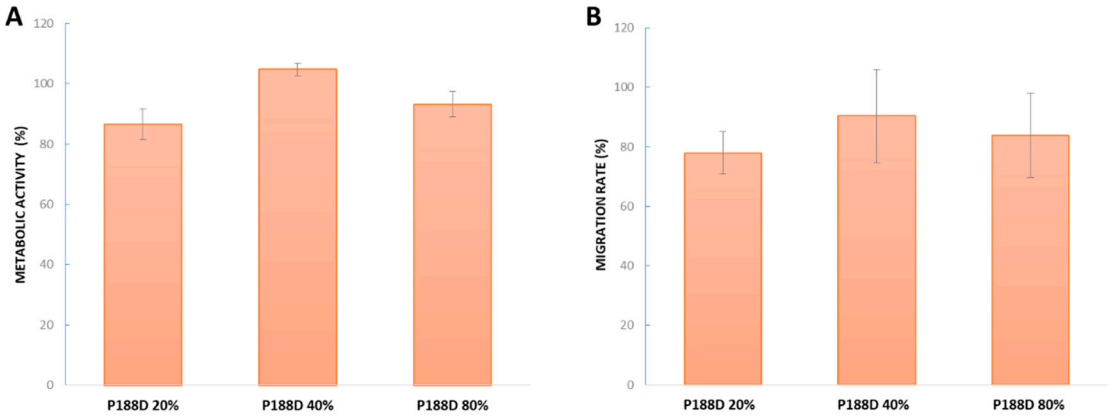

Figure 2. Keratinocyte proliferation (**A**) and migration rate (**B**) (in comparison to the control) in the presence of peptide fractions (45 g/mL).

Table 1. Peptide fractions and respective molecular weight.

Peptide Fraction.	Chromatographic Peak	Molecular Weight (Da)
P188D 20%	P1	9090
	P2	1361
	P3	401
P188D 40%	P1	8082
	P2	4699
	P3	2473
	P4	1199
	P5	382

In this analysis, the results obtained were very distinct (Figure 3) from the previous one (fraction 20%, 40% and 80%). Significant changes were observed in the metabolic activity of cells in the presence of peptides, particularly in the presence of 40% fraction

peptides. Analysing Figure 3A, it is clear that fractions P188D 20% P1, P188D 40% P1 seem to induce a higher metabolic activity of these cells; in contrast, P188D 40% P3 induced a decrease in the metabolic activity. Even though the results showed that sub-fraction P1 from 20% fraction peptide induced the highest metabolic activity in comparison to P2 or P3 from the same fraction, these differences did not reach statistical significance. On the other hand, significant changes were observed for the peptides from the 40% fraction, particularly P188D 40% P1, P3 and P5. While P1 significantly increased the metabolic activity, P3 decreased it.

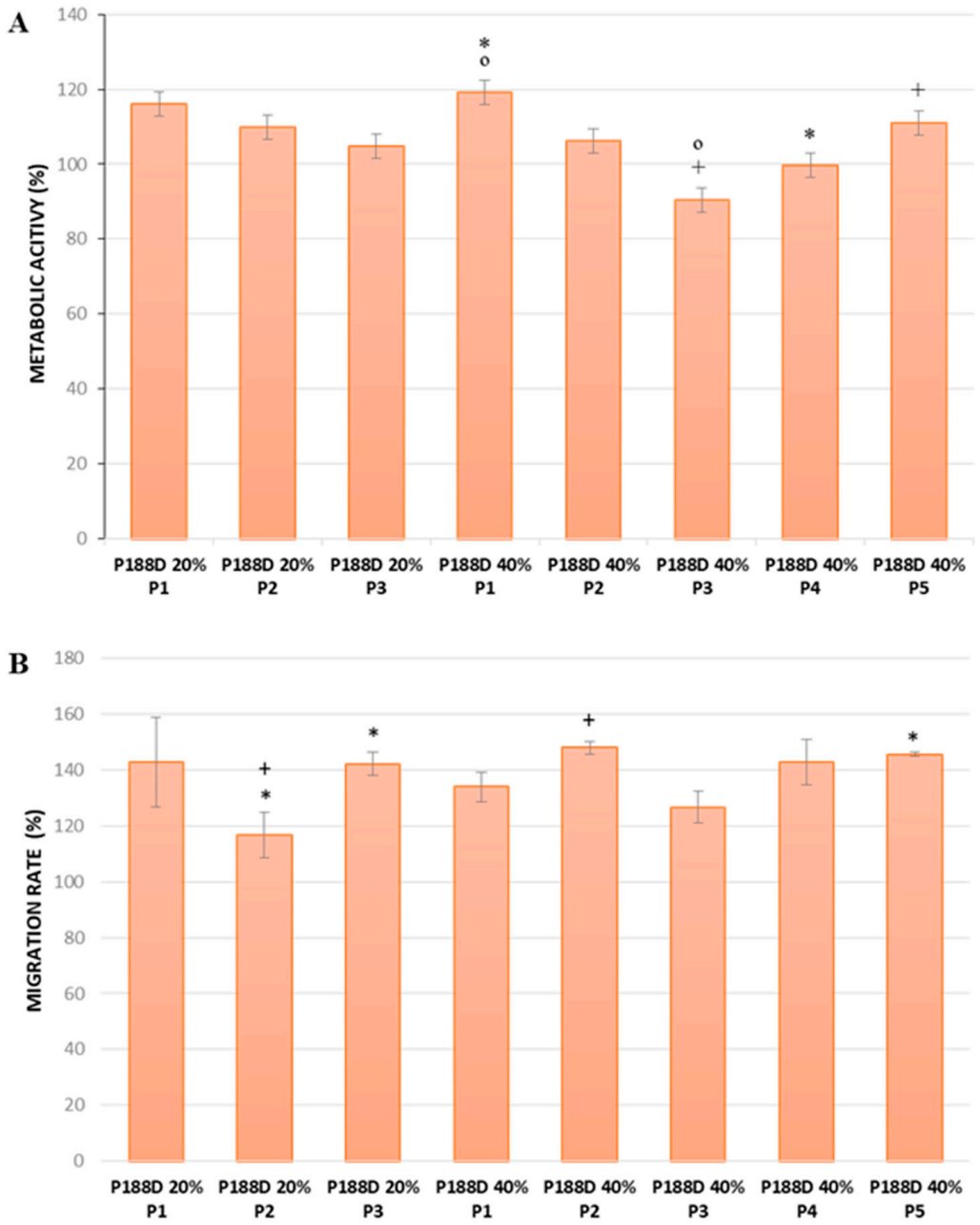

Figure 3. Keratinocyte metabolic activity (**A**) and keratinocyte migration rate (**B**) (in comparison to the control) in the presence of peptide fraction. Same symbol shows significant differences between groups ($p < 0.05$). *. + statistical significant between the groups.

All the peptide sub-fractions induced an increase in the keratinocyte migration rate (Figure 3B), with significant differences. P188D 20%, P2 and P3 are significantly different,

with the latter presenting a higher migration rate in comparison to the former. A similar result was observed for P188D 40% P2, wherein P188D 20% P2 and P188D 40% P2 are significantly different, with the latter inducing a higher migration rate. P188D 40 % P3 also significantly affects migration in comparison with P188D 20% P1 and P188D 20 % P3. It seems that the presence of these peptides has a more significant effect on the migratory ability of these cells than on their metabolic activity. In this case, the use of fractioned peptides is very important for skin reepithelization and therefore for the treatment of chronic wounds.

It is important to mention that resazurin assay is based on the capacity of living cells to transform resazurin to resorufin [29]. This ability is correlated with cell viability and cell number/proliferation. The basis for this understanding is that the higher the conversion of the dye, the higher the cell number. Therefore, taking into consideration this fact, it is possible to interpret the results observed, as the peptides fraction has a higher influence on cellular migration than on proliferation.

There are different physiological or pathological conditions, such as wound repair that induce cells to shift their phenotype from proliferative to migratory in response to similar stimulus (reciprocal control of cell proliferation and migration). It is known that cytokines produced by cells present at the wound area can act as a chemoattractant for distant cells, leading to cell migration [30]. However, upon their arrival, the cells switch from a migrating phenotype to a proliferating one [30]. In this case, the wound inflicted on the cell monolayer may have induced cells to release chemoattractant, signalling cells to migrate to the injury site.

It has been demonstrated that keratin intermediate filaments regulate proliferation, migration, adhesion, and inflammatory phenotype of keratinocytes [31].

Keratins 6, 16 and 17 are very important as they are considered barrier alarmins, rapidly inducing keratinocytes to migrate to the injury site [31]. As in the wound healing process, keratinocytes suffer dramatic changes as they are required to migrate and proliferate during the healing phase. Their expression is founded in the epithelial remodelling phase until the barrier function is repaired, demonstrating the importance of these keratins in wound repair [31]. Among others, the expression of keratin 6, 16, and 17 persist through the epithelial remodelling phase until the barrier function is restored [32]. It is hypothesized that the peptides in the study may have a similar role to these keratins.

3.2. Quantification of TNF-α

Inflammation is a crucial process in several medical conditions, such as psoriasis and wound healing; therefore, the ability of these peptide fractions to influence the inflammatory response in terms of TNF-α release by macrophages was evaluated.

This assay had two distinct steps; the first one was to evaluate if the peptide fractions induced an inflammatory response in non-activated macrophages (0 mg/mL of LPS) and the second one was to evaluate if, in LPS pre-activated macrophages, the peptides would influence the release of the pro-inflammatory cytokine.

As it can be seen in Figure 4A in non-activated macrophages, the peptide fraction P188D 40% induces a significantly high release of TNF-α in comparison to the control (cell culture media only), while the increase in TNF-α in the presence of P188D 20% is significantly lower and similar to the control (no statistically significant difference). In the presence of LPS pre-activated macrophages, the levels of TNF-α with or without the peptides fraction is similar. However, the amount of TNF-α released by peptide fraction P188D 40% was significantly reduced in the presence of LPS. As it is known, the maintenance of an inflammatory microenvironment favours tumorigenesis, particularly in skin cancers, such as the squamous cell carcinoma and basal cell carcinoma, in which there is an important inflammatory component [33]. Even though these peptides are not able to decrease TNF-α release, they do not enhance it. This cytokine is important in the induction of a pro-inflammatory response and it seems that in activated macrophages the presence of these peptides will not contribute to enhancing inflammation, as in some of the traditional

chemotherapy agents, such as cisplatin, paclitaxel, 5-fluorouracil, and doxorubicin [34]. Therefore, the use of these peptides, such as 20% fraction peptides (P188D 20%), which seems to decrease the migration rate as well as the proliferation rate, can be an interesting alternative to the traditional chemotherapy agents.

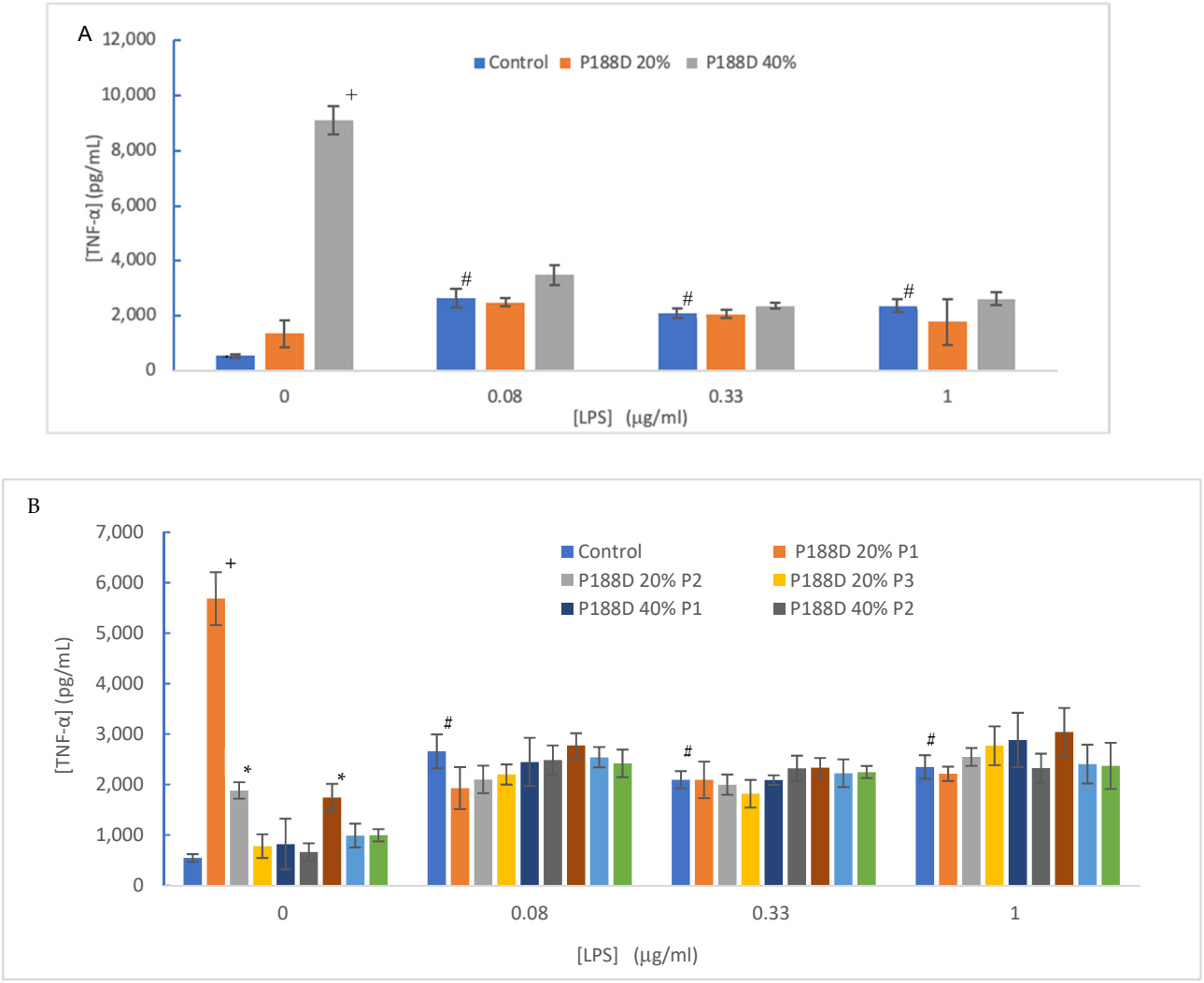

Figure 4. TNF-α release in the presence of the different fractions of the peptide (**A**) 20% and 40% fraction and (**B**) 20% P1, P2, P3 and 40% P1, P2 and P3, P4, P5 in the presence of different concentrations of LPS. * shows statistical difference in comparison to the control and $^{+}$ shows statistical difference in comparison to all other conditions. $^{\#}$ shows statistical difference in comparison to the control of 0 mg/mL LPS ($p < 0.05$). The error bars represent the standard error.

The fractioned peptides from the last fractionation stage (chromatographic peaks by molecular weight) have a similar behaviour when in contact with pre-activated macrophages, where the values of this cytokine can be slightly higher than the negative control (DMEM) but never reaching statistical significance (Figure 4B). On the other hand, the contact of the different fractions with the non-activated macrophages (without LPS) induced distinct results. The P188D 20% P1 and P2, and P188D 40% P3 induced a significantly higher release of TNF-α in comparison to the negative control and all other conditions. Again, these results suggest that these peptides can be used in medical conditions, where there is an inflammatory process involved, as they will not aggravate it, as in the case of cancer or wound healing. These peptides open the possibility to modulate the migration rate of the keratinocytes involved in both processes, to enhance or decrease it, depending on the patient's needs.

Gao et al. [35] demonstrated that the presence of hair keratins in a culture with rat neuronal Schwann cells increased proliferation and increased the number of cells passing through a trans-well pore. In the wound-healing experiment with primary Schwann cells, the damaged areas in the keratin group significantly decreased [35]. The presence of keratins from chicken feathers, when fractioned, also resulted in an increase in the metabolic activity/proliferation, with the exception of one fraction P188D 40% P3. Our results also demonstrated that the peptide fraction stimulates cell proliferation in a wound-healing model similar to the one reported by Gao et al. [35]. However, the keratins extracted from the chicken feathers did not seem to significantly affect the production of the inflammatory cytokine TNF-α, as described by Gao et al. [35]. This difference may be due to the different experimental settings; while in the Gao et al. [35] experimental model the keratin peptides are in contact with the cells prior to LPS activation, in our experimental model, the keratin peptides were added to the cell culture medium after LPS stimulation. Our experimental model allows the evaluation of these peptides as coadjutant molecules for the treatment of skin inflammatory diseases.

The current knowledge regarding macrophage activation in wound healing is the ability of these cells to shift from an M1 classical pro-inflammatory response (stimulated by TNF-α, LPS as an example) to an M2 (polarized by IL-4, for example) anti-inflammatory response activation pathway after microbial infection control [36–38]. It has been described that a biomaterial made from human hair keratins elicit anti-inflammatory responses from naïve macrophages and polarize them towards M2 phenotype [38]. Waters et al. [39] described that keratin influences macrophage behaviour, although the mechanism behind it is extremely complex. Nevertheless, Waters et al. [39] describes that, macrophages are altered when in contact with an immobilized keratin biomaterial surface, and that these changes appear to trend toward an anti-inflammatory phenotype. Even more importantly, it is described that the response of macrophages is dependent on keratin molecular weight. This fact is very important as it can explain the differences observed on the response of the macrophages to the chicken feather keratin hydrolysate.

As the results differ depending on the peptide fraction present, a proteomic study was performed to evaluate the main sequences of peptides present in the solution, as well as comparing it to the keratin structure present in chicken feathers.

3.3. Proteomics

In order to identify an amino-acid sequence that could be responsible for the results observed, the different fractions were analysed using the ProteinPilot™ software and a database containing the sequences of the proteins of interest (*Gallus gallus* feather keratins, from Uniprot 09-2017). Peptide fractions of three proteins were detected, as described in Table 2.

Table 2. Peptide sequence of chicken feather keratin 1, 3 and 4.

Feather Keratin 1
SCYDLCRPSAPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSAAVGSILSEEGVPISSGGFGISGLGSRFSSRRCLPY
Feather Keratin 3
SCFDLCRPCGPTPLANSCNEACVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTLVGSSTSAAVGSILSEEGVPISSGGFGISGLGSRFSGRRCLPC
Feather Keratin 4
SCYDLCRPSAPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSAAVGSILSEEGVPISSGGFGISGLGSRFSSRRCLPY

The proteomics analysis revealed the presence of peptides with different sequences and with homology to three distinct chicken keratin proteins: keratin 1, 3 and 4, as described in Tables 3–6.

Table 3. Peptide sequence of the chicken feather microbial degradation present in the fraction P188D 20% with 99% of confidence and its homology to chicken feather keratin 3 and 4.

Protein	Sequence	Modifications	Number of Repeats
4	SSFPQNTAVGS		2
4	SFPQNTAVGSSTSA		2
4	SFPQNTAVGSSTS		
4	GPILSSFPQNTAVGSSTS		
4	GPILSSFPQNTAVGSSTS	Deamidated(N)@10	
4	GPILSSFPQNTAVGSS		
4	GPILSSFPQNTAVGS		2
4	GPILSSFPQNTAVGS	Pro→Asn@8	
4	GPILSSFPQNTAVGS	Asn→His@10	4
4	GPILSSFPQNTAVGS	Deamidated(N)@10	
4	GPILSSFPQNTAVGS	Asn→His@10	
4	GPILSSFPQNTAVGS	Deamidated(N)@10	3
4	GPILSSFPQNTAVGS	Oxidation(P)@8, Gln→His@9	
4	GPILSSFPQNTAVG	Deamidated(N)@10	
4	GPILSSFPQNTAV		
4	GPILSSFPQNTAV	Deamidated(N)@10	3
4	GPILSSFPQNTAV	Asn→His@10	
4	GPILSSFPQNTA		
4	GPILSSFPQNTA	Deamidated(N)@10	
4	AVGSSTSAAVGSIL		
3	GSRFSGRR	Arg→Ser@7	2
3	GPILSSFPQNTLVGS	Deamidated(N)@10, Leu→Val@12	
3	GPILSSFPQNTLVGS	Deamidated(N)@10, Leu→Val@12	
4, 3	VVVTLPGPILS		2
4, 3	VVTLPGPILS		2
4, 3	VVTLPGPILS	Ser→Thr@10	
4, 3	VVTLPGPIL		2
4, 3	VVIQPSPVVVT		
4, 3	VVIQPSPVVV	Pro→Ser@7	3
4, 3	VVIQPSPVVV	Oxidation(P)@5, Ser→Pro@6	
4, 3	VVIQPSPVVV		4
4, 3	VVIQPSPVVV	Val→Leu@1	2
4, 3	VVIQPSPVVV	Ser→Ala@6	3
4, 3	VVIQPSPVVV	Carbamidomethyl@N-term, Ser→Pro@6	
4, 3	VVIQPSPV		2
4, 3	VVIQPSPV	Ser→Ala@6	
4, 3	VIQPSPVVV	Ser→Ala@5	
4, 3	VIQPSPVV	Ser→Ala@5	
4, 3	TLPGPILSSFPQ		2
4, 3	TLPGPILS	Ser→Thr@8	
4, 3	TLPGPILS	Ser→Thr@8	
4, 3	TLPGPILS	Leu→Phe@2	
4, 3	SSGGFGISGLGSR	Delta:H(2)C(2)@N-term	

Table 3. *Cont.*

Protein	Sequence	Modifications	Number of Repeats
4, 3	SRVVIQPSP		2
4, 3	SPVVVTLPGPILSSFPQN	Pro→Thr@8	
4, 3	SPVVVTLPGPILSSFPQ	Pro→Thr@8	
4, 3	SPVVVTLPGPILSSFPQ	Pro→Asp@8	
4, 3	SPVVVTLPGPILSSFPQ	Pro→Asp@8, Deamidated(Q)@17	
4, 3	SPVVVTLPGPILSSFPQ	Oxidation(P)@8	
4, 3	SPVVVTLPGPILS		
4, 3	SPVVVTLPGPIL		2
4, 3	SPVVVTLPGP		2
4, 3	SPVVVTLPG	Oxidation(P)@8, Gly→Cys@9, Carbamidomethyl(C)@9	
4, 3	SPVVVTLP		
4, 3	SGGFGISGLGSR		
4, 3	SGGFGISGLGSR	Ile→Asn@6	
4, 3	SEEGVPISSGGFGIS	Glu→Ser@3	
4, 3	SEEGVPISSGGFGIS		
4, 3	SEEGVPISSGGFGI	Glu→Ser@3	2
4, 3	SEEGVPISSGGFGI	Glu→Ser@2	
4, 3	SEEGVPISSGGF	Glu→Ser@3	
4, 3	RVVIQPSP	Arg→Asp@1	
4, 3	QPSPVVVTLPGPIL	Gln→Met@1	
4, 3	QDSRVVIQPSP		2
4, 3	PSPVVVTLPGPILS		
4, 3	PSPVVVTLPGPIL		
4, 3	PSPVVVTLPGP		
4, 3	PSPVVVTLP	Pro→Ala@1	
4, 3	PSPVVVTLP		2
4, 3	PISSGGFGISGLGS	Leu→Thr@12	
4, 3	LPGPILSSFPQN		
4, 3	LPGPILSSFPQ		
4, 3	IQPSPVVVTLP		
4, 3	IQPSPVVVTLP	Ser→Pro@4	2
4, 3	IQPSPVVVT	Ser→Pro@4	2
4, 3	IQPSPVVV	Ser→Pro@4	5
4, 3	ILSSFPQN		2
4, 3	GVPISSGGFGISGLGSR		2
4, 3	GVPISSGGFGIS		5
4, 3	GVPISSGGFGI		3
4, 3	GVPISSGGFGI	Ile→Phe@11	
4, 3	GVPISSGGFG		
4, 3	GVPISSGGF		3
4, 3	GVPISSGGF	PhosphoHexNAc(S)@6	
4, 3	GPILSSFPQNT		3
4, 3	GPILSSFPQNT	Deamidated(N)@10	2
4,3	GPILSSFPQNT	Asn→His@10	

Table 3. *Cont.*

Protein	Sequence	Modifications	Number of Repeats
4, 3	GPILSSFPQN		7
4, 3	GPILSSFPQN	Asn→Gln@10	3
4, 3	GPILSSFPQN	Asn→Ser@10	2
4, 3	GPILSSFPQN	Deamidated(N)@10	
4, 3	GPILSSFPQ		2
4, 3	GPILSSFPQ	Ser→Thr@5	3
4, 3	GPILSSFP		4
4, 3	GISGLGSRFS	Phe→Leu@9	
4, 3	GISGLGSRFS		3
4, 3	GISGLGSRF	Phe→Tyr@9	
4, 3	GISGLGSRF	Ser→Gly@7	2
4, 3	GGFGISGLGSRF		2
4, 3	GGFGISGLGSR		
4, 3	GFGISGLGSRF		
4, 3	GFGISGLGSR		2
4, 3	GFGISGLGSR	Ser→Gly@9	
4, 3	EGVPISSGGFGIS		3
4, 3	EGVPISSGGFGI		
4, 3	EGVPISSGGF	Glu→Ser@1	
4, 3	EEGVPISSGGFGI	Glu→Ser@2	

Table 4. Peptide sequence of the chicken feather microbial degradation present in the fraction P188D 40% with 99% of confidence and its homology to Keratin Chicken Feather 4 and 1.

Protein	Sequence	Modifications	Number of Repeats
4	VVVTLPGPILSSFPQN	Pro→Asp@6	
4	VVVTLPGPILSSFPQN		4
4	VVVTLPGPILSSFPQ		7
4	VVVTLPGPILSSFPQ	Pro→Asn@6	
4	VVVTLPGPILSSFP	cGMP + RMP-loss(S)@12	
4	VVVTLPGPILSS		
4	VVVTLPGPILS	Ser→Thr@11	
4	VVVTLPGPILS	Thr→Leu@4	2
4	VVVTLPGPILS	Pro→Met@6	
4	VVVTLPGPILS		31
4	VVVTLPGPIL		3
4	VVVTLPGPI		
4	VVTLPGPILSSFPQN	Leu→Phe@4	
4	VVTLPGPILSSFPQ		5
4	VVTLPGPILSSFPQ	Leu→Phe@4	2
4	VVTLPGPILSS	Ser→Val@11	
4	VVTLPGPILS	Ser→Thr@10	
4	VVTLPGPIL		2
4	VVIQPSPVVVTLPGPILSSF		
4	VVIQPSPVVVTLPGPILSSFPQ		4
4	VVIQPSPVVVTLPGPILSSF	cGMP + RMP-loss(S)@19	

Table 4. *Cont.*

Protein	Sequence	Modifications	Number of Repeats
4	VVIQPSPVVVTLPGPILS	Pro→Ser@7	
4	VVIQPSPVVVTLPGPILS		6
4	VVIQPSPVVVTLPGPILS	Val→Leu@1	
4	VVIQPSPVVVTLPGPILS	Ser→Ala@6	
4	VVIQPSPVVVTLPGPIL		2
4	VVIQPSPVVVTLPGP	Pro→Ala@5, Oxidation(P)@7	
4	VVIQPSPVVVTLPGP	Pro→Thr@7	2
4	VVIQPSPVVVTLP	Pro→Ser@7	2
4	VVIQPSPVVVTLP	Oxidation(P)@7, Thr→Pro@11	3
4	VVIQPSPVVVTLP	Ser→Pro@6, Oxidation(P)@7	
4	VVIQPSPVVVTLP	Pro→Ser@7	
4	VVIQPSPVVVTLP		7
4	VVIQPSPVVVTLP	Val→Leu@1	
4	VVIQPSPVVVTLP	Ser→Ala@6	3
4	VVIQPSPVVVTLP	Pro→Ser@5, Oxidation(P)@7	
4	VVIQPSPVVVTLP	Oxidation(P)@5, Pro→Ser@7	
4	VTLPGPILSSFPQN		
4	VTLPGPILSSFPQ		4
4	VTLPGPILSSFPQ	Leu→Phe@3	3
4	VIQPSPVVVTLP	Ser→Ala@5	
4	VIQPSPVVVTLP	Ser→Ala@5	
4	TLPGPILSSFPQN		2
4	TLPGPILSSFPQ		6
4	TLPGPILSSFPQ	Ser→Thr@8	
4	TLPGPILSSFPQ	Leu→Phe@2	5
4	TLPGPILS	Leu→Phe@2	
4	TLPGPILS	Leu→Phe@2	
4	TLPGPILS	Ser→Phe@8	
4	SRVVIQPSP	Arg→Asp@2	
4	SPVVVTLPGPILSSFPQN	Pro→Thr@8	
4	SPVVVTLPGPILSSFPQ		4
4	SPVVVTLPGPILS		5
4	SPVVVTLPGPILS	Dehydrated(T)@6	
4	SPVVVTLPGPIL		3
4	SPVVVTLPGP		
4	SPVVVTLPG	Oxidation(P)@8, Gly→Cys@9, Carbamidomethyl(C)@9	
4	SGGFGISGLGSRF		
4	SCGGFGISGLGSR	Cys→Leu@2	
4	RVVIQPSPVVVTLPGPILS		
4	PVVVTLPGPILSSFPQ	Pro→Thr@1	
4	PVVVTLPGPILS	Pro→Thr@1	
4	PVVVTLPGPILS	Pro→Ser@1	2
4	PVVVTLPGPIL	Pro→Thr@1	
4	PVVVTLPGP	Pro→Ser@1	

Table 4. *Cont.*

Protein	Sequence	Modifications	Number of Repeats
4	PVVVTLPGP	Pro→Thr@1	2
4	PSPVVVTLPGPILS		3
4	PSPVVVTLPGPIL	Pro→Ala@1	
4	PSPVVVTLPGPIL		3
4	PSPVVVTLPGP		
4	LPGPILSSFPQNTAVGS	Deamidated(N)@12	
4	LPGPILSSFPQ	Ile→Phe@5	3
4	LPGPILSSFPQ	Leu→Phe@1	
4	LPGPILSSFPQ		2
4	IQPSPVVVTLPGPILSSFPQ	Ser→Pro@4	5
4	IQPSPVVVTLPGPILS		3
4	IQPSPVVVTLPGPILS	Ser→Pro@4	5
4	IQPSPVVVTLPGPIL	Ser→Pro@4	
4	IQPSPVVVTLPGP	Ser→Pro@4	3
4	IQPSPVVVTLP	Ser→Ala@4	
4	IQPSPVVVTLP	Ser→Pro@4	5
4	GVPISSGGFGISGLGSR		
4	GVPISSGGFGISGL		
4	GVPISSGGFGIS	Ile→Phe@11	
4	GVPISSGGFGI	2	2
4	GVPISSGGFGI	Ile→Phe@11	
4	GPILSSFPQNTAVGS		
4	GPILSSFPQNTAVGS	Asn→His@10	3
4	GPILSSFPQNTAVGS	Deamidated(N)@10	2
4	GPILSSFPQNTAVGS	Deamidated(N)@10, Ala→Val@12	2
4	GPILSSFPQNTAV	Deamidated(N)@10	3
4	GPILSSFPQNT		2
4	GPILSSFPQNT	Deamidated(N)@10	
4	GPILSSFPQN		5
4	GPILSSFPQ		
4	GPILSSFPQ	Ser→Thr@6	
4	GPILSSFPQ	Ser→Thr@5	2
4	GGFGISGLGSRFS		2
4	GGFGISGLGSRF		3
4	GGFGISGLGSR		3
4	GFGISGLGSRFS	Phe→Leu@11	
4	GFGISGLGSR		2
4	FGISGLGSR		
4	EGVPISSGGFGIS		

Table 5. Peptide sequence of the chicken feather microbial degradation present in the fraction P188D 20% P1, P2 and P3 with 99% of confidence and its homology to chicken feather keratin 4, 1, 3, respectively.

Sequence	Modifications	Protein Modifications	Number of Repeats
P188D 20% P1			
EGVPISSGGF			
EGVPISSGGFGI			
EGVPISSGGFGIS			
GPILSSFPQ			
GPILSSFPQN			
GPILSSFPQNT			
GPILSSFPQNTAV	Deamidated(N)@10		
GVPISSGGF			
GVPISSGGFGI			
GVPISSGGFGIS			
IQPSPVVVTLP			
IQPSPVVVTLPGPILS			
PSPVVVTLPGP			
PSPVVVTLPGPILS			
QDSRVVIQP	Gln→pyro-Glu@N-term		
QDSRVVIQPSP	Gln→pyro-Glu@N-term		
SFPQNTAVGSSTS	Deamidated(N)@5		
SFPQNTAVGSSTSA	Deamidated(N)@5		
SFPQNTAVGSSTSAA	Deamidated(N)@5		
SGGFGISGLGSR			
SPVVVTLPGP			
SPVVVTLPGPILS			
TLPGPILSSFPQ			
VPISSGGFGI			
VPISSGGFGIS			
VVIQPSPV			
VVIQPSPVVV			
VVIQPSPVVVTLPGPILS			
VVTLPGPILS			
VVVTLPGPIL			
VVVTLPGPILS			
GPILSSFPQNTAVGS	Asn→His@10	Asn→His@54	
EEGVPISSGGFGI	Glu→Ser@2	Glu→Ser@72	
SEEGVPISSGGF	Glu→Ser@2	Glu→Ser@71	
SEEGVPISSGGFGI	Glu→Ser@2	Glu→Ser@71	
SEEGVPISSGGFGIS	Glu→Ser@2	Glu→Ser@71	
EEGVPISSGGF	Glu→Ser@2	Glu→Ser@72	

Table 5. *Cont.*

Sequence	Modifications	Protein Modifications	Number of Repeats
EEGVPISSGGFGIS	Glu→Ser@2	Glu→Ser@72	
SFPQNTAVGS	Asn→His@5	Asn→His@54	
SSFPQNTAVGS	Asn→His@6	Asn→His@54	
VVIQPSPVVVTLP	Ser→Val@6	Ser→Val@37	
PVVVTLPGP	Pro→Thr@1	Pro→Thr@38	
PVVVTLPGPIL	Pro→Ser@1	Pro→Ser@38	
PVVVTLPGPILS	Pro→Thr@1	Pro→Thr@38	
IQPSPVVVT	Ser→Pro@4	Ser→Pro@37	
VIQPSPVVVTLP	Pro→Thr@6	Pro→Thr@38	
IQPSPVVV	Ser→Pro@4	Ser→Pro@37	
RVVIQPSP	Arg→Thr@1	Arg→Thr@31	
PSPVVVTLP	Thr→Leu@7	Thr→Leu@42	
SEEGVPISS	PhosphoHexNAc(S)@8	PhosphoHexNAc(S)@77	
VPISSGGF	PhosphoHexNAc(S)@5	PhosphoHexNAc(S)@78	
SPVVVTLPG	Oxidation(P)@8; Gly→Cys@9; Carbamidomethyl(C)@9	Oxidation(P)@44; Gly→Cys@45	
DSRVVIQPSP	Asp→Thr@1; Deamidated(R)@3	Asp→Thr@29; Deamidated(R)@31	
EEGVPISSGGF	Glu→Ser@1	Glu→Ser@71	
EEGVPISSGGFGI	Glu→Ser@1	Glu→Ser@71	2
EEGVPISSGGFGI	Glu→Ser@2	Glu→Ser@72	
EEGVPISSGGFGIS	Glu→Ser@1	Glu→Ser@71	
EGVPISSGGFGI			
EGVPISSGGFGIS			2
GPILSSFPQ			3
GPILSSFPQN			3
GPILSSFPQNT			3
GPILSSFPQNTAV	Deamidated(N)@10		
GPILSSFPQNTAVGS	Deamidated(N)@10		4
GPILSSFPQNTAVGS	Asn→His@10	Asn→His@54	3
GVPISSGGF			2
GVPISSGGFGI			
GVPISSGGFGIS			2
IQPSPVVV	Ser→Pro@4	Ser→Pro@37	
IQPSPVVVT	Ser→Pro@4	Ser→Pro@37	
IQPSPVVVTLP	Pro→Thr@3	Pro→Thr@36	
IQPSPVVVTLP	Pro→Thr@5	Pro→Thr@38	
IQPSPVVVTLP	Thr→Leu@9	Thr→Leu@42	
IQPSPVVVTLP	Pro→Ser@3	Pro→Ser@36	
IQPSPVVVTLP	Ser→Pro@4	Ser→Pro@37	

Table 5. *Cont.*

Sequence	Modifications	Protein Modifications	Number of Repeats
PSPVVVTLP			
PVVVTLPGP	Pro→Ser@1	Pro→Ser@38	
PVVVTLPGP	Pro→Thr@1	Pro→Thr@38	
PVVVTLPGP	Pro→Ser@1	Pro→Ser@38	
PVVVTLPGPIL	Pro→Ser@1	Pro→Ser@38	2
PVVVTLPGPIL	Pro→Thr@1	Pro→Thr@38	2
PVVVTLPGPILS	Pro→Thr@1	Pro→Thr@38	
PVVVTLPGPILS	Pro→Ser@1	Pro→Ser@38	2
RVVIQPSP	Arg→Asp@1	Arg→Asp@31	
SEEGVPISS			
SEEGVPISS	Cation:Na(E)@3		2
SEEGVPISS	Glu→Ser@2	Glu→Ser@71	
SEEGVPISS	Glu→Ser@3	Glu→Ser@72	
SEEGVPISS	Glu→Ser@3	Glu→Ser@72	
SEEGVPISS	Glu→Ser@2	Glu→Ser@71	
SEEGVPISS	Glu→Lys@2	Glu→Lys@71	
SEEGVPISS	PhosphoHexNAc(S)@8	PhosphoHexNAc(S)@77	
SEEGVPISS	PhosphoHexNAc(S)@9	PhosphoHexNAc(S)@78	
SEEGVPISSGGF	Glu→Ser@2	Glu→Ser@71	
SEEGVPISSGGF	Glu→Lys@2	Glu→Lys@71	
SEEGVPISSGGF	Glu→Ser@2	Glu→Ser@71	
SEEGVPISSGGF			3
SEEGVPISSGGF	Glu→Ser@2	Glu→Ser@71	
SEEGVPISSGGFGI	Glu→Ser@3	Glu→Ser@72	
SEEGVPISSGGFGI	Glu→Ser@2	Glu→Ser@71	2
SEEGVPISSGGFGIS	Glu→Ser@2	Glu→Ser@71	
SEEGVPISSGGFGIS			3
SFPQNTAVGS	Asn→His@5	Asn→His@54	4
SFPQNTAVGS	Deamidated(N)@5		4
SFPQNTAVGSSTS	Deamidated(N)@5		2
SFPQNTAVGSSTSA	Deamidated(N)@5		
SPVVVTLPG	Oxidation(P)@8; Gly→Cys@9; Carbamidomethyl(C)@9	Oxidation(P)@44; Gly→Cys@45	
SPVVVTLPGP			
SPVVVTLPGPILS			3
SSFPQNTAVGS	Asn→His@6	Asn→His@54	2
VIQPSPVVVTLP	Pro→Thr@4	Pro→Thr@36	
VIQPSPVVVTLP	Ser→Ala@5	Ser→Ala@37	
VPISSGGFGIS			

Table 5. *Cont.*

Sequence	Modifications	Protein Modifications	Number of Repeats
VVIQPSPV			2
VVIQPSPVVV	Ser→Ala@6	Ser→Ala@37	2
VVIQPSPVVV			
VVIQPSPVVVTLP	Pro→Ser@5; Oxidation(P)@7	Pro→Ser@36; Oxidation(P)@38	
VVIQPSPVVVTLP	Oxidation(P)@5; Pro→Ser@7	Oxidation(P)@36; Pro→Ser@38	
VVIQPSPVVVTLP			
VVIQPSPVVVTLP	Ser→Val@6	Ser→Val@37	4
VVIQPSPVVVTLP	Pro→Ser@7	Pro→Ser@38	
VVIQPSPVVVTLP	Pro→Thr@5	Pro→Thr@36	
VVIQPSPVVVTLP	Pro→Thr@7	Pro→Thr@38	2
VVIQPSPVVVTLP	Pro→Ser@5	Pro→Ser@36	
VVIQPSPVVVTLP			
VVIQPSPVVVTLPGPILS			
VVTLPGPILS			2
VVVTLPGPILS			3
P188D 20% P2			
GPILSSFPQN			
GLGSRFSGR	Arg→Ser@9	Arg→Ser@93	
PVVVTLPGPILS	Pro→Thr@1	Pro→Thr@38	2
PVVVTLPGPILS	Pro→Ser@1	Pro→Ser@38	2
P188D 20% P3			
GFGISGLGSR	Gly→Asn@3	Gly→Asn@82	
GLGSRFSGR	Arg→Ser@9	Arg→Ser@93	

Table 6. Peptide sequence of the chicken feather microbial degradation present in the fraction P188D 40% P2 and P3 with 99 % of confidence and its homology to chicken feather keratin 4.

Sequence	Modifications	Protein Modifications	Number of Repeats
P188D 40% P2			
EGVPISSGGFGIS			
GFGISGLGSR			
GGFGISGLGSR			
GPILSSFPQ			
GPILSSFPQN			
GPILSSFPQNT			
GPILSSFPQNTAVGS	Deamidated(N)@10		
GVPISSGGF			
GVPISSGGFGI			
GVPISSGGFGIS			
IQPSPVVV			

Table 6. *Cont.*

Sequence	Modifications	Protein Modifications	Number of Repeats
PSPVVVTLP			
QDSRVVIQPSP		Gln→pyro-Glu@N-term	
SEEGVPISSGGF	Glu→Ser@2	Glu→Ser@71	
SEEGVPISSGGFGIS			
SFPQNTAVGSSTS	Deamidated(N)@5		
SGGFGISGLGSR			
SPVVVTLPGP			
TLPGPILSSFPQ			
VVIQPSPV			
VVIQPSPVVV			
VVIQPSPVVVTLP			
VVTLPGPILS			
VVVTLPGPIL			
VVVTLPGPILS			
PVVVTLPGP	Pro→Ser@1	Pro→Ser@38	
SFPQNTAVGS	Asn→His@5	Asn→His@54	
IQPSPVVVTLPGP	Ser→Pro@4	Ser→Pro@37	
EEGVPISSGGF	Glu→Ser@2	Glu→Ser@72	
VIQPSPVVVTLP	Ser→Ala@5	Ser→Ala@37	
SRVVIQPSP	Arg→Asp@2	Arg→Asp@31	
SPVVVTLPG	Oxidation(P)@8; Gly→Cys@9; Carbamidomethyl(C)@9	Oxidation(P)@44; Gly→Cys@45	
VPISSGGF	PhosphoHexNAc(S)@5	PhosphoHexNAc(S)@78	
IQPSPVVVTLP	Oxidation(P)@3; Pro→Ala@5	Oxidation(P)@36; Pro→Ala@38	
EEGVPISSGGF	Glu→Ser@1	Glu→Ser@71	
EGVPISSGGFGIS			2
EGVPISSGGFGIS			
GGFGISGLGSR			
GPILSSFPQ			4
GPILSSFPQN			4
GPILSSFPQNT			2
GPILSSFPQNTAVGS	Deamidated(N)@10		2
GPILSSFPQNTAVGS	Asn→His@10	Asn→His@54	3
GVPISSGGF			3
GVPISSGGFGIS			3
IQPSPVVV	Ser→Pro@4	Ser→Pro@37	
IQPSPVVV	Pro→Glu@5	Pro→Glu@38	
SEEGVPISSGGF	Glu→Lys@2	Glu→Lys@71	

Table 6. *Cont.*

Sequence	Modifications	Protein Modifications	Number of Repeats
SEEGVPISSGGF			2
SEEGVPISSGGFGIS			0.038
SFPQNTAVGS	Asn→His@5	Asn→His@54	
SFPQNTAVGS	Deamidated(N)@5		
SPVVVTLPG	Oxidation(P)@8; Gly→Cys@9; Carbamidomethyl(C)@9	Oxidation(P)@44; Gly→Cys@45	
SPVVVTLPGP			2
TLPGPILSSFPQ			
TLPGPILSSFPQ	Leu→Phe@2	Leu→Phe@43	
VVIQPSPVVV	Ser→Ala@6	Ser→Ala@37	3
VVTLPGPILS			4
VVTLPGPILS	Leu→Phe@4	Leu→Phe@43	
VVVTLPGPIL			
VVVTLPGPILS			4
P188D 40% P3			
GFGISGLGSR	Gly→Asn@3	Gly→Asn@82	

Analysing the fraction P188D 20% it was detected the presence of amino-acid sequences similar to the ones present in keratin 3 and 4 (Table 3). The presence of the two keratins (3 and 4) seems to decrease the migration and metabolic activity of the keratinocytes. In the P188D 40% fraction only the presence of sequences related to keratin 4 were found (Table 4). The loss of peptides with the homology to keratin 3 in P188D 40% results in keratinocytes with a behaviour similar to the control. It can be hypothesized that the presence of peptides with homology to keratin 3 may be responsible for cell migration and metabolic activity decrease. Keratins are known to be intermediate filament-forming proteins that are related to differentiation status [40] and are known to be crucial for the motile processes, although is not fully clear [41].

The proteomics analysis detected any peptides for the fractions P188D 40% P1, P4 and P5 related to chicken keratin feathers. In the case of fractions P188D 20% P1, 40% P2 and 40% P3, all the peptides present had homology to chicken feather keratin 4 with some modifications on the sequence resulting in changes in the protein, as can be seen in Tables 5 and 6.

The peptides present in the fraction P188D 20% P2 had homology to feather keratin 1 and 4, while 20% P3 has a homology to the chicken feather keratin 3. The results obtained for migration or metabolic activity were not possible to correlate with the presence or absence of the peptides with homology to the keratin 3; this may be due to the loss of some of the peptide with particular sequences.

Nevertheless, this study demonstrates the ability to modulate keratinocyte migration and metabolic activity in the presence of different peptide sequences with homology to keratin.

4. Conclusions

This study demonstrated that the microbial degradation of chicken feathers has significant advantages. It is an environmentally friendly method to obtain bioactive peptides. The resulting peptides induced significant changes in the viability and migration rate of keratinocytes. The effect observed was dependent on the size distribution (fraction). The distinct effect of the peptides, depending on their fraction, opens doors to tailor-made

medicine, where it is possible to modulate the cell's response, either to increase or decrease their migration rate and metabolic activity. The production of TNF-α was not exacerbated by the presence of the keratin peptides if there was a pre-activation of macrophages. On the other hand, P188D 40% and P188D 20% P1 significantly increased TNF- α production when placed in contact with non-activated macrophages. If these peptides are placed in contact with macrophages in their non-activated state, they will induce the production of an inflammatory cytokine. On the other hand, if the macrophages are already activated, the presence of the peptides will not exacerbate the condition.

The size as well as the sequence of the peptides present influences the cellular response, so on this project, the sequence of the peptides was determined and compared to *Gallus gallus* feather keratin. It was not possible to correlate the result obtained in terms of migration rate or metabolic activity, even though it was observed that the different peptides collections had homology to different *Gallus gallus* feather keratin. This fact may be due to changes present in a particular peptide sequence. Nevertheless, this study demonstrates the ability to modulate keratinocyte migration and metabolic activity in the presence of different peptide sequences with homology to keratin.

The work presented here is a step forward on the understanding of keratin peptides' influence on keratinocytes and immune cells system cells, macrophages, opening doors to tailor-made medicine.

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